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労災疾病臨床研究事業費補助金
総括研究報告書

職業性胆管癌に対する総合的診断法の確立

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研究要旨

（1）ジクロロメタン（DCM）や 1,2-ジクロロプロパン（DCP）に暴露された従業員や健康管理手帳交付者の検診において、昨年度に加えて健康管理手帳交付者 1 名と元同社従業員であった 1 名が、今年度新たな検診受診者として加わった。昨年度の検診において大阪の印刷事業場 S 社で 18 例目となる胆管癌と診断された患者に対して、外科治療を施行し、その病理検査結果、臨床成績や曝露状況に基づいて、同患者は 2015 年末に職業性胆管癌として労災認定を受けた。これまでの S 社従業員に対する調査や検診の結果、胃癌 2 名（1 例はすでに死亡、1 例は検診実施中）、皮膚ボーエン病 1 名（検診実施中）および腎癌 1 名（他院で経過観察中）の既往を有する従業員が判明した。

（2）外科切除および病理解剖施行 16 例の病理学的検討によって、ほとんどの比較的大型胆管において、慢性胆管傷害像、胆管癌の前癌病変あるいは早期癌病変と考えられている Biliary intraepithelial neoplasia（BilIN)や intraductal papillary neoplasm of bile duct

（IPNB）病変がみられた。特に職業性胆管癌症例の画像所見上の特徴である癌を伴わない限局性胆管拡張像を示す胆管にはこのような種々の病変がみられた。また、広範囲の胆管がγ-H2AX 陽性であった。すなわち、DNA 傷害を伴う慢性胆管傷害から BilIN や IPNB の前癌病変を経て、浸潤癌に至ると考えられた。全国での多くの職業性胆管癌症例においても同様の所見を確認しえた。

（3）当科および関連病院で加療した職業性胆管癌と通常の胆管癌症例の比較および全国の労災病院のデータベースを用いた若年性胆管癌症例の比較によって、職業性胆管癌症例では、若年、γ-GTP 高値、限局性胆管拡張像、BilIN や IPNB 病変がみられることが特徴であることが確認された。

（4）外科治療例を検討すると、術後、腹腔内感染などの合併症を伴う症例が多く、胆管傷害が術後合併症の発症に影響している可能性が考えられた。また、多中心性再発を疑わせる症例がみられ、この再発形式は高い発癌ポテンシャルが広範囲の胆管にみられる職業性胆管癌の特徴と考えられた。同再発病巣の再切除により、良好な成績が得られた症例があり、積極的な治療が奏功する可能性が示唆された。

（5）以上のような検診結果や職業性胆管癌患者の臨床成績、病理学的検討から、肝機能検査および腫瘍マーカーの測定、腹部超音波検査を行い、それらの検査で異常所見がみられた場合、MRI（MRCP）や CT を実施し、さらに内視鏡的逆行性胆道造影による胆道造影、細胞診および組織診によって確定診断を得るスクリーニング法が望ましいと考えられた。また、外科的治療後においても、転移再発に加えて多中心性再発のリスクがあるため、定期的な血液検査と画像診断が必要であると考えられた。

（6）職業性胆管癌における DNA メチル化異常の関与、および胆管硬化の病態に関して病理組織学的な検討を行った結果、職業性胆管癌では腫瘍部だけではなく非腫瘍部においても DNA メチル化を媒介する DNA methyltransferases（DNMTs）の発現が亢進していることが明らかとなった。このことから職業性胆管癌の多段階発癌過程において DNA メチル化異常が蓄積し、エピジェネティックな発がんの素地が形成されている可能性が示唆された。職業性胆管癌の胆管周囲線維化の組織学的特徴として、胆管周囲にα-smooth muscle actin （α-SMA）陽性の紡錘形細胞を多く認めた所見は原発性硬化性胆管炎に類似していた。一方で、職業性胆管癌のα-SMA 陽性細胞の分布状況や胆管周囲線維化の程度は閉塞性黄疸とは異なっていた。これらの所見は、職業性胆管癌の発癌機序および胆管硬化の機序解明に有用と考えられた。

（7）職業性胆管癌症例 4 例の全エクソン解析により、通常型胆管癌ゲノムと著しく異な

る①きわめて高頻度の体細胞変異、②一塩基置換のセンス・アンチセンス鎖間のバイアスに加え、③特異的な 3 塩基置換パターンが認められた。これらの特徴が解析例全例に認められ、これまでの大規模な胆管癌のゲノム解析でも未報告のものであることから、網羅的ゲノム解析により得られる変異プロファイルが本疾患を特徴づけるゲノムバイオマーカーになりうると考えられた。

（8）次世代シークエンサーを用いて、血清エクソソーム中 miRNA を解析した結果、肝内胆管がんと肝細胞がん、正常肝それぞれのエクソソーム中の塩基は平均 770 万、880 万、800 万リード得られた。解析に使用した miRNA は 36 種で診断率は 74.5%であり、エクソソーム中 miRNA は胆管がん診断マーカーとして使用できることを示した。

（9）厚生労働省によって新たに職業性胆管癌と認定された 6 名について、使用した化学物質の種類を特定するとともに、各種の情報を基にして曝露濃度を推定した。診断年齢は 40 歳代が 4 名、50 歳代が 2 名である。6 名中 5 名は印刷作業者であり、DCP および DCM に曝露されており、最高曝露濃度は DCP が 190～560 ppm、DCM が 300～980 ppm と推定され、1 日労働時間の時間荷重平均濃度は DCP が 13～230 ppm、DCM が 20～470 ppm と推定された。他の 1 人は IC カードに接着剤および帯電防止剤をコーティングする業務に従事しており、コーティング機のロール洗浄に DCP を使用していた。DCP の最高曝露濃度は 150 ppm と推定され、1 日労働時間の時間荷重平均濃度は 5～19 ppm と推定された。

A．研究目的

職業性胆管癌の臨床像および病理学的所見および分子生物学的特徴を検討し、通常の胆管癌症例のそれらと比較することにより職業性胆管癌の特徴を明らかにする。これらにより職業性胆管癌の診断法を明確にする。また分子生物学的検討により職業性胆管癌の発癌メカニズムの解明と新規バイオマーカーを検討する。さらに臨床病理学的所見、分子生物学的所見、曝露状況や過去および今後の健康診断結果の解析により職業性胆管癌のハイリスクグループの設定と胆管癌発症予測を含めた健康診断法を検討する。

B. 研究方法

職業性胆管癌と診断された症例における臨床像や検診結果を検討し、通常の胆管癌症例のそれらと比較する。また、全国労災病院のデータベースを用いて若年性（50 歳未満）胆管癌症例のそれらと比較する。職業性胆管癌の切除標本や病理解剖標本を用いた免疫組織学的検討を含む病理学的検討において、職業性胆管癌の発癌過程を検討するとともに、通常の胆管癌症例との比較を行う。さらに、職業性胆管癌症例と通常の胆管癌症例の癌部および非癌部標本のメタボロームおよびトランスクリプトーム解析を行う。また、職業性胆管癌の切除標本を用いてゲノム解析を行い、職業性胆管癌の特徴を明らかにする。さらに次世代シークエンサーを用いて、血清中エクソソーム

中マイクロ RNA を解析し、胆管癌診断マーカーとしての意義を検討した。一方、職業性胆管癌症例の曝露状況を明らかにし、その環境因子を同定した。

「印刷労働者にみられる胆管癌発症の疫学研究」（承認番号　2368）として大阪市立大学医学部倫理委員会および「ゲノム異常解析に基づく胆管癌の発生・進展の分子機構の解明」（承認番号 2840）として大阪市立大学ヒトゲノム遺伝子解析研究に関する倫理委員会の承認を得て行った。また、胆管がん等の職業性発癌の原因解明とバイオマーカー開発」のためヒトゲノム・遺伝子解析に関する倫理指針に則り研究を計画し、国立がん研究センター研究倫理審査委員会の承認（2014-072）を得て実施した。

C.　研究結果

（１）検診結果の解析

大阪の印刷 S 社の元あるいは現従業員のうち DCM や DCP に曝露したと考えられる従業員および健康管理手帳交付者（DCP 業務従事者）に対して検診を行った。昨年度に加えて、健康管理手帳交付者 1 名と元同社従業員であった 1 名が、本年度新たな検診受診者として加わった。職業性胆管癌の特徴であるγ-GTP 持続高値例が 4 例にみられ、うち 2 例では MRCP や CT による精査を行ったが、新たな胆管癌発症者はみられなかった。

昨年度の検診において同社で 18 例目となる胆管癌と診断された患者に対して、外科治療を施行した。その病理検査結果や臨床成績に基づいて、同患者は 2015 年末に労災認定を受けた。

これまでの同社従業員に対する調査や検診の結果、同社従業員において胃癌 2 名（1 例はすでに死亡、1 例は検診実施中）、皮膚ボーエン病 1 名（検診実施中）および腎癌 1 名（他院で経過観察中）の既往を有する従業員が判明した。なお、腎癌の 1 名は、少量であったが、2014 年に International Agency for Research on Cancer (IARC)において発癌リスクが group 1 と改定されたトリクロロエチレンの曝露歴を有する可能性を否定できなかった。さらにトリクロロエチレンが原因と考えられた重症肝炎既往を有する 1 例（Kubo S, et al. J Occup Health 2015;57;87-90）および他院で加療中である B 型肝炎患者 1 名に対しても検診実施中である。

（２）臨床病理学的特徴の解析

現在、職業性胆管癌と認定されている症例は、大阪 S 社の 18 例を含めて、全国で 38 例である。これらの症例のうち、患者あるいは患者家族より承諾を得た後、病理学的検討が可能であった 16 例の病理学的所見を解析した。

切除標本の全割面（最大 124 切片）を解析しえた 3 例において、全割面の病理学的所見と画像診断における胆管像を比較し、マッピングを施行した。その結果、主腫瘍は高分化から低分化腺癌を示す腫瘤形成型肝内胆管癌や胆管内発育型肝内胆管癌であった。3 例とも比較的大型胆管（肝外胆管から肝内第 3 次分枝）のほとんどの部位で、胆管や付属線の腫瘍性増殖、胆管消失像、胆管周囲の著明な硬化像（線維化）などの慢性胆管像、胆管癌の前癌病変あるいは早期癌病変と考えられている Biliary intraepithelial neoplasia (BilIN) や

intraductal papillary neoplasm of bile duct （IPNB）がみられた。特に、以前より職業性胆管癌の画像的特徴と指摘してきた、原発性硬化性胆管炎（PSC）の胆管像に類似した、癌による胆管狭窄を伴わない限局性肝内胆管拡張像を示す胆管ではBilIN と IPNB 病変や胆管癌の伸展がみられ、これらの病理学的変化が画像上での特徴と関連していると考えられた。さらに、DNA 傷害を示すγ-H2AX による免疫組織学的検討を行うと、ほぼ正常にみえる胆管、慢性胆管傷害、BilIN、IPNB および浸潤癌の部位に陽性であり、胆管全体にわたってDNA 傷害が生じていることが判明した。また、癌化を示す S100P による免疫組織学的検討を行うと、BilIN、IPNB と浸潤癌で陽性であり、BilIN や IPNB の段階で癌化がみられることが判明した。すなわち、ほとんどの胆管で DNA 傷害がみられ、慢性胆管傷害から BilIN や IPNB の前癌病変を経て、浸潤癌に至ると考えられた（Kinoshita M, et al. J Hepatobiliary Pancreat Sci 2016;23:92-101）。なお、同社胆管癌症例の 1 例が、胆管癌に対する外科治療後、胆管癌の再発はみられないものの肝不全で死亡したが、病理解剖の結果、胆管周囲を含む肝線維化の著明な進行がみられたことが判明した（Tomimaru Y, et al. Hepatol Res 2015;45:488-93）

全国の職業性胆管癌症例のうち、大型胆管の病理学的検討が可能であった外科治療あるいは病理解剖が行われた 16 例では、全例に慢性胆管傷害および BilIN がみられ、IPNB が多くの症例で確認された。すなわち、広範囲の胆管に慢性胆管傷害や前癌病変が認められることが、職業性胆管癌の病理学的特徴であることが判明した（Kinoshita M, et al. J Hepatobiliary Pancreat Sci 2016;23:92-101）。

職業性胆管癌の胆管硬化について、組織標本に含まれる主に隔壁レベルの肝内胆管を評価対象とした。α-smooth muscle actin (α-SMA)の免疫染色では、職業性胆管癌の硬化した胆管周囲に α-SMA 陽性の紡錘形細胞を多数認め、このα-SMA 陽性細胞の分布状況は PSC に類似していた。対照とした閉塞性黄疸とウイルス性肝炎/肝硬変では胆管周囲に散在性にα-SMA 陽性細胞を認めるのみであった。肝実質の α-SMA 陽性細胞の分布は同一症例でも不均一であったが、ウイルス性肝炎/肝硬変で最も多くの陽性細胞を認めた。職業性胆管癌の肝実質での α-SMA 陽性細胞の分布はPSCと比較的類似しており、実質内の局所的に肝星細胞の活性化を認めた。閉塞性黄疸は検討した 4 群の中で最も肝星細胞の活性化が目立たなかった。シリウスレッド染色では，職業性胆管癌と PSC、閉塞性黄疸で胆管周囲に輪状の線維化を認め、これはウイルス性肝炎/肝硬変ではみられなかった。線維化の程度を比較すると、PSC と閉塞性黄疸では胆管周囲にほぼ同程度の膠原線維の沈着を認めたが、職業性胆管癌ではこの2つの疾患群より膠原線維化が明らかに緻密で、胆管硬化がより強い傾向にあった。

（3）職業性胆管癌と通常の胆管癌症例との比較

当科および関連病院で切除された職業性胆管癌（肝内胆管癌）5 例と通常型で肝門型肝内胆管癌 46 例の臨床病理学的所見および治療成績を比較した。その結果、通常型肝内胆管癌に比較し、職業性肝内胆管癌

では若年で、γ-GTP 高値例、ICG15 分値低値例、血小板高値例が多かった。また、画像診断上、癌を伴わない限局性肝内胆管拡張像や乳頭状増殖像が多くみられた。病理学的には、職業性肝内胆管癌において胆管内発育型が多く、BilIN、IPNB および慢性胆管傷害像が多くみられた。切除後予後にはついては差がみられなかったが、観察期間が短く、今後の検討が必要と考えられた（Hamano G, et al. J Hepatobiliary Pancreat Sci, in press）。

過去の職業歴が検索可能な全国の労災病院のデータベースを用いて、若年性胆管癌（50 歳未満）と大阪 S 社の職業性胆管癌の臨床病理学的所見を比較した。労災病院のデータベースでは胆管癌の集団発生はみられなかった。若年性胆管癌症例に比較して、職業性胆管癌では肝内胆管癌が多く、臨床検査値異常を示す症例が多かった。また、職業性胆管癌で特徴的である限局性の肝内胆管拡張像は、若年性胆管癌症例ではみられなかった（Kaneko R, et al. Asian Pac J Cancer Prev 2015;16:7195-7200）。

（４）外科治療成績の検討

外科的治療が行われた全国の職業性胆管癌症例のうち、患者あるいは患者家族の承諾を得ることができた 20 例の外科治療成績を検討した。肝切除が 6 例、胆道再建を伴う肝切除が 9 例、膵頭十二指腸切除が 3 例、肝切除および膵頭十二指腸切除が 1 例、肝外胆管切除が 1 例に行われた。しかし、4 例が胆管断端癌陽性であり、そのうち 3 例では同部に放射線照射が行われた。術前および術後補助化学療法が 1 例に、術後補助化学療法が 12 例に行われた。術後、腹腔内感染（9 例）、胆管空腸縫合不全（3 例）、胆汁漏（1 例）、膵液瘻（3 例）、術後出血（2 例）などの合併症がみられ、これらの術後合併症の発症に胆管傷害が影響している可能性が考えられた。20 例中 12 例に胆管癌再発がみられたが、肝内多発再発が 2 例に、胆管断端再発が 5 例にみられた。他の 5 例では、原発部位とは異なる部位に単発の再発病巣がみられた。このうち 4 例に再切除が施行されたが、再発部位と原発巣に連続性はみられず、多中心性再発が疑われた。この 4 例中 3 例は生存中である。職業性胆管癌の特徴として、ほとんどの大型胆管の発癌ポテンシャルが亢進している病態から、根治手術後も多中心性再発のリスクが高いと考えられるものの、積極的な治療が奏功する可能性が示唆された。

（５）職業性胆管癌の総合的診断法

以上のような、検診結果および職業性胆管癌症例の臨床検査値、画像診断結果および病理学的検討から、職業性胆管癌のサーベイランス法およびスクリーニング法として、AST、ALT およびγ-GTP を含む肝機能検査、CEA および CA19-9 の腫瘍マーカーを測定し、さらに侵襲性の少ない腹部超音波検査を実施する。それらの検査で異常所見がみられる場合、MRI（MRCP）や CT を実施し、さらに内視鏡的逆行性胆道造影による胆道造影、細胞診および組織診によって確定診断を得る方法が望ましいと考えられた（Kubo S, et al. Surg Today, in press）。また、外科的治療後においても、多中心性再発のリスクもあるため、定期的な血液検査と画像診断が必要であると考えられた。

(6)職業性胆管癌における DNA メチル化の検討

DNA メチル化について DNA methyltransferases（DNMT3A、DNMT3B、DNMT1)の発現を非腫瘍部(大型胆管上皮、胆管周囲付属腺)、および腫瘍部(BilIN、IPNB、浸潤部)のそれぞれについて評価した。新規メチル化に関与する DNMT3A、DNMT3B の発現は、職業性胆管癌の胆管周囲付属腺、BilIN、浸潤癌の部位において対照群より有意に亢進していた。特に職業性胆管癌の胆管周囲付属腺における DNMT3A、DNMT3B の陽性頻度が高く、DNMT3A は職業性胆管癌の 86%、対照群の 27%の症例が陽性($p < 0.01$)、DNMT3B は職業性胆管癌の 86%、対照群の 7%の症例が陽性を示した($p < 0.01$)。また、職業性胆管癌の非癌部の大型胆管上皮において、DNMT3A の発現が対照群より高い傾向にあった(陽性率は職業性胆管癌 50%、対照群 13%; $p = 0.056$)。

メチル化維持に関与する DNMT1 の発現は、検討した疾患群のいずれにおいても非腫瘍部、腫瘍部とも陽性率は低かったが、職業性胆管癌の BilIN の部位において対照群より高い発現を示す傾向にあった(陽性率は職業性胆管癌 29%, 対照群 0%; $p = 0.060$)。

（7）分子生物学的検討

1．職業性胆管癌症例4例の全エクソン解析により、通常型胆管癌ゲノムと著しく異なる①きわめて高頻度の体細胞変異、②一塩基置換のセンス・アンチセンス鎖間のバイアスが認められた。今後これらの特徴が職業性胆管癌の原因物質と考えられている有機溶剤に起因するか、他地域の類似症例にも共通するものかなどを明らかにすることで、職業性胆管癌の診断補助に有用なゲノムバイオマーカーが開発される可能性が示唆された。以上、網羅的ゲノム解析から職業性胆管癌に特徴的な遺伝子変異プロファイルを見出した。

（8）胆管がん早期発見バイオマーカー

細胞間情報伝達に関与するエクソソームを媒体にした胆管がん早期発見バイオマーカー作成を試みた。その結果、肝内胆管がんと肝細胞がん、正常肝それぞれのエクソソーム中の塩基は平均 770 万、880 万、800 万リード得られた。得られたリード数は 49 で、Q20 の精度であった（Q20＝読み違え率は 1%）。解析に使用した miRNA は 36 種で診断率は 74.5%であった。したがって、次世代シークエンサー解析によるエクソソーム中 miRNA は胆管がん診断マーカーとして使用できることを示した。

（9）曝露状況の評価

新たに労災認定された職業性胆管癌 6 例について、使用した化学物質の種類を特定するとともに、各種の情報を基にして曝露濃度を推定した。

症例 N:1953 年生まれの男性。1989 年から 2002 年まで事業所 II においてオフセット校正印刷に従事し、2002 年に胆管がんと診断された。この事業所では、他に 2 人が胆管がんを発症し、業務上認定を受けている。事業所 II では 2 つの作業場で働いたが、作業場 1 の気積は 170 m^3、換気量は 1790 m^3/h、作業場 2 の気積は 180 m^3、換気量は 1100 m^3/h であった。印刷機には局所排気装置は設置されていなかった。ブランケッ

ト洗浄剤には 1,2-DCP、DCM、1,1-ジクロロ-1-フルオロエタンおよびミネラルスピリッツを使用した。作業場全体で使用した DCP は 230–400 g/h、DCM は 56–310 g/h であった。また洗浄作業中に使用した DCP は 330–630 g/h、DCM は 100–500 g/h であった。作業環境濃度は DCP が 28–78 ppm、DCM が 15–50 ppm と推定された。洗浄作業中の曝露濃度は DCP が 170–370 ppm、DCM が 77–330 ppm と推定された。1 日の労働時間は 9 時間であり、時間荷重平均濃度は DCP が 74–170 ppm、DCM が 35–140 ppm と推定された。呼吸保護具は使用しなかった。

症例 O:1949 年生まれの男性。1982 年から 1983 年まで事業所 XII、1983 年から 1986 年まで事業所 XIII、1986 年から 1994 年まで事業所 XIV においてオフセット校正印刷に従事し、1993 年に胆管がんと診断された。いずれの事業所にも印刷作業場は 1 つであった。作業場 3 の気積は 210 m^3、作業場 4 の気積は 240 m^3、作業場 4 の気積は 130 m^3 であった。全体換気装置は設置されていなかったので、換気回数を 1 時間当たり 1 回とし、換気量をそれぞれ 210 m^3/h、240 m^3 および 130 m^3/h と仮定した。印刷機には局所排気装置は設置されていなかった。ブランケット洗浄剤には、DCP および DCM を使用した。作業場全体で使用した DCP は 90–180 g/h、DCM は 110–210 g/h であった。また洗浄作業中に使用した DCP は 260 g/h、DCM は 300 g/h であった。作業環境濃度は DCP が 160–170 ppm、DCM が 240–250 ppm と推定された。洗浄作業中の曝露濃度は DCP が 340–560 ppm、DCM が 520–850 ppm と推定された。1 日の労働時間は 10 時間であり、時間荷重平均濃度は DCP が 200–230 ppm、DCM が 300–350 ppm と推定された。呼吸保護具は使用しなかった。

症例P:1971年生まれの男性。1991年から2014年まで事業所XVにおいてオフセット校正印刷に従事したが、2013年に胆管がんと診断された。事業所XVには1つの印刷作業場があり、気積は350 m^3であった。全体換気装置は設置されていなかったので、換気量を350 m^3/hと仮定した。印刷機には局所排気装置は設置されていなかった。ブランケット洗浄剤には、DCP、DCM、鉱油およびノナンを使用した。作業場全体で使用したDCPは0–210 g/h、DCMは200–450 g/hであった。また洗浄作業中に使用したDCPは0–280 g/h、DCMは240–720 g/hであった。作業環境濃度はDCPが0–130 ppm、DCMが160–370 ppm と推定された。洗浄作業中の曝露濃度はDCPが0–280 ppm、DCMが320–950 ppm と推定された。1日の労働時間は10時間であり、時間荷重平均濃度はDCPが0–160 ppm、DCMが240–470 ppm と推定された。呼吸保護具は使用しなかった。

症例 Q:1970 年生まれの男性。1998 年から 2013 年まで事業所 XVI においてオフセット校正印刷に従事したが、2012 年に胆管がんと診断された。事業所 XVI には印刷作業場は 2 つであり、作業場 7 の気積は 250 m^3、換気量は 600 m^3/h、作業場 8 の気積は 290 m^3 であった。作業場 8 には全体換気装置は設置されていなかったので、換気量を 290 m^3/h と仮定した。印刷機には局所排気装置は設置されていなかった。ブランケット洗浄剤には、DCP、DCM、1,1-ジクロロ

-1-フルオロエタン、トリクロロエチレン、トルエン、キシレン、ヘキサンおよび灯油を使用した。またインキロール洗浄剤も同じものを使用した。作業場全体で使用したDCPは19–120 g/h、DCMは21–130 g/hであった。また洗浄作業中に使用したDCPは110–260 g/h、DCMは130–300 g/hであった。作業環境濃度はDCPが7–42 ppm、DCMが10–66 ppmと推定された。洗浄作業中の曝露濃度はDCPが83–190 ppm、DCMが120–300 ppmと推定された。1日の労働時間は9–9.5時間であり、時間荷重平均濃度はDCPが13–65 ppm、DCMが20–98 ppmと推定された。呼吸保護具は使用しなかった。

症例R：1956年生まれの男性。1981年から2011年まで事業所XVIIにおいてオフセット印刷に従事したが、2011年に胆管がんと診断された。事業所XVIIには4つの印刷作業場があり、そのうち3つの作業場でDCPとDCMを使用した。作業場10の気積は510 m^3、換気量は9540 m^3/h、作業場11の気積は910 m^3、作業場12の気積は710 m^3、換気量は1620 m^3/hであった。作業場11には全体換気装置が設置されていなかったため、換気量を910 m^3/hと仮定した。印刷機には局所排気装置は設置されていなかった。ブランケット洗浄剤には、DCP、DCM、トリクロロエチレンおよび1,1,1-トリクロロエタンを使用した。作業場全体で使用したDCPは0–96 g/h、DCMは200–1880 g/hであった。また洗浄作業中に使用したDCPは0–370 g/h、DCMは780–1600 g/hであった。作業環境濃度はDCPが0–13 ppm、DCMが28–99 ppmと推定された。洗浄作業中の曝露濃度はDCPが0–190 ppm、DCMが530–980 ppmと推定された。1日の労働時間は9.5時間であり、時間荷重平均濃度はDCPが0–59 ppm、DCMが170–370 ppmと推定された。呼吸保護具は使用しなかった。

症例S：1958年生まれの男性。1996年から2001年まで事業所XVIIIにおいて、2001年から2005年まで事業所XIXにおいて、ICカードに接着剤および帯電防止剤をコーティングする業務に従事したが、2008年に胆管がんと診断された。事業所XVIIIにはコーティング作業場は1つであり、作業場13の気積は510 m^3、換気量は300 m^3/hであった。事業所XIXにはコーティング作業場は1つであり、作業場14の気積は160 m^3、換気量は1650 m^3/hであった。局所排気装置は設置されていなかった。コーティング機のロール洗浄剤には、DCPおよび1,1-ジクロロ-1-フルオロエタンを使用した。作業場全体で使用したDCPは8–16 g/h、洗浄作業中に使用したDCPは140 g/hであった。DCPの作業環境濃度は1–11 ppm、洗浄作業中の曝露濃度は72–150 ppmと推定された。1日の労働時間は9時間であり、DCPの時間荷重平均濃度は5–19 ppmと推定された。呼吸保護具は使用しなかった。厚生労働省が収集した情報（印刷作業場の気積と換気量、印刷機の種類、ブランケットとインキロールの洗浄剤の化学成分と使用量、洗浄時間）を取得した。さらにそれらの情報を基にして曝露濃度を推定した。7人中4人はDCPおよびDCMに曝露されており、最高曝露濃度はDCPが230～420 ppm、DCMが58～720 ppmと推定され、1日労働時間の時間荷重平均濃度はDCPが0～210 ppm、DCMが15～270 ppmと推

定された。一方、残りの 3 人は DCM への曝露はあるが、DCP への曝露はなかった。DCM の最高曝露濃度は 600～1300 ppm と推定され、1 日労働時間の時間荷重平均濃度は 84～440 ppm と推定された。これらの結果は、DCP 曝露だけでなく、DCM 曝露もヒトに胆管癌を引き起こす可能性を示唆した。

D. 考察

DCM や DCP に暴露された従業員や健康管理手帳交付者の検診において新たに診断された胆管癌患者の治療を行った。また、検診結果や職業性胆管癌患者の臨床成績から、職業性胆管癌に対するスクリーニング法として、暴露歴に加えて、AST、ALT および γ-GTP を含む肝機能検査、CEA および CA19-9 の腫瘍マーカーの測定、腹部超音波検査を行い、それらの検査で異常所見がみられる場合、MRI（MRCP）や CT を実施し、さらに内視鏡的逆行性胆道造影による胆道造影、細胞診および組織診によって確定診断を得る方法が望ましいと考えられた。切除標本や病理解剖標本の病理学的検討によって、比較的大型胆管のほとんどの部位で、胆管や付属線の腫瘍性増殖、胆管消失像、胆管周囲の著明な硬化像（線維化）などの慢性胆管像、胆管癌の前癌病変あるいは早期癌病変と考えられている BilIN や IPNB 病変がみられた。特に職業性胆管癌症例の画像所見での特徴である癌を伴わない限局性胆管拡張像を示す胆管にはこのような病変がみられた。また、DNA 傷害を示す γ-H2AX による免疫組織学的検討を行うと、ほぼ正常にみえる胆管、慢性胆管傷害、BilIN、IPNB および浸潤癌の部位に陽性であり、胆管全体にわたって DNA 傷害が生じていることが判明した。すなわち、ほとんどの胆管で DNA 傷害がみられ、慢性胆管傷害から BilIN や IPNB の前癌病変を経て、浸潤癌が至ると考えられた。同様の所見は、全国での職業性胆管癌症例の多くにおいても確認しえた。当科および関連病院で加療した職業性胆管癌と通常の胆管癌症例の比較および全国の労災病院のデータベースを用いた若年性胆管癌の比較によって、職業性胆管癌症例では、若年、γ-GTP 高値、限局性胆管拡張像、BilIN や IPNB 病変がみられることが特徴であることが確認された。

外科治療例を検討すると、術後、腹腔内感染などの合併症を伴う症例が多く、胆管傷害が術後合併症の発症に影響している可能性が考えられた。また、原発部位とは異なる部位に単発の再発病巣がみられる症例があり、これらは多中心性再発を疑わせた。この再発形式は高い発癌ポテンシャルが広範囲の胆管にみられる職業性胆管癌の特徴と考えられた。しかし同再発病巣の再切除により、良好な成績が得られた症例があり、積極的な治療が奏功する可能性が示唆された。

DNMT を介した遺伝子プロモーター領域の DNA メチル化異常は、癌抑制遺伝子を不活性化し発癌に寄与する。一見正常にみえる細胞でも、慢性炎症や化学物質などへの曝露により DNA メチル化異常が蓄積していることがあるとされる．膵癌では，膵管内上皮内腫瘍（pancreatic intraepithelial neoplasia、PanIN）を介した多段階発癌の過程において、DNMT3A、DNMT3B、DNMT1 の発現が段階的に亢進し、これら DNMTs の高発現群の膵

癌は低発現群より予後不良であることが報告されている。また、膵癌での DNMT1 発現はがん関連遺伝子のDNAメチル化の蓄積と有意に相関することが示されている。

これまで胆管癌の多段階発癌過程における DNMT の発現は検討されていなかったが、今回の検討結果から、職業性胆管癌では腫瘍部だけではなく非腫瘍部においてもDNMTsの発現が亢進していることが明らかとなった。このことから職業性胆管癌の多段階発癌過程において DNA メチル化異常が蓄積し、エピジェネティックな発がんの素地が形成されている可能性が示唆された。特に胆管周囲付属腺において DNMT3A、DNMT3B の発現亢進を顕著に認めたが、近年、胆管周囲付属腺にステム細胞ニッチが存在することが報告されており、胆管周囲付属腺の組織再生や胆管癌発癌における関与が注目されている。

職業性胆管癌の胆管周囲線維化の組織学的特徴として、胆管周囲に α-SMA 陽性の紡錘形細胞を多く認めた所見は PSC に類似していた. しかし、PSC より膠原線維沈着が緻密で胆管硬化がより強い傾向にあり、職業性胆管癌の胆管周囲線維化は PSC と同質なものではなかった。一方で、職業性胆管癌の α-SMA 陽性細胞の分布状況や胆管周囲線維化の程度は閉塞性黄疸とは異なっており。単に胆管狭窄や閉塞に伴う 2 次性線維化とは異なることが示唆された。

われわれの以前の検討で、職業性胆管癌の非腫瘍部の大型～隔壁胆管, 胆管周囲付属腺では γ-H2AX や p53 の発現をしばしば認めたが、こうした細胞は DNA 損傷から細胞老化を起こしている可能性がある。老化細胞は senescence-associated secretory phenotype（SASP）を分泌することが知られており、SASP には炎症や線維化、発癌に関連した分子も多い。例えば、SASP である IL-6 や MCP-1 は胆管細胞から分泌されうるが、これらは肝星細胞を活性化する作用を有するとされている。また、IL-6 は胆管癌の発癌に関連が深いことが従来から知られている。職業性胆管癌では末梢側の小型胆管に γ-H2AX や p53 の発現は通常みられないが、肝門側において DCP や DCM などへのばく露により胆道系の上皮細胞が傷害され、傷害上皮の胆汁への接触などが誘因となり徐々に細胞老化を生じ、それにより分泌された SASP が一連の症例の胆管病変の病態形成に関与している可能性があると思われる。また、SASP は血清からも検出可能なものが多く、発癌予測マーカーとなりうる SASP が存在するかも知れない。

職業性胆管癌ゲノムには、昨年度報告したきわめて高頻度の体細胞変異、一塩基置換のセンス・アンチセンス鎖間のバイアスに加え、新規の特異的な3塩基置換パターンが見いだされた。特に特徴的な 3 塩基置換パターンは最近論文報告された胆管癌の大規模ゲノム解析でも報告がなく、本疾患を特徴づけるゲノムバイオマーカーになりうると考えられた。DCP を曝露したサルモネラ菌ゲノムの解析で一部類似した特徴が認められたことから、疫学研究で示唆されていた発がんメカニズムとの関連が予想されたが、モデル系と臨床例ではゲノム変異プロファイルに一致しないところもあり、生体内ではさらに複雑なメカニズム（化合物間の相互作用、胆管上皮における化合物代謝機構、炎症等宿主因子との相互作用など）が加味されている可能性も考慮すべきである。臨床例の解析は大阪市の一事業所で発症した症例に限られており、職業性胆管癌が疑われる他の地域の症例や、

発症原因が不明な胆管癌症例との比較について解析を追加する予定である。

次世代シークエンサーN を用いた胆管癌診断は多くのパラメータが必要であるが、CEA や CA19-9 のみで胆管癌と肝細胞癌が区別できない現状に比べると血液中の情報のみで診断することが期待できる。今後検体数を増やし、データの再現を確認することと、ステージ別の miRNA 発現プロファイルを作成し、早期診断マーカーや、治療効果予測マーカーの確立が期待される。

完全混合モデルでは、作業場内で発生した化学物質は瞬間的に拡散混合し、気中濃度は均一であると仮定している。また、近接場–遠隔場モデルでは、2 つの場の内部の気中濃度は均一であると仮定している。現実には、気中濃度には空間的な変動があるので、これらの仮定は正しくない。しかしながら、対象者が勤務した作業場内の気中濃度の空間的な変動に関する情報はないので、これらのモデルを使用することとした。したがって、本研究で算出された濃度は粗い推定値である。

本研究の対象者 6 人中 5 人は印刷作業者であり、DCP および DCM の高濃度長期間曝露を受けており、これまで報告された事例と同様であることが確認された。一方、他の 1 人は IC カードへの接着剤および帯電防止剤のコーティング機の洗浄に DCP を使用しており、印刷以外であっても、DCP の高濃度長期間曝露であれば、胆管癌が発症する可能性のあることが示唆された。

E. 結論

検診結果および職業性症例の解析、および通常の胆管癌との比較から、若年、γ-GTP 高値、限局性胆管拡張像、乳頭状増殖などが職業性胆管癌の特徴と考えられた。病理学的検討から、DNA 傷害を伴う慢性胆管傷害、BilIN や IPNB の前癌病変を経て、浸潤癌が至るメカニズムが推測された。さらに、外科治療では術後合併症をきたしやすいものの、高い発癌ポテンシャルに基づく多中心性発癌を踏まえた積極的な治療が望ましいと考えられた。これらを結果に基づいた職業性胆管癌のスクリーニング法およびフォローアップ法を確立した。

今回の検討結果から、職業性胆管癌では腫瘍部だけではなく非腫瘍部においても DNMTs の発現が亢進していることが明らかとなった。このことから職業性胆管癌の多段階発癌過程において DNA メチル化異常が蓄積し、エピジェネティックな発がんの素地が形成されている可能性が示唆された。職業性胆管癌の胆管周囲線維化の組織学的特徴として、胆管周囲に α-SMA 陽性の紡錘形細胞を多く認めた所見は PSC に類似していた。一方で、職業性胆管癌の α-SMA 陽性細胞の分布状況や胆管周囲線維化の程度は閉塞性黄疸とは異なっていた。これらの所見は、職業性胆管癌の発癌機序および胆管硬化の機序解明に有用と考えられた。

網羅的ゲノム解析から職業性胆管癌に特徴的な遺伝子変異プロファイルを見出した。今後バイオマーカーとしての実用可能性を検討する。

新たに労災認定されて 6 人中 5 人は印刷労働者であり、DCP および DCM の高濃度長期間曝露を受けていた。一方、他の 1 人は IC カードへの接着剤および帯電防止剤のコーティング機の洗浄に DCP を使用しており、印刷以外であっても、DCP の高濃度長期間曝露であれば、胆管癌が発症する

可能性のあることが示唆された。

F. 健康危険情報

本研究は介入試験等ではないため、健康危険情報はない。

G. 研究発表

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1. 特許取得　　なし
2. 実用新案登録　なし
3. その他　　なし

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H. 知的財産権の出願・登録状況

（予定を含む。）

労災疾病臨床研究事業費補助金
分担研究報告書

１．職業性胆管癌の検診結果と臨床像

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研究要旨

（1）ジクロロメタンや 1,2-ジクロロプロパンに暴露された従業員や健康管理手帳交付者の検診に、昨年度に加えて健康管理手帳交付者 1 名と元同社従業員であった 1 名が、今年度新たな検診受診者として加わった。昨年度の検診において大阪の印刷事業場 S 社で 18 例目となる胆管癌と診断された患者に対して、外科治療を施行し、その病理検査結果、臨床成績や曝露状況に基づいて、同患者は 2015 年末に職業性胆管癌として労災認定を受けた。これまでの S 社従業員に対する調査や検診の結果、胃癌 2 名（1 例はすでに死亡、1 例は検診実施中)、皮膚ボーエン病 1 名（検診実施中）および腎癌 1 名（他院で経過観察中）の既往を有する従業員が判明した。

（2）外科切除および病理解剖施行 16 例の病理学的検討によって、ほとんどの比較的大型胆管において、慢性胆管傷害像、胆管癌の前癌病変あるいは早期癌病変と考えられている Biliary intraepithelial neoplasia（BilIN)や intraductal papillary neoplasm of the bile duct （IPNB）病変がみられた。特に職業性胆管癌症例の画像所見での特徴である癌を伴わない限局性胆管拡張像を示す胆管にはこのような種々の病変がみられた。また、広範囲の胆管がγ-H2AX 陽性であった。すなわち、DNA 傷害を伴う慢性胆管傷害から BilIN や IPNB の前癌病変を経て、浸潤癌に至ると考えられた。全国での多くの職業性胆管癌症例においても同様の所見を確認しえた。

（3）当科および関連病院で加療した職業性胆管癌と通常の胆管癌症例の比較および全国の労災病院のデータベースを用いた若年性胆管癌症例の比較によって、職業性胆管癌症例では、若年、γ-GTP 高値、限局性胆管拡張像、BilIN や IPNB 病変がみられることが特徴であることが確認された。

（4）外科治療例を検討すると、術後、腹腔内感染などの合併症を伴う症例が多く、胆管傷害が術後合併症の発症に影響している可能性が考えられた。また、多中心性再発を疑わせる症例がみられ、この再発形式は高い発癌ポテンシャルが広範囲の胆管にみられる職業性胆管癌の特徴と考えられた。同再発病巣の再切除により、良好な成績が得られた症例があり、積極的な治療が奏功する可能性が示唆された。

（5）以上のような検診結果や職業性胆管癌患者の臨床成績、病理学的検討から、肝機能検査および腫瘍マーカーの測定、腹部超音波検査を行い、それらの検査で異常所見がみられた場合、MRI（MRCP）や CT を実施し、さらに内視鏡的逆行性胆道造影による胆道造影、細胞診および組織診によって確定診断を得るスクリーニング法が望ましいと考えられた。また、外科的治療後においても、転移再発に加えて多中心性再発のリスクがあるため、定期的な血液検査と画像診断が必要であると考えられた。

A. 研究目的

検診結果、職業性胆管癌の臨床像および病理学的所見の検討および通常の胆管癌症例のそれらとの比較から、職業性胆管癌の特徴を明らかにする。また、病理学的検討から職業性胆管癌の発癌メカニズムを解明する。これらにより職業性胆管癌の総合的診断法を確立する。

B. 研究方法

（1）大阪の印刷事業場 S 社の元あるいは現従業員のうちジクロロメタン（DCM）や 1,2-ジクロロプロパン（DCP）に曝露したと考えられる従業員および健康管理手帳交付者（DCP 業務従事者）に対して行った検診結果を解析した。（2）職業性胆管癌症例の臨床像および病理学的所見の解析から、それらの特徴を検討した。（3）職業性胆管癌の特徴を通常の胆管癌や全国の労災病院のデータベースを用いた胆管癌症例のそれらと比較した。（4）外科治療成績の検討から、職業性胆管癌症例の特徴と適切な治療法を検討した。（5）これらの結果より職業性胆管癌の総合的診断法や治療法を考案する。

C. 研究結果

（1）検診結果の解析

大阪の印刷 S 社の元あるいは現従業員のうち DCM や DCP に曝露したと考えられる従業員および健康管理手帳交付者（DCP 業務従事者）に対して検診を行った。昨年度に加えて、健康管理手帳交付者 1 名と元同社従業員であった 1 名が、今年度新たな検診受診者として加わった。職業性胆管癌の特徴であるγ-GTP 持続高値例が 4 例にみ

られ、うち 2 例では MRCP や CT による精査を行ったが、新たな胆管癌発症者はみられなかった。

昨年度の検診において同社で 18 例目となる胆管癌と診断された患者に対して、外科治療を施行した。その病理検査結果、臨床成績や曝露状況に基づいて、同患者は 2015 年末に職業性胆管癌として労災認定を受けた。

これまでの同社従業員に対する調査や検診の結果、同社従業員において胃癌 2 名（1 例はすでに死亡、1 例は検診実施中）、皮膚ボーエン病 1 名（検診実施中）および腎癌 1 名（他院で経過観察中）の既往を有する従業員が判明した。なお、腎癌の 1 名は、少量であったが、2014 年に International Agency for Research on Cancer (IARC)において発癌リスクが group 1 と改定されたトリクロロエチレンの曝露歴を有する可能性を否定できなかった。さらにトリクロロエチレンが原因と考えられた重症肝炎既往を有する 1 例（Kubo S, et al. J Occup Health 2015;57;87-90）および他院で加療中である B 型肝炎患者 1 名に対しても検診実施中である。

（2）臨床病理学的特徴の解析

現在、職業性胆管癌と認定されている症例は、大阪 S 社の 18 例を含めて、全国で 38 例である。これらの症例のうち、患者あるいは患者家族より承諾を得た後、病理学的検討が可能であった 16 例の病理学的所見を解析した。

切除標本の全割面（最大 124 切片）を解析しえた 3 例において、全割面の病理学的所見と画像診断における胆管像を比較し、マッピングを施行した。その結果、主腫瘍は高分化から低分化腺癌を示す腫瘤形成型肝内胆管癌や胆管内発育型肝内胆管癌であった。3 例とも比較的大型胆管（肝外胆管から肝内第 3 次分枝）のほとんどの部位で、胆管や付属線の腫瘍性増殖、胆管消失像、胆管周囲の著明な硬化像（線維化）などの慢性胆管像、胆管癌の前癌病変あるいは早期癌病変と考えられている Biliary intraepithelial neoplasia (BilIN) や intraductal papillary neoplasm of the bile duct (IPNB) がみられた。特に、以前より職業性胆管癌の画像的特徴と指摘してきた、原発性硬化性胆管炎の胆管像に類似した、癌による胆管狭窄を伴わない限局性肝内胆管拡張像を示す胆管では BilIN と IPNB 病変や胆管癌の伸展がみられ、これらの病理学的変化が画像上での特徴と関連していると考えられた。さらに、DNA 傷害を示すγ-H2AX による免疫組織学的検討を行うと、ほぼ正常にみえる胆管、慢性胆管傷害、BilIN、IPNB および浸潤癌の部位に陽性であり、胆管全体にわたって DNA 傷害が生じていることが判明した。また、癌化を示す S-100P による免疫組織学的検討を行うと、BilIN、IPNB と浸潤癌で陽性であり、BilIN や IPNB の段階で癌化がみられることが判明した。すなわち、ほとんどの胆管で DNA 傷害がみられ、慢性胆管傷害から BilIN や IPNB の前癌病変を経て、浸潤癌に至ると考えられた（Kinoshita M, et al. J Hepatobiliary Pancreat Sci 2016;23:92-101）。なお、同社胆管癌症例の 1 例が、胆管癌に対する外科治療後、胆管癌の再発はみられないものの肝不全で死亡したが、病理解剖の結果、胆管周囲を含む

肝線維化の著明な進行がみられたことが判明した（Tomimaru Y, et al. Hepatol Res 2015;45:488-93）

全国の職業性胆管癌症例のうち、大型胆管の病理学的検討が可能であった外科治療あるいは病理解剖が行われた 16 例では、全例に慢性胆管傷害および BilIN がみられ、IPNB が多くの症例で確認された。すなわち、広範囲の胆管に慢性胆管傷害や前癌病変が認められることが、職業性胆管癌の病理学的特徴であることが判明した（Kinoshita M, et al. J Hepatobiliary Pancreat Sci 2016;23:92-101）。

（３）職業性胆管癌と通常の胆管癌症例との比較

当科および関連病院で切除された職業性胆管癌（肝内胆管癌）5 例と通常型で肝門型肝内胆管癌 46 例の臨床病理学的所見および治療成績を比較した。その結果、通常型肝内胆管癌に比較し、職業性肝内胆管癌では若年で、γ-GTP 高値例、ICG15 分値低値例、血小板高値例が多かった。また、画像診断上、癌を伴わない限局性肝内胆管拡張像や乳頭状増殖像が多くみられた。病理学的には、職業性肝内胆管癌において胆管内発育型が多く、BilIN、IPNB および慢性胆管傷害像が多くみられた。切除後予後にはついては差がみられなかったが、観察期間が短く、今後の検討が必要と考えられた（Hamano G, et al. J Hepatobiliary Pancreat Sci, in press）。

過去の職業歴が検索可能な全国の労災病院のデータベースを用いて、若年性胆管癌（50 歳未満）と大阪 S 社の職業性胆管癌の臨床病理学的所見を比較した。労災病院のデータベースでは胆管癌の集団発生はみられなかった。若年性胆管癌症例に比較して、職業性胆管癌では肝内胆管癌が多く、臨床検査値異常を示す症例が多かった。また、職業性胆管癌で特徴的である限局性の肝内胆管拡張像は、若年性胆管癌症例ではみられなかった（Kaneko R, et al. Asian Pac J Cancer Prev 2015;16:7195-7200）。

（４）外科治療成績の検討

外科的治療が行われた全国の職業性胆管癌症例のうち、患者あるいは患者家族の承諾を得ることができた 20 例の外科治療成績を検討した。肝切除が 6 例、胆道再建を伴う肝切除が 9 例、膵頭十二指腸切除が 3 例、肝切除および膵頭十二指腸切除が 1 例、肝外胆管切除が 1 例に行われた。しかし、4 例が胆管断端癌陽性であり、そのうち 3 例では同部に放射線照射が行われた。術前および術後補助化学療法が 1 例に、術後補助化学療法が 12 例に行われた。術後、腹腔内感染（9 例）、胆管空腸縫合不全（3 例）、胆汁漏（1 例）、膵液瘻（3 例）、術後出血（2 例）などの合併症がみられ、それらの術後合併症の発症に胆管傷害が影響している可能性が考えられた。20 例中 12 例に胆管癌再発がみられたが、肝内多発再発が 2 例に、胆管断端再発が 5 例にみられた。他の 5 例では、原発部位とは異なる部位に単発の再発病巣がみられた。このうち 4 例に再切除が施行されたが、再発部位と原発巣に連続性はみられず、多中心性再発が疑われた。この 4 例中 3 例は生存中である。職業性胆管癌の特徴として、ほとんどの大型胆管の発癌ポテンシャルが亢進している病態から、根治手術後も多中心性再発のリスクが高い

と考えられるものの、積極的な治療が奏功する可能性が示唆された。

（5）職業性胆管癌の総合的診断法

以上のような、検診結果および職業性胆管癌症例の臨床検査値、画像診断結果および病理学的検討から、職業性胆管癌のサーベイランス法およびスクリーニング法として、AST、ALT およびγ-GTP を含む肝機能検査、CEA および CA19-9 の腫瘍マーカーを測定し、さらに侵襲性の少ない腹部超音波検査を実施する。それらの検査で異常所見がみられる場合、MRI（MRCP）や CT を実施し、さらに内視鏡的逆行性胆道造影による胆道造影、細胞診および組織診によって確定診断を得る方法が望ましいと考えられた（Kubo S, et al. Surg Today, in press）。また、外科的治療後においても、多中心性再発のリスクもあるため、定期的な血液検査と画像診断が必要であると考えられた。

D. 考察

DCM や DCP に暴露された従業員や健康管理手帳交付者の検診において新たに診断された胆管癌患者の治療を行った。また、検診結果や職業性胆管癌患者の臨床成績から、職業性胆管癌に対するスクリーニング法として、暴露歴に加えて、AST、ALT およびγ-GTP を含む肝機能検査、CEA および CA19-9 の腫瘍マーカーの測定、腹部超音波検査を行い、それらの検査で異常所見がみられる場合、MRI（MRCP）や CT を実施し、さらに内視鏡的逆行性胆道造影による胆道造影、細胞診および組織診によって確定診断を得る方法が望ましいと考えられた。

切除標本や病理解剖標本の病理学的検討によって、比較的大型胆管のほとんどの部位で、胆管や付属線の腫瘍性増殖、胆管消失像、胆管周囲の著明な硬化像（線維化）などの慢性胆管傷害像、胆管癌の前癌病変あるいは早期癌病変と考えられている BilIN や IPNB 病変がみられた。特に職業性胆管癌症例の画像所見での特徴である癌を伴わない限局性胆管拡張像を示す胆管にはこのような病変がみられた。また、DNA 傷害を示すγ-H2AX による免疫組織学的検討を行うと、ほぼ正常にみえる胆管、慢性胆管傷害、BilIN、IPNB および浸潤癌の部位に陽性であり、胆管全体にわたって DNA 傷害が生じていることが判明した。すなわち、ほとんどの胆管で DNA 傷害がみられ、慢性胆管傷害から BilIN や IPNB の前癌病変を経て、浸潤癌が至ると考えられた。同様の所見は、全国での職業性胆管癌症例の多くにおいても確認しえた。当科および関連病院で加療した職業性胆管癌と通常の胆管癌症例の比較および全国の労災病院のデータベースを用いた若年性胆管癌の比較によって、職業性胆管癌症例では、若年、γ-GTP 高値、限局性胆管拡張像、BilIN や IPNB 病変がみられることが特徴であることが確認された。

外科治療例を検討すると、術後、腹腔内感染などの合併症を伴う症例が多く、胆管傷害が術後合併症の発症に影響している可能性が考えられた。また、原発部位とは異なる部位に単発の再発病巣がみられる症例があり、これらは多中心性再発を疑わせた。この再発形式は高い発癌ポテンシャルが広範囲の胆管にみられる職業性胆管癌の特徴

と考えられた。しかし同再発病巣の再切除により、良好な成績が得られた症例があり、積極的な治療が奏功する可能性が示唆された。

E. 結論

検診結果および職業性症例の解析、および通常の胆管癌との比較から、若年、γ-GTP 高値、限局性胆管拡張像、乳頭状増殖などが職業性胆管癌の特徴と考えられた。病理学的検討から、DNA 傷害を伴う慢性胆管傷害、BilIN や IPNB の前癌病変を経て、浸潤癌が至るメカニズムが推測された。さらに、外科治療では術後合併症をきたしやすいものの、高い発癌ポテンシャルに基づく多中心性発癌を踏まえた積極的な治療が望ましいと考えられた。これらを結果に基づいた職業性胆管癌のスクリーニング法およびフォローアップ法を確立した。

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G. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

労災疾病臨床研究事業費補助金
分担研究報告書

２．印刷事業場・胆管癌症例の病理病態に関する免疫組織化学的検討

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研究要旨

職業性胆管癌における前癌/前浸潤性病変の出現や胆管上皮傷害，胆管硬化を生じる機序については未解明な点が多い. 今回, 職業性胆管癌の発癌における i)DNA メチル化異常の関与、および ii)胆管硬化の病態に関して病理組織学的な検討を行った．DNA メチル化を媒介する DNA methyltransferases（DNMTs）を介した遺伝子プロモーター領域の DNA メチル化異常は、癌抑制遺伝子を不活性化し発癌に寄与する．これまで胆管癌の多段階発癌過程における DNMT の発現は検討されていなかったが、今回の検討結果から、職業性胆管癌では腫瘍部だけではなく非腫瘍部においても DNMTs の発現が亢進していることが明らかとなった。このことから職業性胆管癌の多段階発癌過程において DNA メチル化異常が蓄積し、エピジェネティックな発がんの素地が形成されている可能性が示唆された。職業性胆管癌の胆管周囲線維化の組織学的特徴として、胆管周囲に α-smooth muscle actin (α-SMA)陽性の紡錘形細胞を多く認めた所見は PSC に類似していた。一方で、職業性胆管癌の α-SMA 陽性細胞の分布状況や胆管周囲線維化の程度は閉塞性黄疸とは異なっていた。これらの所見は、職業性胆管癌の発癌機序および胆管硬化の機序解明に有用と考えられた。

A. 研究目的

職業性胆管癌では、病理組織学的に前癌/前浸潤性病変である胆管内上皮内腫瘍（biliary intraepithelial neoplasia, BilIN）や胆管内乳頭状腫瘍（intraductal papillary neoplasm of bile duct, IPNB）が特徴的に認められ、多段階の発癌過程で癌が進展していることが示唆される（1)。発癌には塩素系有機溶剤である 1,2-ジクロロプロパン（1,2- dichloropropane, DCP）やジクロロメタン（dichloromethane, DCM）

への長時間、高濃度のばく露との関連が推定されている。また、一連の症例の病理所見の特徴として，非癌部における胆管上皮傷害像や胆管硬化もしばしば観察される。われわれは，これまでに職業性胆管癌では DNA2 本鎖切断を示すγ-H2AX の免疫組織化学的な発現が高頻度にみられることを報告した(2)。γ-H2AX の発現は BilIN、IPNB や浸潤癌の部位だけではなく、非腫瘍部の大型～隔壁胆管や胆管周囲付属腺にも認めた。また、職業性胆管癌では免疫組織化学的に非腫瘍部の大型～隔壁胆管、胆管周囲付属腺に p53 の発現をみることがあった。通常の胆管癌とは明らかに異なる病理所見であり、胆道系の上皮細胞において DNA 損傷を伴った特異な過程で癌化が進展していることが示唆された。

職業性胆管癌における前癌/前浸潤性病変の出現や胆管上皮傷害，胆管硬化を生じる機序については未解明な点が多い. 今回，職業性胆管癌の発癌における（１）DNA メチル化異常の関与，および（２）胆管硬化の病態に関して病理組織学的な検討を行った.

B. 研究方法

（１） DNA メチル化異常の検討

職業性胆管癌 8 例，対照として BilIN を合併した肝内結石合併胆管癌 16 例、通常の IPNB14 例の外科的切除材料、ホルマリン固定パラフィン包埋切片を使用した。DNA メチル化を媒介する DNA methyltransferases（DNMTs）の発現を免疫染色で検討した。DNMTs のうち新規メチル化に関与する DNMT3A と DNMT3B、細胞分裂時のメチル化維持に関与する DNMT1 に対する免疫染色を行った。陽性染色の程度を半定量的に評価し、統計学的解析を行った。

（２）胆管硬化の検討

職業性胆管癌（非癌部）6 例、対照として原発性硬化性胆管炎（PSC）4 例、閉塞性黄疸 5 例、ウイルス性肝炎/肝硬変 5 例のホルマリン固定パラフィン包埋切片を用い、α-smooth muscle actin （SMA）に対する免疫染色を行った。線維化の程度はシリウスレッド染色で評価した。

C. 研究結果

（１） 職業性胆管癌における DNMTs の発現

DNMT3A、DNMT3B、DNMT1 の発現を非腫瘍部（大型胆管上皮、胆管周囲付属腺）、および腫瘍部（BilIN、IPNB、浸潤部）のそれぞれについて評価した．新規メチル化に関与する DNMT3A、DNMT3B の発現は，職業性胆管癌の胆管周囲付属腺、BilIN、浸潤癌の部位において対照群より有意に亢進していた。特に職業性胆管癌の胆管周囲付属腺における DNMT3A、DNMT3B の陽性頻度が高く、DNMT3A は職業性胆管癌の 86%，対照群の 27%の症例が陽性（$p < 0.01$）、DNMT3B は職業性胆管癌の 86%、対照群の 7%の症例が陽性を示した（$p < 0.01$）。また、職業性胆管癌の非癌部の大型胆管上皮において、DNMT3A の発現が対照群より高い傾向にあった（陽性率は職業性胆管癌 50%、対照群 13%; $p = 0.056$）。

メチル化維持に関与する DNMT1 の発現は、検討した疾患群のいずれにおいても非腫瘍部、腫瘍部とも陽性率は低かったが、職業性胆管癌の BilIN の部位において対照群より高い発現を示す傾向にあった（陽性率は職業性胆管癌 29%，対照群 0%; $p = 0.060$）。

（２） 職業性胆管癌の胆管硬化

組織標本に含まれる主に隔壁レベルの肝内胆管を評価対象とした。α-SMA の免疫染色では、職業性胆管癌の硬化した胆管周囲に α-SMA 陽性の紡錘形細胞を多数認め、この α-SMA 陽性細胞の分布状況は PSC に類似していた。対照とした閉塞性黄疸とウイルス性肝炎/肝硬変では胆管周囲に散在性に α-SMA 陽性細胞を認めるのみであった。

肝実質の α-SMA 陽性細胞の分布は同一症例でも不均一であったが、ウイルス性肝炎/肝硬変で最も多くの陽性細胞を認めた。職業性胆管癌の肝実質での α-SMA 陽性細胞の分布は PSC と比較的類似しており、実質内の局所的に肝星細胞の活性化を認めた。閉塞性黄疸は検討した 4 群の中で最も肝星細胞の活性化が目立たなかった。

シリウスレッド染色では，職業性胆管癌と PSC、閉塞性黄疸で胆管周囲に輪状の線維化を認め、これはウイルス性肝炎/肝硬変ではみられなかった。線維化の程度を比較すると、PSC と閉塞性黄疸では胆管周囲にほぼ同程度の膠原線維の沈着を認めたが、職業性胆管癌ではこの 2 つの疾患群より膠

原線維化が明らかに緻密で、胆管硬化がより強い傾向にあった。

D. 考察

DNMT を介した遺伝子プロモーター領域の DNA メチル化異常は、癌抑制遺伝子を不活性化し発癌に寄与する。一見正常にみえる細胞でも、慢性炎症や化学物質などへの曝露により DNA メチル化異常が蓄積していることがあるとされる．膵癌では，膵管内上皮内腫瘍（pancreatic intraepithelial neoplasia、PanIN）を介した多段階発癌の過程において、DNMT3A、DNMT3B、DNMT1 の発現が段階的に亢進し、これら DNMTs の高発現群の膵癌は低発現群より予後不良であることが報告されている（3,4)。また、膵癌での DNMT1 発現はがん関連遺伝子の DNA メチル化の蓄積と有意に相関することが示されている。

これまで胆管癌の多段階発癌過程における DNMT の発現は検討されていなかったが、今回の検討結果から、職業性胆管癌では腫瘍部だけではなく非腫瘍部においても DNMTs の発現が亢進していることが明らかとなった。このことから職業性胆管癌の多段階発癌過程において DNA メチル化異常が蓄積し、エピジェネティックな発がんの素地が形成されている可能性が示唆された。特に胆管周囲付属腺において DNMT3A、DNMT3B の発現亢進を顕著に認めたが、近年、胆管周囲付属腺にステム細胞ニッチが存在することが報告されており、胆管周囲付属腺の組織再生や胆管癌発癌における関与が注目されている（5)。

職業性胆管癌の胆管周囲線維化の組織学的特徴として、胆管周囲に α-SMA 陽性の紡錘形細胞を多く認めた所見は PSC に類似していた．しかし、PSC より膠原線維沈着が緻密で胆管硬化がより強い傾向にあり、職業性胆管癌の胆管周囲線維化は PSC と同質なものではなかった。一方で、職業性胆管癌の α-SMA 陽性細胞の分布状況や胆管周囲線維化の程度は閉塞性黄疸とは異なっており、単に胆管狭窄や閉塞に伴う 2 次性線維化とは異なることが示唆された。

われわれの以前の検討で、職業性胆管癌の非腫瘍部の大型～隔壁胆管、胆管周囲付属腺では γ-H2AX や p53 の発現をしばしば認めたが、こうした細胞は DNA 損傷から細胞老化を起こしている可能性がある。老化細胞は senescence-associated secretory phenotype （SASP）を分泌することが知られており、SASP には炎症や線維化、発癌に関連した分子も多い。例えば、SASP である IL-6 や MCP-1 は胆管細胞から分泌されうるが、これらは肝星細胞を活性化する作用を有するとされている（6)。また、IL-6 は胆管癌の発癌に関連が深いことが従来から知られている。職業性胆管癌では末梢側の小型胆管に γ-H2AX や p53 の発現は通常みられないが、肝門側において DCP や DCM などへのばく露により胆道系の上皮細胞が傷害され、傷害上皮の胆汁への接触などが誘因となり徐々に細胞老化を生じ、それにより分泌された SASP が一連の症例の胆管病変の病態形成に関与している可能性があると思われる。また、SASP は血清からも検出可能なものが多く、発癌予測マーカーとなりうる SASP が存在するかも知れない。

E. 結論

今回の検討結果から、職業性胆管癌では腫瘍部だけではなく非腫瘍部においても DNMTs の発現が亢進していることが明らかとなった。このことから職業性胆管癌の多段階発癌過程において DNA メチル化異常が蓄積し、エピジェネティックな発がんの素地が形成されている可能性が示唆された。職業性胆管癌の胆管周囲線維化の組織学的特徴として、胆管周囲に α-SMA 陽性の紡錘形細胞を多く認めた所見は PSC に類似していた。一方で、職業性胆管癌の α-SMA 陽性細胞の分布状況や胆管周囲線

維化の程度は閉塞性黄疸とは異なっていた。これらの所見は、職業性胆管癌の発癌機序および胆管硬化の機序解明に有用と考えられた。

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G. 研究発表

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なし

H. 知的財産権の出願・登録状況

1. 特許取得 なし
2. 実用新案登録　なし
3. その他　なし

なし

労災疾病臨床研究事業補助金
分担研究報告書

３．職業性胆管癌に対する総合的診断法の確立（分子生物学的検討）に関する研究

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研究要旨
印刷事業場S社の労働者に多発した職業性胆管癌症例4例の全エクソン解析により、通常型胆管癌ゲノムと著しく異なる①きわめて高頻度の体細胞変異、②一塩基置換のセンス・アンチセンス鎖間のバイアスに加え、③特異的な3塩基置換パターンが認められた。これらの特徴が解析例全例に認められ、これまでの大規模な胆管癌のゲノム解析でも未報告のものであることから、網羅的ゲノム解析により得られる変異プロファイルが本疾患を特徴づけるゲノムバイオマーカーになりうることが期待された。

Ａ．研究目的

印刷事業場S社の労働者に多発した職業性胆管癌に生じた体細胞変異を網羅的に解析し、発症の原因と推定されている高濃度の有機溶媒暴露や各症例の臨床病理学的な特徴との因果関係を明らかにする。これらの結果から職業性胆管癌が疑われる症例の診断を補助するゲノムバイオマーカーの探索を行う。

Ｂ．研究方法

前年度までに取得した4例の職業性胆管癌及び対照となる通常型胆管癌全エクソンシークエンスデータから体細胞変異を同定し体細胞変異プロファイル（変異数、DNA塩基置換パターン）を解析した。これらのプロファイルと有機溶媒との関連を検討するため、サルモネラ菌株、ヒト上皮由来培養細胞株に1,2-ジクロロプロパンを曝露し、新たにゲノムDNA上に獲得された変異プロファイルを検討した。

（倫理面への配慮）
「胆管がん等の職業性発がんの原因解明とバイオマーカーの開発」のためヒトゲノム・遺伝子解析研究に関する倫理指針に則り研究を計画し、国立がん研究センター研究倫理審査委員会の承認（2014-072）を得て実施した。

Ｃ．研究結果

解析例において癌部特異的な一塩基置換はタンパク質をコードする遺伝子領域約3100万塩基対に対し1例あたり平均145 1ヶ所と対照とした通常型胆管癌の約30倍の頻度であった。またセンス鎖とアンチセンス鎖における変異頻度の有意差が認められ、高濃度の変異原物質への曝露歴が示唆された。さらに3塩基置換パターンの解析では従来報告されていないGpCpY>GpTpYおよびNpCpY to NpTpY or NpApYの特徴的な変化が検出された。サルモネラ菌株へのDCP曝露により、臨床例に類似した3塩基置換パターンが観察された。

Ｄ．考察

大阪の一事業所で発生した職業性胆管癌ゲノムには、昨年度報告したきわめて高頻度の体細胞変異、一塩基置換のセンス・アンチセンス鎖間のバイアスに加え、新規の特異的な3塩基置換パターンが見いだされた。特に特徴的な3塩基置換パターンは最近論文報告された胆管癌の大規模ゲノム解析でも報告がなく、本疾患を特徴づけ

るゲノムバイオマーカーになりうると考えられた。DCPを曝露したサルモネラ菌ゲノムの解析で一部類似した特徴が認められたことから、疫学研究で示唆されていた発がんメカニズムとの関連が予想されたが、モデル系と臨床例ではゲノム変異プロファイルに一致しないところもあり、生体内ではさらに複雑なメカニズム（化合物間の相互作用、胆管上皮における化合物代謝機構、炎症等宿主因子との相互作用など）が加味されている可能性も考慮すべきである。臨床例の解析は大阪市の一事業所で発症した症例に限られており、職業性胆管癌が疑われる他の地域の症例や、発症原因が不明な胆管癌症例との比較について解析を追加する予定である。

Ｅ．結論

網羅的ゲノム解析から職業性胆管癌に特徴的な遺伝子変異プロファイルを見出した。今後バイオマーカーとしての実用可能性を検討する。

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Ｇ．知的財産権の出願・登録状況

特記なし

労災疾病臨床研究事業費補助金
分担研究報告書

４．胆管がん早期発見バイオマーカーに関する研究

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研究要旨

肝内胆管がんは早期発見が困難で予後不良である。現在使用しているCEAやCA19-9は胆管がんの血液マーカーとして有用であるが、特異性が高くない点や早期診断は現状では困難である。細胞間情報伝達に関与するエクソソームを媒体にした胆管がん早期発見バイオマーカー作成を試みた。その結果、次世代シークエンサー解析によるエクソソーム中マイクロRNAは胆管がん診断マーカーとして使用できることを示した。

A.　研究目的

肝内胆管がんは胆管上皮由来の悪性腫瘍である、リスクファクターとしては、胆石、胆嚢炎、膵胆管合流異常などが考えられている他に、胆管胆嚢粘膜への物理化学的、細菌学的刺激を与えてがん発生母地をつくると考えられている。肝内胆管がんの治療は外科的切除が第一選択であるが、早期発見が困難で発見時には外科的切除の適応ではないことが多い。

近年エクソソームと呼ばれる体液中の小胞体が細胞間情報伝達物質として注目されている。エクソソームはあらゆる細胞が放出しており、中にサイトカインやnon coding RNAなどが含まれている。例えば樹状細胞が放出するエクソソームは免疫担当細胞に情報を伝達し、直接抗原提示能を賦活する、T 細胞の活性化などを誘導する、ことによって免疫応答を調節しているが、がん細胞が放出するエクソソームは同じ細胞に対して免疫提示能を低下させる、T 細胞のアポトーシスを誘導する、などによってがん細胞が免疫応答を回避して増殖することをサポートしている。エクソソームに含まれる情報を明らかにすることは生体内で起こっている状態を把握することが可能で、細胞や組織を採取する生検に匹敵する情報が得られることが期待できる。このようなエクソソームやエクソソームに含まれる遺伝子の情報はリキッドバイオプシーと呼ばれている。我々はエクソソームに含まれるマイクロ RNA を解析し、肝内胆管がんの早期診断に利用できるか否かを検討した。

B.　研究方法

対象

対象は当院ならびに関連施設において外科的に切除した肝内胆管がん 17 例、肝細胞がん 20 例、 正常肝 9 例の血清を用いエクソクイックにてエクソソーム画分を濃縮し、total RNA を抽出し次世代シークエンサー(NGS) Hiseq にてデータ採取を行ない、解析は miRDeep2 にて行なった。解析の再現性を確認するために、生データを miRDeep*にて再度解析を行なった。癌の癌組織と周辺部非癌部組織 10 例(印刷工場関連はそのうち 3 例）と比較対象として HBV と HCV に感染していない肝細胞癌（以下肝細胞癌）の癌部と周辺部非癌部組織 6 例を用いた。

（倫理面への配慮）

この臨床研究はヘルシンキ宣言を遵守し、GCP に基づいて実施している。被検者の個人情報については，個人情報保護法に基づいて適切に取り扱う。また既に大阪市立大学医学研究科の倫理申請を行い、承認を受けている(受付番号 1358)。

C. 研究結果

Hiseq によって肝内胆管がんと肝細胞がん、正常肝それぞれのエクソソーム中の塩基は平均 770 万、880 万、800 万リード得られた。得られたリード数は 49 で、Q20 の精度であった（Q20＝読み違え率は 1%）。解析に使用した miRNA は 36 種で診断率は 74.5%であった。

D. 考察

NGS を利用した胆管がん診断は多くのパラメータが必要であるが、CEA や CA19-9 のみで胆管がんと肝細胞がんが区別できない現状に比べると血液中の情報のみで診断することが期待できる。今後検体数を増やし、データの再現を確認することと、ステージ別の miRNA 発現プロファイルを作成し、早期診断マーカーや、治療効果予測マーカーの確立を目標とする。

E. 結論

今回の解析では NGS 解析によるエクソソーム中 miRNA は胆管がん診断マーカーとして使用できることを示した。

F. 健康危険情報

特記事項なし。

G. 研究発表

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学会発表

なし

H. 知的財産権の出願、登録状況

1. 特許取得 なし
2. 実用新案登録　なし
3. その他　なし

労災疾病臨床研究事業費補助金
分担研究報告書

５．職業性胆管がん患者の化学物質曝露に関する研究

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研究要旨

印刷労働者に発生した胆管がんの原因は、洗浄剤に含まれていた 1,2-ジクロロプロパン（1,2-DCP）あるいはジクロロメタン（DCM）と考えられているが、今後の発生を予防するためには、当該労働者におけるこれらの物質への曝露濃度および曝露期間を明らかにすることが重要である。本研究では、厚生労働省が職業性胆管がんと認定した印刷労働者 6 人について、使用した化学物質の種類を特定するとともに、各種の情報を基にして曝露濃度を推定することを目指した。

対象者は厚生労働省により職業性胆管がんと認定された印刷労働者 6 人である。診断年齢は 40 歳代が 4 人、50 歳代が 2 人である。これらの労働者が使用した化学物質を同定するとともに、曝露濃度を推定するために、厚生労働省が収集した情報（印刷作業場の気積と換気量、印刷機の種類、ブランケットとインキロールの洗浄剤の化学成分と使用量、洗浄時間）を取得した。さらにそれらの情報を基にして曝露濃度を推定した。

6人中5人は印刷作業者であり、1,2-DCPおよびDCMに曝露されており、最高曝露濃度は1,2-DCPが190～560 ppm、DCMが300～980 ppmと推定され、1日労働時間の時間荷重平均濃度は1,2-DCPが13～230 ppm、DCMが20～470 ppmと推定された。他の1人はICカードに接着剤および帯電防止剤をコーティングする業務に従事しており、コーティング機のロール洗浄に1,2-DCPを使用していた。1,2-DCPの最高曝露濃度は150 ppmと推定され、1日労働時間の時間荷重平均濃度は5～19 ppmと推定された。

A．研究目的

印刷労働者に発生した胆管がんの原因は、洗浄剤に含まれていた1,2-ジクロロプロパン（1,2-DCP）あるいはジクロロメタン（DCM）と考えられているが、今後の発生を予防するためには、当該労働者におけるこれらの物質への曝露濃度および曝露期間を明らかにすることが重要である。本研究では、厚生労働省が職業性胆管がんと認定した印刷労働者6人について、使用した化学物質の種類を特定するとともに、各種の情報を基にして曝露濃度を推定することを目指した。なお、本研究は大阪市立大学大学院医学研究科の倫理委員会の承認を得て行った。

B．研究方法

1．対象者

対象者は厚生労働省により職業性胆管がんと認定された印刷労働者 6 人である。診断年齢は 40 歳代が 4 人、50 歳代が 2 人である。6 人中 5 人は従業員が 50 人未満の小規模事業所の労働者であり、1 人は従業員が 50～299 人の中規模事業所の労

働者である。

2. 情報収集

これらの労働者が使用した化学物質を同定するとともに、曝露濃度を推定するために、厚生労働省が収集した情報（印刷作業場の気積と換気量、印刷機の種類、ブランケットとインキロールの洗浄剤の化学成分と使用量、洗浄時間）を取得した。

3. 曝露濃度の推定

印刷作業場の 1,2-DCP および DCM の作業環境濃度を推定するために、完全混合モデルにおける定常状態での濃度（下式）を用いた。

$$C_{\mathrm{En}} = \frac{1000\,G_{\mathrm{T}}}{Q} \times \frac{24.47}{M}$$

ここで、C_{En} は作業環境濃度（ppm）、G_{T} は印刷作業場全体における化学物質の発生速度（g/h）、Q は印刷作業場全体の換気速度（m^3/h）、そして M は化学物質の分子量である。使用した 1,2-DCP と DCM の全量が蒸発すると仮定し、G_{T} は 1 日使用量（g）を 1 日の労働時間（h）で割って求めた。

洗浄作業中の作業者の曝露濃度を推定するために、近接場-遠隔場モデルにおける定常状態での濃度（下式）を用いた。このモデルにおける近接場は発生源を中心とする球と仮定し、洗浄作業中の発生源と作業者の呼吸位置との距離を考慮して、球の半径 r を 0.5m とした。

$$C_{\mathrm{Ex}} = \left(\frac{1000\,G_{\mathrm{Re}}}{Q} + \frac{1000\,G_{\mathrm{Re}}}{\beta}\right) \times \frac{24.47}{M}$$

ここで、C_{Ex} は洗浄作業中の作業者の曝露濃度（ppm）である。また、G_{Re} は洗浄作業中の化学物質の発生速度（g/h）であり、洗浄作業中の化学物質の使用量（g）を洗浄作業時間（h）で割って求めた。β は近接場と遠隔場の間の空気の交換速度（m^3/h）であり、下式で求めた。

$$\beta = v \times 3600 \times 2\pi r^2$$

ここで、v は近接場と遠隔場の境界面を通過する気流の速度（m/sec）である。ただし、事業所 XI の印刷機はブランケットが上下にあり、下のブランケットは半密閉であったので、β（m^3/h）は下式で求めた。

$$\beta = v \times 3600 \times \pi r^2$$

なお、近接場と遠隔場の境界面に直接的に当たる気流はなかったので、v は 0.1 m/sec とした。

さらに洗浄作業以外の時間帯の曝露濃度は作業環境濃度と同一と仮定して、1 日の労働時間における時間荷重平均濃度（TWAs）を算出した。

C．研究結果

症例 N

1953 年生まれの男性である。1989 年から 2002 年まで事業所 II においてオフセット校正印刷に従事し、2002 年に胆管がんと診断された。この事業所では、他に 2 人が胆管がんを発症し、業務上認定を受けている。

事業所 II では 2 つの作業場で働いたが、作業場 1 の気積は 170 m^3、換気量は 1790 m^3/h、作業場 2 の気積は 180 m^3、換気量は 1100 m^3/h であった。印刷機には局所排気装置は設置されていなかった。ブランケット洗浄剤には 1,2-DCP、DCM、1,1-ジクロロ-1-フルオロエタンおよびミネラルスピリッツを使用した。作業場全体で使用した 1,2-DCP は 230–400 g/h、DCM は 56–310 g/h であった。また洗浄作業中に使用した 1,2-DCP は 330–630 g/h、DCM は 100–500 g/h であった。

作業環境濃度は 1,2-DCP が 28–78 ppm、DCM が 15–50 ppm と推定された。洗浄作業中の曝露濃度は 1,2-DCP が 170–370

ppm、DCM が 77–330 ppm と推定された。1 日の労働時間は 9 時間であり、時間荷重平均濃度は 1,2-DCP が 74–170 ppm、DCM が 35–140 ppm と推定された。呼吸保護具は使用しなかった。

症例 O

1949 年生まれの男性である。1982 年から 1983 年まで事業所 XII、1983 年から 1986 年まで事業所 XIII、1986 年から 1994 年まで事業所 XIV においてオフセット校正印刷に従事し、1993 年に胆管がんと診断された。

いずれの事業所にも印刷作業場は 1 つであった。作業場 3 の気積は 210 m^3、作業場 4 の気積は 240 m^3、作業場 4 の気積は 130 m^3 であった。全体換気装置は設置されていなかったので、換気回数を 1 時間当たり 1 回とし、換気量をそれぞれ 210 m^3/h、240 m^3 および 130 m^3/h と仮定した。印刷機には局所排気装置は設置されていなかった。ブランケット洗浄剤には、1,2-DCP および DCM を使用した。作業場全体で使用した 1,2-DCP は 90–180 g/h、DCM は 110–210 g/h であった。また洗浄作業中に使用した 1,2-DCP は 260 g/h、DCM は 300 g/h であった。

作業環境濃度は 1,2-DCP が 160–170 ppm、DCM が 240–250 ppm と推定された。洗浄作業中の曝露濃度は 1,2-DCP が 340–560 ppm、DCM が 520–850 ppm と推定された。1 日の労働時間は 10 時間であり、時間荷重平均濃度は 1,2-DCP が 200–230 ppm、DCM が 300–350 ppm と推定された。呼吸保護具は使用しなかった。

症例 P

1971 年生まれの男性である。1991 年から 2014
年まで事業所 XV においてオフセット校正印刷に従事したが、2013 年に胆管がんと診断された。

事業所 XV には 1 つの印刷作業場があり、気積は 350 m^3 であった。全体換気装置は設置されていなかったので、換気量を 350 m^3/h と仮定した。印刷機には局所排気装置は設置されていなかった。ブランケット洗浄剤には、1,2-DCP、DCM、鉱油およびノナンを使用した。作業場全体で使用した 1,2-DCP は 0–210 g/h、DCM は 200–450 g/h であった。また洗浄作業中に使用した 1,2-DCP は 0–280 g/h、DCM は 240–720 g/h であった。

作業環境濃度は 1,2-DCP が 0–130 ppm、DCM が 160–370 ppm と推定された。洗浄作業中の曝露濃度は 1,2-DCP が 0–280 ppm、DCM が 320–950 ppm と推定された。1 日の労働時間は 10 時間であり、時間荷重平均濃度は 1,2-DCP が 0–160 ppm、DCM が 240–470 ppm と推定された。呼吸保護具は使用しなかった。

症例 Q

1970 年生まれの男性である。1998 年から 2013 年まで事業所 XVI においてオフセット校正印刷に従事したが、2012 年に胆管がんと診断された。

事業所 XVI には印刷作業場は 2 つであり、作業場 7 の気積は 250 m^3、換気量は 600 m^3/h、作業場 8 の気積は 290 m^3 であった。作業場 8 には全体換気装置は設置されていなかったので、換気量を 290 m^3/h と仮定した。印刷機には局所排気装置は設置されていなかった。ブランケット洗浄剤には、1,2-DCP、DCM、1,1-ジクロロ-1-フルオロエタン、トリクロロエチレン、トルエン、キシレン、ヘキサンおよび灯油を使用した。またインキロール洗浄剤も同じものを使用した。作業場全体で使用した 1,2-DCP は 19–120 g/h、DCM は 21–130 g/h であった。また洗浄作業中に使用した 1,2-DCP は 110–260 g/h、DCM は 130–300 g/h であった。

作業環境濃度は 1,2-DCP が 7–42 ppm、

DCM が 10–66 ppm と推定された。洗浄作業中の曝露濃度は 1,2-DCP が 83–190 ppm、DCM が 120–300 ppm と推定された。1 日の労働時間は 9–9.5 時間であり、時間荷重平均濃度は 1,2-DCP が 13–65 ppm、DCM が 20–98 ppm と推定された。呼吸保護具は使用しなかった。

症例 R

1956 年生まれの男性である。1981 年から 2011 年まで事業所 XVII においてオフセット印刷に従事したが、2011 年に胆管がんと診断された。

事業所 XVII には 4 つの印刷作業場があり、そのうち 3 つの作業場で 1,2-DCP と DCM を使用した。作業場 10 の気積は 510 m^3、換気量は 9540 m^3/h、作業場 11 の気積は 910 m^3、作業場 12 の気積は 710 m^3、換気量は 1620 m^3/h であった。作業場 11 には全体換気装置が設置されていなかったため、換気量を 910 m^3/h と仮定した。印刷機には局所排気装置は設置されていなかった。ブランケット洗浄剤には、1,2-DCP、DCM、トリクロロエチレンおよび 1,1,1-トリクロロエタンを使用した。作業場全体で使用した 1,2-DCP は 0–96 g/h、DCM は 200–1880 g/h であった。また洗浄作業中に使用した 1,2-DCP は 0–370 g/h、DCM は 780–1600 g/h であった。

作業環境濃度は 1,2-DCP が 0–13 ppm、DCM が 28–99 ppm と推定された。洗浄作業中の曝露濃度は 1,2-DCP が 0–190 ppm、DCM が 530–980 ppm と推定された。1 日の労働時間は 9.5 時間であり、時間荷重平均濃度は 1,2-DCP が 0–59 ppm、DCM が 170–370 ppm と推定された。呼吸保護具は使用しなかった。

症例 S

1958 年生まれの男性である。1996 年から 2001 年まで事業所 XVIII において、2001 年から 2005 年まで事業所 XIX において、IC カードに接着剤および帯電防止剤をコーティングする業務に従事したが、2008 年に胆管がんと診断された。

事業所 XVIII にはコーティング作業場は 1 つであり、作業場 13 の気積は 510 m^3、換気量は 300 m^3/h であった。事業所 XIX にはコーティング作業場は 1 つであり、作業場 14 の気積は 160 m^3、換気量は 1650 m^3/h であった。局所排気装置は設置されていなかった。コーティング機のロール洗浄剤には、1,2-DCP および 1,1-ジクロロ-1-フルオロエタンを使用した。作業場全体で使用した 1,2-DCP は 8–16 g/h、洗浄作業中に使用した 1,2-DCP は 140 g/h であった。

1,2-DCP の作業環境濃度は 1–11 ppm、洗浄作業中の曝露濃度は 72–150 ppm と推定された。1 日の労働時間は 9 時間であり、1,2-DCP の時間荷重平均濃度は 5–19 ppm と推定された。呼吸保護具は使用しなかった。

D．考察

完全混合モデルでは、作業場内で発生した化学物質は瞬間的に拡散混合し、気中濃度は均一であると仮定している。また、近接場-遠隔場モデルでは、2 つの場の内部の気中濃度は均一であると仮定している。現実には、気中濃度には空間的な変動があるので、これらの仮定は正しくない。しかしながら、対象者が勤務した作業場内の気中濃度の空間的な変動に関する情報はないので、これらのモデルを使用することとした。したがって、本研究で算出された濃度は粗い推定値である。

本研究の対象者 6 人の中で、5 人は印刷作業者であり、1,2-DCP および DCM の高濃度長期間曝露を受けており、これまで報告された事例と同様であることが確認された。一方、他の 1 人は IC カードへの接着剤および帯電防止剤のコーティング

機の洗浄に 1,2-DCP を使用しており、印刷以外であっても、1,2-DCP の高濃度長期間曝露であれば、胆管がんを発症する可能性のあることが示唆された。

E．結論

6 人中 5 人は印刷作業者であり、1,2-DCP および DCM に曝露されており、最高曝露濃度は 1,2-DCP が 190～560 ppm、DCM が 300～980 ppm と推定され、1 日労働時間の時間荷重平均濃度は 1,2-DCP が 13～230 ppm、DCM が 20～470 ppm と推定された。他の 1 人は IC カードに接着剤および帯電防止剤をコーティングする業務に従事しており、コーティング機のロール洗浄に 1,2-DCP を使用していた。1,2-DCP の最高曝露濃度は 150 ppm と推定され、1 日労働時間の時間荷重平均濃度は 5～19 ppm と推定された。

E．研究発表

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なし

F．知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

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Hypermutation and Unique Mutational Signatures of Occupational Cholangiocarcinoma in Printing Workers Exposed to Haloalkanes

Running head: Exome Analysis of Occupational Cholangiocarcinomas

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Abbreviations:

1,2-DCP: 1,2-dichloropropane
AID: activation-induced cytidine deaminase
COSMIC: Catalogue of Somatic Mutations in Cancer
DCM: dichloromethane
GSTT1: glutathione S-transferase theta 1
IARC: The International Agency for Research on Cancer
ICGC: The International Cancer Genome Consortium
INDELs: insertions and deletions
SNVs: single-nucleotide variants

Abstract

Cholangiocarcinoma is a relatively rare cancer, but its incidence is increasing worldwide. Although several risk factors have been suggested, the etiology and pathogenesis of the majority of cholangiocarcinomas remain unclear. Recently, a high incidence of early-onset cholangiocarcinoma was reported among the workers of a printing company in Osaka, Japan. These workers underwent high exposure to organic solvents, mainly haloalkanes such as 1,2-dichloropropan (1,2-DCP) and/or dichloromethane (DCM). We performed whole-exome analysis on four cases of cholangiocarcinoma among the printing workers. An average of 44.8 somatic mutations was detected per Mb in the genome of the printing workers' cholangiocarcinoma tissues, approximately 30-fold higher than that found in control common cholangiocarcinoma tissues. Furthermore, C:G-to-T:A transitions with substantial strand bias as well as unique trinucleotide mutational changes of GpCpY to GpTpY and NpCpY to NpTpY or NpApY were predominant in all of the printing workers' cholangiocarcinoma genomes. These results were consistent with the epidemiological observation that they had been exposed to high concentrations of chemical compounds. Whole-genome analysis of *Salmonella typhimurium* strain TA100 exposed to 1,2-DCP revealed a partial recapitulation of the mutational signature in the printing workers' cholangiocarcinoma. Although our results provide mutational signatures unique to occupational cholangiocarcinoma, the

underlying mechanisms of the disease should be further investigated by using appropriate model systems and by comparison with genomic data from other cancers.

Summary

These occupational cholangiocarcinoma cases shared a high mutation burden, strand bias and unique trinucleotide mutational signatures, suggesting that the patients might have been exposed to a common strong mutagen. The underlying mechanisms of mutagenesis should be further investigated.

Introduction

Cholangiocarcinoma has been recognized as a relatively rare cancer but its incidence is increasing worldwide. The incidence rates are higher in the Japanese population (5.2 cases per 100 000 individuals) and Asian populations (including Japanese) (3.3 cases per 100 000 individuals) than in Western populations (2.1 cases per 100 000 individuals among non-Hispanic whites and blacks) [1-6]. Cholangiocarcinoma is an aging-associated cancer, with its reported average age of diagnosis being between the sixth and seventh decades of life [7]. Although several risk factors, such as primary sclerosing cholangitis, bile duct cystic disorders, liver fluke infection, hepatolithiasis, cirrhosis, hepatitis B or C virus infection, diabetes, obesity and exposure to the chemical carcinogen "Thorotrast" (banned in the 1960s), have been suggested, the majority of cholangiocarcinomas arise sporadically [4,5]. Thus, the etiology and pathogenesis of cholangiocarcinoma remain to be elucidated, and the emergence of other risk factors is a likely supposition.

An outbreak of cholangiocarcinoma among workers in an offset color proof-printing company in Osaka, Japan, was recently reported, and this disease was newly classified as an occupational disease by the Ministry of Health, Labour and Welfare of Japan in 2013

(http://www.mhlw.go.jp/english/policy/employ-labour/labour-standards/Occupational.html). Between 1996 and 2012, 17 of the 111 former or current workers of the company were diagnosed with cholangiocarcinoma [8]. In addition, 13 workers in 11 other printing companies in Japan were acknowledged as exhibiting occupational cholangiocarcinoma by April 2014 [9,10]. A detailed investigation of the 17 patients at the first reported company in Osaka revealed that the printing workers' cholangiocarcinoma exhibited an unusually early onset, ranging from 25 to 45 years. All of the patients had been exposed to high concentrations of chemical compounds, including 1,2-dichloropropan (1,2-DCP) and/or dichloromethane (DCM) [8]. The International Agency for Research on Cancer (IARC) re-evaluated the carcinogenic risks of these compounds, recategorizing 1,2-DCP as a Group 1 (carcinogenic to humans) carcinogen instead of Group 3 (not classifiable as to its carcinogenicity to humans) and DCM as Group 2A (probably carcinogenic to humans) instead of Group 2B (possibly carcinogenic to humans) [11,12]. However, previous *in vitro* and *in vivo* studies suggested that both 1,2-DCP and DCM have limited mutagenic and tumorigenic capabilities, and bile duct tumorigenesis was not induced by 1,2-DCP or DCM in rodent models [13-16]. Thus, the relevance of 1,2-DCP and/or DCM exposure to cholangiocarcinoma carcinogenesis remains to be further elucidated. We performed a

genome-wide mutation analysis of four cases of occupational cholangiocarcinoma using genomic DNA samples derived from surgically resected specimens. We found a significant mutational landscape, including a high mutation burden, strand bias and unique trinucleotide mutation signatures commonly that were observed in all of the investigated cases, suggesting that the workers had been exposed to a common strong mutagen. We further investigated the mutational signatures in the genomic DNA of *Salmonella typhimurium* strain TA100 and human epithelial cells exposed to 1,2-DCP.

Materials and Methods

Preparation of clinical samples

Tumor and matched normal formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained from Osaka National Hospital, Osaka City University Hospital and National Cancer Center Hospital East. This study was approved by the Institutional Review Board of each institution. Four occupational cholangiocarcinoma samples were selected for analysis from 17 patients who were workers at a printing company in Osaka and diagnosed with cholangiocarcinoma between 1996 and 2012. Among the 17 occupational cholangiocarcinoma patients worked at the same printing company in Osaka Japan between 1996 and 2012, 12 were surgically treated. The resected tumor

specimens of 8 of the 12 patients were available. According to the quality and quantity of the genomic DNA samples, 4 cases were selected for further analysis. Control common cholangiocarcinoma samples were randomly selected from patients who were surgically treated between 2012 and 2014 at National Cancer Center Hospital East and whose tissues were abundant enough for sequencing analysis. Twenty sections of 10-µm-thick FFPE tissue samples were subjected to laser-capture microdissection or to manual microdissection. The Absolutely RNA FFPE kit (modified protocol for DNA extraction, Agilent Technologies, Santa Clara, CA, USA) was used to prepare the DNA. DNA quality was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), the Quant-iT PicoGreen dsDNA Reagent and Kit (Life Technologies, Carlsbad, CA, USA) and the Infinium HD FFPE DNA Sample QC Kit (Illumina, San Diego, CA, USA).

Whole-exome sequencing

Using 0.25-1.00 µg of double-stranded DNA, we prepared whole-exome sequencing libraries. The exomes were captured using the SureSelect Human All Exon V4+UTRs or the V5+UTRs Kit (Agilent Technologies, Santa Clara, CA, USA) according to the

manufacturer's instructions. The exome capture libraries were then sequenced using a HiSeq 2000 system (Illumina, San Diego, CA, USA) to generate 100-bp paired-end data.

Identification of somatic mutations

Sequence reads were aligned to the human reference genome UCSC hg19 using the Burrows-Wheeler Aligner program (BWA, http://bio-bwa.sourceforge.net/). Single-nucleotide variants (SNVs) and insertions and deletions (INDELs) were called and annotated using the Genome Analysis Toolkit software package (GATK, http://www.broadinstitute.org/gatk/). Sequencing artifacts were filtered out using custom filters (GATK confidence score ≥50, number of variant reads in each direction ≥1, variant allele frequency ≥10%) and by visual inspection. Germline variants were filtered out using data from dbSNP build 131, the 1000 Genomes Project (Phase 1 exome data, released May 21, 2011), 1 Japanese genome, 299 in-house Japanese exomes and matched normal-tissue exomes of the cholangiocarcinoma cases.

Confirmation of somatic mutations

Somatic mutations in the *ARID1A, BRAF, CDKN2A* and *MLL3* genes, which were detected in the case 1 patient, were confirmed using Sanger sequencing. PCR primers

were designed using the Primer3Plus software (www.bioinformatics.nl/primer3plus/) and are listed in Supplementary Table 1. PCR was performed using HotStarTaq DNA Polymerase (Qiagen, Valencia, CA, USA), and the samples were incubated at 95 °C for 15 min, 95 °C for 30 s, 55 °C or 61 °C for 30 s, and 72 °C for 1 min for 40 cycles and then at 72 °C for a final 10 min extension. PCR amplicons were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and a 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

Mutagenicity assay

The 1,2-DCP (1,2-dichloropropane, >98.0% pure) and DCM (dichloromethane, >99.5% pure) were purchased from Wako Pure Chemical Industries, Ltd. , Osaka, Japan and Nacalai Tesque, Inc., Kyoto, Japan, respectively. The standard plate-incorporation method in the absence of a metabolic activation system was performed to test the mutagenicity of 1,2-DCP and DCM in *Salmonella typhimurium* TA100 with a slight modification for testing volatile chemicals [17]. In brief, appropriately sized filter papers absorbed multiple doses of 1,2-DCP or DCM and were put into plastic bags. Plates in which bacteria had been plated in top agar were placed in the plastic bags without covers and sealed tightly. By this method, the bacteria were exposed to the evaporating

haloalkanes. The chemical vapor concentration in the plastic bags was calculated using calibration curves determined by gas chromatography mass spectrometry analysis. After 2 hours of vapor exposure at 37 °C, the plates were removed from the bags and incubated for another 48 hr. *hisG*$^+$ revertant colonies were counted to evaluate mutagenicity. The mutagenic activity of the samples was calculated from the linear portions of the dose-response curves, which were obtained using three doses in duplicate plates on at least two independent experiments.

Analysis of global mutational profiles of 1,2-DCP and DCM in a Salmonella strain

The *hisG*$^+$ colonies were randomly isolated, and genomic DNA was extracted using a Puregene Cell and Tissue kit (Qiagen, Valencia, CA, USA). The mutational profiles induced by haloalkanes were subsequently examined using whole-genome sequencing with reference to a previous report [18]. Using 3 μg of DNA and the SureSelect XT Library Prep Kit (Agilent Technologies, Santa Clara, CA, USA), we prepared whole-genome sequencing libraries according to the manufacturer's instructions. The prepared libraries were sequenced using a HiSeq 1500 system (Illumina, San Diego, CA, USA) to generate 100-bp paired-end data. The sequence reads were aligned to the reference sequence NC_003197 (*Salmonella enterica* subsp. *enterica serovar*

Typhimurium str. LT2 chromosome, complete genome, 4 857 432 bp) using the Burrows-Wheeler Aligner program (BWA, http://bio-bwa.sourceforge.net/), and the SNVs were called and annotated using the Genome Analysis Toolkit software package (GATK, http://www.broadinstitute.org/gatk/). We selected "PASS" variants annotated by the GATK program as high-confidence acquired variants.

Analysis of mutational profile of 1,2-DCP in mammalian cells

NCC-CC1 cells, which were originally established from human biliary tract carcinoma tissue [19], were kindly provided by Dr. Hidenori Ojima at the National Cancer Center Research Institute in 2013. The cells were maintained in RPMI-1640 medium (Wako Pure Chemicals, Japan) containing 10% fetal bovine serum (Thermo Fisher Scientific Inc., MA, USA). This cell was authenticated by DNA microarray and quantitative RT-PCR and last checked in 2010. HEK293 cell was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and have never been passaged longer than 6 months after receipt or resuscitation. This cell line was not authenticated as they came from national repositories. Cloned human embryonic kidney 293 (HEK293) cells stably expressing an open reading frame of the pQCXIP vector (Clontech Laboratories, Inc., A Takara Bio Company, Mountain View, CA, USA) were cultured in DMEM

medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10 % fetal bovine serum (Biowest, Nuaillé, France). We used an *in vitro* vapor exposure system with slight modifications [20]. In brief, appropriately sized filter papers were put into two rhombus-shaped holes in the center of a 6-well culture plate, and a toxic dose of 1,2-DCP (250 μL/plate for single exposure, once with 180 μL/plate and four times with 120 μL/plate for multiple exposure for NCC-CC1; and 50 μL/plate for single exposure for HEK293) was absorbed onto the filter papers. The culture plates were put into plastic bags without covers and sealed tightly. Using this method, the haloalkanes evaporated and dissolved into the culture medium [20]. After 2 and 4 hours of vapor exposure for NCC-CC1 and HEK293, respectively, the cells were reseeded and cultured for 4 to 6 weeks to isolate individual clones. For multiple exposures, the cells were exposed to 1,2-DCP subsequently after recovery from cytotoxicity, and this procedure was repeated up to five times. Genomic DNA was extracted from 2 to 6 clones per exposure group using a DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA), and the mutational profile induced by the haloalkanes was subsequently examined using whole-exome sequencing.

Statistical analyses

The statistical significance of observed differences between the number of somatic SNVs in the printing worker cholangiocarcinoma cases and the control cholangiocarcinoma cases was evaluated using Student's *t*-test. For strand bias analysis, the significance of the differences in the number of SNVs between the un-transcribed (sense) and transcribed (antisense) strand in the cases and controls was evaluated using the chi-square test. The test evaluated whether the proportion of SNVs in each strand differed from 0.5, which is the value expected by chance. For trinucleotide mutational signature analysis, the significance of differences in the number of mutations between the trinucleotide sequence of interest and any sequence except the one of interest was evaluated by the chi-square test. The test evaluated whether the proportion of mutations in each context differed from the frequency of the context in the targeted genome, which is the expected value by chance.

Results

Patient characteristics and quality of whole-exome sequencing

The characteristics of the printing worker patients upon admission are described in Table 1. The printing worker cholangiocarcinoma patients (cases 1-4) were 31- to 40-year-old males. The case 1 patient used both 1,2-DCP and DCM, and the case 2-4

patients used 1,2-DCP for one to 11 years. The characteristics of the control common cholangiocarcinoma patients are described in Supplementary Table 2. The patients with common late-onset cholangiocarcinoma (controls 1-4) were 55- to 79-year-old males and females, and all patients had intrahepatic cholangiocarcinoma. The patients with common early-onset bile duct carcinomas (controls 5-7) were 26- to 39-year-old males and females, and the subtypes were gallbladder cancer, duodenum papilla cancer and extrahepatic cholangiocarcinoma.

Tumor cells were condensed to approximately 50% by laser-capture microdissection or manual microdissection. Genomic DNA was retrieved and used for whole-exome sequencing. The average base coverage of the targeted regions in the tumor and normal samples of the printing workers' cholangiocarcinomas was 139.7-fold (range: 89.8-197.2) and 105.7-fold (range: 66.8-131.2), respectively. The proportion of the targeted regions with 20 reads or higher was 92.1% (range: 76.3-98.5%) and 86.5% (range: 69.0-96.9%) for the tumor and normal samples, respectively. Among the common late-onset cholangiocarcinomas, the average base coverage in the tumor and normal samples was 241.9-fold (range: 210.7-272.8) and 289.4-fold (range: 267.6-309.0), respectively, and 98.2% (range: 97.2-98.6%) and 98.6% (range: 98.4-98.7%) of the target regions in the tumor and normal samples, respectively, had 20 reads or higher. Among

the common early-onset bile duct carcinomas, the average base coverage in the tumor and normal samples was 142.2-fold (range: 131.4-155.5) and 68.3-fold (range: 64.0-75.6), respectively, and 96.5% (range: 96.2-96.8%) and 84.5% (range: 81.9-85.8%) of the target regions in the tumor and normal samples, respectively, had 20 reads or higher (Supplementary Table 3).

The printing workers' cholangiocarcinomas are hypermutated tumors

We identified 1451±1089 (44.6±33.5/Mb) somatic SNVs and 6.8±5.0 (0.2±0.2/Mb) apparent somatic INDELs in the four printing workers' cholangiocarcinomas (cases 1-4) (Table 2). The number of somatic SNVs in these cases was significantly higher than that in the exomes of the four cases of common late-onset intrahepatic cholangiocarcinoma (controls 1-4) or in the three cases of common early-onset bile duct carcinoma (controls 5-7), which showed an average of 44.8±11.9 (1.4±0.4/Mb) and 50.0±23.4 (1.5±0.7/Mb) SNVs, respectively (p=0.04 and 0.04, respectively; Student's t-test) (Table 2 and Figure 1A). All somatic variants detected in the patients are listed in Supplementary File 1. The number of SNVs in the printing worker cholangiocarcinoma cases was also larger than that in the exomes of liver fluke infection-related and -unrelated cholangiocarcinoma cases [21,22] and that in 260 common biliary tract cancers [23].

Notably, the number of INDELs was significantly smaller than the number of SNVs in the printing worker cholangiocarcinoma cases. This smaller number of INDELs is a unique characteristic compared with other hypermutated solid tumors such as microsatellite-instable colorectal cancers [24].

The printing workers' cholangiocarcinoma genomes harbor mutations in genes frequently mutated in common bile tract carcinomas

We selected 20 genes that are frequently mutated in bile tract carcinoma (registered in Catalogue of Somatic Mutations in Cancer (COSMIC) v74). Each of the printing workers' cholangiocarcinoma had amino acid-altering mutations in two to six genes (Table 3). Among the mutations detected in case 1, we confirmed mutations in *ARID1A, BRAF, CDKN2A* and *MLL3* by Sanger sequencing (Supplementary Table 1 and Supplementary Figure 1).

Predominant DNA substitution patterns in the printing workers' cholangiocarcinomas: C:G-to-T:A transition

We next analyzed the single-nucleotide substitution patterns in the printing worker cholangiocarcinoma cases and found that C:G-to-T:A transitions were predominant,

among the somatic SNVs, followed by C:G to A:T transversion. This mutational spectrum is similar to that in the control cholangiocarcinoma cases (Figure 1B).

C:G-to-T:A transitions in the printing workers' cholangiocarcinomas indicate strand bias

We then examined the mutational signatures for transcriptional strand bias. In all of the printing workers' cholangiocarcinomas, a substantial difference in the prevalence of mutations between the sense and antisense strands was observed for the C:G-to-T:A transition, whereas no significant difference was detected in the control cholangiocarcinoma cases (Figure 1C).

The printing workers' cholangiocarcinomas share characteristic trinucleotide mutational signatures

We further analyzed the mutational signature of the base substitutions by incorporating information regarding the 5' and 3' neighboring sites of each mutated base [25]. The most significant trinucleotide mutational pattern in the printing workers' cholangiocarcinomas was GpCpY to GpTpY (p<0.01, chi-square test) (Figure 1D), a novel signature that was not reported in the International Cancer Genome Consortium

(ICGC) project, which classified more than 20 distinct mutational signatures from a mutational catalogue of 7042 primary cancers [25]. This signature was identical among the four printing worker cases. Following the GpCpY to GpTpY signature, NpCpY to NpTpY and NpApY changes were the next most characteristic mutational signatures (Figure 1E). By contrast, the control late-onset cholangiocarcinoma cases harbored 5-methylcytosine deamination signature mutations (NpCpG to NpTpG changes; classified as Signature 1A and 1B in the ICGC project), and the control early-onset bile duct carcinoma cases harbored both 5-methylcytosine deamination signature mutations (NpCpG to NpTpG changes; classified as Signature 1A and 1B in the ICGC project) and APOBEC signature mutations (TpCpW to TpTpW or TpGpW changes; classified as Signatures 2 and 13 in the ICGC project) (Figure 1D).

1,2-DCP and DCM show mutagenicity in S. typhimurium strain TA100

We examined the mutagenic activity of 1,2-DCP and DCM on *S. typhimurium* strain TA100 by Ames assay. Both haloalkanes showed mutagenicity toward TA100 in a dose-dependent manner. The mutagenic potency of 1,2-DCP was slightly higher than that of DCM (Figure 2).

1,2-DCP and DCM preferentially induced SNVs at C:G residues

To determine the global mutational profiles of 1,2-DCP and DCM, we further performed whole-genome analysis of *S. typhimurium* TA100 with or without haloalkane exposure. Revertant mutations occurring within the *hisG* gene were excluded from the analysis because these mutations are affected by selection bias. The number of mutagenic events in the other bacterial DNA increased depending on the dose of 1,2-DCP. The average mutation rate in clones exposed to 1,2-DCP at 3000 ppm (n=76) and 6000 ppm (n=19) was 0.2 and 0.6/Mb, respectively. These mutation rates are 1.4- and 5.1-fold higher than that for the non-exposed control (p=0.05 and p<0.01, respectively, Student's t-test). C:G-to-T:A transitions were the most predominant single-nucleotide substitutions (57.6% and 77.1% of total SNVs in the genomic DNA exposed to 3000 ppm and 6000 ppm of 1,2-DCP, respectively). In contrast, the average mutation rate of clones exposed to 3500 ppm of DCM (n=47) was 0.5/Mb, which is significantly higher than that for the non-exposed control (p<0.01, Student's t-test), and C:G to A:T transversions were predominant (46.6% of total SNVs), followed by C:G-to-T:A transitions (44.1% of total SNVs) (Figure 3A, B).

The mutational signature of 1,2-DCP in S. typhimurium TA100 partially recapitulates the printing workers' cholangiocarcinoma signature

We further analyzed the trinucleotide mutational pattern. Clones exposed to 1,2-DCP harbored NpCpC to NpTpC changes (Figure 3C, indicated by the asterisks), and these changes were more remarkable at 6000 ppm than at 3000 ppm (Supplementary Figure 2). This signature is similar to the second-most dominant signature of the printing workers' cholangiocarcinoma. With DCM exposure, the clones harbored broad changes in C:G to A:T and C:G to T:A mutations, and no specific trinucleotide mutational patterns were observed (Figure 3C).

1,2-DCP has limited mutagenic capability in mammalian cells

To confirm the mutational signature of 1,2-DCP in mammalian cells, we examined the base substitution profiles using cholangiocarcinoma cell line NCC-CC1 and human embryonic kidney 293 (HEK293) cells. The mutation rate of the two independent NCC-CC1 clones after a single exposure to 1,2-DCP at a cytotoxic dose was 0.1 and 0.5/Mb, and they were not significantly higher than the mutation rate of the non-exposed control. The mutation rate of clones repeatedly exposed to 1,2-DCP was 0.5 to 1.3/Mb, and no significant difference was observed here either (Supplementary Table

4 and Supplementary Figure 3A). Among the SNVs, C:G to T:A transitions and C:G to A:T transversions were predominant (60.0% and 59.4% of total SNVs in genomic DNA with single and repeated exposure to 1,2-DCP, respectively) (Supplementary Figure 3B). Moreover, no specific trinucleotide signatures were observed (Supplementary Figure 3C). The mutation rate in 1,2-DCP-exposed HEK293 cells was 0.3±0.1/Mb, and it was not significantly increased (Supplementary Table 5 and Supplementary Figure 4A). Similarly, in NCC-CC1 cells, C:G to T:A transitions and C:G to A:T transversions were observed to predominate (59.6% of total SNVs) (Supplementary Figure 4B), and the trinucleotide signatures observed in the printing workers' cholangiocarcinomas were not recapitulated (Supplementary Figure 4C).

Discussion

The representative characteristic mutational profile, including a high somatic mutation burden, substantial strand bias in C:G to T:A mutations and unique trinucleotide mutational changes (GpCpY to GpTpY and NpCpY to NpTpY or NpApY), shared in all of the investigated printing workers' cholangiocarcinomas suggests that the patients might have been exposed to a common strong mutagen and that these conditions might increase the chance for mutations in cholangiocarcinoma driver genes. Mutations with

transcriptional strand bias are known to occur in cancer genomes as a result of exposure to abundant mutagens and the formation of bulky DNA adducts, such as in smoking-related lung cancer and ultraviolet-associated melanoma [25,26]. We thus suspected that some agents make abundant adducts on G residues and that the transcription-coupled DNA repair machinery preferentially repairs transcribed G residues, that is, sense C residues [27], inducing strand-biased mutations. These results are consistent with the epidemiological observation that these workers had been exposed to high concentrations of chemical compounds. Based on reconstructed experimental data, Kumagai et al. estimated the concentration of volatile solvent in a proof-printing room to be 100-670 ppm for 1,2-DCP and 80-540 ppm for DCM [28]. Both 1,2-DCP and DCM have been used in industrial processes and household products (such as paint stripper) worldwide. However, as mentioned previously, 1,2-DCP and DCM reportedly have very limited tumorigenic capabilities in animal models [13-16]. Wang et al. recently reported that co-exposure to 1,2-DCP and DCM induced an increase in mutations in a mouse *gpt* mutation model. However, they detected mutations predominantly at A:T pairs rather than C:G pairs, in contrast to the mutations observed in the printing workers' cholangiocarcinomas in this study [29].

To verify the mutagenic features of 1,2-DCP and DCM more directly, we applied a model system using *S. typhimurium* strain TA100. Our data suggest that both 1,2-DCP and DCM exhibited mutagenic activity on the *S. typhimurium* TA100 genome, especially at C:G residues.

However, three of the four printing workers' cholangiocarcinoma cases examined in this study were exposed only to 1,2-DCP and not to DCM. Prior epidemiological studies suggested that 1,2-DCP is a more suspicious causative agent than DCM, because all 17 of the patients in the first cohort in Osaka were exposed to 1,2-DCP, whereas 11 were exposed to DCM [8]. Based on these findings, IARC designated 1,2-DCP as a higher rank carcinogen than DCM. Whole genome analysis of 1,2-DCP- or DCM-exposed *S. typhimurium* TA100 might support these epidemiological assumption. The trinucleotide mutational signature of *S. typhimurium* TA100 exposed to 1,2-DCP revealed preferential mutational changes of NpCpC to NpTpC, which overlaps with the second-most predominant trinucleotide mutational signature observed in the printing workers' cholangiocarcinomas, namely, NpCpY to NpTpY or NpApY changes. These results suggest a contribution of 1,2-DCP to mutagenesis and carcinogenesis in the printing workers' cholangiocarcinomas, at least in some capacity.

In mammalian species, inhaled DCM is supposed to be metabolized via a GSTT1-dependent pathway [27,30-33]. S-(chloromethyl)glutathione from DCM is involved in mutagenesis by forming guanine adducts, inducing C:G to A:T and C:G to T:A substitutions, in bacteria and mammalian cells [17,34,35]. In contrast to DCM, the mode of mutagenicity of 1,2-DCP or its potential metabolites remains unclear. NpCpY (the complement to RpGpN) sites are reported to be targets of electrophilic agents such as alkylating agents and platinum-derived drugs [25,36-38]. The N^7- and O^6-positions of guanine are the most reactive nucleophilic sites [39-41], and electrophilic agents react with these sites to form alkyl-DNA adducts or intra- and inter-strand cross-linked DNA adducts, inducing cytotoxicity and mutagenicity. Whether 1,2-DCP or its potential metabolites possess electrophilic features and form alkyl-DNA adducts should be further investigated.

To strengthen the above findings, we examined the mutagenic profile of 1,2-DCP in human epithelial cell-derived cell lines. However, neither single nor repeated exposure of cultured cells to 1,2-DCP induced significant mutagenesis, and the mutation profiles in the *in vitro* system did not recapitulate the specific trinucleotide signatures observed in the clinical samples and *S. typhimurium* TA100 strain model.

Another remaining riddle regarding the specific mutational landscape of the printing workers' cholangiocarcinomas is the most prominent trinucleotide mutational signature, GpCpY to GpTpY. Because this signature was not recapitulated in the *S. typhimurium* TA100 strain model, host factors might have contributed. Although the GpCpY to GpTpY signature has not been identified in any human clinical genome sequencing data, a similar signature was recently reported by Zavadil et al. They reported that acquired mutations caused by overexpression of activation-induced cytidine deaminase (AID) in immortalized human TP53 knock-in mouse fibroblasts were predominantly C:G to T:A substitutions with a GpCp(A/C/T) to GpTp(A/C/T) trinucleotide signature [42]. AID was originally identified as an inducer of somatic hypermutation in the variable region of immunoglobulin genes in activated B cells [43,44]. In addition, the aberrant expression of AID induces lymphoid and nonlymphoid malignancies with accumulated mutations in known cancer-related genes in animal models [45]. AID is reportedly induced by chronic tissue injury in parenchymal cells such as hepatocytes and gastric epithelial cells [46,47]. Marusawa et al. reported significantly high AID expression in the epithelial cells of cholangiocarcinoma, with massive inflammation in the surrounding liver tissue [48]. Although the tissue toxicity of 1,2-DCP and DCM has been reported mainly in animal models, and whether they

affect biliary epithelia remains unclear [49], massive fibrosis was observed to surround cancerous and non-cancerous bile ducts in the printing workers' cholangiocarcinoma samples [8]. Our preliminary immunohistochemistry data suggested that AID was expressed in the transformed epithelial cells of the printing workers' cholangiocarcinoms (data not shown). Whether the GpCpY to GpTpY changes in the printing workers' cholangiocarcinomas were elicited by inflammation-induced AID expression must be further examined using *in vitro* and *in vivo* model systems.

The above-mentioned discrepancies in mutation signature between the clinical samples and the *in vitro* models suggest that the carcinogenic processes of the printing workers' cholangiocarcinomas might be more complex than we had supposed. To elucidate the mechanisms, we should consider the tumor microenvironment including the metabolism of the inhaled chemical compounds at the biliary epithelia and interactions of tumor cells with surrounding non-tumor cells and possibly bacterial flora. To address these questions, model systems reproducing the actual conditions in biliary epithelial cells and the surrounding microenvironment of the printing worker cholangiocarcinoma patients are necessary.

Although the lack of model systems hampered further investigation, the particular mutational profiles, especially the specific trinucleotide mutation signatures

will be a clue to help elucidate the hidden mechanism of this disease. The mutational profiles of various cancer genomes are systematically collected and the mutational signatures are classified by ICGC. Although the signature presented in this study has not been reported, the accrual of further data may give us a chance to find other cases of biliary and other organ cancers with similar signatures. Referring to their epidemiological and clinicopathological background will provide information that the model systems hardly address.

Supplementary material

Supplementary Tables 1-5, Supplementary Figures 1-4 and Supplementary File 1 can be found at http://carcin.oxfordjournals.org/

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TABLE AND FIGURES LEGENDS

Table 1. Patient characteristics of printing worker cholangiocarcinoma cases

Table 2. Number of somatic mutations identified in printing workers' cholangiocarcinomas (cases) and common cholangiocarcinomas (controls)

Table 3. Identified mutations in commonly mutated genes in biliary tract carcinoma (BTC)

Figure 1. The printing workers' cholangiocarcinomas share a high mutation burden, strand bias and a unique trinucleotide mutational signature. (A) Number of exome SNVs in the printing workers' and control cholangiocarcinoma cases (*p<0.05; Student's t-test). (B) Single-nucleotide DNA substitution profiles in the printing workers' and control cholangiocarcinoma cases. (C) Substantial strand bias in mutation counts in the sense (S) and antisense (AS) strands of the printing workers' cholangiocarcinoma cases. Significant strand bias for C:G to T:A mutations was observed in the printing workers' cholangiocarcinoma cases (**p<0.01, ***p<0.001; chi-square test). (D) Trinucleotide mutational patterns in the printing workers' and control cholangiocarcinoma samples.

The mutational signatures were normalized using the trinucleotide frequency in the genome. (E) Secondary characteristic trinucleotide mutational pattern in the printing workers' cholangiocarcinoma samples that emerged after elimination of the prominent mutational pattern GpCpY to GpTpY. The mutational signatures were normalized using the trinucleotide frequency in the genome.

Figure 2. Both 1,2-DCP and DCM show mutagenic activity in *S. typhimurium* strain TA100. The mutagenic activity levels of 1,2-DCP (closed circle) and DCM (closed square) were estimated from the number of revertant colonies.

Figure 3. The mutational signature of 1,2-DCP in *S. typhimurium* TA100 partially recapitulates the printing workers' cholangiocarcinoma signature. (A) Number of SNVs in 1,2-DCP- and DCM-exposed TA100, except for the *hisG* gene target site. (B) Single-nucleotide DNA substitution profiles in TA100 exposed to 1,2-DCP or DCM except for the *hisG* gene target site. (C) Trinucleotide mutational pattern of TA100 exposed to 1,2-DCP or DCM except for the *hisG* gene target site. Clones exposed to 1,2-DCP harbored NpCpC to NpTpC changes (indicated by the asterisks). This signature partially recapitulates the printing workers' cholangiocarcinoma signature as

shown below the 1,2-DCP-exposed TA100 panel. The printing workers' signature is the mean of the frequency of secondary characteristic mutational changes in four patients shown in Figure 1E. The mutational signatures were normalized using the trinucleotide frequency in the genome. The signature of 1,2-DCP was obtained from the sum of the 3000 ppm and 6000 ppm exposure data.

Table 1 Patient characteristics (Printing workers)

		Printing worker			
		Case 1	Case 2	Case 3	Case 4
(ID)		(CHCOSK001)	(CHCOSK003)	(CHCOSK004)	(CHCOSK005)
Anatomical subtype		Intrahepatic	Intrahepatic	Intrahepatic	Intrahepatic
Age		40 year old	39 year old	31 year old	34 year old
Sex		male	male	male	male
Duration of exposure	1,2-DCP	11 years 11 months	7 years 4 months	6 years 6 months	6 years 1 months
	DCM	1 years 5 months	none	none	none
Smoking habit		20 cigarettes /day	20 cigarettes /day	none	none
Alcohol consumption		1.5 to 1.8 L sake/week	4.4 L beer/week	none	occasionally

Table 2 Number of somatic mutations identified in printing workers' cholangiocarcinomas (cases) and common cholangiocarcinomas (controls)

		# of SNVs		# of	Mutation
		Non-synonymous	Synonymous	INDELs	rate (/Mb)
Printing worker	Case 1	1505	558	4	64.6
	Case 2	178	76	3	7.8
	Case 3	601	252	6	26.1
	Case 4	1862	770	14	80.5
Common (late-onset)	Control 1	28	12	5	1.4
	Control 2	35	12	3	1.5
	Control 3	20	12	8	1.2
	Control 4	45	15	4	1.9
Common (early-onset)	Control 5	26	9	4	1.2
	Control 6	31	7	4	1.3
	Control 7	52	25	3	2.4

1

Table 3 Identified mutations in commonly mutated genes in biliary tract carcinoma (BTC)

	Frequency					Printing worker				Common (late-onset)				Common (early-onset)		
			260													
Gene	COSMIC		Japanese			Case	Case	Case	Case	Control	Control	Control	Control	Control	Control	Control
symbol	v74		BTCs		CCDS ID	1	2	3	4	1	2	3	4	5	6	7
TP53	35	%	26	%	CCDS11118				N131Y		R213*		S241Y			R213*
KRAS	23	%	18	%	CCDS8702.1				G12V	G12D	G12V		A146V		G12D	
CDKN2A	15	%	5	%	CCDS56565.1	P75T,			W15*,							
MLL3	15	%	3	%	CCDS5931.1	E141*,								N2787fs		
ARID1A	11	%	11	%	CCDS285.1	Q586*		Q528H,	E119*,			K2189*		L2088del		
IDH1	9	%	3	%	-											
BAP1	8	%	8	%	CCDS2853.1							D535fs				
SMAD4	8	%	9	%	CCDS11950.1		†G508D				N107fs		D351V	G386R	G270fs	I527fs
AXIN1	7	%	2	%	CCDS10405.1		†S782N	G676V	K165*,							Q386*
CTNNB1	7	%	2	%	-											
GNAS	7	%	6	%	-											
PBRM1	7	%	5	%	-											
PIK3CA	7	%	7	%	CCDS43171.1		†H665L							E545K		
ERBB3	6	%	4	%	-											
ATM	5	%	4	%	CCDS31669.1		†W1933*									
BRAF	5	%	2	%	CCDS5863.1	D594G										
FBXW7	5	%	3	%	CCDS34078.1				Q124L						R347H	
ZNF521	5	%	2	%	-											
TERT	4	%	3	%	-											
IDH2	3	%	1	%	-											

† rescued by visual inspection

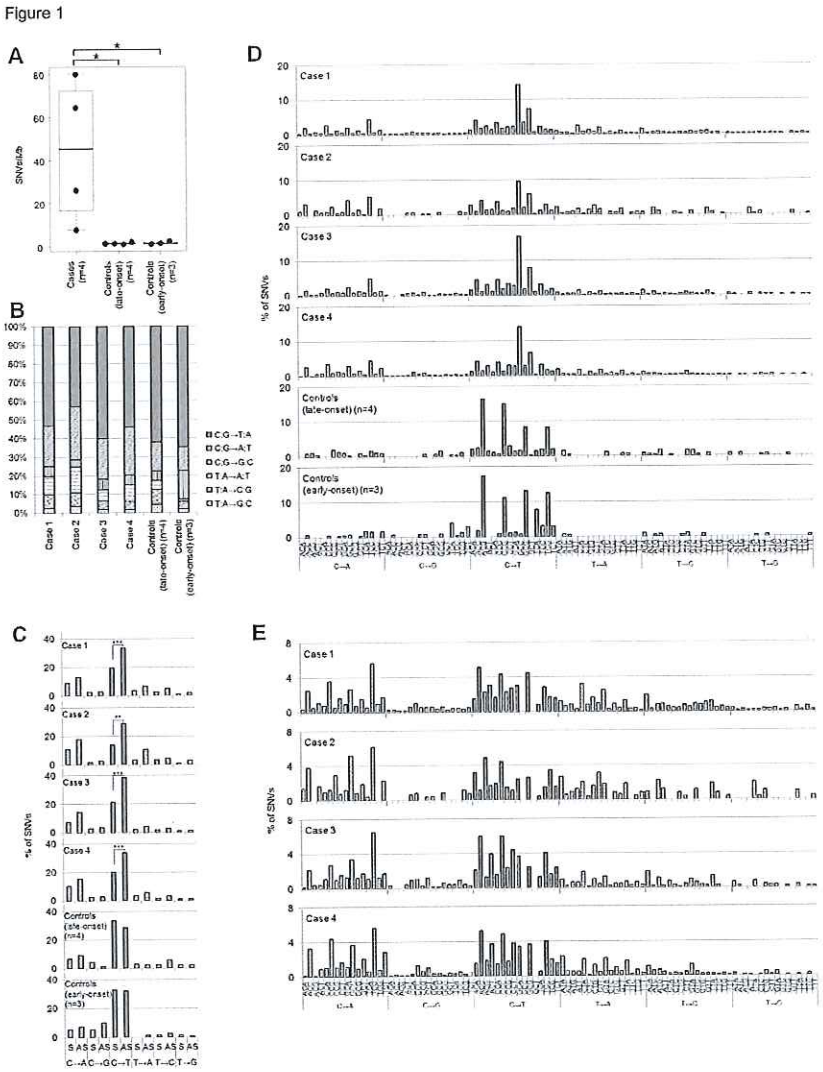

Figure 1. The printing workers' cholangiocarcinomas share a high mutation burden, strand bias and a unique trinucleotide mutational signature. (A) Number of exome SNVs in the printing workers' and control cholangiocarcinoma cases (*p<0.05; Student's t-test). (B) Single-nucleotide DNA substitution profiles in the printing workers' and control cholangiocarcinoma cases. (C) Substantial strand bias in mutation counts in the sense (S) and antisense (AS) strands of the printing workers' cholangiocarcinoma cases. Significant strand bias for C:G to T:A mutations was observed in the printing workers' cholangiocarcinoma cases (**p<0.01, ***p<0.001; chi-square test). (D) Trinucleotide mutational patterns in the printing workers' and control cholangiocarcinoma samples. The mutational signatures were normalized using the trinucleotide frequency in the genome. (E) Secondary characteristic trinucleotide mutational pattern in the printing workers' cholangiocarcinoma samples that emerged after elimination of the prominent mutational pattern GpCpY to GpTpY. The mutational signatures were normalized using the trinucleotide frequency in the genome.
254x334mm (300 x 300 DPI)

Figure 2

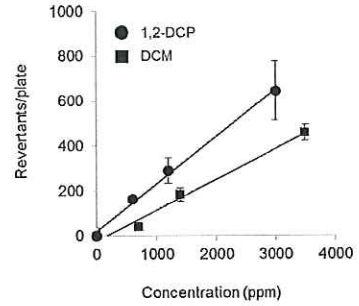

Figure 2. Both 1,2-DCP and DCM show mutagenic activity in S. typhimurium strain TA100. The mutagenic activity levels of 1,2-DCP (closed circle) and DCM (closed square) were estimated from the number of revertant colonies.
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Figure 3

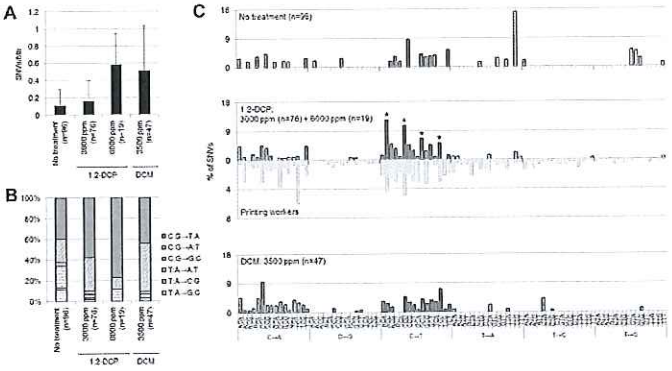


Figure 3. The mutational signature of 1,2-DCP in S. typhimurium TA100 partially recapitulates the printing workers' cholangiocarcinoma signature. (A) Number of SNVs in 1,2-DCP- and DCM-exposed TA100, except for the hisG gene target site. (B) Single-nucleotide DNA substitution profiles in TA100 exposed to 1,2-DCP or DCM except for the hisG gene target site. (C) Trinucleotide mutational pattern of TA100 exposed to 1,2-DCP or DCM except for the hisG gene target site. Clones exposed to 1,2-DCP harbored NpCpC to NpTpC changes (indicated by the asterisks). This signature partially recapitulates the printing workers' cholangiocarcinoma signature as shown below the 1,2-DCP-exposed TA100 panel. The printing workers' signature is the mean of the frequency of secondary characteristic mutational changes in four patients shown in Figure 1E. The mutational signatures were normalized using the trinucleotide frequency in the genome. The signature of 1,2-DCP was obtained from the sum of the 3000 ppm and 6000 ppm exposure data.
190x142mm (300 x 300 DPI)

Supplementary Table 1 Primers and conditions for Sanger sequencing

Gene symbol	Chromosome	Position	Ref.	Variant	AA alteration	Forward primer sequence	Reverse primer sequence	Anneal temperature (°C)
ARID1A	chr1	27,058,048	C	T	Q586*	TATCCTCAGCCCCAGTCTCA	CCCCCAAGCTGTCTGTAGTC	61
BRAF	chr7	140,453,154	T	C	D594G	CCCACTCCATCGAGATTTCA	TGCTTGCTCTGATAGGAAAATG	55
CDKN2A	chr9	21,971,000	C	A	E120*	GCAGGTACCGTGCGACAT	CTTCCTGGACACGCTGGT	61
MLL3	chr7	151,849,845	T	A	L4157F	ACCATTGCTTGTTGGAGGAA	GGGTTTGGAGTATCGACAGC	55
MLL3	chr7	152,012,392	C	A	E141*	GTTGGTTTCTCCATGGCAAG	TGCGCTTTTTGTTACTGTGG	55

1

Supplementary Table 2 Patient characteristics (Common)

	Common (late-onset)				Common (early-onset)		
	Control 1	Control 2	Control 3	Control 4	Control 5	Control 6	Control 7
(ID)	(CHCLO001)	(CHCLO002)	(CHCLO003)	(CHCLO004)	(CHCEO008)	(CHCEO009)	(CHCEO010)
Anatomical subtype	Intrahepatic	Intrahepatic	Intrahepatic	Intrahepatic	Gallbladder	Duodenum papilla	Extrahepatic
Age	55 year old	73 year old	72 year old	79 year old	39 year old	31 year old	26 year old
Sex	male	male	female	female	male	male	female
Smoking habit	none	40 cigarettes /day	none	none	20 cigarettes /day	none	none
Alcohol consumption	none	1.3 L sake/week, 2.5 L distilled spirit/week, 0.6 L beer/week	none	none	0.1 to 0.2 L distilled spirit/day (1 to 3 times/month)	none	<0.1 L distilled spirit/day, <0.3 L beer/day (1 to 3 times/month)

Supplementary Table 3 Summary of the whole-exome sequencing

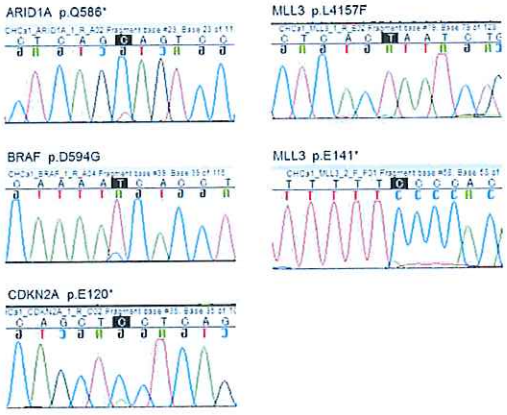
		Whole-exome sequencing					
		Starting dsDNA (μg)	Total read	Ave. depth	% 10x	% 20x	% 30x
Case 1 (CHCOSK001)	Tumor	0.30	278,710,461	89.8	84.0	76.3	71.0
	Liver	1.00	305,562,705	97.3	81.8	74.3	69.1
	Pancreas	0.50	260,723,377	66.8	78.6	69.0	61.7
Case 2 (CHCOSK003)	Tumor	0.50	240,297,905	197.2	99.1	98.5	97.3
	Liver	1.00	129,507,840	116.7	98.4	96.2	92.4
Case 3 (CHCOSK004)	Tumor	1.00	116,863,954	108.3	98.0	95.3	90.8
	Liver	1.00	143,348,319	131.2	98.6	96.9	94.1
Case 4 (CHCOSK005)	Tumor	0.25	210,752,331	163.6	99.1	98.4	96.9
	Liver	1.00	127,408,279	116.4	98.3	95.9	91.8
Control 1 (CHCLO001)	Tumor	1.00	290,671,449	241.7	99.0	98.5	97.6
	Liver	1.00	341,660,701	280.5	99.1	98.7	98.1
Control 2 (CHCLO002)	Tumor	1.00	328,017,605	272.8	99.1	98.6	97.8
	Liver	1.00	372,207,123	309.0	99.1	98.7	98.1
Control 3 (CHCLO003)	Tumor	1.00	290,602,272	242.3	98.9	98.4	97.7
	Liver	1.00	319,228,484	267.6	98.9	98.4	97.7
Control 4 (CHCLO004)	Tumor	1.00	295,371,019	210.7	98.5	97.2	95.2
	Liver	1.00	369,704,901	300.4	98.9	98.5	98.0
Control 5 (CHCEO008)	Tumor	0.50	290,583,119	155.5	98.4	96.8	94.3
	Liver	0.50	120,960,798	75.6	92.1	81.9	72.4
Control 6 (CHCEO009)	Tumor	1.00	154,812,520	131.4	98.5	96.2	92.3
	Liver	1.00	75,055,174	64.0	95.6	85.8	73.4
Control 7 (CHCEO010)	Tumor	1.00	161,954,739	139.7	98.4	96.4	92.9
	Liver	1.00	76,203,472	65.2	95.5	85.8	73.6

Supplementary Table 4 Summary of the whole-exome sequencing of NCC-CC1 cells exposed to 1,2-DCP

	Whole-exome sequencing								
	Starting DNA (μg)	Total read	Ave. depth	% 10x	% 20x	% 30x	# of SNVs	# of INDELs	Mutation rate (/Mb)
Control_clone1	0.11	49,084,725	40.9	92.4	74.9	55.6	19	1	0.6
Control_clone2	1.00	48,674,212	43.0	92.0	74.5	55.9	20	0	0.6
1,2-DCP_clone1	0.08	38,455,656	32.3	88.2	64.6	43.0	16	0	0.5
1,2-DCP_clone2	0.10	38,443,006	32.2	88.2	64.5	42.8	4	0	0.1
1,2-DCP repeated_clone1	1.00	54,419,622	45.2	93.4	77.6	59.3	18	0	0.5
1,2-DCP repeated_clone2	1.00	54,753,997	45.9	93.6	78.2	60.3	23	0	0.7
1,2-DCP repeated_clone3	1.00	46,446,922	38.7	91.4	71.8	51.6	18	0	0.5
1,2-DCP repeated_clone4	1.00	59,692,900	49.7	94.3	80.7	64.1	42	1	1.3

Supplementary Table 5 Summary of the whole-exome sequencing of HEK293 cells exposed to 1,2-DCP

	Whole-exome sequencing								
	Starting DNA (μg)	Total read	Ave. depth	% 10x	% 20x	% 30x	# of SNVs	# of INDELs	Mutation rate (/Mb)
1,2-DCP_clone1	0.20	75,038,948	62.3	96.6	88.5	75.8	7	1	0.2
1,2-DCP_clone2	0.20	73,932,637	60.2	96.4	87.7	74.4	16	0	0.5
1,2-DCP_clone3	0.20	69,992,205	58.2	96.3	87.0	73.1	7	1	0.2
1,2-DCP_clone4	0.20	67,105,745	56.3	96.0	86.1	71.5	3	0	0.1
1,2-DCP_clone5	0.20	77,985,178	62.6	96.7	88.6	75.9	11	0	0.3
1,2-DCP_clone6	0.20	71,622,698	59.2	96.2	86.8	72.9	8	0	0.2



Supplementary Figure 1. The mutations in *ARID1A, BRAF, CDKN2A* and *MLL3* detected in the case 1 patient were confirmed by Sanger sequencing.

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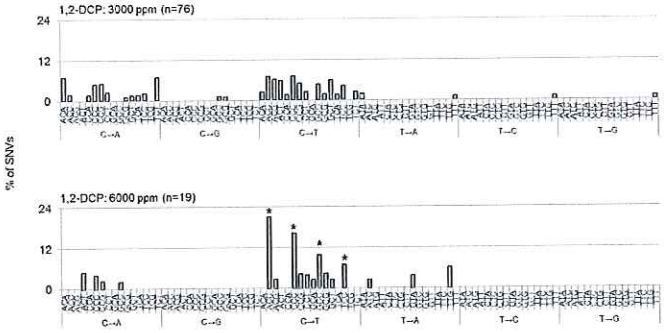

Supplementary Figure 2. The mutational signature of 1,2-DCP in *S. typhimurium* TA100 partially recapitulates the printing workers' cholangiocarcinoma signature represented by NpCpC to NpTpC changes. The clones exposed to 6,000 ppm of 1,2-DCP showed a more remarkable signature of NpCpC to NpTpC changes than the clones exposed to 3,000 ppm (indicated by asterisks).

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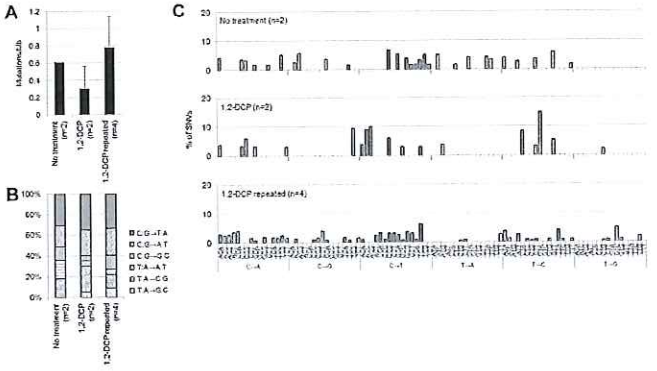

Supplementary Figure 3. Mutational profile of 1,2-DCP-exposed cholangiocarcinoma cells (NCC-CC1). (A) Number of mutations in 1,2-DCP-exposed NCC-CC1 cells. (B) Single-nucleotide DNA substitution profile in NCC-CC1 cells exposed to 1,2-DCP. (C) The trinucleotide mutational pattern of NCC-CC1 cells exposed to 1,2-DCP. The mutational signatures were normalized using the trinucleotide frequency in the genome.

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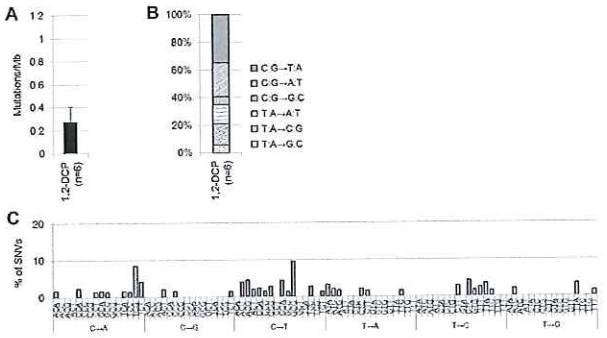

Supplementary Figure 4. Mutational profiles of 1,2-DCP-exposed human embryonic kidney 293 cells (HEK293). (A) Number of mutations in 1,2-DCP-exposed HEK293 cells. (B) Single-nucleotide DNA substitution profile in HEK293 cells exposed to 1,2-DCP. (C) The trinucleotide mutational pattern of HEK293 cells exposed to 1,2-DCP. The mutational signatures were normalized using the trinucleotide frequency in the genome.

190x142mm (300 x 300 DPI)

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ORIGINAL ARTICLE

Screening and surveillance for occupational cholangiocarcinoma in workers exposed to organic solvents

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Abstract
Purpose This study aimed to establish an efficient strategy for screening and surveillance for occupational cholangiocarcinoma.
Methods We evaluated the consecutive changes in laboratory findings during regular health examinations and in abdominal ultrasonography findings before the diagnosis of occupational cholangiocarcinoma in nine patients. The results of laboratory tests and abdominal ultrasonography at the time of diagnosis were also examined.
Results In all patients, the serum γ-glutamyl transpeptidase (γ-GTP) activity increased several years before the diagnosis of cholangiocarcinoma. The serum alanine aminotransferase (ALT) activity also increased several years before the diagnosis, following an increase in the serum aspartate aminotransferase (AST) activity in most patients. Abdominal ultrasonography before the diagnosis revealed regional dilatation of the bile ducts, which continued to enlarge. At the time of diagnosis, the γ-GTP, AST, and ALT activities were increased in nine, seven, and seven patients, respectively. The regional dilatation of bile ducts without tumor-induced stenosis, dilated bile ducts due to tumor-induced stenosis, space-occupying lesions, and/or lymph node swelling were observed. The serum concentrations of carbohydrate antigen 19-9 (CA 19-9) and/or carcinoembryonic antigen (CEA) were increased in all patients.
Conclusions Regular health examinations with a combination of ultrasonography and laboratory tests including the γ-GTP, AST, ALT, CA 19-9, and CEA levels are useful for screening and surveillance for occupational cholangiocarcinoma.

Keywords Occupational cholangiocarcinoma · Screening and surveillance · Health examination · Organic solvent

Abbreviations

γ-GTP	γ-Glutamyl transpeptidase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CA19-9	Carbohydrate antigen 19-9

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CEA Carcinoembryonic antigen
DCM Dichloromethane
DCP 1,2-Dichloropropane
PSC Primary sclerosing cholangitis
MRI Magnetic resonance imaging
MRCP Magnetic resonance cholangiopancreatography
ERCP Endoscopic retrograde cholangiopancreatography

Introduction

An outbreak of cholangiocarcinoma among workers at a printing company was recently reported [1, 2]. Although the mechanism underlying the development of cholangiocarcinoma is still unknown, long-term exposure to chemicals that include high concentrations of dichloromethane (DCM) and/or 1,2-dichloropropane (DCP) is strongly suspected to be a cause of the disease [1–3]. The Ministry of Health, Labour and Welfare of Japan classified this type of cholangiocarcinoma as "occupational cholangiocarcinoma" on October 1, 2013 [3]. Thirty-six patients, some of whom have been described in previous reports [2, 4], had been diagnosed with occupational cholangiocarcinoma as of February 2015.

Early detection of cholangiocarcinoma is essential, because the treatment outcomes for cholangiocarcinoma are still unsatisfactory [5–10]. Of 17 patients diagnosed with occupational cholangiocarcinoma following employment in the aforementioned printing company in Osaka, 11 patients were diagnosed on further examination after a regular health examination revealed abnormal findings on either laboratory tests or ultrasonography [2]. This demonstrates the importance of regular health examinations for screening and surveillance for occupational cholangiocarcinoma. In addition, previous studies revealed that abnormal liver function test results were observed several years before the diagnosis of cholangiocarcinoma [11, 12]. However, an efficient strategy for the detection of occupational cholangiocarcinoma during the health examinations of workers with long-term exposure to high concentrations of organic solvents remains to be elucidated. The aim of this study was to establish an efficient strategy for screening and surveillance for occupational cholangiocarcinoma through the evaluation of changes in the results of laboratory tests in nine patients with occupational cholangiocarcinoma. These patients were selected because the consecutive results of laboratory tests performed prior to the diagnosis of cholangiocarcinoma could be obtained for this group. The consecutive results of abdominal ultrasound studies performed before the diagnosis of cholangiocarcinoma were also available and evaluated in two patients.

Patients and methods

The subjects included in this study were nine patients with occupational cholangiocarcinoma (Table 1). Of the nine patients, six worked at a single printing company in Osaka (Company A) [2], two worked at another company (Company B), and one worked at third company (Company C) [4]. Of the nine patients, seven patients (patients 1, 3–8) were exposed to a high concentration of DCP, one (patient 9) was exposed to a high concentration of DCM and one (patient 2) was exposed to high concentrations of both DCP and DCM. In four patients, cholangiocarcinoma was diagnosed while they were currently working at the printing companies, while the cholangiocarcinoma was diagnosed after the end of the exposure in the remaining patients. The interval between the end of the exposure and the diagnosis of cholangiocarcinoma ranged from 3 years and 10 months to 12 years. The consecutive results of laboratory tests that were performed at regular health examinations before the diagnosis of cholangiocarcinoma were available for all nine patients. The changes in the serum activities of γ-glutamyl

Table 1 Clinical findings and laboratory test results of the nine patients with occupational cholangiocarcinoma

Patient no.	Company	Age	Gender	Exposure	Alcohol abuse	Smoking	T-Bil (mg/dl)	AST (IU/l)	ALT (IU/l)	γ-GTP (U/l)	CEA (ng/ml)	CA19-9 (IU/l)
1	A	25	Male	DCP	Yes	Yes	0.8	75	112	1729	8832	30.5
2	A	35	Male	DCM, DCP	No	Yes	6.4	148	306	2457	1.6	119.2
3	A	31	Male	DCP	No	Yes	1.4	153	311	1196	5.1	1084
4	A	39	Male	DCP	No	No	0.5	45	92	486	5.4	20.6
5	A	39	Male	DCP	No	Yes	0.5	30	34	347	1.5	105
6	A	31	Male	DCP	No	No	0.6	18	14	75	2.1	501
7	B	37	Male	DCP	No	Yes	1.02	114	212	526	2.5	2418
8	B	42	Male	DCP	No	Yes	10	188	321	1404	3.1	23253
9	C	49	Male	DCM	No	No	1.2	84	137	2034	0.7	565.3

DCP 1,2-dichloropropane, *DCM* dichloromethane

transpeptidase (γ-GTP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were evaluated because abnormal results on these tests have often been observed at the diagnosis of occupational cholangiocarcinoma [2, 4].

In two patients, the consecutive results of abdominal ultrasound studies were obtained from two hospitals where these two patients were followed up because of abnormal liver function tests. We evaluated tumor lesions, such as space-occupying lesions in the liver, papillary or protruding tumorous lesions in the bile duct, dilatation of the bile ducts, and lymph node swelling detected by ultrasonography.

This study was approved by the ethics committee of Osaka City University (No. 2368), and all subjects or their legally authorized representatives (for deceased patients) provided written informed consent.

Results

Changes in laboratory test results

The consecutive results of tests of the serum activities of γ-GTP, AST and ALT are shown in Fig. 1. In all nine patients, the serum γ-GTP activity was increased several years before the detection of cholangiocarcinoma. The serum ALT activity also increased several years before the detection of cholangiocarcinoma, following an increase in the serum AST activity in most patients. In patients 3, 4 and 5, the serum activities of γ-GTP, AST and ALT increased gradually after the end of the exposure. In patient 5, cholangitis occurred 1 month before the diagnosis of cholangiocarcinoma. Patient 6 retired from printing company A because of extremely increased activity levels of γ-GTP, AST and ALT, and the activity levels gradually decreased after retirement.

Changes in abdominal ultrasonographic images

In patient 5, regional dilatation of the bile ducts in the posterior segment and the lateral segment was detected 5 months before the diagnosis of cholangiocarcinoma, and these were subsequently enlarged (Fig. 2). At the diagnosis of cholangiocarcinoma, regional dilatation of the bile ducts without tumor-induced stenosis and tumorous lesions in the bile duct with thick walls in the lateral segment was observed. In patient 6, regional dilatation was first detected 5 years and 3 months before the diagnosis of cholangiocarcinoma, and the ducts continued to enlarge gradually (Fig. 3). At the diagnosis of cholangiocarcinoma, regional dilatation of the bile ducts, dilatation of the intrahepatic bile ducts due to tumor-induced stenosis, space-occupying lesions, and/or lymph node swelling were observed in nine patients.

The results of laboratory test results and abdominal ultrasonography

At the time of diagnosis of cholangiocarcinoma, the serum activities of AST and ALT were elevated in seven of the nine patients. The serum γ-GTP activity was elevated in all nine patients (Table 1). The serum concentrations of carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) were elevated in eight and three patients, respectively; the serum concentrations of CA 19-9 and/or CEA were elevated in all nine patients. Among the nine patients, ultrasonography detected tumorous lesions in six patients, space-occupying lesions in the liver (Fig. 3k) in three patients (patients 1, 6, and 7) and tumorous lesions in the bile ducts (Fig. 2f) in four patients (patients 2, 3, 5, and 9). Ultrasonography also showed wall thickening of the bile ducts (hyperechoic wall) (Fig. 2f) in one patient (patient 5). Dilated intrahepatic bile ducts with tumor-induced stenosis (Fig. 3f) were detected by ultrasonography in six patients (patients 1, 3, 4, 6, 8, and 9). Regional dilatation of the intrahepatic bile ducts without tumor-induced obstruction (Figs. 2, 3), which is a characteristic of patients with occupational cholangiocarcinoma [2, 4], was detected in two patients (patients 5 and 6). Lymph node swelling (Fig. 3e) was observed in one patient (patient 6). Abnormal findings indicating the possibility of malignant disease were detected by ultrasonography in all nine patients.

Discussion

Early detection of cholangiocarcinoma, while it is still in the resectable stage, is essential, because complete resection is the most effective and curative treatment. However, the early diagnosis of cholangiocarcinoma is difficult, because the signs and symptoms of the disease are often nonspecific. Of 17 patients who were diagnosed to have occupational cholangiocarcinoma following employment at a printing company in Osaka, 11 patients were diagnosed on further examination after a regular health examination revealed abnormal findings on either laboratory tests or ultrasonography [2]. This demonstrates the importance of regular health examinations for screening and surveillance for occupational cholangiocarcinoma.

Exposure to organic solvents induced liver dysfunction in the patients diagnosed with occupational cholangiocarcinoma. In previous studies, pathological examinations of resected specimens demonstrated chronic bile duct injury and/or cholestasis due to cholangiocarcinoma [2, 4, 13]. In the current study, the serum γ-GTP activity was found to

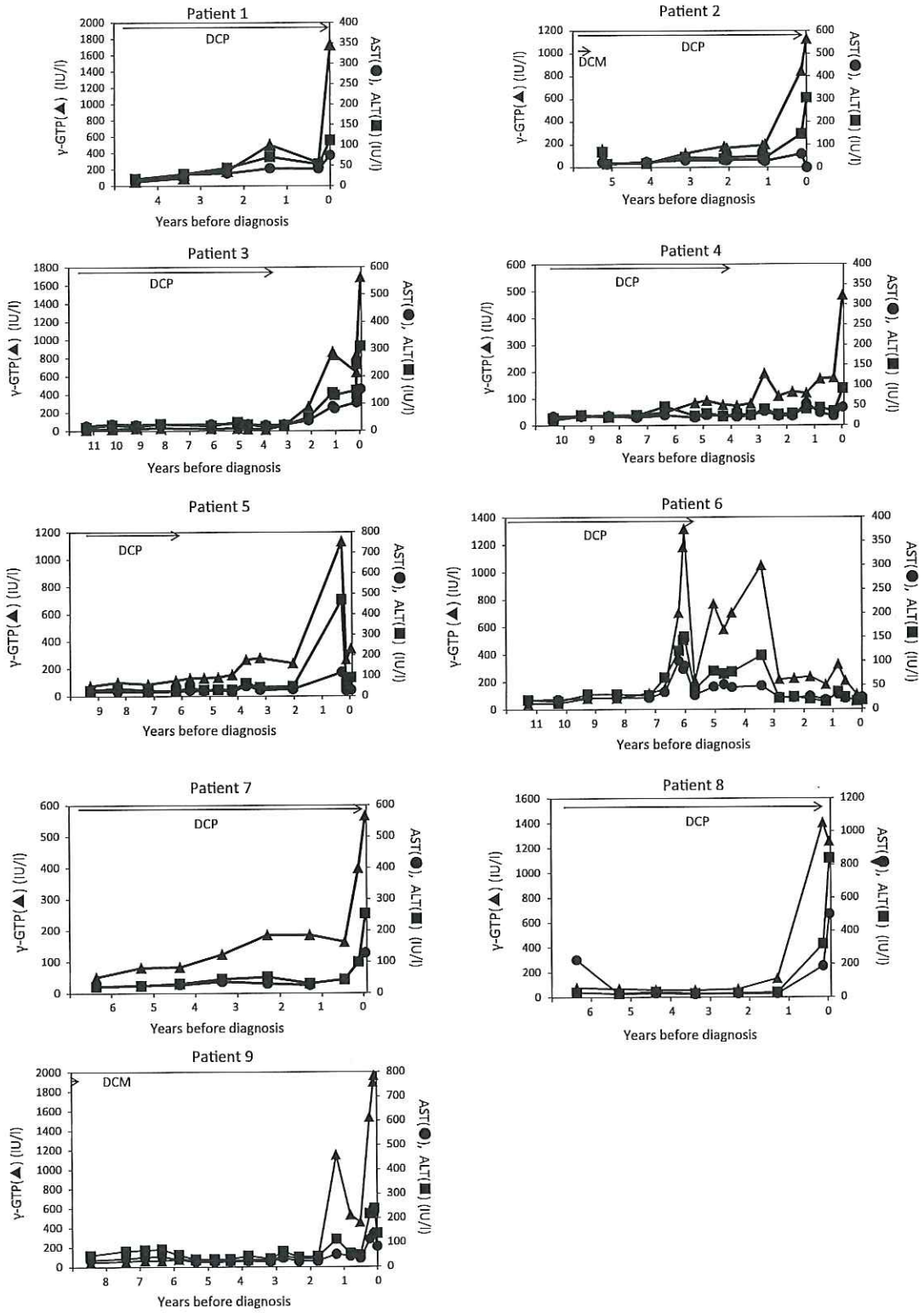

Fig. 1 The changes in the laboratory test results before the diagnosis of cholangiocarcinoma. *Closed triangles* γ-GTP; *closed circles* aspartate aminotransferase; *closed squares* alanine aminotransferase. The *arrows* show the term of the exposure to 1,2-dichloropropane (DCP) and dichloromethane (DCM)

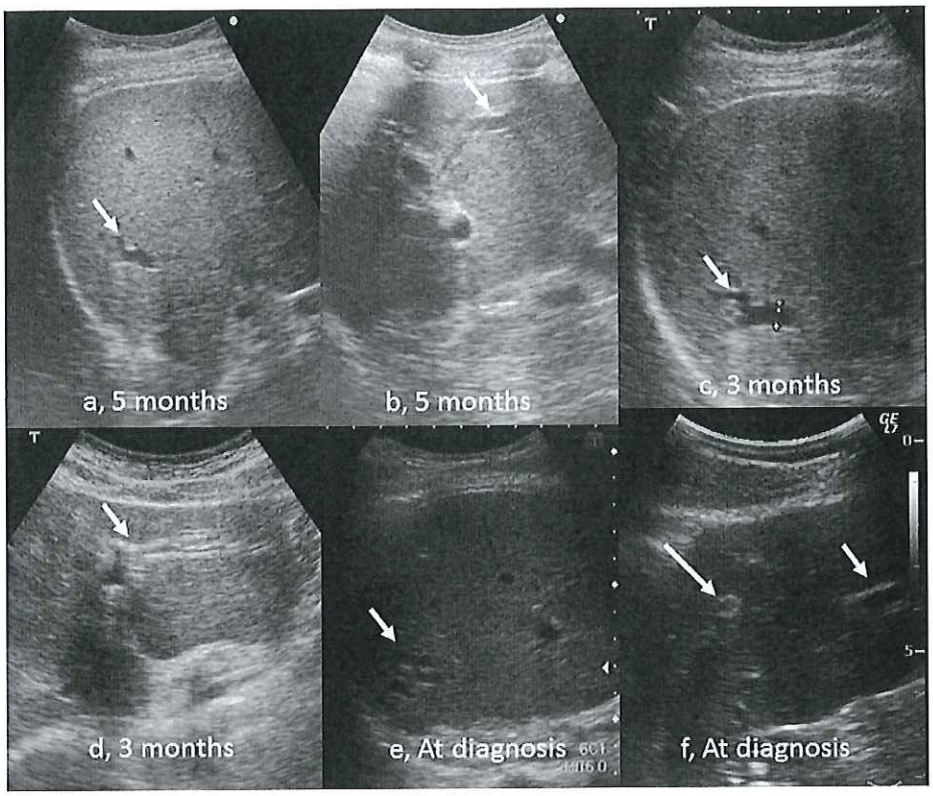

Fig. 2 The changes in the findings of abdominal ultrasound studies before the diagnosis of cholangiocarcinoma in patient 5. The *times* indicate the duration before the detection of cholangiocarcinoma. *Short arrows* show the regional dilatation of the bile ducts without tumor-induced stenosis. The *long arrow* shows a tumorous lesion in the bile duct with wall thickening (hyperechoic wall)

have increased several years before the diagnosis of cholangiocarcinoma. The serum ALT activity was also increased several years before the detection of cholangiocarcinoma, following an increase in the serum AST activity in most patients. As a result, the serum γ-GTP activity was elevated in all nine patients with occupational cholangiocarcinoma. These findings suggest that the observed liver dysfunction might be related to chronic bile duct injury and that the development of precancerous lesions and/or cholangiocarcinoma was due to the exposure to chlorinated organic solvents. Therefore, consecutive assessment of the liver function with tests for AST, ALT and γ-GTP is useful for the evaluation of bile duct and liver injury, and for estimating the risk of cholangiocarcinoma during regular health examinations. However, these tests cannot detect cholangiocarcinoma itself, and are not a definitive method for diagnosing cholangiocarcinoma.

The serum concentration of CA 19-9 is elevated in 60–80 % of patients with intrahepatic cholangiocarcinoma [5–7, 14–16]; testing the serum concentrations of CA 19-9 is currently widely used to detect cholangiocarcinoma, particularly in patients with primary sclerosing cholangitis (PSC), which is a risk factor for cholangiocarcinoma [14, 17–21]. It is also known that the serum concentrations of CEA are often elevated in patients with cholangiocarcinoma [5, 15, 16] and high serum concentrations of CEA suggest intrahepatic cholangiocarcinoma in patients with hepatolithiasis [22, 23]. In the current study, the serum concentrations of CA 19-9 and CEA were increased at the time of diagnosis of cholangiocarcinoma in three patients and eight patients, respectively. The serum concentrations of CA 19-9 and/or CEA were elevated in all nine patients at the time of diagnosis. This demonstrates that measurement of the serum concentrations of CA 19-9 and CEA is useful in indicating the possibility of cholangiocarcinoma.

In this study, abdominal ultrasonography revealed the gradual enhancement of the regional dilatation of the bile ducts before the diagnosis of cholangiocarcinoma in two patients in whom consecutive ultrasonography results were available. Ultrasonography detected abnormal findings (tumorous lesions and/or dilated bile ducts) in all nine patients at the time of diagnosis of cholangiocarcinoma. Thus, although it is difficult to use ultrasonography to obtain a definitive diagnosis of cholangiocarcinoma, ultrasonography is useful for screening and surveillance for occupational

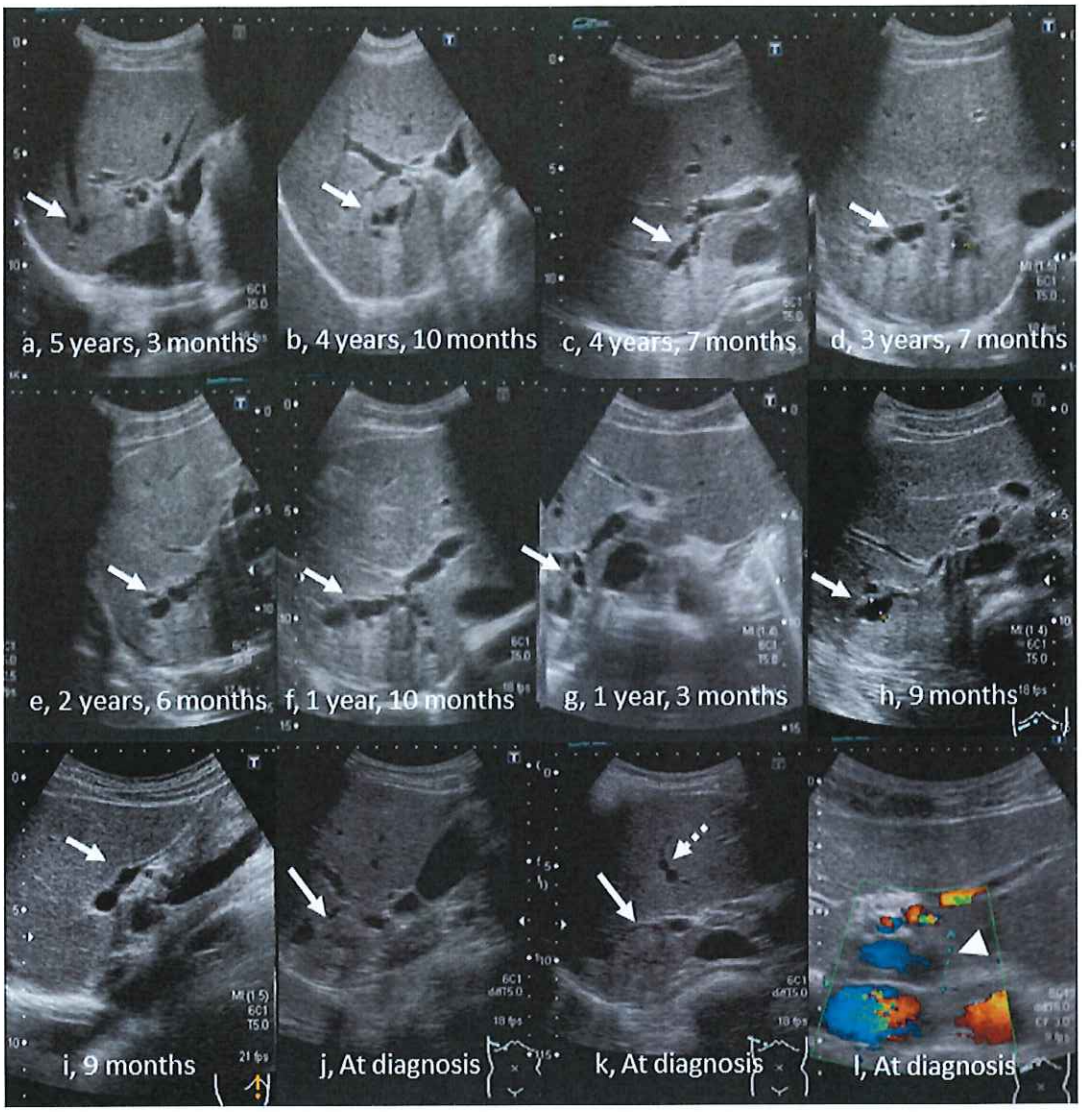

Fig. 3 The changes in the findings of abdominal ultrasound studies before the diagnosis of cholangiocarcinoma in patient 6. The *times* indicate the duration before the detection of cholangiocarcinoma. *Short arrows* show the regional dilatation of the bile ducts without tumor-induced stenosis. *Long arrows* show a space-occupying lesion in the liver. The *dotted arrow* shows dilatation of the bile duct due to tumor-induced stenosis. The *arrow head* shows lymph node swelling

cholangiocarcinoma because of its high rate of detection of abnormal findings and its noninvasive nature.

For the surveillance for cholangiocarcinoma in patients with PSC, a combination of the measurement of the serum concentrations of CA 19-9 and ultrasonography or magnetic resonance imaging/magnetic resonance cholangiopancreatography (MRI/MRCP) is recommended [24]. Endoscopic retrograde cholangiopancreatography (ERCP) is reserved for patients with an increased serum concentration of CA 19-9 or imaging evidence of dominant strictures. For regular health examinations, noninvasive and cost-effective methods are preferred. The results of the current study demonstrate that a combination of ultrasonography and laboratory tests including the γ-GTP, AST, ALT, CA 19-9 and CEA levels is useful for screening and surveillance for occupational cholangiocarcinoma in workers who are exposed to chlorinated organic solvents.

For patients with PSC, measurement of the serum concentrations of CA19-9 and ultrasonography at 12-month intervals is recommended for the screening and surveillance for cholangiocarcinoma [17, 24]. The incidence of cholangiocarcinoma seems to be higher in printing company workers (17 out of 101 workers in the offset color proof-printing department at the printing company in

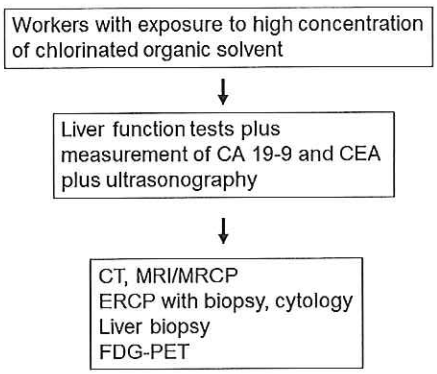

Fig. 4 The proposed program for the screening and surveillance for cholangiocarcinoma in workers exposed to chlorinated organic solvents

Osaka [2]) than in patients with PSC (their risk of subsequent development of cholangiocarcinoma was 0.5–1.5 % per year [25, 26]). Therefore, health examinations with laboratory tests and ultrasonography at least every 6 months may be recommended for workers who are exposed to high concentrations of chlorinated organic solvents. When ultrasonography or laboratory test results are abnormal, computed tomography, MRI, and/or MRCP should be performed to detect possible cholangiocarcinoma. Furthermore, ERCP with biopsy and/or cytology, or a liver biopsy, is recommended to obtain a definitive diagnosis (Fig. 4). However, it is necessary to evaluate this strategy in a prospective cohort study.

The longest period between the end of the exposure to the chlorinated organic solvents and the diagnosis of cholangiocarcinoma was 12 years. Thus, it is necessary to monitor the workers long term even after they switch jobs or retire.

In conclusion, regular health examinations with a combination of ultrasonography and laboratory tests including the γ-GTP, AST, ALT, CA 19-9 and CEA levels is useful for screening and surveillance for occupational cholangiocarcinoma in workers who are exposed to high concentrations of chlorinated organic solvents. Health examinations at least every 6 months, and for the long term even after the discontinuation of exposure are warranted.

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Compliance with ethical standards

Conflict of interest Shoji Kubo and co-authors have no conflicts of interest.

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ORIGINAL ARTICLE

Comparison of clinicopathological characteristics between patients with occupational and non-occupational intrahepatic cholangiocarcinoma

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Abstract

Background An outbreak of cholangiocarcinoma has been reported among workers of an offset color proof-printing department at a printing company in Japan. In this study, we compared the clinicopathological findings of this type of intrahepatic cholangiocarcinoma (occupational ICC) and non-occupational ICC.

Methods The clinical records of 51 patients with perihilar-type ICC who underwent liver resection, including five patients with occupational ICC were retrospectively reviewed. The clinicopathological features were compared.

Results In the occupational group, the patients were significantly younger ($P > 0.01$), while serum γ-glutamyl transpeptidase activity and the proportions of patients with regional dilatation of the bile ducts without tumor-induced obstruction were significantly higher ($P = 0.041$ and $P > 0.01$, respectively); the indocyanine green retention rate at 15 min was significantly lower ($P = 0.020$). On pathological examinations, precancerous or early cancerous lesions, such as biliary intraepithelial neoplasia and intraductal papillary neoplasm of the bile duct, were observed at various sites of the bile ducts in all occupational ICC patients; such lesions were observed in only six patients in the control group ($P > 0.01$).

Conclusions The clinicopathological findings including age, liver function test results, diagnostic imaging findings, and pathological findings differed between the occupational and control groups.

Keywords Biliary intraepithelial neoplasia · Intraductal papillary neoplasm of the bile duct · Occupational cholangiocarcinoma · Organic solvent · Printing company

Introduction

Cholangiocarcinoma arises from the epithelium of the intra- and extrahepatic bile ducts. Many studies have shown that the risk factors for cholangiocarcinoma include hepatolithiasis, primary sclerosing cholangitis (PSC), liver fluke infection, and hepatitis B and C virus infections [1–5]. Recently, an outbreak of cholangiocarcinoma has been reported among young adult workers of an offset color proof-printing department at a printing company in Osaka, Japan [6, 7]. These 17 patients with cholangiocarcinoma had been exposed to chlorinated organic solvents that were used to remove ink residues, including 1,2-dichloropropane (DCP) and/or dichloromethane (DCM) [6, 7]. This type of cholangiocarcinoma in these 17 patients and in 19 other workers who were exposed to high concentrations of DCP and/or DCM for a long term is recognized as "occupational cholangiocarcinoma (occupational biliary tract cancer)" by the Japanese Ministry of Health, Labour and Welfare, until August 2015 [8].

We have previously described the clinical findings, laboratory test results, diagnostic imaging results, pathological findings, treatments, and prognosis of patients with occupational intrahepatic cholangiocarcinoma (ICC) [7, 9–11]. However,

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in the previous studies, we investigated only patients with occupational cholangiocarcinoma; a comparison between patients with occupational cholangiocarcinoma and those with non-occupational cholangiocarcinoma was not performed. We have also reported that the main and most invasive cholangiocarcinoma lesions in patients with occupational cholangiocarcinoma were located in the large bile duct [7, 9, 10]; these were classified as perihilar type ICC [12–14]. Therefore, in this study, we compared the clinicopathological findings of the patients with occupational ICC and patients with non-occupational perihilar type ICC to clarify the characteristics of the patients with occupational ICC.

Methods

The records of 93 patients who underwent liver resection for ICC at two hospitals (i.e. Osaka City University Hospital and Ishikiriseiki Hospital) between January 1997 and December 2014 were retrospectively reviewed. Of the 93 patients, ICCs in 51 patients were classified as the perihilar type, and ICCs in the other 42 patients were classified as the peripheral type. The 42 patients with peripheral type ICC were excluded, because the occupational ICCs in five patients were classified into perihilar type ICC and because the pathogenesis and clinicopathological characteristics of perihilar large duct type ICC and peripheral small duct type ICC are different [12–14]. We investigated the clinical features, laboratory test results, diagnostic imaging results, pathological findings, treatments, and outcomes between patients with occupational ICC (i.e. the occupational group) and those with non-occupational ICC (i.e. the control group). Patient data, including the medical history, alcohol intake history, clinical findings, and laboratory test results, and diagnostic imaging results were obtained from the medical records and films, respectively.

The pathological findings were recorded and described according to the World Health Organization classification of ICC [13]. Tumors involving the large bile ducts comparable in size to the intrahepatic second branches were considered the perihilar type, and tumors involving the smaller ducts comparable in size to the segmental branches were considered the peripheral type [12–14]. The macroscopic types of ICC were grossly classified as the mass-forming (MF), periductal infiltrating (PI), or intraductal growth (IG) type. Surgical specimens were cut into 5-mm slices and fixed in 10% formalin. Permanent sections were prepared after staining with hematoxylin and eosin. Preneoplastic or early preinvasive neoplastic lesions of the biliary tree were classified as flat dysplastic epithelial tumors (biliary intraepithelial neoplasia: BilIN) or grossly visible papillary tumors (intraductal papillary neoplasm of the bile duct: IPNB) [13, 15–18]. BilIN lesions were histologically classified according to their cellular and structural features as BilIN-1 (mild atypia), BilIN-2 (moderate atypia), or BilIN-3 (severe atypia corresponding to in situ carcinoma). BilIN-1 lesions presented with mild atypical cellular and nuclear features, such as nuclear membrane irregularities or nuclear enlargement with only minimal disturbances to cellular polarity. BilIN-2 lesions had evident aberrant cellular and nuclear features not sufficient to suggest overt carcinoma; these lesions also had focal disturbance in cellular polarity. BilIN-3 lesions presented with diffuse disturbances in cellular polarity with or without distinct atypical cellular and nuclear features that corresponded to carcinoma in situ. In this study, only BilIN-2 and BilIN-3 lesions were examined because it is controversial whether BilIN-1 lesions contain any reactive hyperplastic changes. "Chronic bile duct injury" was used as a collective term to describe duct injuries such as epithelial damage, fibrosis of the duct wall and periductal tissue, and chronic inflammatory cell infiltration, in various combinations [7, 9]. In this study, two patients with biliary cystoadenocarcinoma were excluded.

Two or more segmentectomies were considered major hepatectomy while one segmentectomy or limited resection was considered minor hepatectomy. Lymph node involvement was diagnosed on pathologic examination of the resected lymph nodes. After hospital discharge, patients were followed-up at the outpatient clinic. For each patient, biochemical test and tumor marker measurements, including carcinoembryonic antigen and carbohydrate antigen 19–9 (CA19–9), and diagnostic imaging using computed tomography, ultrasonography, or magnetic resonance imaging were performed every 3 months.

Statistical analysis

The Student's *t*-test or the Mann–Whitney *U*-test was used to determine differences in age and laboratory test results. The χ^2 test or Fisher's exact test was used to evaluate significant differences in the categorical data between groups. Survival data were calculated from the date of resection until 30 September 2015 or the date of death. The cumulative survival rates were calculated by using the Kaplan–Meier method, and the significant differences between groups were evaluated by using the log-rank test. Differences with $P > 0.05$ were considered statistically significant. Statistical analysis was performed with JMP 9.0 (SAS Institute, Cary, NC, USA).

This study was approved by the ethics committee of Osaka City University, and all subjects or their legally authorized representatives provided written informed consent.

Results

Patient characteristics

The 51 patients with perihilar type ICC included 33 men and 18 women, with the age ranging from 31 to 83 years (median, 65 years). Of the 51 patients, five patients had occupational ICC (i.e. the occupational group); the other 46 patients were included in the control group. The five patients in the occupational group were former or current workers of an offset color proof-printing department at a printing company in Osaka [7]. Of the five patients, one patient was exposed to a high concentration of DCP and DCM and the other four patients were exposed to a high concentration of DCP alone. The period of the exposure ranged from 6 years and 1 month to 16 years and 1 month. The median observation period between the date of surgery and death or the study endpoint (September 2015) was 1057 days (range: 371–1799 days) in the occupational group and 643 days (range: 67–4739 days) in the control group.

Risk factors for ICC

Of the 46 patients in the control group, 10 patients (22%) were positive for anti-hepatitis C virus antibody, three patients (6.5%) were positive for hepatitis B virus surface antigen, two patients (4.4%) had been diagnosed with alcoholic hepatitis, and three patients (6.5%) had hepatolithiasis. No underlying liver diseases were detected in the remaining 28 patients (61%).

Clinical findings and laboratory test results

The mean age at diagnosis of the patients in the occupational group was 35 years (range, 31–39 years), and the mean age

Table 1 Clinical features and laboratory test results

Variables	Occupational (n = 5)	Control (n = 46)	P-value
Background			
Age	34 (31–39)	68 (32–83)	<0.01
Gender: male/female	5/0	28/18	0.15
BMI (kg/m^2)	23.8 (19.0–27.5)	22.4 (15.0–35.8)	0.34
Habitual alcoholic drinker[a]	0	7 (15%)	1.00
Diabetes mellitus	0	6 (13%)	1.00
Viral infection	0	13 (28%)	0.31
HBsAg positive	0	3 (6.5%)	1.00
HCVAb positive	0	10 (22%)	0.57
First momentum			
Presence of symptoms	1 (20%)	18 (39%)	0.64
Abnormal of laboratory data	4 (80%)	16 (35%)	0.071
Abnormal findings of diagnostic imaging	0	12 (26%)	0.32
Laboratory data			
Total bilirubin (mg/dl)	0.6 (0.5–1.4)	0.8 (0.3–22.7)	0.70
Albumin (g/dl)	4.4 (3.5–4.9)	4.1 (3.0–4.9)	0.34
Prothrombin time (%)	103 (91–120)	96 (58–142)	0.27
ICGR15 (%)	4.7 (3.0–7.6)	9.0 (2.3–31.5)	0.020
AST (IU/l)	30 (18–39)	29 (19–164)	0.68
ALT (IU/l)	46 (14–100)	34 (9–556)	0.28
Platelet counts (x10 /μl)	23.9 (19.1–32.5)	18.8 (5.4–38.2)	0.044
γ-GTP (IU/l)	347 (75–785)	105 (17–620)	0.041
γ-GTP > 60 (IU/l)	5 (100%)	31 (67%)	0.30
CEA > 5 (ng/ml)	1 (20%)	12 (26%)	1.00
CA19–9 > 37 (U/ml)	4 (80%)	28 (61%)	0.64

[a] > 60 g/day of ethanol consumption

Continuous variables including age, body mass index (BMI), and laboratory test results were expressed as median and range. *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *CA* carbohydrate antigen, *CEA* carcinoembryonic antigen, *γ-GTP* γ-glutamyl transpeptidase, *HBsAg* hepatitis B surface antigen, *HCVAb* anti-hepatitis C virus antibody, *ICGR15* indocyanine green retention rate at 15 min

was significantly lower in the occupational group than in the control group ($P > 0.01$, Table 1). Although all patients in the occupational group were men, there was no significant difference in sex distribution between the groups. In addition, there were no significant differences in the proportion of patients with habitual alcohol intake, diabetes mellitus, and infections with the hepatitis B or C virus. In the occupational group, one patient visited to the hospital because of right hypochondralgia and the four other patients visited the hospitals because of abnormal results for the liver function tests such as elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT), and/or γ-glutamyl transpeptidase (γ-GTP) activities during regular health examinations. In the control group, ICC was diagnosed by further examination for some symptoms such as abdominal pain and jaundice in 18 patients. In other 28 patients in the control group, ICC was diagnosed by further examination of abnormal laboratory examination results (16 patients) or abnormal findings on diagnostic imaging (12 patients) during treatment for other diseases including chronic hepatitis C, chronic hepatitis B, diabetes mellitus, hypertension, and hyperlipidemia. Although there was no significant difference, the proportion of patients who showed abnormal results on the laboratory tests tended to be higher in the occupational ICC group ($P = 0.071$).

At the diagnosis of cholangiocarcinoma, the platelet count was significantly higher in the occupational group than in the control group ($P = 0.044$). In the occupational group, γ-GTP activity was elevated in all the five patients; the serum γ-GTP activity was significantly higher in the occupational group than in the control group ($P = 0.041$). The indocyanine green retention rate at 15 min (ICGR15) was significantly lower in the occupational group than in the control group ($P = 0.020$). Although the serum CA19–9 concentrations of four patients in the occupational group were elevated, the proportion of patients with elevated serum CA19–9 concentration was not significantly different between the groups.

Diagnostic imaging results

On diagnostic imaging such as computed tomography, magnetic resonance imaging, and ultrasonography, space-occupying lesion(s) was detected in two patients in the occupational group (Table 2 and Fig. 1a) and in 38 patients (83%) in the control group. The bile duct with papillary, villous, or protruding tumors was detected in three patients in the occupational group (Fig. 1b,c) and in four patients (8.7%) in the control group. The proportion of patients in whom such tumors were detected was significantly higher in the occupational group than in the control group ($P = 0.015$). Stenosis or obstruction of the bile ducts was detected in one patient in the occupational group (Fig. 1d) and in 27 patients (59%) in the control group. Dilatation of the peripheral bile ducts due to tumor-induced obstruction was detected in one patient in the occupational group and in 30 patients (65%) in the control group.

The regional dilatation of the bile ducts without tumor-induced obstructions (Fig. 1d) – a characteristic of occupational cholangiocarcinoma [7, 9] – was detected in four patients in the occupational group and in one patient (2.2%) in the control group. The proportion of patients with regional dilatation of the bile ducts without tumor-induced obstructions was significantly higher in the occupational group than in the control group ($P > 0.01$).

Table 2 Diagnostic imaging results and treatment

Findings	Occupational ($n = 5$)	Control ($n = 46$)	P-value
Space occupying lesions	2 (40)	38 (83)	0.061
Bile ducts with papillary, villous, or protruding tumors	3 (60)	4 (8.7)	0.015
Stenosis or obstruction of the bile ducts	1 (20)	27 (59)	0.16
Dilated bile ducts due to tumor-induced obstruction	1 (20)	30 (65)	0.071
Dilated bile ducts without tumor-induced obstruction	4 (80)	1 (2.2)	<0.01
Operative methods			
Major hepatectomy	5 (100)	44 (96)	1.00
Resection of the extrahepatic bile duct	2 (40)	12 (26)	0.61
Resection of vessels	0	3 (6.5)	1.00
Dissection of lymph nodes	2 (40)	13 (28)	0.62
Adjuvant chemotherapy	4 (80)	23 (50)	0.35

Numbers in parentheses show percent.

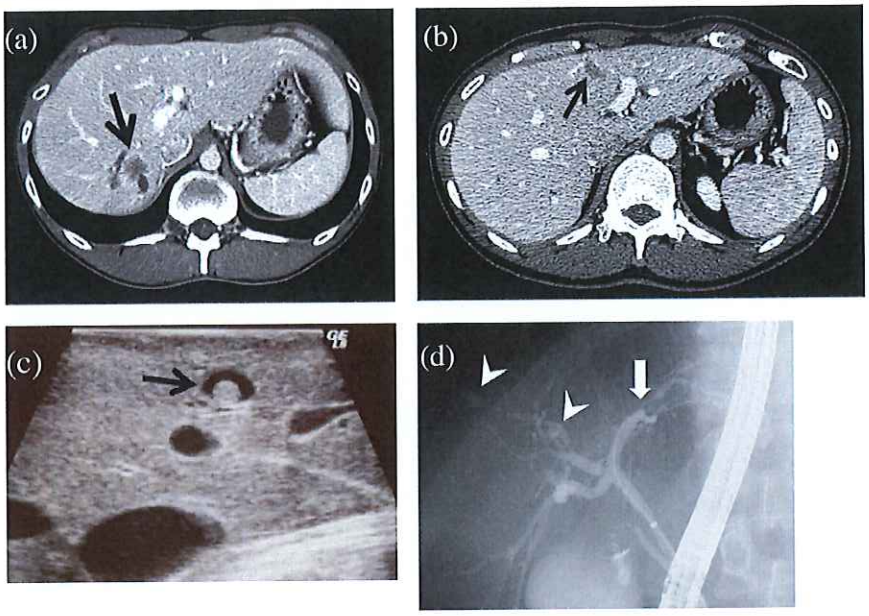

Fig. 1 Findings of diagnostic imaging results. (**a**) Space occupying lesion in a patient with occupational intrahepatic cholangiocarcinoma (arrow). Modified from [7]. (**b**, **c**) Bile duct with papillary tumor in a patient with occupational intrahepatic cholangiocarcinoma (arrow). (**d**) Stenosis of the bile duct (arrow), and dilated bile ducts without tumor induced obstruction (arrow heads). Modified from [7]

Treatment

Major hepatectomy was performed in all five patients in the occupational group and in 44 patients (96%) in the control group (Table 2). Resection of the extrahepatic bile duct and reconstruction of the biliary tract were performed in two patients in the occupational group and in 12 patients (26%) in the control group. Adjuvant chemotherapy using gemcitabine, tegafur/gimeracil/oteracil, cisplatin, or tegafur/uracil was administered to four patients in the occupational group and to 23 patients (50%) in the control group.

Pathological findings

In the occupational group, the main lesion of ICC was macroscopically classified into the MF type in two patients and the IG type in three patients (Table 3). In the control group, the main lesion of ICC was classified into the MF type in 39 patients, the PI type in four, and the IG type in three. The distribution of the types was significantly different between the groups; the proportion of the IG type was significantly higher in the occupational group than in the control group ($P > 0.012$). The proportions of patients with serosal invasion, vascular invasion, and lymph node metastasis were not

Table 3 Pathological findings

Findings	Occupational ($n = 5$)	Control ($n = 46$)	P-value
Macroscopic findings			
MF/PI/IG	2/0/3	39/4/3	0.012
Tumor size (cm, median + range)	2.1 (0.5–20)	3.9 (0.8–12.5)	0.23
Histological findings			
Serosal invasion	0	14 (30)	0.31
Vascular invasion	1 (20)	24 (52)	0.35
Lymph node metastasis	1 (20)	17 (37)	0.64
Precancerous or early cancerous lesion	5 (100)	6 (13)	<0.01
BilIN-2/3	5 (100)	4 (8.7)	<0.01
IPNB	5 (100)	2 (4.4)	<0.01
Chronic bile duct injury	5 (100)	21 (46)	0.051
Cirrhosis	0	1 (2.2)	1.00

In histological findings, numbers in parentheses show percent.
IG intraductal growth, *IPNB* intraductal papillary neoplasm of the bile duct, *MF* mass-forming, *PI* periductal infiltrating

different between the groups. All five patients in the occupational group had BilIN-2/3 lesions and IPNB or invasive IPNB. In the control group, BilIN-2/3 lesions were observed in four patients. Of the four patients with BilIN-2/3 lesions in the control group, three patients had hepatolithiasis and one patient had a history of exposure to organic solvents while working at a metalworking company. IPNB or invasive IPNB was observed in two patients in the control group. The proportions of patients with BilIN-2/3 lesions and patients with IPNB (or invasive IPNB) were significantly higher in the occupational group than in the control group ($P > 0.01$ for both). Chronic bile duct injury was observed in all five patients in the occupational group and in 21 patients in the control group. The proportion of patients with chronic bile duct injury tended to be higher in the occupational group ($P = 0.051$). No cirrhotic changes were detected in the noncancerous hepatic tissues in the occupational group; one patient in the control group had cirrhosis.

Surgical outcomes

One patient in the occupational ICC group died of ICC recurrence 1 year after surgery. ICC recurred in 29 patients in the control group. The shortest survival day in the control group was 67 days, and the patient died of sepsis (hospital death). The cumulative survival rates at 1 and 3 years in the control group were 76% and 44%, respectively. The cumulative survival rates did not differ between the groups ($P = 0.17$, Fig. 2).

Discussion

We have previously reported that relatively young age; elevated AST, ALT, and γ-GTP activities at diagnosis; regional dilatation of the intrahepatic bile ducts without tumor-induced obstruction of the bile duct; and PSC-like appearance such as multiple strictures of the bile ducts with or without fusiform dilatation on diagnostic imaging are characteristics of occupational cholangiocarcinoma; furthermore, the main tumor or most invasive lesions were located in the large bile duct [7, 9]. We also reported that AST, ALT, and γ-GTP activities had been observed to be increased several years before the diagnosis of cholangiocarcinoma in most patients with occupational ICC, and that regional dilatation of the intrahepatic bile ducts without tumor-induced obstruction of the bile ducts had been observed on imaging several years before the diagnosis in some patients [7, 9, 10, 19, 20]. On pathological examination, chronic bile duct injuries and precancerous or early cancerous lesions, such as BilIN and IPNB, are observed at various sites of the bile ducts [7, 9, 10]. In this study, we compared the characteristics of patients with occupational ICC and those with non-occupational ICC of the perihilar type who underwent surgical treatments at the same hospitals during the same period.

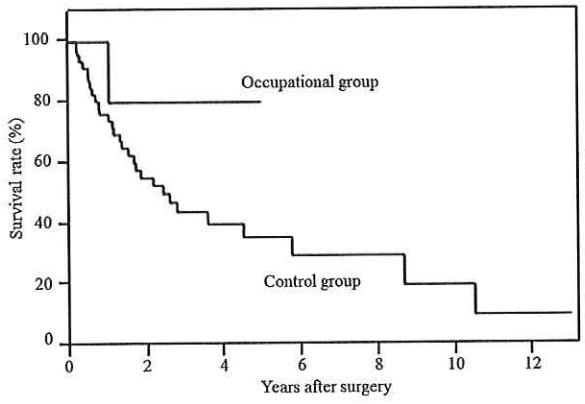

Fig. 2 Cumulative survival rate after surgery in patients with occupational intrahepatic cholangiocarcinoma and non-occupational intrahepatic cholangiocarcinoma

Intrahepatic cholangiocarcinoma usually occurs in patients in their sixth or seventh decade of life [1, 3, 21, 22]. In this study, the mean patient age was 35 years in the occupational group and 68 years in the control group. The mean age in the occupational group was relatively low. This finding is related to the relatively young age of most workers in the printing company and suggests that the exposure to chlorinated organic solvents can induce malignant transformation during a short time.

Four of the five patients in the occupational group visited the hospital because of abnormal findings such as elevated AST, ALT, and γ-GTP activities noted during regular health examinations at the company. The proportion of patients who visited the hospitals for abnormal results on laboratory tests tended to be higher in the occupational group than in the control group. In addition, in some patients in the occupational group, AST, ALT, and γ-GTP activities increased several years before the diagnosis of ICC [19, 20]. These findings indicate that regular health examination is important to detect abnormality of the biliary tract for workers exposed to organic solvents.

In the laboratory test results, the platelet count was significantly lower and the ICGR15 was significantly higher in the control group than in the occupational group. The control group included patients with chronic hepatitis C, chronic hepatitis B, and alcoholic liver disease. On the other hand, no patients had in the occupational group these underlying liver diseases. The differences in the platelet count and ICGR15 between the groups were related to the differences in the underlying diseases.

On diagnostic imaging, the proportion of patients with regional dilatation of the bile ducts without tumor-induced obstructions, that were noted on cholangiography and appeared

to correspond to those of PSC, was significantly higher in the occupational group than in the control group. Such characteristics of occupational ICC that have been reported in previous studies [7, 9, 10] were confirmed in this study.

On pathological examination, the proportions of patients with chronic bile duct injury, BilIN, and IPNB were significantly higher in the occupational group than in the control group. These BilIN and IPNB lesions have been reported in patients with hepatolithiasis, liver fluke infection, and chronic hepatitis C [14–17, 23–25]. Therefore, chronic inflammation caused by hepatolithiasis, liver fluke infection, and hepatitis virus induces neoplastic transformation through dysplastic changes, subsequently resulting in cholangiocarcinoma. Although such lesions are detected in the affected hepatic segments in patients with hepatolithiasis, such lesions were detected at the various sites of the bile ducts in patients in the occupational group. These findings indicated that the exposure to chlorinated organic solvents caused bile duct injury and induced carcinogenic processes at the various sites of the bile ducts; these findings are important characteristics of occupational ICC. In this study, BilIN lesions were detected in four patients in the control group; three of the four patients had hepatolithiasis and one patient had a history of exposure to organic solvents in a metalworking company. Thus, the exposure to organic solvents may induce cholangiocarcinoma in workers except at the printing company.

Although IPNB lesions were detected in all patients in the occupational group, IPNB lesion(s) (invasive IPNB) was detected in only two patients in the control group. In the occupational group, two patients had MF type ICC and three patients had IG type ICC. Aishima et al. reported that ICC can be classified into the perihilar large bile duct type and the peripheral small duct type [14]. In the perihilar large bile duct type, chronic biliary inflammation caused by PSC, hepatolithiasis, and liver flukes may induce BilIN or PI type cholangiocarcinoma via one pathway. These tumor type progress with invasion to the liver parenchyma, then adopt the morphologic features of the MF and PI type tumors (i.e. the MF + PI type). Another pathway shows the intraductal papillary or tubular growth of the tumors (IG type cholangiocarcinoma and IPNB with invasion). Thus, the carcinogenic pathway through BilIN proceeded mainly in patients with MF type ICC, and the pathway through IPNB proceeded mainly in patients with the IG type. In this study, the proportion of patients with IPNB was significantly higher in the occupational group than in the control group. These findings were related to the higher proportion of patients with papillary, villous, or protruding tumors on the diagnostic imaging and that with IG type ICC in the occupational group.

Although the cumulative survival rates were not different between the groups, the number of patients was too small and the observation period was too short to evaluate the prognosis. Further studies are necessary to compare the difference in prognosis.

In conclusion, the clinicopathological findings including age, the results of liver function tests, and pathological findings differed between the occupational and control groups. Such differences in the clinicopathological findings might be based on the different carcinogenic processes between occupational ICC and non-occupational ICC. Therefore, it is necessary to investigate the detailed mechanisms of the development of occupational ICC.

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Conflict of interest None declared.

Author contribution Study design: GH and SK designed the study. Acquisition of data: GH, SK, S. Takemura, S. Tanaka, HS, MK, TI, TY. Pathological aspect of the study: KW and TY analyzed pathological findings. Data analysis: GH, SK, S. Takemura, S. Tanaka, MK, TI, TS. Manuscript drafted by GH, SK, and TS. All authors reviewed the manuscript.

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ORIGINAL ARTICLE

Pathological spectrum of bile duct lesions from chronic bile duct injury to invasive cholangiocarcinoma corresponding to bile duct imaging findings of occupational cholangiocarcinoma

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Abstract

Background We aimed to identify the pathological characteristics of occupational cholangiocarcinoma.

Methods We examined the location and distribution of the carcinomas: atypical epithelium including biliary intraepithelial neoplasia (BilIN) and intraductal papillary neoplasm of the bile duct (IPNB); and chronic bile duct injuries in operative or autopsy liver specimens from 16 patients. We examined the detailed pathological findings and diagnostic imaging of three patients. Immunohistochemical analysis using primary antibodies against γH2AX and S100P was performed.

Results BilIN and chronic bile duct injury were observed in 16 patients, and IPNB or invasive IPNB was observed in 11 patients. BilIN, IPNB, and/or chronic bile duct injury were observed in almost all the large bile ducts. Regional dilatation of the bile ducts without tumor-induced obstruction revealed such pathological changes. Highly positive results for the γH2AX and S100P markers were noted in invasive carcinoma, BilIN, and IPNB, whereas positive results for γH2AX and negative results for S100P were noted in non-neoplastic biliary epithelium.

Conclusions The carcinogenic process of occupational cholangiocarcinoma comprised chronic bile duct injury and DNA damage in almost all the large bile ducts, along with induction of precancerous lesions and development of invasive carcinoma. Such pathological findings reflected radiological changes on diagnostic imaging.

Keywords 1,2-dichloropropane · Biliary intraepithelial neoplasia · Intraductal papillary neoplasm of the bile duct · Occupational cholangiocarcinoma · Printing company

Introduction

An outbreak of cholangiocarcinoma in printing companies in Japan was recently reported [1–3], and such cholangiocarcinoma was first recognized as an occupational disease by the Japanese Ministry of Health, Labour and Welfare in 2013 [4]. By September 2015, 36 patients, including 17 patients at the printing company in Osaka [1], were diagnosed with occupational cholangiocarcinoma by the Ministry. It was reported that 1,2-dichloropropane (DCP) and dichloromethane (DCM) play an important role in the development of this type of cholangiocarcinoma [1–4]. In June 2014, the International Agency for Research on Cancer decided to classify DCP as group 1 (carcinogenic to humans) and DCM as group 2A (probably carcinogenic to humans) [5]. We previously reported that an increased serum

concentration of gamma–glutamyl transpeptidase (γ-GTP) activity, the regional dilatation of the intrahepatic bile ducts without tumor-induced obstruction and biliary images similar to the appearance of primary sclerosing cholangitis (PSC), such as multiple strictures of the bile ducts with or without fusiform dilatation [6] on diagnostic imaging, papillary proliferating tumor, and the presence of precancerous or early cancerous lesions, such as biliary intraepithelial neoplasia (BilIN) and intraductal papillary neoplasm of the bile ducts (IPNB), and non-specific bile duct injuries, such as fibrosis, are characteristic of patients with occupational cholangiocarcinoma [1, 3, 7]. However, the number of subjects was small, and an extensive pathological examination and comparison between pathological findings and radiological findings have not been performed in previous studies [1, 3, 7].

In this study, we evaluated the pathological features of 16 patients with occupational cholangiocarcinoma. In addition, for three of the 16 patients, whose detailed radiological findings and whole operative specimens for extensive pathological examination were available, we examined the radiological and pathological findings more closely. We also discuss the possible carcinogenic progression of occupational cholangiocarcinoma.

Subjects and methods

Subjects

The subjects in this study consisted of 16 men with occupational cholangiocarcinoma who were former and current workers at printing companies in Japan (Hokkaido, Miyagi, Osaka, and Fukuoka prefectures) and whose operative or autopsy specimens, including hepatic tissue, were available for pathological examination (Table 1). They were exposed to various types of chlorinated organic solvents, including DCP, DCM, and 1,1,1-trichloroethane (TCE). We evaluated the clinicopathological findings in the 16 patients using surgical specimens from 14 patients (patients no. 1, 2, 4–12, and 14–16) and autopsy specimens from two patients (patients no. 3 and 13). We also performed a detailed pathological examination of the whole resected liver, including immunohistochemical analysis corresponding to the bile duct imaging findings, while making anatomical charts of the large bile ducts in three patients (patients no. 10–12). "Large bile duct" is used as a collective term that refers to the common hepatic duct, the left or right hepatic duct, or to the first to third branches of the intrahepatic bile duct [8].

This study was approved by the ethics committee of Osaka City University, and all subjects or their legally authorized representatives (for deceased patients) provided written informed consent. This multicenter occupational cholangiocarcinoma study group consisted of investigators at 15 institutes.

Pathological examination

We evaluated the clinicopathological characteristics, including the presence of BilIN or IPNB, in non-cancerous hepatic tissue, and the presence of chronic bile duct injury in the 16 patients. The pathological findings were evaluated by pathologists (Y.N. and Y.S.) according to the World Health Organization classifications for intrahepatic cholangiocarcinoma [9]. Pre- or early neoplastic lesions of the bile ducts were classified as BilIN or IPNB. BilIN lesions were histologically classified according to their cellular and structural features as BilIN-1 (mild atypia), BilIN-2 (moderate atypia), or BilIN-3 (severe atypia corresponding to *in situ* carcinoma). In this study, we mainly surveyed BilIN-2 and BilIN-3 lesions because it was unclear whether BilIN-1 lesions included reactive hyperplastic changes. "Chronic bile duct injury" is a collective term that refers to various combinations of duct injuries such as epithelial damage, fibrosis of the bile duct wall and periductal tissue, and chronic inflammatory cell infiltration.

We evaluated the detailed pathological findings of the whole resected liver while making anatomical charts of the large bile ducts in three patients (patients no. 10–12) as follows. Left hepatectomy and excision of segment 7 were performed in patient 10. Right hepatectomy and resection of the extrahepatic bile duct with hepaticojejunostomy and reconstruction were performed in patient 11. Extended left hepatectomy was performed in patient 12. To enable the extensive pathological examination of all the operative specimens, 56 histological sections (5-mm each) were obtained from the specimen of patient 10, 85 sections from the specimen of patient 11, and 124 sections from the specimen of patient 12. After macroscopic observation of the specimens, the sections were cut, embedded in paraffin, and stained with hematoxylin-eosin (HE). Observed lesions including the main tumor(s), precancerous lesions such as BilIN-2/3 and IPNB, and chronic bile duct injury on HE-stained specimens were cross-referenced with the anatomical charts of the bile ducts. The pathological findings were mapped to evaluate the correlation between the diagnostic imaging and pathological findings.

Immunohistochemistry

Immunological staining was performed using primary antibodies against S100P (1:100 rabbit monoclonal, Epitomics) and γH2AX (1:100 rabbit monoclonal; Novus Biologicals, Littleton, CO, USA) to evaluate neoplastic changes and DNA injury locations, respectively, in three patients

Table 1 Clinicopathological finding in 16 patients

Patients	Age	Gender	Organic solvents	Location and type of cholangiocarcinoma	Treatments	Specimens	BilIN-2/3	IPNB/ invasive IPNB	Chronic bile duct injury
1	34	M	DCP, DCM, TCE	ICC, mass-forming	Extended rt. hepatectomy, resection of extrahepatic bile duct	Surgery	Yes	Yes	Yes
2	34	M	DCP, DCM, TCE	ICC, mass-forming	Rt. trisegmentectomy	Surgery	Yes	Yes	Yes
3	25	M	DCP	ICC, mass-forming	Chemotherapy	Autopsy	Yes	ND	Yes
4	35	M	DCP, DCM, TCE	ECC (perihilar), papillary	Extended rt. hepatectomy, resection of extrahepatic bile duct	Surgery	Yes	Yes	Yes
5	40	M	DCP, DCM, TCE	ICC, mass-forming ECC (distal), papillary	Rt. Hepatectomy, Pancreaticoduodenectom	Surgery	Yes	Yes	Yes
6	38	M	DCP, DCM	ICC, mass-forming	Segmentectomy 8	Surgery	Yes	ND	Yes
7	40	M	DCP, DCM, TCE	ICC, intraductal growth	Extended lt. hepatectomy	Surgery	Yes	Yes	Yes
8	31	M	DCP	ECC (perihilar), papillary	Extended rt. hepatectomy, resection of extrahepatic bile duct	Surgery	Yes	Yes	Yes
9	39	M	DCP	ICC, intraductal growth ECC (perihilar), papillary	Lt. hepatectomy, resection of extrahepatic bile duct	Surgery	Yes	Yes	Yes
10	39	M	DCP	ICC, intraductal growth	Lt. hepatectomy, Segmentectomy 7	Surgery	Yes	Yes	Yes
11	31	M	DCP	ICC, mass-forming	Rt. hepatectomy, resection of extrahepatic bile duct	Surgery	Yes	Yes	Yes
12	34	M	DCP	ICC, intraductal growth	Extended lt. hepatectomy	Surgery	Yes	Yes	Yes
13	37	M	DCP, DCM[a], TCE	ICC, mass-forming	Chemotherapy	Autopsy	Yes	Yes	Yes
14	42	M	DCP, DCM[a], TCE	ECC (perihilar), scirrhous constricting	Extended rt. hepatectomy withpreoperative chemotherapy and radiotherapy	Surgery	Yes	No	Yes
15	57	M	DCP, DCM	ICC, mass-forming	Extended rt. hepatectomy, resection of extrahepatic bile duct	Surgery	Yes	No	Yes
16	47	M	DCP, DCM	ICC, mass-forming	Rt. trisegmentectomy	Surgery	Yes	ND	Yes

BilIN biliary intraepithelial neoplasia, *DCM* dichloromethan, *DCP* 1,2-dichloropropane, *ECC* extrahepatic cholangiocarcinoma, *ICC* intrahepatic cholangiocarcinoma, *IPNB* intraductal papillary neoplasm of the bile duct, *invasive IPNB* IPNB with an associated invasive carcinoma, *ND* not determined because of small amount of non-cancerous hepatic tissue, *TCE* 1,1,1-trichloroethane.

[a] The amount of DCM used was small

(patients no. 10–12). After deparaffinization, antigen retrieval was performed by autoclaving the sections in citrate buffer 10 mmol/l (pH 6.0). The sections were then immersed in 0.3% hydrogen peroxidase in purified water for 20 min at room temperature to block endogenous peroxidase activity. After pretreatment with blocking serum (Blocking One; NACALAI TESQUE), the sections were incubated overnight at 4°C with each of the primary antibodies. The sections were then incubated with a secondary antibody conjugated to peroxidase-labeled polymer using the HISTOFINE system (Nichilei, Tokyo, Japan). Color development was performed using 3,3′-diaminobenzidine

tetrahydrochloride, and the sections were lightly counterstained with hematoxylin. A semiquantitative analysis of the immunostained sections was performed. According to the mapping charts of invasive carcinoma, BilIN-2/3, IPNB, peribiliary gland, and chronic bile duct injuries were observed in fields at × 200 magnification, and the area of highest labeling of γH2AX and S100P nuclear expression were selected for each focus in the sections. The proportion of stained cells was evaluated as follows: – (negative); + (≤20%); and ++ (>20%).

Anatomical charts of the bile duct

Dynamic computed tomography (CT), ultrasonography (US), magnetic resonance cholangiopancreatography (MRCP), and endoscopic retrograde cholangiopancreatography (ERCP) studies were performed in these three patients. Anatomical charts of the large bile ducts were created to depict the pathological changes of the large bile ducts by reference to these diagnostic images in each case. The spectrum of biliary tract lesions including stricture or obstruction of the bile duct, regional dilatation of the intrahepatic bile ducts without tumor-induced obstruction were evaluated on these diagnostic images.

The liver anatomy and operative methods were classified according to Brisbane 2000 terminology [10].

Results

Clinicopathological findings of 16 patients

The age of the patients at diagnosis ranged from 25 to 57 years (mean, 37 years), and all patients were men (Table 1). Of the 16 patients, seven patients (patients no. 1, 2, 4, 5, 7, 13, and 14) had been exposed to DCP, DCM, and TCE; three patients (patients no. 6, 15, and 16) had been exposed to DCP and DCM; and six patients (patients no. 3, and 8–12) had been exposed to only DCP. The main tumor was classified as intrahepatic cholangiocarcinoma in 11 patients, and extrahepatic cholangiocarcinoma (perihilar) in three patients. One patient was diagnosed as having intrahepatic and extrahepatic (perihilar) cholangiocarcinoma, and another patient was diagnosed as having intrahepatic and extrahepatic (distal side) cholangiocarcinoma.

The histopathological examination showed that the main tumor in the 16 patients was adenocarcinoma. BilIN-2/3 lesions and chronic bile duct injury were observed in all 16 patients (Table 1). IPNB, including IPNB with an associated invasive carcinoma (invasive IPNB) and some mucus production, was observed in 11 patients (patients no. 1, 2, 4, 5, and 7–13). In three patients (patients no. 3, 6, and 16), the presence of IPNB could not be evaluated sufficiently because of the presence of small amounts of non-cancerous hepatic tissue. In four patients in whom the whole resected liver (patients no. 10–12) or the whole liver by autopsy (patients no. 13) were available for pathological examination, BilIN-2/3, IPNB, and chronic bile duct injury were observed in almost all of the large bile ducts.

Extensive pathological examination of the whole resected specimens

Patient 10

On the preoperative MRCP (Fig. 1a), regional dilatation of the peripheral bile ducts without tumor-induced obstruction was observed in segment 7 (B7). Multiple strictures and regional dilatation of the bile ducts in the left lobe were also seen. Macroscopic examination of the operative specimens showed the dilatation of B7 (Fig. 1b) and thickening of Glisson's sheath in the left lobe (Fig. 1c), corresponding to the MRCP findings. On the histopathological examination, well differentiated papillary adenocarcinoma with mucus-producing eosinophilic cytoplasm (intraductal growth type) was observed in the B7 dilatation (Fig. 2b,c), and it was diagnosed to be an oncocytic type invasive IPNB with minimal invasion. BilIN2/3 lesions with different morphology from that of the main lesion were observed in the peripheral portions of B7 (Fig. 2a). In the left lobe, BilIN-2/3 was observed throughout the bile ducts, which were not abnormal on the preoperative imaging (Fig. 2d). IPNB was observed in the peripheral bile ducts in segment 2 (B2), corresponding to the regional dilatation of the bile ducts seen on the preoperative image (Fig. 2e). Chronic multifocal bile duct injuries were found throughout the bile ducts.

Patient 11

A space-occupying lesion was observed in segment 6 on the preoperative CT (Fig. 1d). Dilated bile ducts in the posterior segment (peripheral side of the tumor) as well as multiple strictures and dilatation of the bile ducts in the anterior segment were observed on MRCP and ERCP (Fig. 1e). Macroscopic examination of the operative specimens showed a tumor in segment 6 (mass-forming type) and dilatation of the bile ducts (Fig. 1f). Histopathologically, a space-occupying lesion on preoperative diagnostic imaging was an oncocytic type of invasive adenocarcinoma with micropapillary lesions on the luminal sides and extramural invasion and periductal infiltration to the posterior branch (Fig. 3a,e,f). BilIN-2/3 lesions were observed in the peripheral portions of the bile ducts in segments 6 and 7 (B6, B7)

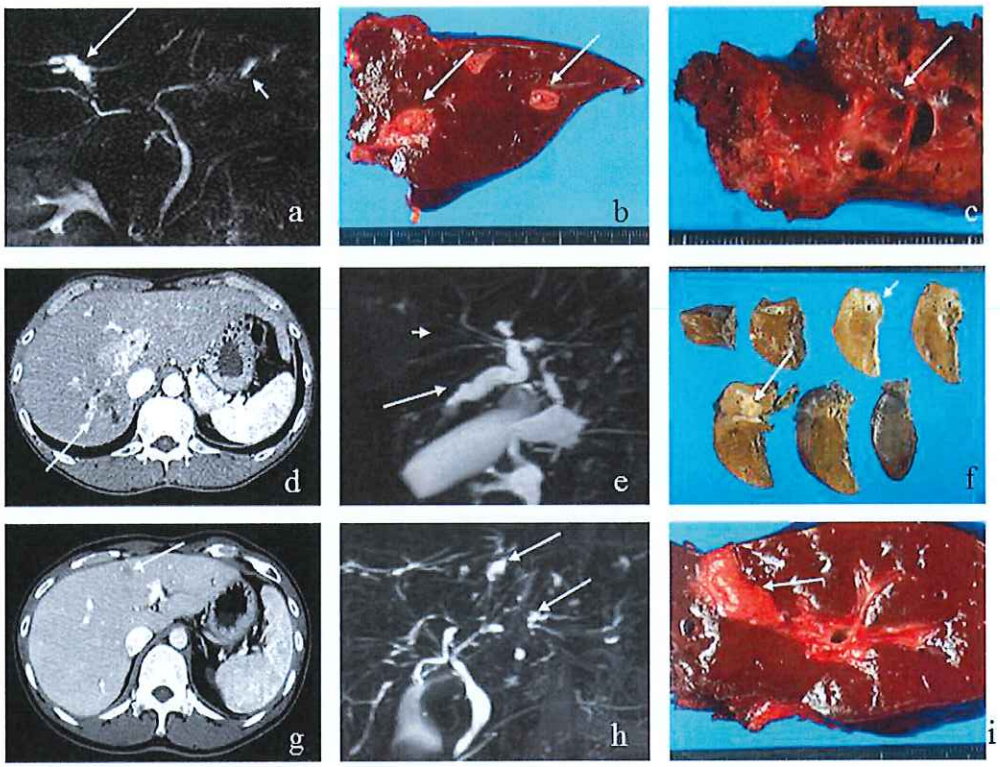

Fig. 1 Diagnostic imaging and surgical specimens. Regional dilatation of the bile ducts without tumor-induced obstruction (long arrow) is observed in the bile ducts of segment 7 (B7) and the peripheral bile ducts in the left lobe (short arrow) on preoperative magnetic resonance cholangiopancreatography (MRCP) of patient 1 (**a**). Macroscopic examination of patient 1's operative specimens (b and c) showing thickening of Glisson's sheath in the left lobe (arrow, **b**) and dilatation of B7 (arrow, **c**) [1]. A space-occupying lesion (arrow) is observed in the proximal side of segment 6 on the preoperative computed tomography (CT) image of patient 2 (**d**). The dilated bile ducts in the posterior segment (peripheral part of the tumor, long arrow) and multiple strictures and dilatation of the bile ducts in the anterior segment (short arrow) are observed on MRCP of patient 2 (**e**). Macroscopic examination of patient 2's operative specimens shows a tumor in segment 6 (long arrow) and dilatation of the bile ducts (short arrow) (**f**). Preoperative CT of patient 3 (**g**) showed tumor lesions in the bile ducts of segment 4 (B4; arrow), while MRCP (**h**) shows dilatation in the peripheral and proximal bile ducts in segment 4 (arrows). Macroscopic examination of patient 3's operative specimens (**i**) showing a tumorous lesion in the proximal side of B4 (arrow)

(Fig. 3b). In the peripheral parts of B6, a mixture of atypical epithelium with the same form as the main tumor and atypical epithelium with a different form (Fig. 3c,d) were observed. BilIN-3 lesions were observed in the hepatic duct, which was stenotic on the preoperative diagnostic imaging (Fig. 3g). Chronic bile duct injuries were observed in the bile ducts of the anterior segment and hepatic duct that corresponded to the stricture and dilatation of the bile duct on preoperative diagnostic imaging (Fig. 3h).

Patient 12

Preoperative CT showed tumors in the bile ducts in segment 4 (B4), MRCP showed dilatation in the peripheral and proximal sides of B4 (Fig. 1g,h). The macroscopic findings of the operative specimens showed a tumorous lesion on the proximal side of B4 (Fig. 1i) that was further identified as a papillary adenocarcinoma with intraductal growth (intrductal growth type). Histopathologically, the carcinoma cells showed acidophilic cytoplasm, and the tumor was classified as an oncocytic type invasive IPNB (Fig. 4a,b). The tumor invaded outside the wall and the atypical epithelium spread to the proximal side of the bile ducts in segment 2 (B2), corresponding to the bile duct dilatation seen on the preoperative diagnostic imaging (Fig. 4e,f). Moreover, IPNB lesions differed from the main tumor observed in the peripheral portions of B4 and B3 (Fig. 4c,d). BilIN-2/3 lesions with gastric metaplasia were observed throughout the bile ducts (Fig. 4g,h). Chronic bile duct injuries were detected in various sites of the bile ducts, although these ducts appeared normal on preoperative diagnostic imaging.

Immunohistochemical analysis

In three patients (patients no. 10–12), positive expressions of γH2AX and S100P were detected in almost all portions of the invasive carcinoma, BilIN-2/3, and IPNB (Fig. 5a–f, Table 2) in the large bile ducts. Weakly positive expression was also

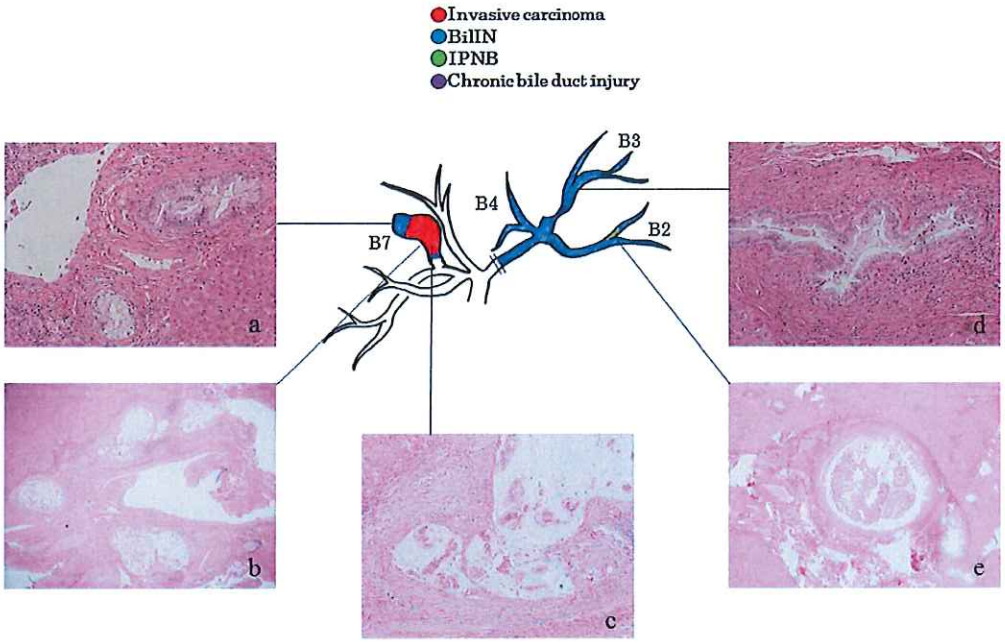

Fig. 2 Mapping chart of the atypical epithelium of patient 10. (**a**) hematoxylin and eosin (HE) staining (×200). In the peripheral parts of segment 7 (B7), biliary intraepithelial neoplasia (BilIN)-2/3 lesions with different morphology from that of the main tumor are observed. (**b**) HE staining (×40) and (**c**) HE staining (×100). Papillary proliferating oncocytic invasive intraductal papillary neoplasm of the bile duct (IPNB) with mucus production is observed in the dilated B7. (**d**) HE staining (×200). BilIN-2/3 lesions are observed throughout the entire bile ducts that did not present as abnormal on preoperative imaging, and gastric metaplasia occurred in many areas. (**e**) HE staining (×40). IPNB without invasion was observed in the peripheral parts of segment 2 that showed regional dilatation of the bile ducts on preoperative imaging

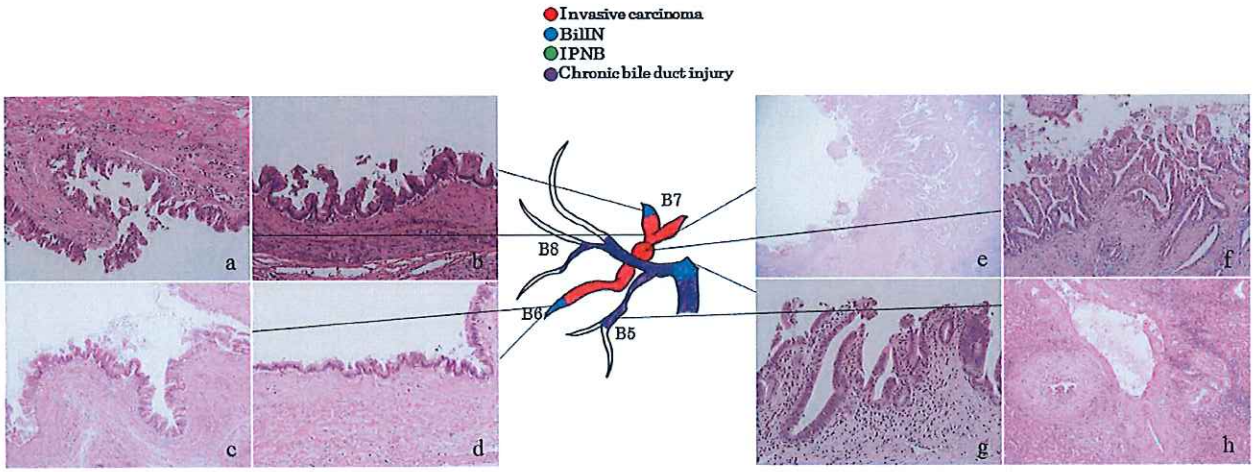

Fig. 3 Mapping chart of the atypical epithelium of patient 11. (**a**) hematoxylin and eosin (HE) staining (×200). Papillary adenocarcinoma invading the bile ducts in the posterior segment. (**b**) HE staining (×200). In the peripheral portions of the bile ducts in segments 6 and 7 (B6 and B7), biliary intraepithelial neoplasia (BilIN)-3 lesions with gastric metaplasia are visible. (**c**,**d**) HE staining (×200). In the peripheral portions of B6, a mixture of atypical epithelium with the same form as the main tumor (**c**) and atypical epithelium with a different form (**d**). (**e**) HE staining (×40) and (**f**) HE staining (×200). An oncocytic type papillary adenocarcinoma with extramural invasion and periductal infiltration to the posterior branch is visible. (**g**) HE staining (×200). BilIN-3 lesions are observed in the hepatic duct that was seen as stenosis of the bile duct on the preoperative image. (**h**) HE staining (×100). Chronic bile duct injury is observed in the bile ducts in the anterior segment and common hepatic duct that were seen as strictures and dilatation of the bile duct on preoperative diagnostic imaging

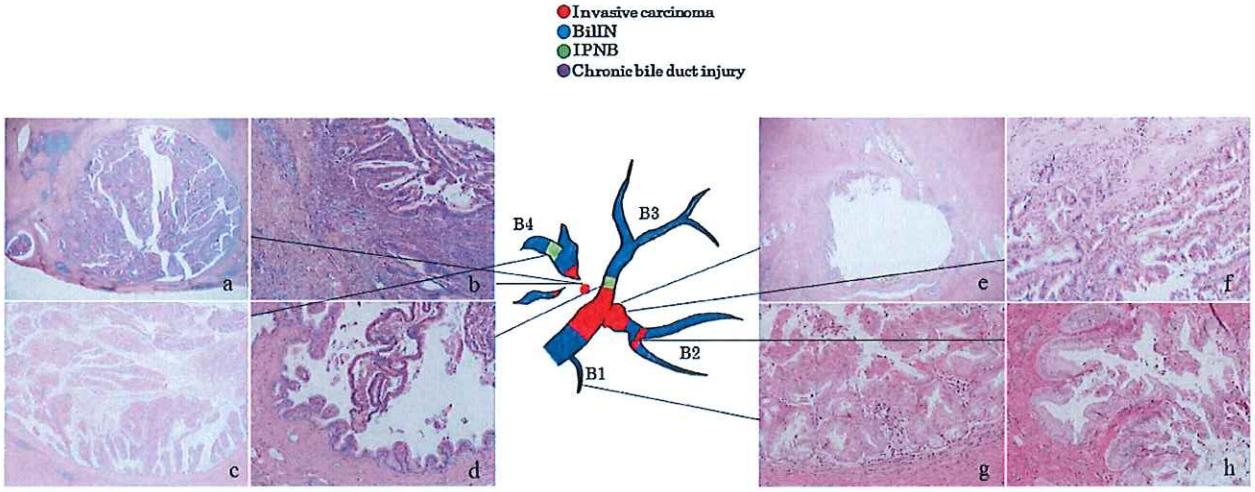

Fig. 4 Mapping chart of atypical epithelium in patient 12. (**a**) hematoxylin and eosin (HE) staining (×40). (**b**) HE staining (×100). The main lesion in segment 4 (B4) was a papillary adenocarcinoma growing in the bile duct. The tumor cells had eosinophil granules, and the tumor was diagnosed as an oncocytic type intraductal papillary neoplasm of the bile duct (IPNB) with invasion. There were findings of partial invasion outside the wall. (**c**,**d**) HE staining (×100). IPNB with a different form from that of the main tumor was observed in the periphery of B4 (**c**) and the proximal side of segment 3 (**d**). (**e**) HE staining (×40). (**f**) HE staining (×200). The main tumor invaded the proximal side of segment 2 that was observed as biliary dilatation on the preoperative imaging. (**g**,**h**) HE staining (×200). Biliary intraepithelial neoplasia-2/3 lesions with gastric metaplasia are visible throughout the entire excised specimen

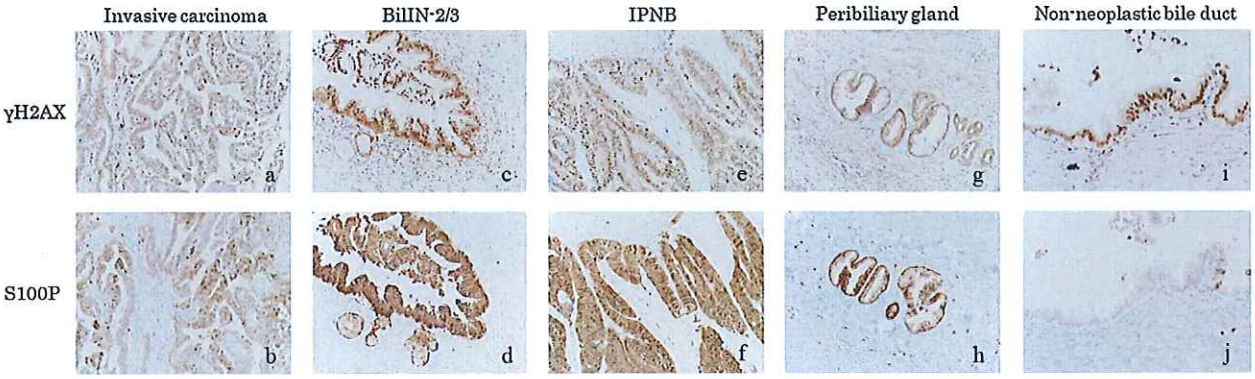

Fig. 5 Immunohistochemical analysis. (**a-h**) (×200). Immunohistochemical expressions of γH2AX and S100P in invasive carcinoma, biliary intraepithelial neoplasia-2/3, IPNB, and peribiliary gland. (**i**,**j**) (×200). In chronically injured areas of the bile duct, although S100P expression is relatively weak or absent, γH2AX expression is observed, similar to that in the neoplastic epithelium

Table 2 Immunohistochemical analysis of three patients

Enzyme	Patients	Invasive carcinoma	BilIN-2/3	IPNB	Peribiliary gland	Non-neoplastic bile duct
γH2AX	10	++	++	++	+	+
	11	++	++	++	++	+
	12	++	++	++	+	ND
S100P	10	++	++	++	+	–
	11	++	++	++	+	–
	12	++	++	++	+	ND

BilIN biliary intraepithelial neoplasia, *IPNB* intraductal papillary neoplasm of the bile duct, *ND* not determined because there was not non-neoplastic epithelium in large bile ducts.
–, negative expression; +, positive expression but not exceeding 20%; ++, positive expression more than 20%

seen at the peribiliary glands (Fig. 5g,h). In two patients (patients no. 10 and 11) who could evaluated for non-neoplastic biliary epithelium, although γH2AX expression was also detected within the non-neoplastic biliary epithelium similar to BilIN, IPNB, and invasive carcinoma, S100P expression was absent or relatively weak in non-neoplastic epithelium (Fig. 5i,j). The specific expression of neither γH2AX nor S100P was detected in the hepatocytes of any of these patients.

Discussion

The findings obtained in this study of the 16 patients of occupational cholangiocarcinoma are summarized as follows: (1) A spectrum of pathological changes such as chronic bile duct injury and early neoplastic and pre-invasive lesions such as BilIN and/or IPNB and invasive cholangiocarcinoma was observed in the biliary tree in all 16 patients. Such pathological findings were observed in almost all of the large bile ducts with or without sclerotic changes through extensive pathological observation while referring to biliary imaging in three patients. (2) This spectrum of biliary tract lesions reflected biliary tract images such as PSC-like changes including regional bile duct dilatation without tumor-induced obstruction in these patients. (3) Aberrant expression of S100P was seen in pre-invasive neoplastic lesions in addition to invasive cholangiocarcinoma, and DNA damage was even found in chronic bile duct injuries in addition to neoplastic biliary lesions.

BilIN and IPNB are currently regarded as precancerous or early cancerous lesions as per the World Health Organization's classifications of cholangiocarcinoma [9], and are considered to be involved in the multistep carcinogenesis of cholangiocarcinoma in patients with hepatolithiasis [11–13]. Papillary or invasive carcinoma, precancerous or early cancerous lesions such as BilIN and IPNB, and non-specific bile duct injuries such as fibrosis are reportedly pathological characteristics of occupational cholangiocarcinoma [1, 3, 7]. In a previous study [11], BilIN-2/3 was observed in nine of 19 patients with cholangiocarcinoma associated with hepatolithiasis, and IPNB was observed in 10 of the 19 patients. In addition, BilIN-2/3 was observed in 24 of 55 patients with hepatolithiasis without cholangiocarcinoma, and IPNB was observed in nine of 55 of these patients. Such lesions were observed in the large bile ducts containing stones and in the adjacent bile ducts [11]. Multistep carcinogenesis from BilIN was also suggested in PSC [14]. Lewis et al. reported that BilIN-2/3 lesions were observed in 50% of patients with end-stage PSC who underwent liver transplantation [15]. In this study, BilIN lesions were observed in all 16 patients, whereas occupational cholangiocarcinoma and IPNB or invasive IPNB were observed in 11 of the 16 patients; however, it was difficult to examine pathological findings because of the presence of insufficient materials from three patients (patients no. 3, 6, and 16). In addition, BilIN and IPNB were observed in almost all of the large bile ducts in the whole liver obtained by autopsy (patients no. 13) as well as the whole resected liver (patients no. 10–12). Thus, the high frequency of BilIN and/or IPNB and the wide distribution of such lesions were characteristic of the patients with occupational cholangiocarcinoma in comparison with patients with hepatolithiasis or PSC.

Intrahepatic cholangiocarcinomas in patients 10 and 12 were classified as intraductal growth type and the carcinoma in patient 11 was classified as a mass-forming type. Intrahepatic cholangiocarcinoma is classified into the perihilar large bile duct type and the peripheral small bile duct type [16, 17]. In the perihilar large bile duct type, chronic biliary inflammation caused by PSC, hepatolithiasis, and liver flukes may induce BilIN followed by periductal infiltrating carcinoma as well as the mass-forming type cholangiocarcinoma and IPNB followed by intraductal papillary tumor growth (intraductal growth type cholangiocarcinoma and invasive IPNB). In this study, BilIN was observed in all 16 patients and IPNB was observed in 11 of the 16 patients, which indicates that the patients with occupational cholangiocarcinoma could either follow the BilIN or IPNB pathway. The extensive pathological examination in three patients (patients no. 10–12) showed that although the three patients had the potential for following either of these pathways, the carcinogenic process in the pathway involving BilIN was primarily followed in patient 11, whereas the carcinogenic process in the pathway involving IPNB was primarily followed in patients 10 and 12.

S100P is a 95-amino-acid protein that has been shown to mediate tumor growth, metastasis, and invasion through the binding of Ca^{2+} ions and receptors for advanced glycation end products [18]. Increased S100P levels have been observed in carcinoma of the pancreas, biliary tract, lung, breast, and ovary [19]. In addition, the previous reports showed that S100P was aberrantly expressed in perihilar cholangiocarcinoma [20–22], and that it was detected in BilIN-2/3 as well as invasive carcinoma in patients with hepatolithiasis [21, 22]. In this study, S100P was aberrantly and strongly positive in BilIN-2/3 lesions and IPNB, while invasive carcinoma was also positive to S100P. However, in the non-neoplastic epithelium expressing γH2AX (see below), S100P expression was relatively weak or absent.

One of the earliest steps in the cellular response to DNA double-strand breaks is the phosphorylation of histone H2AX at serine 139, the γ-phosphorylation site, which results in γH2AX [23]. On immunohistochemical analyses, γH2AX was highly expressed in many premalignant lesions, cancer cells, and solid tumors [24–28]. In our previous study, γH2AX expression was observed in the foci of BilIN, IPNB, and/or invasive cholangiocarcinoma in seven of 16 patients

with cholangiocarcinoma associated with hepatolithiasis [29]. In this study, γH2AX expression was observed in the foci of BilIN, IPNB, and invasive carcinoma in all of the three patients (patients no. 10–12) with occupational cholangiocarcinoma. In addition, in two patients (patients no. 10 and 11) who could be evaluated for non-neoplastic biliary epithelium, γH2AX expression was also detected within the non-neoplastic biliary epithelium similar to BilIN, IPNB, and invasive carcinoma. However, in non-neoplastic epithelium positively expressing γH2AX, S100P expression was absent or relatively weak, indicating that precancerous or cancerous lesions developed in the bile ducts affected by DNA injury. In addition, one previous study reported that non-neoplastic biliary epithelial cells of the large bile duct and peribiliary glands were negative for γH2AX in patients with cholangiocarcinoma associated with hepatolithiasis [29]. The findings in the present study and previous study [29] indicate that DCP or its metabolites might induce DNA damage and neoplastic changes in almost all of the large bile ducts, and that the frequent expression of γH2AX is another characteristic of occupational cholangiocarcinoma, in comparison with cholangiocarcinoma associated with hepatolithiasis.

In this study, the biliary tree was extensively examined by using several histological sections from whole liver specimens and through the use of biliary tract images. We observed that the regional dilatation of intrahepatic bile ducts as well as bile duct sclerosis with or without multiple strictures similar to PSC corresponded to invasive cholangiocarcinoma, including papillary adenocarcinoma (invasive IPNB), precancerous or early cancerous lesions such as BilIN and IPNB, and chronic bile duct injury with fibrosis. In patients 10 and 11, a PSC-like appearance on the diagnostic images and changes in laboratory test results were observed several years before the diagnosis of cholangiocarcinoma was made [7]. Thus, the identification of such changes on diagnostic imaging can be useful for screening and surveillance for occupational cholangiocarcinoma [30].

It appears plausible that chlorinated organic solvents (DCP and DCM) or their products induce chronic bile duct injury with DNA damage, gradually followed by precancerous lesions and invasive carcinoma—this is indicative of the multistep carcinogenesis process in occupational cholangiocarcinoma. The wide distribution of biliary epithelium with DNA injury and pre- or early cancerous lesions expressing S100P indicates that the various sites of the bile ducts of patients exposed to chlorinated organic solvents have malignant potential, and may eventually develop multifocal carcinogenesis. Although studies concerning occupational cholangiocarcinoma are rare because the number of patients with occupational cholangiocarcinoma is very small and the materials for the studies including resected specimens are limited, further analyses are necessary to understand the detailed mechanism of the development of occupational cholangiocarcinoma.

In conclusion, extensive pathological observation of the biliary tree of the surgically resected livers of patients with occupational cholangiocarcinoma showed a unique spectrum of biliary lesions including chronic bile duct injury, proliferative changes, pre-invasive neoplastic lesions, and invasive cholangiocarcinoma, which reflects the regional dilatation of the intrahepatic bile ducts and PSC-like changes seen on biliary tract imaging. Furthermore, a carcinogenic process consisting of chronic bile duct injury with DNA damage that occurred within almost all of the large bile ducts, the induction of pre- or early cancerous lesions such as BilIN and IPNB, and the development of invasive carcinoma is proposed. More studies are mandatory to evaluate the chronic biliary and DNA damage as well as the progression to neoplastic lesions.

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Conflict of interest None declared.

Author contributions Study design: MK, SK, and YN designed the study. Acquisition of data: MK, SK, YN, Y. Sato, S. Takemura, S. Tanaka, GH, TI, HT, T. Yamada, SN, AA, MF, Y. Sugawara, T. Yamamoto, MA, KN, MU, TM, KT, and KS. Pathological aspect of the study: YN and Y. Sato analyzed pathological findings. Data analysis: MK, SK, YN, Y. Sato, and TS. Manuscript drafted by MK, SK, YN, and TS. All authors reviewed the manuscript.

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RESEARCH ARTICLE

Comparison of Clinical Characteristics between Occupational and Sporadic Young-Onset Cholangiocarcinoma

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Abstract

Background: Since seventeen employees of an offset printing company in Osaka, Japan developed cholangiocarcinoma it has become recognized as an occupational cancer. This study investigated the differences of clinical features between occupational cholangiocarcinoma and sporadic young-onset cholangiocarcinoma. Materials and Methods: Thirty-four young adults (<50 years old) with sporadic cholangiocarcinoma were extracted from the Rosai Hospital Group database (sporadic group) and their clinical features were compared with those of 17 patients with occupational cholangiocarcinoma (occupational group). Results: The 34 patients in the sporadic group were treated for cholangiocarcinoma at 16 different Rosai hospitals. There were significant differences of age (p<0.01), gender (p<0.01), abnormal laboratory tests (p<0.01), and tumor location (p<0.01) between the two groups. The percentage of patients with abnormal laboratory tests was significantly higher in the occupational group than in the sporadic group (p<0.001). Regional dilation of bile ducts, which is a characteristic of occupational cholangiocarcinoma, was not observed in the sporadic group. Conclusions: No cluster of cholangiocarcinoma cases was identified in the Rosai Hospital database. There were differences of clinical features between occupational and sporadic cholangiocarcinoma, which might be helpful for diagnosing occupational cholangiocarcinoma in the future.

Keywords: Occupational cholangiocarcinoma - juvenile cholangiocarcinoma - organic solvent

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Introduction

Recently, a cluster of cholangiocarcinoma cases was reported among relatively young workers in the offset color proof printing department of a printing company in Osaka, Japan (Kumagai et al., 2013; Kubo et al., 2014). In 2013, this type of cholangiocarcinoma was recognized as an occupational disease by the Japanese Ministry of Health, Labour and Welfare (Ministry of Health, Labour and Welfare. 2013). At the printing company in question, large amounts of dichloromethane (DCM) and 1,2-dichloropropane (DCP) were used in the printing process and the workers were exposed to high concentrations of these chlorinated organic solvents (Kumagai et al., 2013; National Institute of Occupational Safety and Health. 2012). From the estimated concentrations of DCP and DCM to which the workers were exposed and assessment of genotoxicity (Suzuki et al., 2014; Yamada et al., 2014), it was concluded that long-term exposure to high concentrations of DCP caused the development of cholangiocarcinoma. Although there is no definite evidence of carcinogenicity for DCM, it might also play a role in the development of cholangiocarcinoma (Yamada et al., 2015). Because of this event, the International Agency for Research on Cancer (IARC) upgraded the carcinogenicity of dichloromethane from Group 2B (possibly carcinogenic to humans) to Group 2A (probably carcinogenic to humans) and that of 1,2-dichloropropane from Group 3 (not classifiable as to carcinogenicity for humans) to Group 1 (carcinogenic to humans) in 2014 (Benbrahim et al., 2014).

The patients with occupational cholangiocarcinoma at the printing company were typically relatively young male workers with high serum levels of γ -glutamyl transpeptidase (γ -GTP) and regional dilatation of the intrahepatic bile ducts without obstruction by the tumor. In these patients, the primary cancer arose from a large bile duct (common hepatic duct, left or right hepatic ducts, or the first to third branches of the intrahepatic bile ducts) and they had co-existing precancerous lesions such as biliary intraepithelial neoplasia (BilIN) and intraductal neoplasms of the bile ducts (Kubo et al., 2014). These characteristics seemed to be different from the typical features of cholangiocarcinoma.

Up to October 30, 2014, 34 patients in Japan were recognized to have occupational cholangiocarcinoma by the Ministry of Health, Labour and Welfare. Therefore, it is important to determine whether occupational

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cholangiocarcinoma is widespread in Japan and if there is any doubt about the occupational etiology.

The Rosai Hospitals comprise 34 hospitals located throughout Japan from Hokkaido to Kyushu that treat patients with or without occupational diseases. At these hospitals, the occupational history of each patient is noted in the medical records, with the three previous occupations being registered in the database at the time of admission. This is a unique feature of the database for the Rosai hospitals (Rosai database).

The present study was performed to investigate the differences of clinical features between patients who developed occupational cholangiocarcinoma at the printing company in Osaka and young-onset patients with sporadic cholangiocarcinoma treated at the Rosai hospitals nationwide.

Materials and Methods

Cholangiocarcinoma is defined as young-onset cholangiocarcinoma if it develops in patients under 50 years old

A total of 34 patients with young-onset cholangiocarcinoma were extracted from the Rosai database by using keywords from the International Classification of Disease (9th and 10th edition), which were intrahepatic bile duct, extrahepatic bile duct, and biliary tract and parts unknown [ICD9:1551 (intrahepatic cholangiocarcinoma), ICD9:1561 (extrahepatic cholangiocarcinoma), ICD9:1569 (part undetectable); ICD10: C221 (intrahepatic cholangiocarcinoma), ICD10: C240 (extrahepatic cholangiocarcinoma), ICD10: C249 (part undetectable)]. The Rosai database contains 5.27 million medical records from April 1, 1984 to May 31, 2014, among which 2.79 million records include data on the occupational history. Cholangiocarcinoma was diagnosed in 7717 patients (including 5910 patients with occupational data) and 265 of them were under 50 years old (including 205 with occupational data).

Detailed medical records, including laboratory data and diagnostic imaging findings, were only available for 34 of the young patients. These 34 patients were employed in this study.

Exposure of the patients to DCM and/or DCP was estimated by using the Pollutant Release and Transfer Register (Ministry of Economy., 2014), indicating that five of the 34 patients had possible to exposure to DCM and/or DCP.

In this study, the 17 patients with occupational cholangiocarcinoma from the Osaka printing company were classified as the occupational group and the 34 patients with young-onset cholangiocarcinoma identified in the Rosai database formed the sporadic group.

We compared clinical features between the occupational group and the sporadic group, including lifestyle factors such as smoking and drinking (habitual drinking with an ethanol intake of 80g / day or more), laboratory data such as γ-GTP, carbohydrate antigen (CA) 19-9, and carcinoembryonic antigen (CEA), serum markers of hepatitis B and C virus, known risk factors for cholangiocarcinoma, diagnostic imaging findings (computed tomography, magnetic resonance imaging, endoscopic retrograde cholangiopancreatography, and magnetic resonance cholangiopancreatography), tumor location (intrahepatic or extrahepatic), and histological findings. We also compared clinical features between the occupational group and the 5 patients from the sporadic group with possible exposure to DCM and/or DCP (possible exposure group).

The diagnosis of cholangiocarcinoma was re-evaluated by a gastroenterologist from Kanto Rosai Hospital, who reviewed the medical records and imaging findings. Tumor

Table 1. Comparison of Characteristics between the Occupational Group and the Sporadic Group

	Occupational group (n = 17)	Sporadic group (n = 34)	P value
Age (years, mean)	25-45 (36)	23-49 (44)	0.009**
Gender (M:F)	17:0	23:11	0.009**
Alcohol abuse	3	14	0.12
Smoking	13	19	0.22
Symptoms	5	21	0.029*
Abnormal laboratory tests	12	4	0.001>**
Elevated γ-GTP	17	14(16)	0.23
Elevated CEA	10	5(14)	0.2
Elevated CA 19-9	13	9(16)	0.28
Tumor location			0.008*
Intrahepatic	10	7	
Extrahepatic	5	25	
Intra and Extrahepatic	2	0	
Unknown	0	2	
Tumor stage			0.07
I-III	2	9	
IVA and IVB	15	12	
Unknown	0	13	
Treatment			0.5
Surgery	12	12	
Chemotherapy and/or radiation	5	8	

*Elevation of γ-GTP, CA19-9, CEA indicates the number of patients with enzymes elevated at, the first admission. γ-GTP, γ-glutamyl transpeptidase; CA19-9, carbohydrate antigen19-9; CEA, carcinoembryonic antigen, Symptoms indicate the number of patients with complaints at the onset (abdominal pain, jaundice, pruritus, and anorexia), Abnormal laboratory tests shows the number of patients with abnormal test results.

location was classified according to the 'General Rules for Clinical and Pathological Studies on Cancer of the Biliary Tract (6th Edition). Tumors arising in a bile duct peripheral to the secondary branches were classified as intrahepatic cholangiocarcinoma, while tumors that developed in the so-called perihilar or distal sites were classified as extrahepatic cholangiocarcinoma. Pathological findings were recorded and described according to the World Health Organization classification of intrahepatic and extrahepatic cholangiocarcinoma (Bosman et al., 2010).

The χ^2 test or Fisher's exact test was used to evaluate significance of differences in categorical data between the groups. Statistical analysis was performed with Stata VER13 (Stata Corp. Texas, USA).

This study was approved by the ethics committee of Japan Labour Health and Welfare Organization Kanto Rosai Hospital.

Results

Clinical features of the sporadic group

The 34 patients with cholangiocarcinoma were treated at 16 different Rosai hospitals. Therefore, a cluster of young-onset cholangiocarcinoma was not found in the Rosai database accumulated from hospitals distributed throughout Japan.

In the sporadic group, 23 patients were male and 11 were female. The chief presenting complaint was abdominal pain in 11 patients, jaundice in 7, pruritus in 2, and anorexia in 1. Four patients presented to hospital because of abnormal laboratory test results. In the remaining 9 patients, the chief complaint was not specified. Two patients were positive for serum HBs antigen. Known risk factors for cholangiocarcinoma, such as primary sclerosing cholangitis, hepatolithiasis, pancreaticobiliary maljunction, or liver fluke infection, were not observed in the sporadic group.

The clinical stage at the time of diagnosis was stage 1 in 2 patients, stage 2 in 4, stage 3 in 3, stage 4A in 4, and stage 4B in 8. Detailed information about staging was not available for the other 13 patients. Seven patients had intrahepatic cholangiocarcinoma and 25 patients had extrahepatic cholangiocarcinoma, while the location of the primary tumor was unknown in 2 patients.

Surgical treatment was performed in 12 patients (pancreaticoduodenectomy in 8, right lobectomy in 1, and unknown operative procedure in 3). Seven patients underwent chemotherapy (tegafur-gimeracil-oteracil potassium in 3, gemcitabin in 2, cisplatin in 1, and intra-arterial fluorouracil in 1). The histologic diagnosis was adenocarcinoma in 26 patients, while the histology was unknown in the other 8 patients.

Comparison between the occupational group and the sporadic group.

Table 1 shows a comparison of clinical features between the occupational group and the sporadic group. The mean age was significantly younger in the occupational group than the sporadic group ($p<0.01$). All patients in the occupational group were male, while 23

Table 2. Comparison of Characteristics between the Occupational Group and the Possible Exposure Group

	Occupational group (n = 17)	Possible Exposure group (n = 5)	P value
Age (years, mean)	25-45 (36)	44-49 (46)	0.001**
Gender (M:F)	17:0	4:1	0.23
Symptoms	5	4	0.12
Tumor location			0.03*
Intrahepatic	10	0	
Extrahepatic	5	5	
Intra and Extrahepatic	2	0	
Unknown	0	0	
Tumor stage			0.009**
I-III	2	4	
IVA and IVB	15	1	
Unknown	0	0	
Treatment			1
Surgery	12	4	
Chemotherapy and/or radiation	5	1	

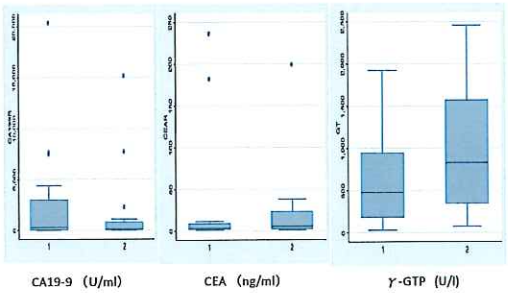

Figure 1. Comparison of Laboratory Data At Diagnosis. γ-GTP, γ-glutamyl transpeptidase; CA19-9, carbohydrate antigen19-9; CEA, carcinoembryonic antigen. Three abnormal outliers in the sporadic group were not incorporated in the main plot (CA19-9, 216394 U/ml; CEA, 8832 ng/mg; CEA, 2530 ng/mg). Although γ-GTP levels were higher in the occupational group, there were no significant differences

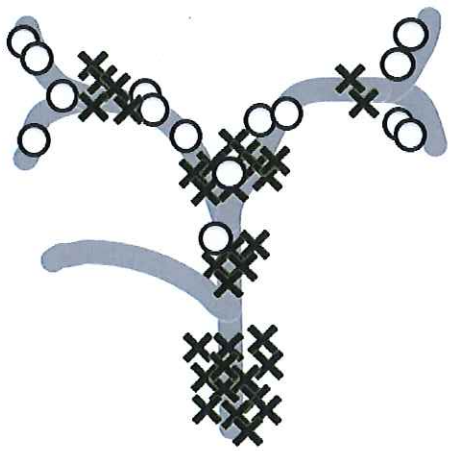

Figure 2. Tumor Locations. The sites of the main tumors are shown by open circles (occupational group) or by crosses (sporadic group). Two patients with both intrahepatic and extrahepatic tumors are not shown in the figure

patients were male and 11 were female in the sporadic group ($p<0.01$). With regard to lifestyle factors, there was no statistical difference in the number of patients with alcohol abuse or habitual smoking. Five patients in the occupational group presented to hospital because of symptoms, whereas 21 of 34 patients in the sporadic group presented because of symptoms ($p<0.05$). Twelve patients in the occupational group presented to hospital because of abnormal laboratory test results (tests performed for regular health checks in 11 patients and during treatment for another disease in 1 patient). The proportion of patients with symptoms was significantly higher in the sporadic group than in the occupational group ($p<0.05$). Serum γ-GTP was elevated in all patients from the occupational group, while it was elevated in 14 out of 16 patients tested in the sporadic group. The proportion of patients with elevation of CEA and CA 19-9 did not differ between the groups. Although there were no significant differences of γ-GTP, CA19-9, and CEA levels between the two groups, there was a trend for γ-GTP to be higher in the occupational group (Figure 1). On diagnostic imaging, regional bile duct dilatation without tumor obstruction was not observed in the sporadic group, unlike the occupational group.

In the occupational group, 15 patients had stage IV cholangiocarcinoma, while the carcinoma was classified as stage IV in 12 of 21 patients with data from the sporadic group. In the occupational group, 10 patients had intrahepatic cholangiocarcinoma, 5 had extrahepatic cholangiocarcinoma, and 2 had both intrahepatic and extrahepatic tumors (Figure 2). Most of the extrahepatic tumors were classified as so-called perihilar cholangiocarcinoma. In 13 of the 27 patients with extrahepatic cholangiocarcinoma from the sporadic group, the tumor was classified as distal cholangiocarcinoma. The proportion of intrahepatic cholangiocarcinoma was significantly higher in the occupational group than in the sporadic group ($p<0.05$). Eleven patients in the occupational group underwent hepatectomy and 1 underwent hepatectomy combined with pancreaticoduodenectomy. The proportion of patients undergoing hepatectomy was significantly higher in the occupational group than in the sporadic group ($p<0.01$).

Comparison between the occupational group and the sporadic group

Table 2 shows a comparison of clinical features between the 17 patients in the occupational group and five patients in the possible exposure group (a subgroup of the sporadic group). The mean age was significantly younger in the occupational group than the possible exposure group ($p<0.01$). In the possible exposure group, 4 patients were male and 1 was female. Although 5 of 17 patients in the occupational group presented to hospital because of symptoms, 4 patients visited hospital because of symptoms in the possible exposure group (jaundice, pruritus, and anorexia). Little data on γ-GTP, CEA, and CA19-9 levels was obtained from the medical records, so statistical analysis was not performed. All patients in the possible exposure group had extrahepatic cholangiocarcinoma, unlike the occupational group ($p<0.05$). In the possible exposure group, tumor stages were lower than in the occupational group.

Discussion

This study showed that there was no regional clustering of young-onset cholangiocarcinoma in the Rosai database for 16 hospitals around Japan. A previous study based on data from the Osaka Cancer Registry revealed that there was neither a change in trend nor regional clustering of cholangiocarcinoma in Osaka (Ikeda et al., 2013). It was also reported that the cluster of cholangiocarcinoma cases detected in Osaka may not be reproduced in the printing industry nationwide from analysis of the Japan Health Insurance Association claims database (Okamoto et al., 2013). Thus, the occurrence of occupational cholangiocarcinoma among workers of the Osaka company was not indicative of a wider problem.

This study also identified some differences of clinical features between occupational and sporadic cholangiocarcinoma. It is known that cholangiocarcinoma usually occurs in patients in their 60s-70s, while the patients with occupational cholangiocarcinoma were under 50 years old. When patients under 50 years old were extracted from the Rosai database for the sporadic group, the mean age of the occupational group was still significantly lower. Thus, the 17 patients with occupational cholangiocarcinoma were extremely young compared with patients developing sporadic cholangiocarcinoma. In addition, the proportion of male patients was significantly higher in the occupational group than in the sporadic group. In the relevant section of the printing company, most employees were young men and their for 6 to 19 years induced cholangiocarcinoma (Kubo et al., 2014). Thus, the gender difference between the occupational and sporadic groups is considered to reflect the low ratio of female workers exposed to organic solvents at the printing company.

The proportion of patients with symptoms was higher in the sporadic group than in the occupational group, whereas the proportion of patients with abnormal laboratory test results identified during regular health checks was higher in the occupational group. In some patients from the occupational group, elevation of the serum concentrations of γ-GTP, aspartate aminotransferase, and alanine aminotransferase was observed several years before the detection of cholangiocarcinoma (Kumagai et al., 2014; Kubo et al., 2014). These findings indicate that patients in the sporadic group generally presented to hospital after the onset of symptoms and that regular health checks are important for detecting occupational cholangiocarcinoma.

Although cholangiocarcinoma can develop at any site in the bile duct, more than 60 % are extrahepatic and only a small percentage of these tumors arise in the intrahepatic bile ducts (Razumilava et al., 2014). While these trends were seen in the sporadic group, many of the tumors developed in the large intrahepatic bile ducts in the occupational group. As a result, the proportion of patients who underwent hepatectomy was higher in the occupational group than in the sporadic group, while the proportion of patients undergoing

pancreaticoduodenectomy was higher in the sporadic group.

The characteristic findings in patients with occupational cholangiocarcinoma include regional dilatation of the intrahepatic bile ducts related to chronic bile duct injury, precancerous lesions including biliary intraepithelial neoplasia, and early cancerous lesions (Kaneko et al., 2014; Kubo et al., 2014; Sato et al., 2014; Suzuki et al., 2014; Tomimaru et al., 2015). In contrast, regional dilatation of the intrahepatic bile ducts was not observed by diagnostic imaging in the sporadic group. Thus, the process of carcinogenesis seems to differ between the occupational group and the sporadic group.

The differences that we identified between the occupational group and the possible exposure group were similar to those between the occupational group and the sporadic group. Although there is no detailed information about exposure to organic solvents in the possible exposure group, the extent of exposure might be lower in this group than in the occupational group. Occupational cholangiocarcinoma develops after long-term exposure to high concentrations of organic solvents. It is important to know the occupational history (including exposure to organic solvents) and the detailed clinical features of patients with cholangiocarcinoma because this information is useful for diagnosing occupational cholangiocarcinoma.

The present study identified some differences of clinical data between the occupational and the sporadic group, but evaluation was limited by the small number of young adult patients with cholangiocarcinoma. In fact, sufficient data were available for only 34 patients with sporadic young-onset cholangiocarcinoma, accounting for a mere 0.4% of all cholangiocarcinoma patients in the Rosai database. Therefore, the influence of selection bias cannot be ruled out. An unexpected finding was that clinical data did not include the occupational history in many cases. This may be due to technical problems such as different methods of data processing by each member of the Rosai Hospital group. In addition, availability of medical records is limited to five years in compliance with the law. Therefore, further improvements are necessary to allow studies to be performed with more precision.

In conclusion, this study showed that the cluster of occupational cholangiocarcinoma in Osaka was an isolated event and that there are differences of clinical features between occupational and sporadic cholangiocarcinoma. These findings might be helpful for diagnosing occupational cholangiocarcinoma in the future.

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LETTER TO THE EDITOR

Re: Occupational cholangiocarcinoma

Shoji Kubo

Known etiological factors for cholangiocarcinoma include hepatolithiasis, primary sclerosing cholangitis, pancreaticobiliary maljunction, and liver flukes such as *Opisthorchis viverrini* and *Clonorchis sinensis*. Recently, we have reported relatively young workers with cholangiocarcinoma who were exposed to extremely high concentrations of 1,2-dichloropropane (DCP) and/or dichloromethane (DCM) for a long-term period [1–3]. These chlorinated organic solvents were used to clean ink residue in the offset proof-printing process. The cholangiocarcinoma in such workers was recognized as an occupational disease (occupational cholangiocarcinoma) by the Japanese Ministry of Health, Labour and Welfare on 1 October 2013 [4].

Humans are infected with liver flukes by ingesting the metacercariae in raw, fermented and/or partially cooked freshwater fish such as carp [5]. In northwest Thailand, there is a high incidence of cholangiocarcinoma due to *Opisthorchis viverrini* caused by infections from the consumption of raw freshwater fish such as genera *Puntius*, *Cyclocheilichthys* and *Hampala*. In Japan, *Clonorchis sinensis* were occasionally found in patients with biliary diseases many years ago in a limited area where people commonly ate raw freshwater fish. Usually, Japanese people eat raw fish from the sea, but not raw freshwater fish. The number of patients infected with *Clonorchis sinensis* has been dramatically decreased due to the improvements in health supervision and the decrease in the number of shellfish (*Bithynia* sp) serving as a first intermediate host.

So far, only 12 patients with cholangiocarcinoma associated with liver flukes have been reported in Japan. All of these patients were older than 50 years old (10 patients were older than 60 years old) and liver flukes were found in the bile or the resected specimens in all patients. The 17 patients in our previous first report [2] and the eight of nine patients in our second report [3] were younger than 50 years (one patient was 57 years old). Liver flukes were not detected in the bile, the resected specimen or the feces in any of the 26 patients. On diagnostic imaging, regional dilatation of the intrahepatic bile ducts without tumor-induced obstruction, like primary sclerosing cholangitis, was found to be a characteristic of patients with occupational cholangiocarcinoma [2, 3]. However, such findings have not been reported in cholangiocarcinoma patients with *Clonorchis sinensis*. As a result, the liver fluke infection could not be considered to have been the cause of the cholangiocarcinoma in the 26 previously reported patients [2, 3].

Recently, the International Agency for Research on Cancer assessed the carcinogenicity of DCP and DCM, and decided that DCP should be classified as carcinogenic to humans (group 1) on the basis of sufficient evidence in humans that exposure to DCP causes cholangiocarcinoma (biliary tract cancer), including our report [2]. DCM was classified as probably carcinogenic to humans (Group 2A) on the basis of limited evidence [6]. The epidemiologic relationship between chlorinated organic solvents and the development of cholangiocarcinoma has not been clarified. A large cohort set-up for four Nordic countries (Finland, Iceland, Norway, and Sweden) over a period of 45 years showed an increased risk of cholangiocarcinoma among individuals employed at the printing companies, although the study included various types of workers, such as typographers, printers, lithographers and bookbinders [7]. The results showed that the increased risk of cholangiocarcinoma among workers in the printing company in Osaka possibly extends beyond the specific company and country. It is therefore important to recognize that chlorinated organic solvents have possible carcinogenic effects, including the potential of development of cholangiocarcinoma.

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Case Study

Severe acute hepatitis in a printing company worker: A case study

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Abstract: Severe acute hepatitis in a printing company worker: A case study: Shoji KUBO, *et al.* Department of Hepato-Biliary-Pancreatic Surgery, Osaka City University Graduate School of Medicine—Objectives: It has been reported that chlorinated organic solvent is a cause of hepatitis. **Methods:** we investigate clinical and pathological findings of a patient with severe acute hepatitis who was exposed to chlorinated organic solvents. **Results:** A 34-year-old man who was exposed to chlorinated organic solvents including dichloromethane, 1,2-dichloropropane, and trichloroethylene, presented with general fatigue, vomiting, and diarrhea. At admission, his laboratory test results showed extremely elevated aspartate aminotransferase (4,872 IU/l), alanine aminotransferase (3,000 IU/l), and lactate dehydrogenase (11,600 IU/l) levels and a prothrombin level below normal (41%). No encephalopathy was noted. These findings were indicative of severe acute hepatitis. Viral hepatitis, autoimmune hepatitis, alcoholic disease, bile duct disease, and viral infection were excluded as causes of hepatitis by clinical, laboratory, and imaging findings. After diagnosis, the patient was administered fresh frozen plasma and glucagon-insulin therapy. Liver function recovered within a few weeks, and a liver biopsy performed 25 days after admission showed the recovery phase after acute liver damage. **Conclusions:** These clinical and pathological findings indicate that exposure to chlorinated organic solvents may have induced severe acute hepatitis in this patient.
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Key words: 1,2-Dichloropropane, Printing company, Severe acute hepatitis, Trichloroethylene

The toxicities of various chemicals, including organic solvents, have been reported, and methods for protection and occupational exposure limits have been established. Most organic solvents are mainly absorbed into the body via inhalation, ingestion, and the skin, and they often directly affect the eyes, skin, and respiratory tract. Absorbed organic solvents are toxic to the nervous system, liver, kidney, and heart[1, 2]. Hepatic damage after exposure to certain organic solvents has been described[3–9]. Here, we report a case of severe acute hepatitis in a printing company worker who was exposed to various chemicals, including organic solvents such as 1,2-dichloropropane (DCP), dichloromethane (DCM), and trichloroethylene (TCE). This study was performed according to the Declaration of Helsinki (2008), and the patient provided written informed consent.

Case Report

The patient started work in an offset color proof-printing department of a company in 1986. The present building was constructed in 1991. The printing room was located in the first basement floor of the building, with a front room adjacent to the printing room. The ventilation rates of these rooms were very low because of the basement location and the low capacity of the installed ventilation equipment. The patient made printing plates in the front room. In the process, he used high-purity TCE to remove stains from glass plates for about one year just before developing severe acute hepatitis. The amount of TCE he used per day was estimated to be 1-2 l based on his memory. Because no respiratory protection was

provided, he have been exposed to high levels of TCE.

In the printing room, proof-printing workers used large amount of organic solvent cleaner to remove ink residue from a rubber transcription roller. The cleaners was a mixture of gasoline (50% by weight) and 1,1,1-trichloroethane (50%) before 1989; it was a mixture of DCP (50–60%), DCM (15–25%), and 1,1,1-trichloroethane (15–25%) from approximately 1985 to 1992-1993; and it was a mixture of DCP (40–50%), DCM (40–50%) and petroleum hydrocarbons (1–10%) from 1992–1993 to March 1996; and it was nearly pure DCP solvent (98%) from April 1996 to October 2006. Airborne solvent concentrations in the printing room were estimated to be extremely high, which was confirmed in an experiment conducted by the Japanese National Institute of Occupational Safety and Health[10].

Furthermore, because the contaminated air of the printing room flowed into the front room due to positive pressure in the printing room, the airborne solvent concentrations were also estimated to have been high in the front room. Consequently, the patient was also exposed to these chemicals when working in the front room. In addition to making printing plates, he also supervised the progress of printing mainly in the front room but frequently went into the printing room to provide guidance and occasionally to conduct proof-printing. When working in the printing room, he was exposed to high levels of the abovementioned chlorinated organic solvents. Other chemicals such as kerosene and inks were also used in the department.

The patient (at 34 years of age) experienced general fatigue, vomiting, and diarrhea and visited a hospital in December 1996. According to the period of solvent use, he had been exposed to DCP and TCE just before the onset of symptoms and to DCM within 1 year before onset. He had drank 350 m*l* of beer per day during previous 10 years (<80 g of ethanol daily, which is the lower limit for alcoholic liver disease[11]) and smoked 20 cigarettes/day during the previous 14 years. He had no history of blood transfusion, sometimes took vitamins and aspirin for headaches, and had a body mass index of 18.3.

At admission, the patient was lucid with no abnormal neurological system or respiratory tract findings. The liver was palpable in the right infracostal region at a two finger widths. No dermatitis was noted. The laboratory test results at admission are shown in Table 1. The aspartate aminotransferase (AST, 4,872 IU/l), alanine aminotransferase (ALT, 3,000 IU/l); and lactate dehydrogenase (LDH, 11,160 IU/l) levels were markedly elevated, and the prothrombin test value was 41%. The concentrations of total bilirubin and direct bilirubin were 1.2 mg/d*l* and 0.2 mg/d*l*, respectively.

Table 1. Laboratory test results at admission

Red blood cell ($\times 10^4$/mm^3)	448
Hemoglobin (g/d*l*)	13.6
White blood cell (/mm^3)	6,800
Prothrombin test (%)	41
Aspartate aminotransferase (U/*l*)	4,872
Alanine aminotransferase (U/*l*)	3,000
Alkaline phosphatase (U/*l*)	140
Total bilirubin (mg/d*l*)	1.2
Direct bilirubin (mg/d*l*)	0.2
Lactate dehydrogenase (U/*l*)	11,160
γ-Glutamyl transpeptidase[a] (U/*l*)	45
BUN[a] (mg/d*l*)	18
Creatine[a] (mg/d*l*)	0.6
Na[a] (mEq/m*l*)	137
K[a] (mEq/m*l*)	3.6
Cl[a] (mEq/m*l*)	105
CRP (mg/d*l*)	1.9

[a]Tests performed the day after admission.

The serum alkaline phosphatase and γ-glutamyl transpeptidase (γ-GTP) levels were within the reference range. Eosinophilia was not detected. The results for hepatitis viral markers (IgM-HA antibody, hepatitis B e antigen and antibody, hepatitis B surface antigen and antibody, hepatitis B core antibody, hepatitis B virus DNA polymerase, hepatitis C virus [HCV] antibody, HCV RNA, hepatitis D virus antibody, and GBV-C RNA) were negative. The patient was positive for cytomegalovirus IgG and Epstein-Barr (EB) virus IgG antibody, but negative for cytomegalovirus IgM antibody and EB virus IgM antibody, indicating previous infections with cytomegalovirus and EB virus. Serum anti-nuclear antibody and lupus erythematosus (LE) test results were negative. Serum complement (CH50; 50% hemolytic unit of complement), carcinoembryonic antigen, and carbohydrate antigen 19–9 levels were within the reference ranges. Ultrasonography and computed tomography showed mild hepatomegaly but no abnormal findings in the biliary system. From these findings, severe acute hepatitis was diagnosed, and the patient was treated with fresh frozen plasma and glucagon-insulin therapy. Liver function recovered within a few weeks (Fig. 1). A liver biopsy performed 25 days after admission showed size inequality of hepatocytes and multinuclear hepatocytes (Fig. 2). Many phagocytes were present in the hepatic lobules, and mild lymphocytes infiltration and fatty droplets in a few hepatocytes were seen. Fibrous expansion of portal areas and cholestasis were not observed. These findings are indicative of the recovery phase after acute liver damage. The

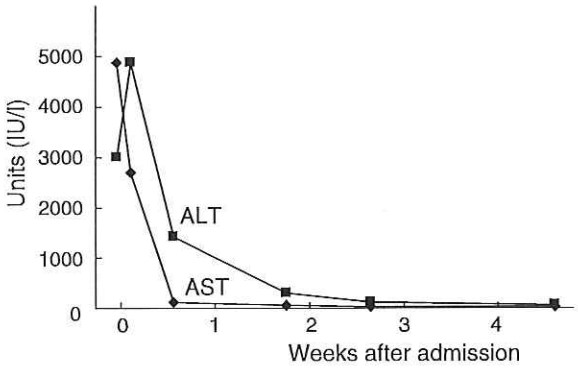

Fig. 1. Changes in alanine and aspartate aminotransferase levels after admission.

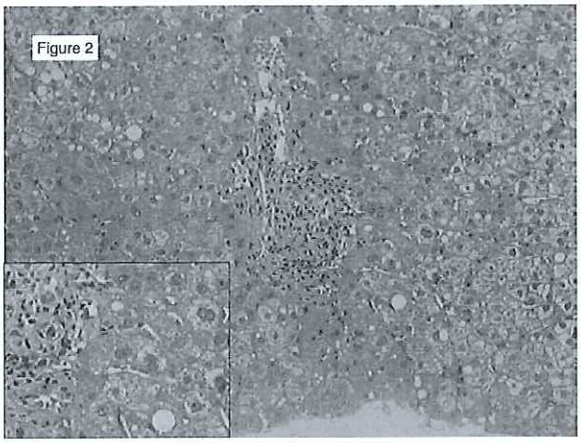

Fig. 2. Pathological findings of liver biopsy (hematoxylin and eosin, 20×, and inset, 40×).

clinical course and pathological findings indicate that the patient's severe acute hepatitis was not caused by viral hepatitis, autoimmune hepatitis, alcoholic disease, bile duct disease, or viral infection (cytomegalovirus and EB virus) but instead was caused by exposure to chlorinated organic solvents.

Use of TCE was stopped at the printing company after this event. Since his discharge from the hospital, he has not been exposed to high concentrations of chlorinated organic solvents. The patient is now in good health.

Discussion

Severe acute hepatitis developed in a worker in an offset color proof-printing department of a company. The three criteria for the diagnosis of toxic hepatitis include the following: (1) liver damage after occupational exposure to a substance, considering the patient's work history and current workplace; (2) elevated liver enzyme activity to at least double the upper limit of the reference range; and (3) exclusion of tertiary conditions such as other causes of liver damage[12, 13]. The patient in this study was exposed to various solvents, including DCP, DCM, and TCE. His serum AST, ALT and LDH levels were remarkably elevated at the time of admission to the hospital and improved rapidly after admission (stopping exposure) and treatment. The patient did not have any known cause of severe acute hepatitis, such as viral hepatitis, autoimmune hepatitis, alcoholic liver disease, viral infection (adenovirus, cytomegalovirus, or EB virus), or biliary tract disease.

Acute toxicity cause by DCP, DCM and TCE has been reported by several investigators[3–9]. The International Chemical Safety Cards produced of the International Labour Organization[2] warn that long-term or repeated exposure to DCM or DCP may affect the liver and kidneys. Repeated or prolonged contact of skin with TCE may cause dermatitis. This solvent may affect the central nervous system, liver, and kidneys. Recently, Chang *et al.*[14] reported that exposure to both lead and organic solvents is dangerous, even if exposure to each of the individual components is within the respective permissible limit. In the present case, the patient developed symptoms during exposure to DCP and TCE and within 1 year of exposure to DCM. There were few workers exposed to both DCP and TCE. Therefore, DCP and TCE were suspected to be the causative agents of the severe acute hepatitis in the patient. DCM also might have contributed to the development of hepatitis. In addition, mixed exposure to such organic solvents might synergize towards the development of the hepatitis.

Toxic hepatitis after exposure to chemicals can be divided into three types: hepatocellular, cholestatic, and mixed type[15]. Laboratory test results in this patient showed elevated AST, ALT, and LDH levels and a decreased prothrombin value, whereas the serum alkaline phosphatase and γ-GTP levels were within the reference ranges. These results indicate that the hepatitis in this patient should be classified into as a hepatocellular type. Recently, an outbreak of cholangiocarcinoma occurred in this same company, and chlorinated organic solvents, particularly DCM and DCP, were suspected to play a causative role[16, 17]. In patients with occupational cholangiocarcinoma, laboratory test results showed elevated γ-GTP levels (with or without elevated AST and/or ALT levels), and pathological findings demonstrated chronic bile duct injury and non-injured hepatocytes[17]. In addition, the patients with occupational cholangiocarcinoma were not exposed to TCE. Therefore, the mechanism causing severe acute hepatitis in the present patient seemed to be different from that causing occupational cholangiocarcinoma.

Although this patient's liver function improved rapidly after restricting further exposure and administering fresh frozen plasma and glucagon-insulin therapy, death due to acute liver failure was reported in a patient with suspected TCE exposure[9]. Thus, regular assessment of liver function in workers exposed to such chlorinated organic solvents is important because excessive exposure may induce lethal acute hepatitis.

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Comprehensive analysis of transcriptome and metabolome analysis in Intrahepatic Cholangiocarcinoma and Hepatocellular Carcinoma

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Intrahepatic cholangiocarcinoma (ICC) and hepatocellular carcinoma (HCC) are liver originated malignant tumors. Of the two, ICC has the worse prognosis because it has no reliable diagnostic markers and its carcinogenic mechanism is not fully understood. The aim of this study was to integrate metabolomics and transcriptomics datasets to identify variances if any in the carcinogenic mechanism of ICC and HCC. Ten ICC and 6 HCC who were resected surgically, were enrolled. miRNA and mRNA expression analysis were performed by microarray on ICC and HCC and their corresponding non-tumor tissues (ICC_NT and HCC_NT). Compound analysis was performed using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). Principle component analysis (PCA) revealed that among the four sample groups (ICC, ICC_NT, HCC, and HCC_NT) there were 14 compounds, 62 mRNAs and 17 miRNAs with two distinct patterns: tumor and non-tumor, and ICC and non-ICC. We accurately (84.38%) distinguished ICC by the distinct pattern of its compounds. Pathway analysis using transcriptome and metabolome showed that several pathways varied between tumor and non-tumor samples. Based on the results of the PCA, we believe that ICC and HCC have different carcinogenic mechanism therefore knowing the specific profile of genes and compounds can be useful in diagnosing ICC.

Intrahepatic cholangiocarcinoma (ICC) is the second most common hepatic cancer and accounts for 10–25% of all hepatic malignant tumors[1,2]. Hepatocellular carcinoma (HCC) represents the major histological subtype of primary liver malignancies, accounting for 70% to 85% of the total liver cancer burden[3]. Most cases of HCC (75% to 90%) are found in patients with liver cirrhosis resulting from chronic hepatitis B or C infection, alcoholic injury, and recently in non-alcoholic steatohepatitis (NASH)[4,5]. ICC is labeled as a malignant tumor arising from the peripheral intrahepatic bile duct epithelium[1]. High

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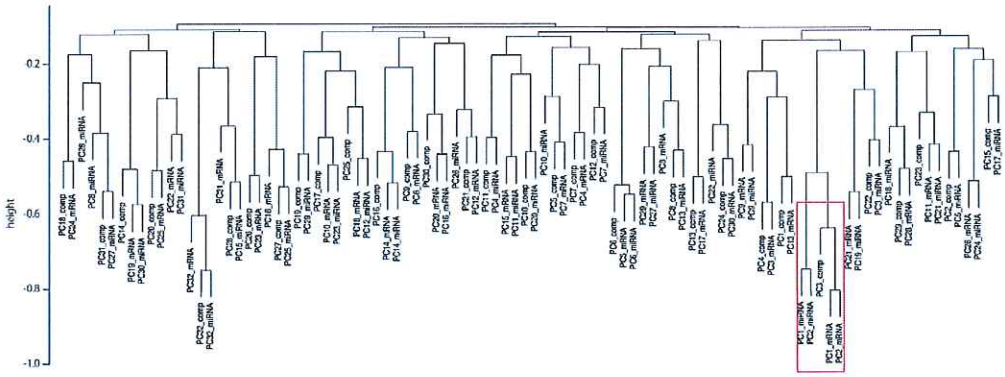

Figure 1. Hierarchical Clustering using correlation coefficients. Hierarchical clustering of 96 PCs: consisting of 32 PCs each obtained from mRNAs, miRNAs and compounds. Each PC consists of 32 dimensional vectors with 32 elements, each of which corresponds to the contribution of each sample to each PC. The correlation coefficients between PCs were computed using these 32 elements. On the Vertical axis are absolute negative correlation coefficients that are used as distance for hierarchical clustering (lower pairs have larger absolute correlations). Red rectangle indicates 5PCs which were chosen by hierarchical clustering.

risk factors associated with ICC includes being male[6] being over age 65[7], and having primary sclerosing cholangitis (PSC)[8], biliary duct cysts[9], hepatolithiasis[10], and chemical toxin overload[11]. It was also previously reported that genetically impaired biliary excretion of phospholipids is an underlying mechanism of ICC[12,13]. Metabolomic investigations support this view, as lower phosphatidylcholine and elevated glycine- and taurine-conjugated bile acids have been reported in the bile of ICC patients[14,15].

Several pathways and genetic alternations have been associated with ICC development. For example, aberrant glucose and lipid metabolism as well as 15-hydroxyprostaglandin dehydrogenase-mediated 15-keto-prostaglandin E2 signaling cascade inhibited ICC cell growth via peroxisome proliferator-activated receptor-gamma, Smad2/3, and TAP63 pathway[16]. The endocannabinoid anandamide exerts an anti-proliferative effect on ICC through one of the cannabinoid G-protein coupled receptor 55, and by stabilizing lipid rafts. This allows for the recruitment and activation of the Fas receptor complex[17]. Simvastatin is known to induce ICC cell death by disrupting Rac1/lipid raft co-localization and by depressing Rac1 activity[18]. Aberrant miRNA expression has also been associated with ICC; notably, miR-21 has been implicated in cell proliferation, apoptosis, metastasis, and migration[19,20]. let-7a was also found to be up-regulated in ICC and contributes to the survival of cholangiocytes via enforced IL-6 activity[21,22].

ICC is characterized by histological observations of dissected tumors. It is difficult to conclude whether ICC is truly derived from cholangiocytes as it was reported that patients with hepatitis C virus infection often develop ICC, suggesting that ICC is derived from transformed hepatocytes[23]. Several reports have suggested that Notch activation is critical for hepatocytes to convert into biliary lineage cells during the onset of ICC and its subsequent malignancy and progression. In these said studies therefore, ICC was generated by biliary lineage cells derived from hepatocytes, rather than from cholangiocytes[24]. ICC is known to have a poorer survival outcome than HCC mainly due to the advanced tumor stage of patients with intrahepatic metastasis at presentation and early postoperative recurrence[25]. New biomarkers that can detect ICC, especially in its early stages, and that can help clarify the mechanism of ICC are needed to increase the survival rate for ICC patients.

In this study, we performed a comprehensive analysis using transcriptome and metabolome to discover (1) if there is a difference in the carcinogenic process between ICC and HCC, and (2) accurate and sensitive molecular markers to diagnose ICC.

Result

Variable selection using principal component. Each mRNA, miRNA or compound taken from the thirty two samples (10 pairs of ICC and ICC-NT, and 6 pairs of HCC and HCC-NT) (Supplementary Table 1), was considered as a point in a 32 dimensional space and embedded in a low dimensional space using principal component analysis (PCA). Figure 1 shows the hierarchical clustering of the principal components (PCs). The vertical axis exhibits the negative correlation coefficients used to define the distance measures of the clusters. Since each CX_j^k (j,k = 1, 2,,, 32) is a composite of the 32 samples, $CX^k = (CX_1^k, CX_2^k, \ldots, CX_{32}^k)$ was also expressed as 32-dimensional vectors (see supplementary method). To reiterate, the primary aim of this study was to perform an integrated analysis of compounds, mRNA, and miRNA, to identify those that are related to ICC and HCC. The numbers attached to PCs represent the order of PCs while smaller numbers indicate larger contributions to overall variances. Using

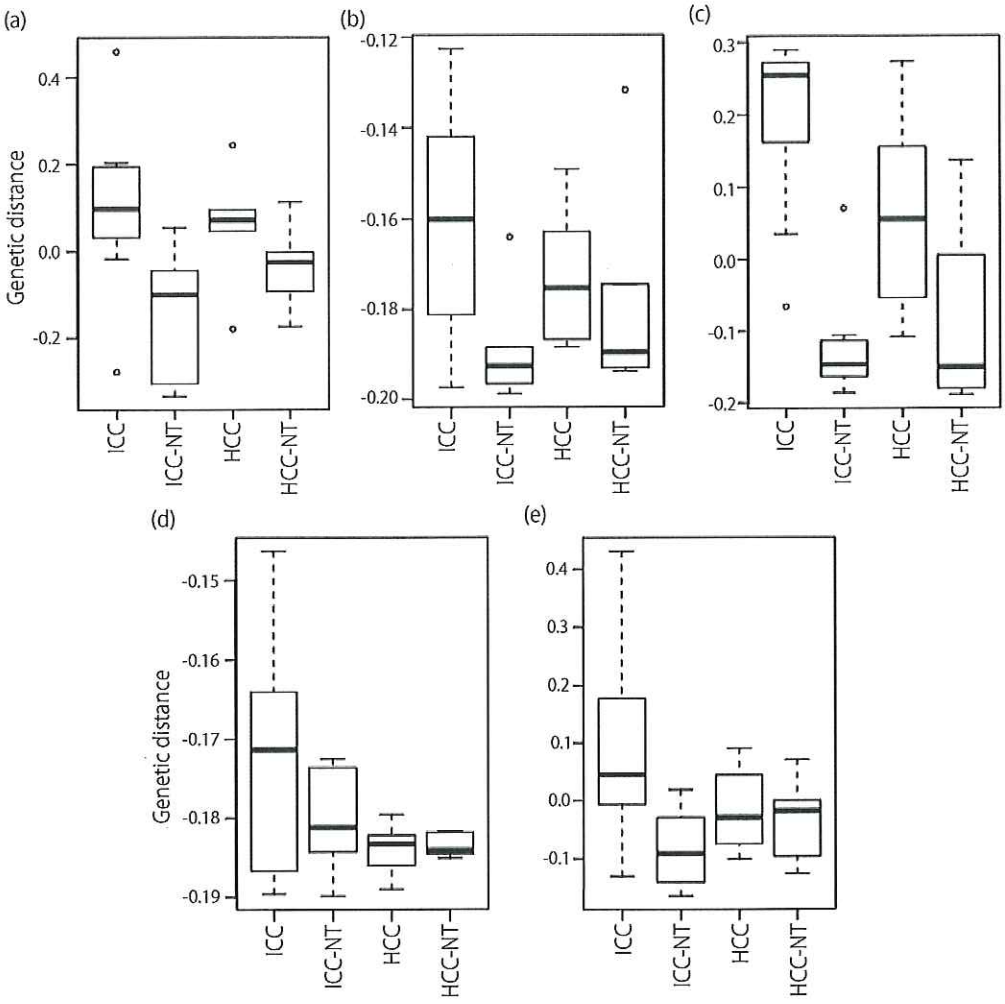

Figure 2. Scatter diagram. Lower triangle: Scatter plot between five PCs (**a**) PC3_comp, (**b**) PC1_mRNA, (**c**) PC2_mRNA, (**d**) PC1_miRNA, and (**e**) PC2_miRNA, selected based on the hierarchical clustering shown in Fig. 1. Each row and column corresponds to the PC displayed diagonally. Open rectangle in the second row and third column indicates the correlation coefficients (upper numerical value) and their P-values (lower numerical value) associated with the corresponding scatter plots.

Unweighted Pair Group Method with Arithmetic Mean (UPGMA) we performed separate PCA for compounds, mRNA and miRNA. The PCs were chosen based on the following two criteria: (1) The PC cluster should be located in the lower right position, in other words, the absolute value of the correlation coefficient should be the largest. (2) k of selected CX^k should be as small as possible, since a smaller k indicates more contributions. Five PC (loading)s fulfilled these criteria: PC3_comp (PC3_ compound), PC1_mRNA, PC2_mRNA, PC1_miRNA and PC2_miRNA (Fig. 3).

In order to validate the correlation of each selected PC, we performed a scatter plot analysis (Fig. 2). A comparison was done of the expression patterns of compound, miRNA, and mRNA among ICC, ICC-NT, HCC, and HCC-NT. As expected ICC and HCC had expressions that were markedly distinct from their non-tumor (NT) counterpart; however, the difference between ICC and ICC-NT was greater than HCC and HCC-NT (Fig. 2). Each row and column corresponds to the PCs displayed diagonally. For example, the scatter plot in the third row and second column is between PC1_mRNA and PC2_mRNA. Open rectangles in the second row and third column indicate correlation coefficients (upper numerical value) and their P-values (lower numerical value) associated with the corresponding scatter plots.

Next, to quantitatively confirm that selected PCs were significantly distinct among HCC, ICC, HCC-NT and ICC-NT, we performed the following categorical regression analysis.

$$CX_j^k = C_{X0} + C_{ICC}\delta_{ICC,j} + C_{ICC-NT}\delta_{ICC-NT,j} + C_{HCC}\delta_{HCC,j} + C_{HCC-NT}\delta_{HCC-NT,j}$$

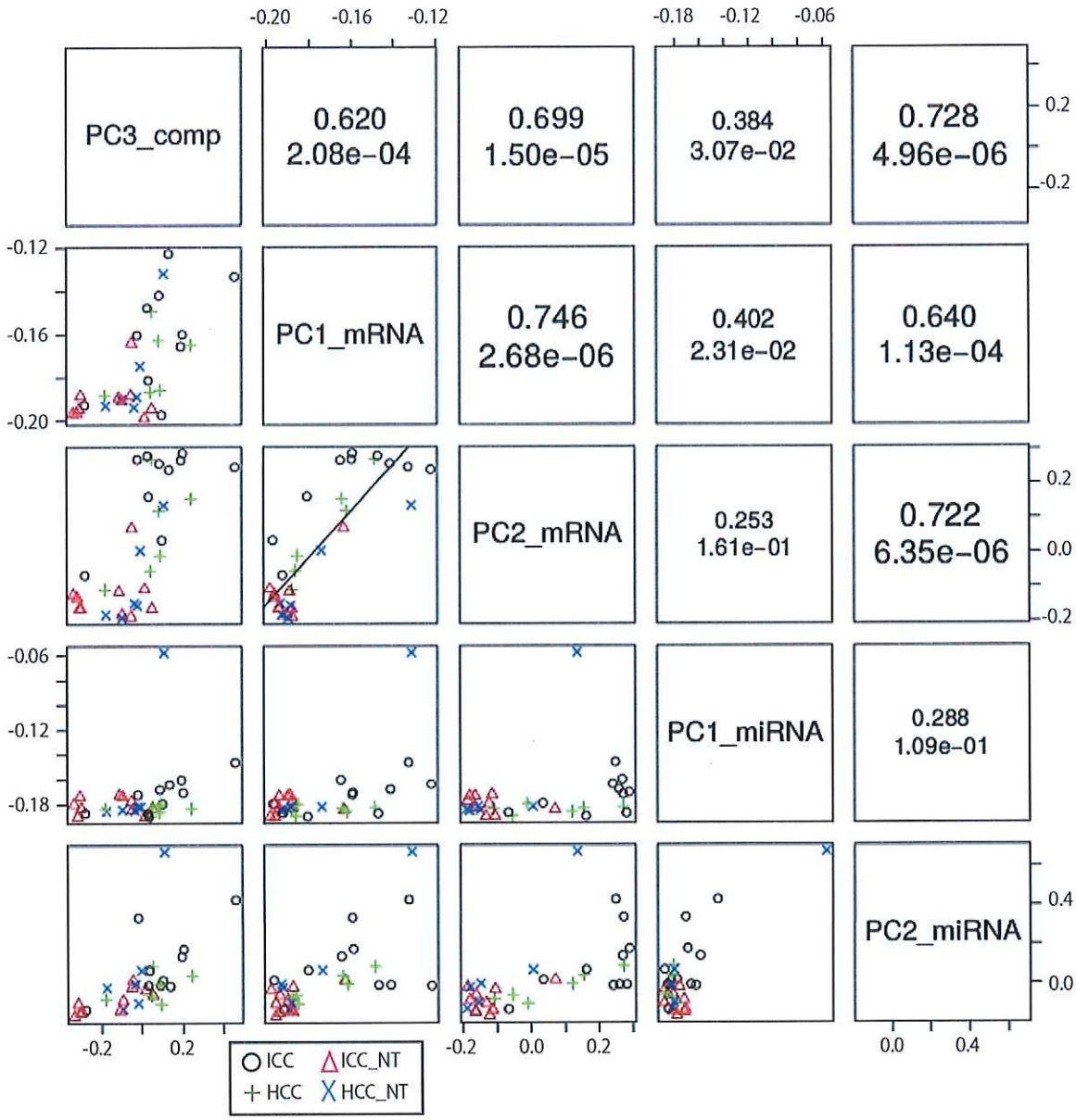

Figure 3. Box plot in 5 PCA. Box Plot of PCs is shown in Fig. 2. The sample contribution to each PC are shown using boxplot classified based on four sample classes. The correlation coefficients used for the hierarchical clustering tree represented in Fig. 1 and shown in the upper triangle in Fig. 2 are for the distances found among the five plots. Vertical line represents genetic distance. Red dots are depicted as measured value of each probe after normalization. P values are shown in a scatter plot (P value of PC3_comp, PC1_mRNA, PC2_mRNA, PC1_miRNA, and PC2_miRNA, correspond to 8.68e-03, 1.69e-02, 3.98e-06, 4.74e-02, and 9.42e-03, respectively).

Here $\delta_{a,i}$ takes one if the i-th sample is equivalent to category a (a represents ICC, ICC-NT, HCC, or HCC-NT), otherwise it had a value of zero. CX_i^k reflects the contribution of the sample i to the kth principal component. We employed categorical regression instead of ordinary multivariate analysis because the order and magnitude among the four groups (ICC, ICC/-NT, HCC, and HCC-NT) was unknown. The genetic distance between ICC and ICC-NT appeared larger than between HCC and HCC-NT (Fig 3). All together, this data suggests that we successfully selected PCs that accurately distinguished two distinct patterns (tumor/non-tumor and ICC/non-ICC) among the four groups. More detailed methodological and theoretical background can be found in our previous publications[26,27] and supplementary methods.

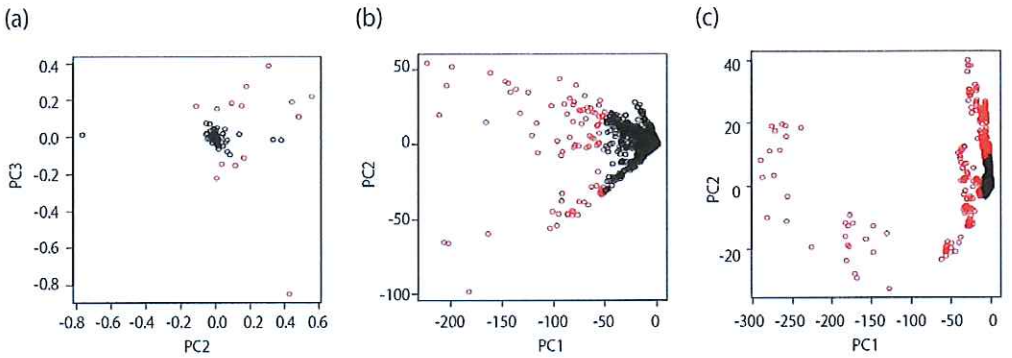

Figure 4. Embedding of compound, mRNA, and miRNA. (a) Two-dimensional embedding of compounds spanned by PC2 and PC3. (b) Two-dimensional embedding of mRNA spanned by PC1 and PC2. (c) Two dimensional embedding of miRNA spanned by PC1 and PC2. We chose compounds, mRNA and miRNAs as "outliers" for further analysis.

Selection of ICC related compound, mRNA, and miRNA. We used PCA based unsupervised FE to select the ICC related compounds, mRNA, and miRNA[28]. Results from scatter plots in five PCs, showed that each of the four groups had compound levels or gene expression patterns that were distinct. Figure 4 shows two-dimensional embedding of mRNA/miRNA spanned by PCXi1 and PCXi2 and compounds spanned by PCX_i2 and $PCX_i^{3\ all}$ obtained by PCA (see supplementary method). Each circle in Fig. 4 corresponds to an individual compound detected by CE-TOFMS or individual mRNA/miRNA expression (probe) on microarray plate. Red circles are "outliers" selected for further analysis: compounds along PC3, mRNA along PC1 and miRNAs along PC1 and PC2. In total 14 outlying compounds, 62 mRNAs, and 17 miRNAs, were selected since they had larger contributions towards the 5PCs (red circle in figure selection) (Fig. 4), (Supplementary Table 2, 3, 4).

Fourteen compounds (Fig. 5 and Supplementary Table 2), were selected that could separately distinguish each of our four sample groups. For example, Glycerol 3−phosphate, Succinic acid and Glycerophosphocholine were differentially expressed in the tumor (HCC and ICC) and non-tumor (HCC-NT and ICC-NT) population. A distinction between tumor and non-tumor samples was also seen in PC3 of compound (Fig. 3). Thus we surmise that it is due to these three compounds that a distinction can be made between tumors and non-tumors. On the other hand, four amino acids (Lys, Pro, Leu and Ile) were more diversely expressed in ICC/ICC-NT than in HCC and HCC-NT. Since this was also observed in PC3 (Fig. 3), we believe these four amino compounds are primarily responsible for ICC/ICC-NT having a larger genetic distance than HCC and HCC-NT. Hypoxanthine and Taurine were the only compounds that had distinct expressions that differentiated ICC from HCC, HCC-NT and ICC-NT which suggests that these two compounds allowed ICC to be distinguished from other tumor and non-tumor samples.

Multiple probes can be mapped to a singular gene in order to increase sensitivity and specificity; several mRNAs (APOA1, MTRNR2L2, and RPS2) in this study had multiple probes resulting in a total of 67 probes for 62 mRNAs. Abbreviations for genes are shown in Table S3. Among these 62 mRNAs (Fig. 6 and Supplementary Table 3), there were several groups that share similar expression with compounds. For example, HRP, HP, APOA1, ALDOB, ITIH4, ORM1, SERPINA1, HRG, and MT2A are mRNAs that express differentially between tumors (HCC and ICC) and non-tumors (ICC-NT and HCC-NT), while ALB, APOE, RBP4, TTR, AMBP, APOA2, APOC3, APOH, CES1 and APOC1 are expressed in ICC distinctly from the other three samples. We observed two varying patterns among mRNA: one is the distinction between tumors and non-tumors reflected by PC2_mRNA; the other is the distinction between ICC and the other three groups, which are reflected by PC1_mRNA (Fig. 3).

Similarly, two clear distinctions were observed between tumors and non-tumors, and ICC and non-ICC among 17 miRNAs (Fig. 7 and Table S4). The former group consisted of miR-21-5p, miR-122-5p, miR-451a, and miR-4286, while let-7b-5p and miR-16-5p fell in the latter. These two tendencies were also observed in PC2 and PC1 in miRNAs (Fig. 3).

ICC related carcinogenetic pathway. In order to clarify the biological significance of the selected compounds and genes Integrated Molecular Pathway Level Analysis (IMPaLA) (http://impala.molgen.mpg.de) was used to calculate the pathway enrichment of the compounds and mRNA chosen from pathways, such as KEGG and REACTOME as well as other data sets. The list of pathways with P values for which multiple comparisons and adjustments were carried out is shown ($p < 0.05$) in supplementary Table 5 and 6.

In the heatmap in Supplementary Fig. 1 and 2 the black boxes represent either compound or mRNAs. Similar pathways were classified under one color cluster using UPGMA. For example, C00819

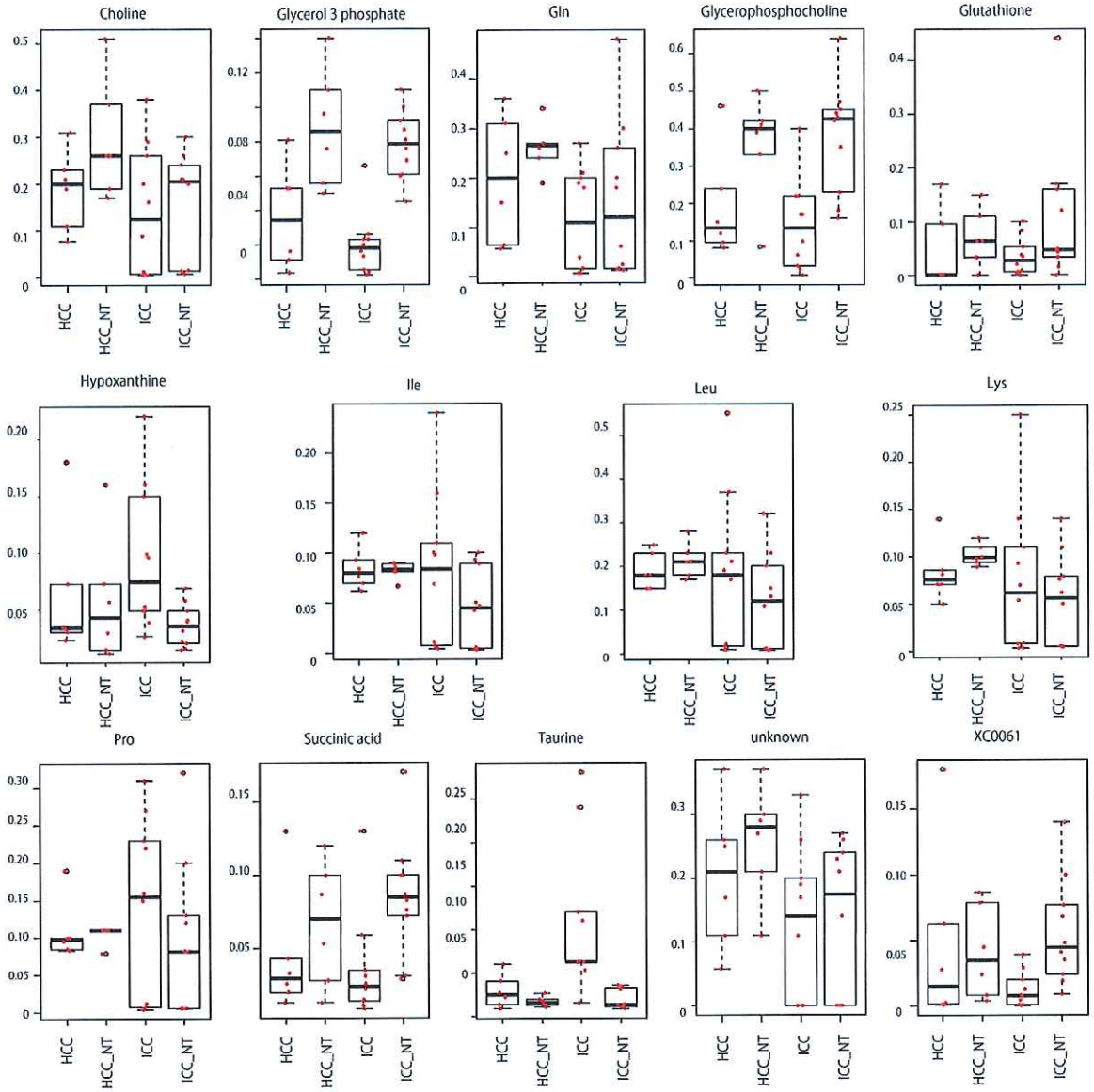

Figure 5. Box plot of selected compounds. Amount of 14 compounds used to discriminate between tumor (ICC and HCC) and non-tumor (ICC_NT and HCC_NT) or between ICC and non-ICC (ICC-NT, HCC, and HCC-NT). Left vertical axis shows the amount of compound. Red dots are depicted as measured value of each probe after normalization. Each p-value is indicated in Table S2. XC00061 is only known as Pubchem accession number (http://www.ncbi.nlm.nih.gov/pccompound). Unknown: neither the name of component nor the Pubchem accession number is known.

(Glutamine) contributes to D-glutamine and D-glutamate metabolism. The largest cluster (red) represents pathways related to tRNA synthesis (tRNA charging, tRNA aminoacylation, Cytosolic tRNA aminoacylation, Mitochondrial tRNA amicoacylation, Aminoacyl-tRNA biosynthesis), and amino acid synthesis (glutathione synthesis and recycling, amino acid transport across the plasma membrane, amino acid and oligopeptide SLC transporters, transport of inorganic cations/anions and amino acids/oligopeptide). The green cluster shows lipoprotein related pathways: metabolism of lipids and lipoproteins, acetylcholine Synthesis, glycerophospholipid metabolism, phospholipid metabolism, and hydrolysis of LPC (Supplementary Fig. 1).

In the mRNA analysis performed using DIANA-miRPath (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index), the yellow cluster represents the lipoprotein related pathways (PPAR signaling pathway, HDL—mediated lipid transport, lipoprotein metabolism, lipid digestion_ mobilization_ and transport, statin pathway, chylomicron—mediated lipid transport, scavenging of heme from

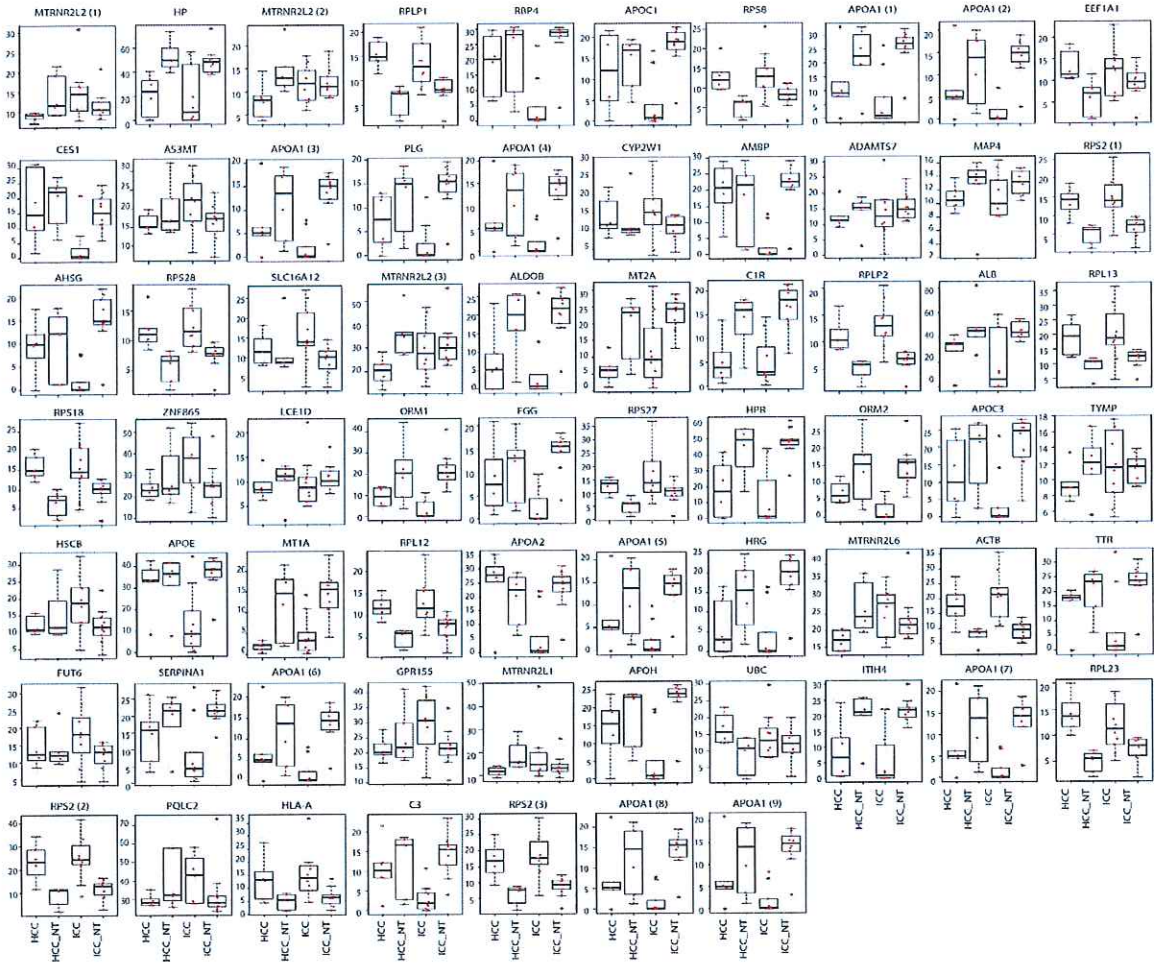

Figure 6. Box plot of selected mRNAs. Expression pattern of 62 mRNA used to discriminate between tumor (ICC and HCC) and non-tumor (ICC_NT and HCC_NT) or between ICC and non-ICC (ICC-NT, HCC, and HCC-NT). In cases where there are multiple probes for one mRNA, pleural box plots with the same mRNA are listed. Vertical left axis shows the expression level of mRNAs. Red dots are depicted as measured value of each probe after normalization. P-values are indicated in Table S3.

plasma, binding and uptake of ligands by scavenger receptors, FOXA2 and FOXA3 transcription factor networks). The red cluster shows the transcription related pathways including the tRNA relation pathway (activation of the mRNA upon cap−binding, ribosomal scanning and start codon recognition, peptide chain elongation, eukaryotic translation elongation, metabolism of RNA, metabolism of mRNA, ribosome, eukaryotic translation initiation, cap−dependent translation initiation, SRP−dependent cotranslational protein targeting to GTP hydrolysis and joining of the 60S ribosomal subunit, L13a−mediated translational silencing of ceruloplasmin 3−UTR−mediated translational regulation) (Supplementary Fig. 2).

Pathway analysis of the validated miRNAs using DIANA-miRPath pointed to 62 cancer-related pathways ($p < 0.05$). RNA synthesis related pathway (RNA transport, Ribosome biogenesis in eukaryotes, Ribosome, RNA polymerase, Aminoacyl-tRNA biosynthesis) and glucose-lipid synthesis pathway (Adipocytokine signaling pathway, Insulin signaling pathway) were found to be associated with ICC carcinogenesis (Supplementary Table 7).

Diagnosing ICC using compounds and miRNA expression pattern. We attempted to identify diagnostic biomarkers for ICC. We used leave one out cross validation (LOOCV) to classify samples into three groups: ICC, HCC, and non-tumorous. Using the profile of 14 compounds (Supplementary Table 2), we classified ICC, HCC and non-tumorous tissue with 84.38% accuracy (Table 1). ICC was distinguished from the other 3 sample groups with 78.13% of accuracy (Table 1) using the expression profile of 17 miRNAs (Supplementary Table 4). However mRNA expression combined with the characteristics

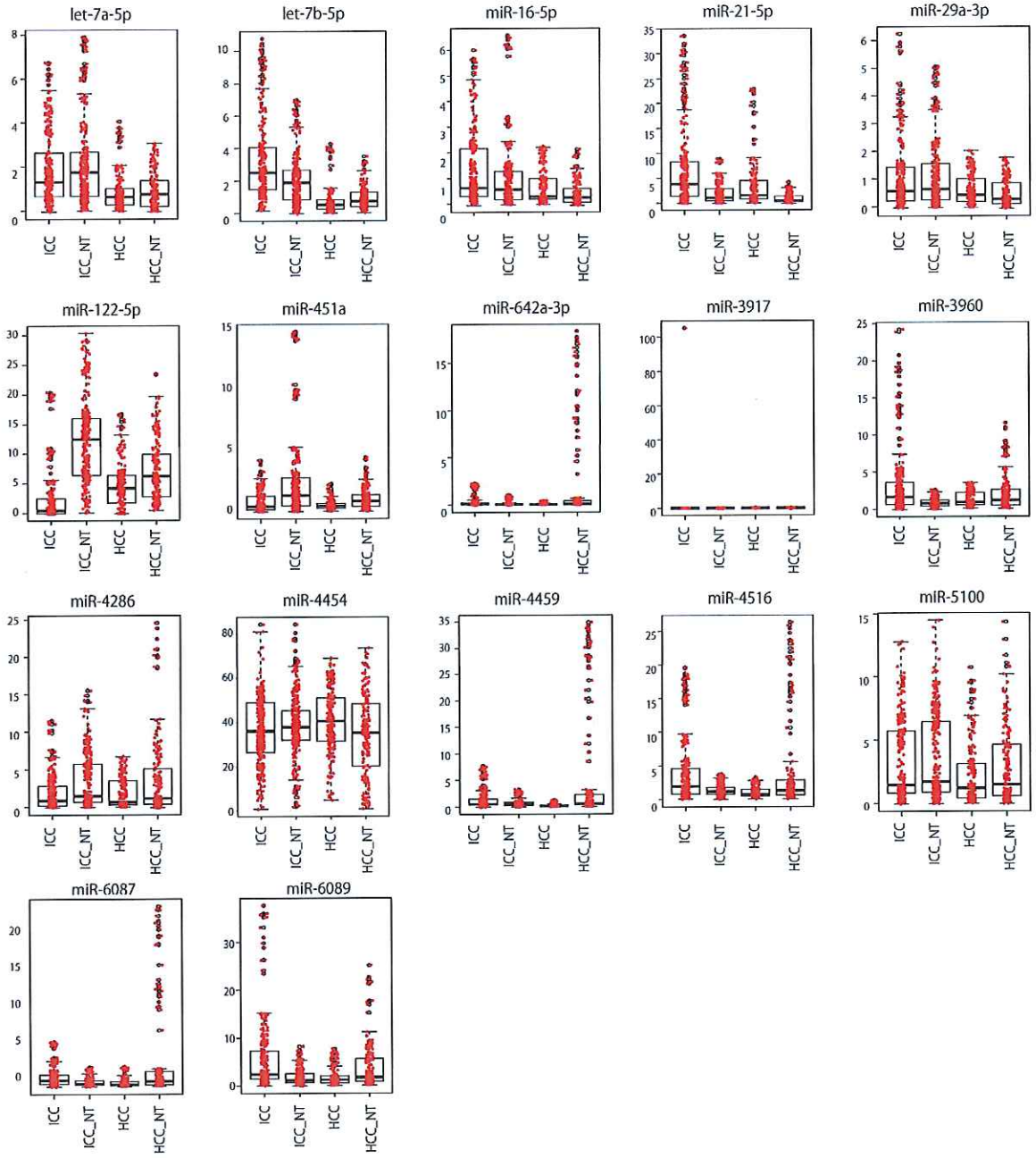

Figure 7. Box plot of selected miRNAs. Expression pattern of 17 miRNAs used to discriminate between tumor (ICC and HCC) and non-tumor (ICC_NT and HCC_NT) or between ICC and non-ICC (ICC-NT, HCC, and HCC-NT). Vertical left axis shows miRNA expression levels. P-values are indicated in Table S4.

of the compounds or miRNA expression profile was unable to diagnose ICC with high accuracy (date not shown).

Discussion

In this study, we performed an integrated analysis of transcriptome and metabolome to clarify the mechanism of ICC and HCC carcinogenesis and to discover novel diagnostic markers for ICC. Using five PCs comprising of 14 compounds, 62 mRNAs and 17 miRNAs, we observed two distinct patterns: tumor/non-tumor, and ICC/non-ICC.

It was difficult to uncover the carcinogenetic mechanism for ICC although each gene expression and the level of each compound could be determined. We used Integrated Molecular Pathway Level Analysis

		Result		
		non-tumor	HCC	ICC
A. Using profiling of compounds				
Prediction	non-tumor	14	0	2
	HCC	0	5	0
	ICC	2	1	8
B. Using miRNA expression pattern				
Prediction	non-tumor	13	1	1
	HCC	2	4	1
	ICC	1	1	8

Table 1. Classifying CC, HCC and non-tumor.

(IMPaLA) to analyze compounds and mRNA and the DIANA-miRPath for miRNA analysis. Aberrant expression of mRNA in ICC was related to aberrant tRNA metabolism, amino acid metabolism, and lipoprotein metabolism (supplementary Fig. 1). Elevated compound levels coincided with lipoprotein and tRNA metabolism (Supplementary Fig. 2). Finally a connection was found between ICC and several miRNA related pathways namely: RNA transport, ribosome biogenesis in eukaryotes, protein export, RNA polymerase, and Aminoacyl-tRNA biosynthesis. Although we did not find that lipoprotein to be a miRNA related pathway, it had a strong connection to miRNA. However lipid metabolism, amino acid metabolism, and RNA metabolism are associated with ICC formation.

In our analysis, there were two primary pathways implicated in ICC. The first is lipid and glucose related pathway. A large number of metabolomic changes were observed in HCC that were relative to cirrhosis or to control subjects (review in[29]). Lipids, bile juice secretion, and ICC were found to be closely inter-related. Taurocholate and phosphatidylcholine have no effect on apolipoprotein B (apo B) secretion but has been known to significantly increase the basolateral secretion of APOA1[30]. Elevated taurine level in ICC in this study coincides with previous reports[14,15]. Signs of metabolic remodeling has been detected by metabolomics in the livers of HCC patients, namely a decrease in glucose, citrate, and glycerol 3-phosphate coupled with an increase in pyruvate (signs of the Warburg effect[31]), and a switch from mitochondrial respiration to cytosolic aerobic glycolysis[32,33]. In our study, down regulated amino acid metabolism and up regulated G-3-P metabolism were observed in both HCC and ICC. Thus, metabolic reprogramming in HCC and ICC appeared to exhibit a modest Warburg shift towards glycolytic metabolism and a major upregulation of fatty acid catabolism in some tumor types.

The second pathway implicated in ICC is the tRNA related pathway. Aminoacyl-tRNA synthetases (ARSs) are essential and ubiquitous 'house-keeping' enzymes responsible for charging amino acids to their cognate tRNAs with a high fidelity and providing the substrates for global protein synthesis[34]. Defects in either canonical or noncanonical ARS functions can cause or contribute to human diseases. ARSs suspected involvement in several types of cancer through aberrant expression and interactions have been previously reported[35,36]. Our analysis indicated that in addition to ARS, many RNA regulated pathways were involved in ICC. Many therapeutic reagents that are highly potent also produce adverse side effects such as non steroidal anti-inflammatory drugs associated with increased risk of coronary heart disease, and anti-VEGF-A inhibitors implicated in the disruption of blood vessel maintenance. Novel cancer drug related to ARS might provide a new set of physiologic extracellular or intracellular pathways as the basis for developing novel therapeutics with minimal side effects. The advantage of applying natural secretory or endogenous ARSs (e.g., GARS and EPRS) to clinical use is that they catalyze the ligation of amino acids to their cognate tRNAs with a high fidelity. For example, RS and ARS-interacting multifunctional proteins participate in the formation of Glioblastoma multiforme, therefore these compounds are possible candidates to be used in the development of innovative drugs[36]. Another candidate is human LARS which has the ability to correct mitochondrial dysfunctions caused by $tRNA^{Leu}UUR^{A3233G}$ mutation-related neurodegenerative disorder in MELAS syndrome[37].

Several reports on the origin of hepatocyte and cholangiocyte have led to the understanding that hepatoblasts are bipotent precursors that develop into either hepatocyte (the main epithelial cells in the liver) or cholangiocyte (the epithelial cells lining the intrahepatic biliary ducts). The formation of hepatocyte and cholangiocyte is temporally and spatially separated, which suggests that localized inducers or repressing mechanisms operate to direct the fate of both[38]. Hepatocytes can change into biliary lineage cells when intrahepatic bile duct regeneration is induced, but cholangiocytes cannot proliferate owing to toxic influences[39]. Hepatocytes can transdifferentiate into biliary lineage cells regardless of their position in the hepatic lobule. The location of lineage-converting hepatocytes is likely decided by the nature of the toxins used. Notch-mediated conversion of hepatocytes into biliary lineage cells accelerates ICC formation[24]. Moreover non-B non-C HCC patients were chosen for this study as there would be are no influenced by gene expression or the amount of compounds in hepatic virus B or C infection. In

this study, embryological similarity between cholangiocyte and hepatocyte coincided with the similarity between the carcinogenic mechanism of ICC and HCC. Moreover, the difference in clinical malignancy grade between ICC and HCC also coincided with the genetic differences between ICC and HCC.

In this study we were able to diagnose ICC with high accuracy using molecular information from the tumor tissue. Specifically, the serum values of AFP and DCP, and CEA and CA19-9 were used to distinguish HCC and ICC, respectively. However, the specificity and sensitivity for diagnosing HCC by AFP and DCP, and diagnosing ICC by CEA and CA19-9 were not satisfactory. Pathological diagnosis of ICC was complicated because poorly differentiated HCC and ICC had similar pathological findings; as well, there was a type of HCC which had the characteristics of both ICC and HCC. ICC could be diagnosed using miRNA or compounds with an approximate 80% accuracy. To our knowledge this is the first report that has identified a highly specific tumor marker for ICC. Although our sample was not as large as would have been ideal, integrating transcriptome and metabolome analysis creates more reliable results than performing a single analysis such as transriptome or metabolome. We propose that in the future a similar study be done with a larger sample.

In conclusion, we found that there were several common pathways involved in ICC and HCC formation and the clinical and genetic malignant potential of ICC was higher than HCC. Using PCA we also revealed that ICC could be distinguished from non-ICC using the biomarkers we identified. These mRNA, miRNAs, and compounds are a promising start to uncovering novel biomarkers that may lead to therapeutic applications in the future.

Methods

Sample preparation. Ten ICC and six HCC samples were obtained by surgical resection (Supplementary Table 1). We created four groups using the ICC/HCC samples and their respective surrounding non-tumor tissues. ICC and HCC were diagnosed using tumor markers (AFP, CEA, CA19-9 and DCP), CT and pathological examination. All samples were negative for HBs-Ag and anti-HCV. All patients provided written informed consent, and the Faculty of Medicine Ethics Committee of Osaka City University approved all aspects of this study in accordance with the Helsinki Declaration.

RNA preparation and miRNA. Total RNA from tissue samples was prepared using a mirVana miRNA extraction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. To detect miRNA, 100 ng of RNA was labeled and hybridized using the Human microRNA Microarray Kit (Rel. 12.0) (Agilent Technologies, CA, USA) according to the manufacturer's protocol for use with Agilent microRNA microarrays Version 1.0. Hybridization signals were detected with Agilent DNA microarray scanner G2505B and the scanned images were analyzed using Agilent feature extraction software (v10.10.1.1). All data were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE57555.

mRNA microarray. To detect mRNA, 100 ng of RNA was labeled and hybridized using the SurePrint G3 Human GE Microarray Kit (Ver 2.0) (Agilent Technologies, CA, USA) according to the manufacturer's protocol for use with Agilent microRNA microarrays Version 2.0. Hybridization signals were detected with Agilent DNA microarray scanner G2539A and the scanned images were analyzed using Agilent feature extraction software (v10.10.1.1). All data were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE57555.

Measurement of metabolites. After surgical resection, the sample aliquots were frozen in liquid nitrogen and then tissue was made into a fine powder using a pestle and mortar in liquid nitrogen. Approximately 50 mg of frozen powder tissue was plunged into 1,500 μL of 50% acetonitrile/Milli-Q water containing internal standards (H3304-1002, Human Metabolome Technologies, Inc., Tsuruoka, Japan) at 0 °C in order to inactivate the enzymes. The tissue was homogenized thrice at 1,500 rpm for 120 sec using a tissue homogenizer (Microsmash MS100R, Tomy Digital Biology Co., Ltd., Tokyo, Japan) and then the homogenate was centrifuged at 2,300 × *g* and 4 °C for 5 min. Subsequently, 800 μL of upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter at 9,100 × *g* and 4 °C for 120 min to remove proteins. The filtrate was centrifugally concentrated and re-suspended in 50 μL of Milli-Q water for CE-MS analysis.

Capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) was carried out using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 6210 Time of Flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). The systems were controlled by Agilent G2201AA ChemStation software version B.03.01 for CE (Agilent Technologies, Waldbronn, Germany). The metabolites were analyzed using a fused silica capillary (50 μm *i.d.* × 80 cm total length), with commercial electrophoresis buffer (Solution ID: H3301-1001 for cation analysis and H3302-1021 for anion analysis, Human Metabolome Technologies) as the electrolyte. The sample was injected at a pressure of 50 mbar for 10 sec (approximately 10 nL) in cation analysis and 25 sec (approximately 25 nL) in anion analysis. The spectrometer was scanned from *m/z* 50 to 1,000. Other conditions were as previously described[40–42].

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Author Contributions

Y.M. and Y.T. designed the research. Y.M., S.K., A.T., S.I., E.K., K.Iwaisako., K.Ikeda, N.K., T.O. and Y.T. performed the research. Y.M., N.K., T.O. and Y.T. wrote the paper.

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Supplementary information

Comprehensive analysis of transcriptome and metabolome analysis in Intrahepatic Cholangiocarcinoma and Hepatocellular Carcinoma

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Supplementary tables

Supplementary table 1. Clinical information

Supplementary table 2. List of compounds selected by PCA

Supplementary table 3. List of mRNAs selected by PCA

Supplementary table 4. List of miRNAs selected by PCA

Supplementary table 5. List of cancer related pathways based on compound information.

Supplementary table 6. List of cancer related pathways based on mRNA information.

Supplementary table 7. List of cancer related pathways based on miRNA information.

Supplementary figure

Supplementary figure 1. Heatmap of compound

Supplementary figure 2. Heatmap of mRNA

Supplementary methods

Supplementary table 1. Clinical information

Code No.	Sex	Age	CEA (ng/ml)	CA19-9 (ng/ml)	AFP (ng/ml)	DCP (U/ml)	Histology	Tumor size (mm x mm)
ICC								
1	M	74	9.4	34	6	24	M	35 x 45
2	M	32	1.7	1223	3.1	39	M	125 x 95
3	M	79	6.1	15	3.5	306	M	35 x 25
4	M	57	2.5	891	1.7	ND	M	23 x 15
5	M	63	8.6	8	4.9	16	M	21 x 16
6	F	62	3.1	28	3.7	17	M	20 x 18
7	M	39	1.5	105	4.6	21	W	ND
8	M	31	2.1	799	7.5	25	M	21x19
9	F	50	12.8	43566	2	NI	M	54x48
10	M	34	4	44	5.7	29	W	10x15
all		52.1±17.6	5.2±3.9	4671±13673	4.3±1.82	59.6±99.8		39.9±34.5
HCC								
11	M	74	4.9	9	4010.7	515	P	40 x 25
12	M	72	3.4	3	4.3	23	M	55 x 45
13	M	77	20.8	160	1750	8177	M	45 x 30
14	M	71	2.3	10	3.6	767	M	45 x 40

15	M	69	1.5	2	6.1	39365	M	80 x 60
16	F	76	2	2.6	2.9	48911	M	90 x 70
all		73.2±3.1	5.8±7.4	31.1±63.2	962.9±1629.3	16293±21986		59.2±20.8

Abbreviations NI; no information, ND; not determined the size of tumor, Histology; W; well differentiated, M; moderately differentiated, P; poorly differentiated

Supplementary table 2. List of compounds selected by PCA

Accession No.	Name	P-value
C00042	Succinic acid	4.61E-02
C00047	Lysine	3.87E-01
C00051	Glutathione	2.77E-01
C00064	Glutamine	1.37E-01
C00093	Glycerol 3 phosphate	7.67E-06
C00114	Choline	9.61E-02
C00123	Leucine	4.36E-01
C00148	Proline	7.79E-01
C00245	Taurine	1.58E-03
C00262	Hypoxanthine	1.14E-01
C00303	Glutamine	1.37E-01
C00407	Isoleucine	2.90E-01
C00670	Glycerophosphocholine	2.14E-03
C00739	Lysine	3.87E-01
C00763	Proline	7.79E-01
C00819	Glutamine	1.37E-01
C01570	Leucine	4.36E-01
C06418	Isoleucine	2.90E-01

C16434	Isoleucine	2.90E-01
C16435	Proline	7.79E-01
C16439	Leucine	4.36E-01
C16440	Lysine	3.87E-01
unknown	XC0061	1.31E-01
unknown	unknown	1.18E-01

Abbreviation, Accession No. is based on Pubchem.

Supplementary table 3. List of mRNAs selected by PCA

Accession No.	Gene name	Abbreviation	P-value
NM_000035	aldolase B, fructose-bisphosphate	ALDOB	3.95e-04
NM_000039	apolipoprotein A-I	APOA1	3.80e-04
NM_000040	apolipoprotein C-III	APOC3	8.34e-04
NM_000041	apolipoprotein E	APOE	1.30e-03
NM_000042	apolipoprotein H	APOH	1.71e-04
NM_000064	complement component 3	C3	9.44e-04
NM_000150	fucosyltransferase 6	FUT6	1.79e-01
NM_000301	plasminogen	PLG	1.01e-04
NM_000371	transthyretin	TTR	3.16e-03
NM_000412	histidine-rich glycoprotein	HRG	1.81e-04
NM_000477	albumine	ALB	3.89e-03
NM_000509	fibrinogen gamma chain	FGG	2.41e-04
NM_000607	orosomucoid 1	ORM1	7.24e-05
NM_000608	orosomucoid 2	ORM2	3,22e-05
NM_000976	ribosomal protein L12	RPL12	8.38e-04
NM_000978	ribosomal protein L23	RPL23	1.20e-04
NM_001002236	serpin peptidase inhibitor, clade A	SERPINA1	3.10e-03
NM_001003	ribosomal protein, large, P1	RPLP1	6.19e-05

NM_001004	ribosomal protein, large, P2	RPLP2	1.49e-05
NM_001012	ribosomal protein S8	RPS8	2.57e-03
NM_001025195	carboxylesterase 1	CES1	1.38e-03
NM_001030	ribosomal protein S27	RSP27	8.87e-03
NM_001031	ribosomal protein S28	RPS28	2.24e-04
NM_001033045	G protein-coupled receptor 155	GPR155	9.62e-02
NM_001040125	PQ loop repeat containing 2	PQLC2	2.59e-01
NM_001101	actin beta	ACTB	4.22e-05
NM_001113755	thymidine phosphorylase	TYMP	5.12e-01
NM_001190452	MT-RNR2-like 1	MTRNP2L1	2.54e-01
NM_001190470	MT-RNR2-like 2	MTRNP2L2	1.35e-01
NM_001190487	MT-RNR2-like 6	MTRNR2L6	7.29e-02
NM_001195605,	zinc finger protein 865	ZNF865	1.36e-01
NM_001402	eukaryotic translation elongation factor 1 alpha 1	EEF1A1	4.14e-02
NM_001622	alpha-2-HS-glycoprotein	AHSG	8.46e-05
NM_001633	alpha-1-microglobulin/bikunin precursor	AMBP	6.78e-05
NM_001643	apolipoprotein A-II	APOA2	3.78e-04
NM_001645	apolipoprotein C-I	APOC1	5.72e-04
NM_001733	complement component 1, r subcomponent	C1R	2.54e-05
NM_002116	major histocompatibility complex, class I, A	HLA-A	1.28e-02

NM_002218	inter-alpha-trypsin inhibitor heavy chain family, member 4	ITIH4	2.79e-05
NM_002952	ribosomal protein S2	RPS2	3.27e-05
NM_005143	haptoglobin	HP	3.90e-04
NM_005946	metallothionein 1A	MT1A	1.03e-04
NM_005953	metallothionein 2A	MT2A	9.58e-04
NM_006744	retinol binding protein 4, plasma	RBP4	1.04e-04
NM_014272	ADAM metallopeptidase with thrombospondin type 1 motif, 7	ADAMTS7	8.27e-01
NM_017781	cytochrome P450, family 2, subfamily W, polypeptide 1	CYP2W1	4.30e-01
NM_020682	arsenite methyltransferase	AS3MT	1.97e-01
NM_020995	haptoglobin-related protein	HRP	2.60e-05
NM_021009	ubiquitin C	UBC	1.14e-01
NM_022551	ribosomal protein S18	RPS18	1.36e-01
NM_030885	microtubule-associated protein 4	MAP4	3.69e-01
NM_033251	ribosomal protein L1	RPL13	3.08e-03
NM_172002	HscB mitochondrial iron-sulfur cluster co-chaperone	HSCB	1.08e-01
NM_178352	late cornified envelope 1D	LCE1D	8.73e-01
NM_213606	solute carrier family 16, member 12	SLC16A12	1.21e-01

Abbreviation: This list includes some overlapping microarray probe (9 APOA1, 2 MTRNR2L2, and 2 RPL2

Supplementary table 4. List of miRNAs selected by PCA

miRNA	p-value	miRNA	p-value
hsa-let-7a-5p	0.00E+00	hsa-miR-4454	2.85E-04
hsa-let-7b-5p	0.00E+00	hsa-miR-4459	0.00E+00
hsa-miR-122-5p	0.00E+00	hsa-miR-4516	0.00E+00
hsa-miR-16-5p	6.66E-16	hsa-miR-451a	0.00E+00
hsa-miR-21-5p	0.00E+00	hsa-miR-5100	9.73E-06
hsa-miR-29a-3p	1.84E-14	hsa-miR-6087	1.60E-01
hsa-miR-3917	5.40E-01	hsa-miR-6089	0.00E+00
hsa-miR-3960	0.00E+00	hsa-miR-642a-3p	0.00E+00
hsa-miR-4286	3.24E-12		

Supplementary table 5. List of cancer related pathway based on compound information.

Name	Source	Overlapping metabolites	No. of metabolites	P-value
Amine compound SLC transporters	Reactome	C00114;C00064;C00148;C00245;C00407;C00047;C00123	35 (35)	4.31E-08
ABC transporters - Homo sapiens (human)	KEGG	C00064;C00114;C00148;C00245;C00093;C00407;C00051;C00047;C00123	122 (122)	9.47E-08
Transport of glucose and other sugars_ bile salts and organic acids_ metal ions and amine compounds	Reactome	C00064;C00114;C00148;C00245;C00407;C00042;C00047;C00123	78 (83)	9.47E-08
leukotriene biosynthesis	HumanCyc	C00064;C00148;C00407;C00051;C00047;C00123	29 (30)	2.71E-07
γ-glutamyl cycle	HumanCyc	C00064;C00148;C00407;C00051;C00047;C00123	29 (29)	2.71E-07
Glutathione synthesis and recycling	Reactome	C00064;C00148;C00407;C00051;C00047;C00123	30 (31)	2.82E-07
Na+/Cl- dependent neurotransmitter transporters	Reactome	C00064;C00148;C00245;C00407;C00047;C00123	31 (31)	2.88E-07
Amino acid transport across the plasma membrane	Reactome	C00064;C00148;C00245;C00407;C00047;C00123	32 (32)	2.88E-07
SLC-mediated transmembrane transport	Reactome	C00114;C00064;C00148;C00262;C00245;	158 (164)	2.88E-07

		C00407;C00042;C00047;C00123		
Glutathione conjugation	Reactome	C00064;C00148;C00407;C00051;C00047;C00123	36 (40)	5.48E-07
Transmembrane transport of small molecules	Reactome	C00114;C00064;C00148;C00262;C00245;C00407;C00042;C00047;C00123	184 (195)	9.27E-07
Endosomal/Vacuolar pathway	Reactome	C00064;C00148;C00047;C00123;C00407	20 (20)	1.49E-06
Amino acid and oligopeptide SLC transporters	Reactome	C00064;C00148;C00245;C00407;C00047;C00123	45 (45)	1.67E-06
Proton/oligonucleotide cotransporters	Reactome	C00064;C00148;C00047;C00123;C00407	21 (21)	1.67E-06
Transport of inorganic cations/anions and amino acids/oligopeptides	Reactome	C00064;C00148;C00245;C00407;C00047;C00123	48 (48)	2.25E-06
tRNA charging	HumanCyc	C00064;C00148;C00047;C00123;C00407	24 (24)	3.03E-06
Antigen processing-Cross presentation	Reactome	C00064;C00148;C00047;C00123;C00407	29 (29)	7.89E-06
Transport of inorganic cations-anions and amino acids-oligopeptides	Wikipathways	C00064;C00148;C00407;C00123;C00047	31 (32)	1.06E-05
Metabolism of amino acids and derivatives	Reactome	C00064;C00148;C00245;C00407;C00042;C00051;C00047;C00123	181 (190)	1.32E-05
S-methyl-5-thio-α-D-ribose 1-phosphate degradation	HumanCyc	C00064;C00148;C00047;C00123;C00407	35 (35)	1.72E-05
Class I MHC mediated antigen processing &	Reactome	C00064;C00148;C00047;C00123;C00407	35 (35)	1.72E-05

presentation				
Immune System	Reactome	C00114;C00064;C00148;C00407;C00047;C00123	87 (102)	5.84E-05
Protein digestion and absorption - Homo sapiens (human)	KEGG	C00064;C00148;C00407;C00123;C00047	47 (47)	7.25E-05
Adaptive Immune System	Reactome	C00064;C00148;C00047;C00123;C00407	48 (48)	7.74E-05
Gene Expression	Reactome	C00064;C00148;C00407;C00042;C00047;C00123	94 (100)	8.17E-05
Phase II conjugation	Wikipathways	C00064;C00148;C00407;C00051;C00047;C00123	95 (110)	8.37E-05
Aminoacyl-tRNA biosynthesis - Homo sapiens (human)	KEGG	C00064;C00148;C00407;C00123;C00047	52 (52)	0.000104
Metabolism	Reactome	C00114;C00064;C00148;C00262;C00245;C00093;C00407;C00670;C00042;C00051;C00047;C00123	794 (1000)	0.000107
Phase II conjugation	Reactome	C00064;C00148;C00407;C00051;C00047;C00123	114 (140)	0.000222
Hydrolysis of LPC	Reactome	C00114;C00093;C00670	8 (8)	0.000254
tRNA Aminoacylation	Wikipathways	C00064;C00148;C00407;C00123;C00047	65 (65)	0.000254
Cytosolic tRNA aminoacylation	Reactome	C00064;C00148;C00047;C00123;C00407	65 (65)	0.000254

Mitochondrial tRNA aminoacylation	Reactome	C00064;C00148;C00047;C00123;C00407	65 (65)	0.000254
tRNA Aminoacylation	Reactome	C00064;C00148;C00047;C00123;C00407	65 (65)	0.000254
Mineral absorption - Homo sapiens (human)	KEGG	C00064;C00148;C00407;C00123	29 (29)	0.000276
Metabolism of amino acids and derivatives	Wikipathways	C00064;C00148;C00245;C00042;C00051;C00047	173 (186)	0.00203
One carbon donor	Wikipathways	C00114;C00051;C00245	19 (23)	0.00275
Doxycycline Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Demeclocycline Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Oxytetracycline Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Minocycline Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Lymecycline Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Tetracycline Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Clomocycline Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Clarithromycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Clindamycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Azithromycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Streptomycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Spectinomycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Kanamycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Gentamicin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275

Netilmicin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Neomycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Roxithromycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Erythromycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Amikacin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Telithromycin Action Pathway	SMPDB	C00148;C00407;C00123	20 (20)	0.00275
Biological oxidations	Reactome	C00064;C00148;C00407;C00051;C00047;C00123	202 (252)	0.00306
Glucose Homeostasis	Wikipathways	C00262;C00407;C00047	21 (21)	0.0031
Urea cycle and metabolism of arginine_ proline_ glutamate_ aspartate and asparagine	EHMN	C00042;C00064;C00148;C00051;C00047	125 (125)	0.0036
Amino acid conjugation	Wikipathways	C00064;C00245	5 (5)	0.00715
Transport of glucose and other sugars_ bile salts and organic acids_ metal ions and amine compounds	Wikipathways	C00114;C00042;C00148	29 (29)	0.00795
Neurotransmitter Release Cycle	Reactome	C00114;C00042;C00064	30 (30)	0.00867
Glycine_ serine_ alanine and threonine metabolism	EHMN	C00114;C00064;C00051;C00047	88 (88)	0.013
Glycerophospholipid metabolism	EHMN	C00114;C00064;C00093;C00670	96 (96)	0.0179
GABAergic synapse - Homo sapiens (human)	KEGG	C00064;C00042	9 (9)	0.0232

Acetylcholine Synthesis	Wikipathways	C00114;C00670	9 (9)	0.0232
Homocarnosinosis	SMPDB	C00042;C00064;C00051	48 (48)	0.0306
Hyperinsulinism-Hyperammonemia Syndrome	SMPDB	C00042;C00064;C00051	48 (48)	0.0306
Succinic semialdehyde dehydrogenase deficiency	SMPDB	C00042;C00064;C00051	48 (48)	0.0306
4-Hydroxybutyric Aciduria/Succinic Semialdehyde Dehydrogenase Deficiency	SMPDB	C00042;C00064;C00051	48 (48)	0.0306
Glutamate Metabolism	SMPDB	C00042;C00064;C00051	48 (48)	0.0306
2-Hydroxyglutric Aciduria (D And L Form)	SMPDB	C00042;C00064;C00051	48 (48)	0.0306
Transmission across Chemical Synapses	Reactome	C00064;C00114;C00042	51 (51)	0.0318
Neuronal System	Reactome	C00064;C00114;C00042	51 (51)	0.0318
Prolinemia Type II	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
Prolidase Deficiency (PD)	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
Arginine and Proline Metabolism	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
Hyperprolinemia Type I	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
Hyperprolinemia Type II	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
Ornithine Aminotransferase Deficiency (OAT Deficiency)	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
Arginine: Glycine Amidinotransferase Deficiency (AGAT Deficiency)	SMPDB	C00042;C00148;C00763	52 (52)	0.0318

Glycerophospholipid metabolism - Homo sapiens (human)	KEGG	C00114;C00093;C00670	52 (52)	0.0318
Hyperornithinemia with gyrate atrophy (HOGA)	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
Creatine deficiency_ guanidinoacetate methyltransferase deficiency	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
L-arginine:glycine amidinotransferase deficiency	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
Hyperornithinemia-hyperammonemia-homocitrullinuria [HHH-syndrome]	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
Guanidinoacetate Methyltransferase Deficiency (GAMT Deficiency)	SMPDB	C00042;C00148;C00763	52 (52)	0.0318
D-Glutamine and D-glutamate metabolism - Homo sapiens (human)	KEGG	C00064;C00819	12 (12)	0.0318
Synthesis of PG	Reactome	C00114;C00093	13 (13)	0.0354
Glycerophospholipid biosynthesis	Wikipathways	C00114;C00093;C00670	56 (64)	0.0354
Trans-sulfuration pathway	Wikipathways	C00051;C00245	14 (14)	0.0354
Isovaleric Aciduria	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
3-Methylcrotonyl Coa Carboxylase Deficiency Type I	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
Propionic Acidemia	SMPDB	C00042;C00407;C00123	58 (58)	0.0354

Maple Syrup Urine Disease	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
3-Hydroxy-3-Methylglutaryl-CoA Lyase Deficiency	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
Isobutyryl-coa dehydrogenase deficiency	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
3-hydroxyisobutyric aciduria	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
3-hydroxyisobutyric acid dehydrogenase deficiency	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
Isovaleric acidemia	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
Methylmalonate Semialdehyde Dehydrogenase Deficiency	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
Methylmalonic Aciduria	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
3-Methylglutaconic Aciduria Type IV	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
3-Methylglutaconic Aciduria Type III	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
Beta-Ketothiolase Deficiency	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
Glycerophospholipid biosynthesis	Reactome	C00114;C00093;C00670	58 (63)	0.0354
3-Methylglutaconic Aciduria Type I	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
Valine_ Leucine and Isoleucine Degradation	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
2-Methyl-3-Hydroxybutryl CoA Dehydrogenase Deficiency	SMPDB	C00042;C00407;C00123	58 (58)	0.0354
Metabolism of lipids and lipoproteins	Reactome	C00114;C00245;C00093;C00670;C00042;	358 (443)	0.0372

		C00051		
Synthesis of PA	Reactome	C00114;C00093	15 (15)	0.04
Phospholipid metabolism	Reactome	C00114;C00093;C00670	66 (71)	0.0498
Purine metabolism	Reactome	C00064;C00051;C00262	66 (67)	0.0498

Supplementary table 6. List of cancer related pathway based on mRNA information.

Name	Source	Overlapping genes	No. of genes	p-value
Peptide chain elongation	Reactome	NM_001003;NM_000978;NM_002952;NM_022551;NM_001030;NM_000976;NM_033251;NM_001012;NM_001402;NM_001031;NM_001004	95 (95)	5.64E-10
Eukaryotic Translation Elongation	Reactome	NM_001003;NM_000978;NM_002952;NM_022551;NM_001030;NM_000976;NM_033251;NM_001012;NM_001402;NM_001031;NM_001004	100 (100)	5.64E-10
Cytoplasmic Ribosomal Proteins	Wikipathways	NM_001003;NM_000978;NM_002952;NM_022551;NM_001030;NM_000976;NM_033251;NM_001012;NM_001031;NM_001004	88 (88)	3.62E-09
Eukaryotic Translation Termination	Reactome	NM_001003;NM_000978;NM_002952;NM_022551;NM_001030;NM_000976;NM_033251;NM_001012;NM_001031;NM_001004	94 (94)	5.34E-09
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	Reactome	NM_001003;NM_000978;NM_002952;NM_022551;NM_001030;NM_000976;NM_033251;NM_001012;NM_001031;NM_001004	99 (99)	7.25E-09
Formation of a pool of free 40S subunits	Reactome	NM_001003;NM_000978;NM_002952;NM_022551;NM_001030;NM_000976;NM_033251;NM_001012;NM_001031;NM_001004	106 (106)	1.21E-08
Nonsense Mediated Decay (NMD) enhanced	Reactome	NM_001003;NM_000978;NM_002952;NM_0225	110 (110)	1.32E-08

by the Exon Junction Complex (EJC)		51;NM_001030;NM_000976;NM_033251;NM_0 01012;NM_001031;NM_001004		
Nonsense-Mediated Decay (NMD)	Reactome	NM_001003;NM_000978;NM_002952;NM_0225 51;NM_001030;NM_000976;NM_033251;NM_0 01012;NM_001031;NM_001004	110 (110)	1.32E-08
3_ -UTR-mediated translational regulation	Reactome	NM_001003;NM_000978;NM_002952;NM_0225 51;NM_001030;NM_000976;NM_033251;NM_0 01012;NM_001031;NM_001004	116 (116)	1.64E-08
L13a-mediated translational silencing of Ceruloplasmin expression	Reactome	NM_001003;NM_000978;NM_002952;NM_0225 51;NM_001030;NM_000976;NM_033251;NM_0 01012;NM_001031;NM_001004	116 (116)	1.64E-08
GTP hydrolysis and joining of the 60S ribosomal subunit	Reactome	NM_001003;NM_000978;NM_002952;NM_0225 51;NM_001030;NM_000976;NM_033251;NM_0 01012;NM_001031;NM_001004	117 (117)	1.64E-08
Translation	Reactome	NM_001003;NM_000978;NM_002952;NM_0225 51;NM_001030;NM_000976;NM_033251;NM_0 01012;NM_001402;NM_001031;NM_001004	160 (160)	1.64E-08
SRP-dependent cotranslational protein targeting to membrane	Reactome	NM_001003;NM_000978;NM_002952;NM_0225 51;NM_001030;NM_000976;NM_033251;NM_0 01012;NM_001031;NM_001004	118 (118)	1.64E-08
Cap-dependent Translation Initiation	Reactome	NM_001003;NM_000978;NM_002952;NM_0225	124 (124)	2.34E-08

		51;NM_001030;NM_000976;NM_033251;NM_001012;NM_001031;NM_001004		
Eukaryotic Translation Initiation	Reactome	NM_001003;NM_000978;NM_002952;NM_022551;NM_001030;NM_000976;NM_033251;NM_001012;NM_001031;NM_001004	124 (124)	2.34E-08
Ribosome - Homo sapiens (human)	KEGG	NM_001003;NM_001004;NM_002952;NM_022551;NM_000976;NM_001030;NM_033251;NM_001012;NM_001031;NM_000978	133 (134)	4.42E-08
Scavenging of heme from plasma	Reactome	NM_005143;NM_020995;NM_001633;NM_000477;NM_000039	12 (12)	2.06E-07
Metabolism of mRNA	Reactome	NM_001003;NM_001004;NM_002952;NM_022551;NM_001030;NM_000976;NM_033251;NM_001012;NM_001031;NM_000978	178 (178)	6.87E-07
Metabolism of proteins	Reactome	NM_001003;NM_172002;NM_001004;NM_000301;NM_000978;NM_002952;NM_022551;NM_000976;NM_001030;NM_033251;NM_001031;NM_001402;NM_001101;NM_001012;NM_014272	598 (598)	3.06E-06
Binding and Uptake of Ligands by Scavenger Receptors	Reactome	NM_001633;NM_005143;NM_000039;NM_020995;NM_000477;NM_000041	41 (41)	3.52E-06
Retinoid metabolism and transport	Reactome	NM_000371;NM_000039;NM_001643;NM_0000	42 (42)	3.90E-06

		40;NM_000041;NM_006744		
Metabolism of RNA	Reactome	NM_001003;NM_001004;NM_002952;NM_022551;NM_001030;NM_000976;NM_033251;NM_001012;NM_001031;NM_000978	226 (226)	5.57E-06
Lipoprotein metabolism	Reactome	NM_001643;NM_000040;NM_000041;NM_000477;NM_000039	30 (30)	2.59E-05
Statin Pathway	Wikipathways	NM_001643;NM_000040;NM_000041;NM_001645;NM_000039	31 (31)	2.95E-05
HDL-mediated lipid transport	Reactome	NM_000040;NM_000041;NM_000477;NM_000039	15 (15)	6.02E-05
Chylomicron-mediated lipid transport	Reactome	NM_001643;NM_000040;NM_000041;NM_000039	17 (17)	0.0001
Platelet degranulation	Reactome	NM_000412;NM_000039;NM_001002236;NM_000477;NM_000509;NM_000301	82 (82)	0.000178
Response to elevated platelet cytosolic Ca2+	Reactome	NM_000412;NM_000039;NM_001002236;NM_000477;NM_000509;NM_000301	87 (87)	0.000243
Lipid digestion_ mobilization_ and transport	Reactome	NM_001643;NM_000040;NM_000041;NM_000477;NM_000039	50 (50)	0.000286
Diseases associated with visual transduction	Reactome	NM_000371;NM_000039;NM_001643;NM_000040;NM_000041;NM_006744	96 (96)	0.000382

Visual phototransduction	Reactome	NM_000371;NM_000039;NM_001643;NM_000040;NM_000041;NM_006744	96 (96)	0.000382
Complement and Coagulation Cascades	Wikipathways	NM_001643;NM_001002236;NM_001733;NM_000301;NM_000064	54 (54)	0.000382
Formation of the ternary complex_ and subsequently_ the 43S complex	Reactome	NM_001030;NM_001031;NM_002952;NM_001012;NM_022551	55 (55)	0.000397
Statin Pathway_ Pharmacodynamics	PharmGKB	NM_000040;NM_000041;NM_001645;NM_000039	25 (25)	0.000397
Translation initiation complex formation	Reactome	NM_001030;NM_001031;NM_002952;NM_001012;NM_022551	62 (62)	0.000677
Ribosomal scanning and start codon recognition	Reactome	NM_001030;NM_001031;NM_002952;NM_001012;NM_022551	62 (62)	0.000677
Activation of the mRNA upon binding of the cap-binding complex and eIFs_ and subsequent binding to 43S	Reactome	NM_001030;NM_001031;NM_002952;NM_001012;NM_022551	63 (63)	0.000713
Complement and coagulation cascades - Homo sapiens (human)	KEGG	NM_001002236;NM_000509;NM_001733;NM_000301;NM_000064	69 (69)	0.00109
FOXA2 and FOXA3 transcription factor networks	PID	NM_000477;NM_000371;NM_000035;NM_000039	45 (45)	0.00383
Complement	Wikipathways	NM_000509;NM_000301;NM_000477;NM_0000	95 (95)	0.0049

		64;NM_000039		
Vitamin B12 Metabolism	Wikipathways	NM_000301;NM_000041;NM_000477;NM_000039	51 (51)	0.00599
Staphylococcus aureus infection - Homo sapiens (human)	KEGG	NM_000509;NM_001733;NM_000301;NM_000064	57 (57)	0.00907
Capecitabine Metabolism Pathway	SMPDB	NM_001113755;NM_001025195	5 (5)	0.0152
Capecitabine Action Pathway	SMPDB	NM_001113755;NM_001025195	5 (5)	0.0152
PPAR signaling pathway - Homo sapiens (human)	KEGG	NM_001643;NM_000040;NM_021009;NM_000039	69 (69)	0.0178
Platelet activation_ signaling and aggregation	Reactome	NM_000412;NM_000039;NM_001002236;NM_000477;NM_000509;NM_000301	208 (208)	0.0207
Gene Expression	Reactome	NM_001003;NM_001004;NM_002952;NR_003287;NM_022551;NM_000976;NM_021009;NM_001030;NM_033251;NM_001031;NM_001402;NM_001012;NM_000978	1103 (1104)	0.0471

Supplementary table 7. The pathway related to miRNAs

KEGG pathway	p-value	#genes	#miRNAs
p53 signaling pathway	2.54E-25	32	7
RNA transport	8.37E-23	52	6
Hepatitis B	1.10E-19	49	7
Prion diseases	1.13E-19	11	5
Ribosome biogenesis in eukaryotes	1.25E-17	30	5
Prostate cancer	1.27E-15	32	7
Bladder cancer	1.15E-14	18	6
Colorectal cancer	1.78E-13	23	7
Small cell lung cancer	1.22E-12	28	7
Cell cycle	1.41E-10	35	7
Pancreatic cancer	2.29E-10	25	7
Pathways in cancer	3.30E-10	74	7
Chronic myeloid leukemia	3.30E-10	24	7
Protein export	9.00E-10	11	4
Ribosome	1.99E-09	27	5
Insulin signaling pathway	7.04E-09	36	7
Legionellosis	8.10E-09	19	5
PI3K-Akt signaling pathway	1.92E-08	72	7

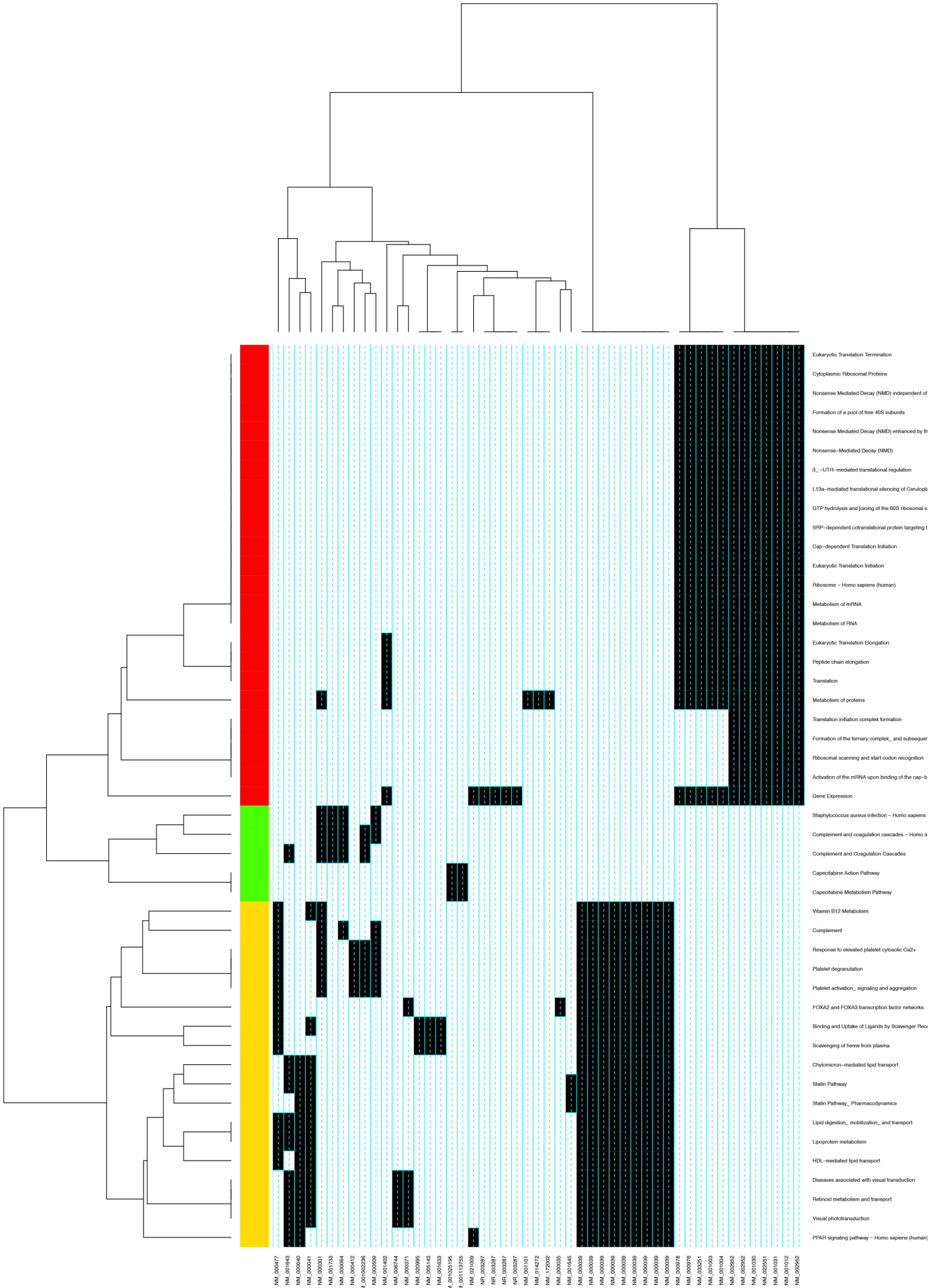
Glioma	1.94E-08	23	7
Non-small cell lung cancer	2.97E-08	18	7
Melanoma	3.49E-07	20	7
Epstein-Barr virus infection	5.74E-07	47	7
Protein processing in endoplasmic reticulum	1.99E-06	40	7
Ubiquitin mediated proteolysis	3.21E-06	34	7
Endometrial cancer	5.73E-06	16	7
Viral carcinogenesis	5.31E-05	47	7
Acute myeloid leukemia	5.51E-05	16	6
Chagas disease (American trypanosomiasis)	5.51E-05	25	7
RNA polymerase	1.05e-04	10	4
HIF-1 signaling pathway	2.00e-04	26	7
Influenza A	2.00e-04	37	7
Focal adhesion	3.08e-04	41	7
Herpes simplex infection	4.47e-04	40	7
Lysine degradation	5.56e-04	14	5
Toxoplasmosis	1.28e-03	27	7
Hepatitis C	1.96e-03	27	7
Progesterone-mediated oocyte maturation	2.00e-03	19	7
Aminoacyl-tRNA biosynthesis	2.82e-03	15	4

mTOR signaling pathway	2.82e-03	15	8
Amyotrophic lateral sclerosis (ALS)	2.93e-03	14	7
Citrate cycle (TCA cycle)	4.16e-03	9	4
ErbB signaling pathway	4.46e-03	18	7
Oocyte meiosis	4.47e-03	23	7
Salmonella infection	4.84e-03	18	7
Viral myocarditis	4.88e-03	16	7
TGF-beta signaling pathway	5.18e-03	20	6
Thyroid cancer	6.04e-03	8	4
Measles	6.07e-03	29	7
Neurotrophin signaling pathway	6.31e-03	25	7
HTLV-I infection	6.61e-03	47	7
Basal transcription factors	7.16e-03	11	5
Shigellosis	7.16e-03	15	7
B cell receptor signaling pathway	7.81e-03	17	6
Spliceosome	7.94e-03	28	5
Epithelial cell signaling in Helicobacter pylori infection	1.13e-02	15	7
Adipocytokine signaling pathway	1.51e-02	15	6
GnRH signaling pathway	2.38e-02	18	7
Mineral absorption	2.60e-02	12	6

Butanoate metabolism	2.64e-02	7	5
Pyruvate metabolism	3.96e-02	10	5
Hypertrophic cardiomyopathy (HCM)	4.15e-02	16	7
Transcriptional misregulation in cancer	4.63e-02	34	7

Abbreviations, #genes: number of genes related to this pathway, #miRNA: number of miRNA related to this pathway


Transmission across Chemical Synapses
Neurotransmitter Release Cycle
Neuronal System
GABAergic synapse – Homo sapiens (human)
Hyperinsulinism–Hyperammonemia Syndrome
Homocarnosinosis
Succinic semialdehyde dehydrogenase deficiency
4–Hydroxybutyric Aciduria/succinic Semialdehyde Dehydroc
Glutamate Metabolism
2–Hydroxyglutric Aciduria (D And L Form)
Purine metabolism
D–Glutamine and D–glutamate metabolism – Homo sapiens
Amino acid oxidation
Glycine,_ serine,_ alanine and threonine metabolism
Trans–suturation pathway
One carbon donor
Urea cycle and metabolism of arginine,_ proline,_ glutamate,_
Metabolism of amino acids and derivatives
Metabolism of amino acids and derivatives
Prolidase Deficiency (PD)
Prolinemia Type II
Arginine and Proline Metabolism
Hyperprolinemia Type I
Hyperprolinemia Type II
Ornithine Aminotransferase Deficiency (OAT Deficiency)
Arginine: Glycine Amidinotransferase Deficiency (AGAT Defi
Hyperornithinemia with gyrate atrophy (HOGA)
Creatine deficiency_ guanidinoacetate methyltransferase de
L–arginine:glycine amidinotransferase deficiency
Hyperornithinemia–hyperammonemia–homocitrullinuria (HH
Guanidinoacetate Methyltransferase Deficiency (GAMT Defi
Transport of glucose and other sugars,_ bile salts and organi
Transport of glucose and other sugars,_ bile salts and organi
Amine compound SLC transporters
Transmembrane transport of small molecules
SLC–mediated transmembrane transport
Proton/oligonucleotide cotransporters
Endosomal/Vacuolar pathway
tRNA charging
Antigen processing–Cross presentation
Transport of inorganic cations–anions and amino acids–oligo
5–methyl–5–thio–α–D–ribose 1–phosphate de
Class I MHC mediated antigen processing & presentation
Protein digestion and absorption – Homo sapiens (human)
Adaptive Immune System
Aminoacyl–tRNA biosynthesis – Homo sapiens (human)
tRNA Aminoacylation
Cytosolic tRNA aminoacylation
Mitochondrial tRNA aminoacylation
tRNA Aminoacylation
γ–glutamyl cycle
leukotriene biosynthesis
Glutathione synthesis and recycling
Glutathione conjugation
Phase II conjugation
Phase II conjugation
Biological oxidations
Amino acid transport across the plasma membrane
Na+/Cl– dependent neurotransmitter transporters
Amino acid and oligopeptide SLC transporters
Transport of inorganic cations/anions and amino acids/oligo
Immune System
Gene Expression
Demeclocycline Action Pathway
Doxycycline Action Pathway
Oxytetracycline Action Pathway
Minocycline Action Pathway
Lymecycline Action Pathway
Tetracycline Action Pathway
Clomocycline Action Pathway
Clarithromycin Action Pathway
Clindamycin Action Pathway
Azithromycin Action Pathway
Streptomycin Action Pathway
Spectinomycin Action Pathway
Kanamycin Action Pathway
Gentamicin Action Pathway
Netilmicin Action Pathway
Neomycin Action Pathway
Roxithromycin Action Pathway
Erythromycin Action Pathway
Amikacin Action Pathway
Telithromycin Action Pathway
Mineral absorption – Homo sapiens (human)
3–Methylcrotonyl Coa Carboxylase Deficiency Type I
Isovaleric Aciduria
Propionic Acidemia
Maple Syrup Urine Disease
3–Hydroxy–3–Methylglutaryl–CoA Lyase Deficiency
Isobutyryl–coa dehydrogenase deficiency
3–hydroxyisobutyric aciduria
3–hydroxyisobutyric acid dehydrogenase deficiency
Isovaleric acidemia
Methylmalonate Semialdehyde Dehydrogenase Deficiency
Methylmalonic Aciduria
3–Methylglutaconic Aciduria Type IV
3–Methylglutaconic Aciduria Type III
Beta–Ketothiolase Deficiency
3–Methylglutaconic Aciduria Type I
Valine,_ Leucine and Isoleucine Degradation
2–Methyl–3–Hydroxybutiryl CoA Dehydrogenase Deficiency
Glucose Homeostasis
Glycerophospholipid metabolism – Homo sapiens (human)
Hydrolysis of LPC
Glycerophospholipid biosynthesis
Glycerophospholipid biosynthesis
Phospholipid metabolism
Glycerophospholipid metabolism
Acetylcholine Synthesis
Synthesis of PA
Synthesis of PG
Metabolism of lipids and lipoproteins
Metabolism
ABC transporters – Homo sapiens (human)
C00042
C00051
C00763
C00245
C00114
C00819
C00262
C00670
C00093
C00047
C00064
C00148
C00123
C00407


Eukaryotic Translation Termination
Cytoplasmic Ribosomal Proteins
Nonsense Mediated Decay (NMD) independent of
Formation of a pool of free 40S subunits
Nonsense Mediated Decay (NMD) enhanced by th
Nonsense−Mediated Decay (NMD)
3_ −UTR−mediated translational regulation
L13a−mediated translational silencing of Ceruloplz
GTP hydrolysis and joining of the 60S ribosomal s
SRP−dependent cotranslational protein targeting t
Cap−dependent Translation Initiation
Eukaryotic Translation Initiation
Ribosome − Homo sapiens (human)
Metabolism of mRNA
Metabolism of RNA
Eukaryotic Translation Elongation
Peptide chain elongation
Translation
Metabolism of proteins
Translation initiation complex formation
Formation of the ternary complex_ and subsequer
Ribosomal scanning and start codon recognition
Activation of the mRNA upon binding of the cap−b
Gene Expression
Staphylococcus aureus infection − Homo sapiens
Complement and coagulation cascades − Homo s
Complement and Coagulation Cascades
Capecitabine Action Pathway
Capecitabine Metabolism Pathway
Vitamin B12 Metabolism
Complement
Response to elevated platelet cytosolic Ca2+
Platelet degranulation
Platelet activation_ signaling and aggregation
FOXA2 and FOXA3 transcription factor networks
Binding and Uptake of Ligands by Scavenger Rece
Scavenging of heme from plasma
Chylomicron−mediated lipid transport
Statin Pathway
Statin Pathway_ Pharmacodynamics
Lipid digestion_ mobilization_ and transport
Lipoprotein metabolism
HDL−mediated lipid transport
Diseases associated with visual transduction
Retinoid metabolism and transport
Visual phototransduction
PPAR signaling pathway − Homo sapiens (human)
NM_000477
NM_001643
NM_000040
NM_000041
NM_000301
NM_001733
NM_000064
NM_000412
M_001002296
NM_000509
NM_001402
NM_006744
NM_000371
NM_020995
NM_005143
NM_001633
M_001025195
M_001113755
NM_021009
NR_003287
NR_003287
NR_003287
NM_001101
NM_014272
NM_172002
NM_000035
NM_001645
NM_000039
NM_000039
NM_000039
NM_000039
NM_000039
NM_000039
NM_000039
NM_000039
NM_000039
NM_000978
NM_000976
NM_032251
NM_001003
NM_001004
NM_002952
NM_002952
NM_001050
NM_022551
NM_001031
NM_001012
NM_002952

Supplementary figure legend

Supplementary figure 1. Heatmap of compound

Horizontal line indicates ICC related compounds. Vertical line represents the pathways.

Supplementary figure 2. Heatmap of mRNA

Horizontal line indicates mRNAs related to ICC. Vertical line indicates pathways.

Supplementary methods

1 Data Normalization

1.1 mRNA/microRNA

"gProcessedSignal" was extracted from the microarray data and was normalized so as to have mean zero and standard deviation of one.

1.2 Compounds

No normalization was applied to the amount of compounds.

2 Principal Component Analysis and hierarchical clustering in order to choose PCs for feature extraction

Suppose expression matrix X is

$$(\vec{x}_1, \vec{x}_2, \cdots, \vec{x}_p)$$

where $\vec{x}_i$ is n dimensional vector. n is the number of mRNAs, miRNAs or componuds, and p is number of saples, i.e., 32. PCA allows to go from n points in a space of dimension $p(= 32)$ to n points in a space of dimension k (number of PCs considered). Each PC is a combination of p variables (p-dimensional vector); the number of PCs extracted is p. PCA was applied to matrix X.

`prcomp` function in [1] was used for principal component analysis and mRNAs, miRNAs and compounds were separately embedded into low dimensional space. Since there were 16 patients from which two samples (tumor and normal tissue) were extracted, in total 32 samples of mRNA, miRNA an compounds expression. Thus, there were up to 32 PCs for each of mRNA, miRNA and compounds expression. Each PCX ($X = 1, ..., 32$) should have weight CX_j^k from jth sample among 32 samples for kth expression (k stands for either mRNA, miRNA or compounds),

$$PCX_i^k = \sum_{j=1}^{32} CX_j^k x_{ij}$$

where PCX_i^k is PC score of ith probe (i.e., coordinate in the embedding space) of kth expression and $x_{ij}k$ is the expression of jth sample of ith probe. CX_j^k is also known as PC loadings. Negative signed absolute correlation coefficient $- \mid \rho_{X,X'}^{k,k'} \mid$ between $(CX_1^k, CX_2^k, ..., CX_{32}^k)$ and $(CX'^{k'}_1, CX'^{k'}_2, ..., CX'^{k'}_{32})$ were used as distance for hierarchical clustering. Hierarchical clustering was performed by `hclust` function in R[1] with `method="average"` option, thus it is Unweighted Pair Group Method using arithmetic Average (UPGMA).

3 PCA based unsupervised FE (PCAFE)

Using PCAFE [2, 3], mRNAs, miRNAs and compounds were extracted. mRNAs whose PC1 < -50, miRNAs whose $\sqrt{PC1^2 + PC2^2} > 10$, and compounds whose $\mid PC3 \mid > 0.1$ was extracted for further analysis.

4 Pathway analysis of obtained mRNAs, miRNAs, and compounds

In order to perform pathway enriched analyses, two servers were employed. For mRNAs and compounds, IMPaLA[4, 5] was used. For miRNAs, DIANA-mirpath[6, 7] with using Tarbase as target identification was used. For both servers, files including miRNAs (mature miRNA names), mRNAs (RefSeq mRNA) or compounds (KEGG compound ID) were uploaded. Enriched pathways were automatically extracted.

5 Discrimination between ICC, HCC and controls

As done before [2, 3], using extracted mRNAs, miRNAs, and compounds, we have tried to discriminate HCC, ICC and normal tissues (three class discrimination problem).

1. 32 samples are embedded into low dimensional space using either extracted mRNAs, miRNAs or compounds.

2. PC scores (i.e., coordinates in the embedded space) of each sample were used for discrimination.

3. Using PC scores up to optimal number of PCs, three classes were discriminated using linear discriminant analysis (LDA), where LDA was

performed using `lda` function in `MASS` package in R[1] with the options `CV=T, prior=rep(1/3,3)`.

4. Performance was evaluated by comparing true labels and `class` variables obtained by `lda` (`class` variables were obtained by leave one out cross validation since option `CV=T` was set).

6 Categorical regression performed for mRNAs, miRNAs and compounds in supplementary tables 2, 3, and 4

Categrical regression was performed to each of extracted mRNAs, miRNAs and compounds as

$$x_{ij} = C_i + C_{ICC}\delta_{j,ICC} + C_{ICC-NT}\delta_{j,ICC-NT} + C_{HCC}\delta_{j,HCC} + C_{HCC-NT}\delta_{j,HCC-NT}$$

and P-values associated with the regression analysis was provided. Here $\delta_{a,j}$ takes one if the j-th sample is equivalent to category a (a represents ICC, ICC-NT, HCC, or HCC-NT), otherwise it had a value of zero.

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Case Study

Chemical exposure levels in printing and coating workers with cholangiocarcinoma (third report)

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Abstract: Chemical exposure levels in printing and coating workers with cholangiocarcinoma (third report): Kenichi YAMADA, *et al.* Occupational Health Research and Development Center, Japan Industrial Safety and Health Association—Objective: This study aimed to identify the chemicals used by five printing workers and one coating worker who developed cholangiocarcinoma and estimate the workers' levels of chemical exposure. **Methods:** We obtained information on chemicals from the Ministry of Health, Labour and Welfare, Japan, and estimated working environment concentrations of the chemicals in printing and coating rooms and exposure concentrations during the ink and dirt removal processes. We also calculated shift time-weighted averages of exposure concentrations. **Results:** All five printing workers were exposed to both 1,2-dichloropropane (1,2-DCP) and dichloromethane (DCM). The estimated maximum exposure concentrations for each of the five workers were 190 to 560 ppm for 1,2-DCP and 300 to 980 ppm for DCM, and the estimated shift average exposure concentrations were 0 to 230 ppm for 1,2-DCP and 20 to 470 ppm for DCM. The coating worker was exposed to 1,2-DCP, but not DCM. He did not use ink, and thus was subjected to different conditions than the printing workers. The estimated maximum exposure concentration of 1,2-DCP was 150 ppm, and the estimated shift time-weighted average exposure concentration was 5 to 19 ppm. **Conclusions:** Our findings support the notion that 1,2-DCP contributes to the development of cholangiocarcinoma in humans and the notion that DCM may also be a contributing factor. The finding that the coating worker was exposed to 1,2-DCP at a lower exposure concentration is important for determining the occupational exposure limit. Furthermore, the subject did not use ink, which suggests that ink did not contribute to the development of cholangiocarcinoma.
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Key words: 1,2-dichloropropane, Cholangiocarcinoma, Coating worker, Dichloromethane, Printing worker

In May 2012, five employees (including former employees) of an offset proof-printing plant in Osaka, Japan, were reported to have developed intrahepatic or extrahepatic bile duct cancer (cholangiocarcinoma)[1, 2]. Subsequently, other employees from this plant were found to have developed cholangiocarcinoma, reaching a total of 17 individuals by the end of 2012[3]. All had been exposed for a long term to 1,2-dichloropropane (1,2-DCP) at very high levels, and 11 had also been exposed to dichloromethane (DCM)[3]. The Ministry of Health, Labour and Welfare (MHLW) recognized these individuals as having developed an occupational disease.

After this incident became widely known through mass media, workers who developed cholangiocarcinoma at other printing plants filed workers' compensation claims, with the total number of workers reaching 76 (excluding the aforementioned 17) as of May 2015[4]. By June 2015, 19 of the 76 workers were recognized as having developed an occupational disease[4]. We previously reported that 13 of the 19 employees had experienced long-term exposure to very high concentrations of 1,2-DCP and/or DCM[5, 6]. The present study aimed to identify the chemicals that the remaining six workers were exposed to and estimate the levels of chemical exposure using mathematical models. This study was approved by the Ethics Committee of Osaka City University.

Subjects and Methods

Subjects

Subject characteristics are summarized in Table 1.

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Table 1. Subject characteristics

Subject	Birth year	Employment in printing or coating company				Year of diagnosis	Day of recognition*2
		Plant	Duration	Location	Scale*1		
N	1953	II*3	1989–2002	Fukuoka	Small	2002	Dec 2, 2014
O	1949	XII XIII XIV	1982–1983 1983–1986 1986–1994	Aichi	Small	1993	Jun 10, 2014
P	1971	XV	1991–2014	Aichi	Small	2013	Jun 10, 2014
Q	1970	XVI	1998–2013	Tokyo	Small	2012	Feb 26, 2015
R	1956	XVII	1981–2011	Tokyo	Middle	2011	Sep 11, 2014
S	1958	XVIII XIX	1996–2001 2001–2005	Kyoto	Small	2008	Jul 24, 2014

*1: Small, fewer than 50 employees; Middle, 50–299 employees. *2: Day when cholangiocarcinoma was recognized as an occupational disease. *3: This plant is the same as the plant where Subjects C and D (described in our previous study[5]) had worked.

The alphabetical letters N to S were used to identify subjects, in keeping with the identification scheme described in our previous reports[5,6]. The subjects included four printing workers and one coating worker who were employed at small-scale plants (fewer than 50 employees) and one printing worker who was employed at a middle-scale plant (50–299 employees). Subject N had worked in Plant II (the plant that Subjects C and D of our previous report worked at[5]). The Roman numbers XII to XIX were used to identify plants in the present study, in keeping with the identification scheme described in our previous reports[5,6]. All subjects were diagnosed with cholangiocarcinoma and were recognized as having developed an occupational disease by the MHLW. At diagnosis, four subjects were in their 40s, and two subjects were in their 50s.

Collection of information regarding working conditions and chemicals used

In order to identify the chemicals used and to estimate chemical exposure concentrations, the following information was obtained from the MHLW: volumes and ventilation rates of the printing and coating rooms, types of printing and coating machines operated by the subjects, components of chemicals used to remove ink from the ink transcription roll (blanket) and ink roll of the printing machines and to remove dirt from the cleaning roll of the coating machines, and duration of the removal operation. Information on amounts of 1,2-DCP and dichloromethane (DCM) used was also obtained from the MHLW.

Estimation of working environment and exposure concentrations

As described in our previous reports[5,6], we used a well-mixed model[7,8] to estimate working environment concentrations of 1,2-DCP and DCM in the printing and coating rooms and used a near-field and far-field model[7,8] to estimate exposure concentrations during the removal operation. Furthermore, we calculated shift time-weighted averages (TWAs) of exposure concentrations.

Results

Subject N

Subject N was a male born in 1953 (Table 1). He was employed at a glass production company from 1984 to 1989 and used silica, limestone, and kerosene. Thereafter, he was employed at the same printing company as Subjects C and D[5] from 1989 to 2002 and engaged in offset proof printing at Plant II from 1991 to 1997. He had no other occupational history of chemical handling. He was diagnosed with cholangiocarcinoma in 2002.

Table 2 shows basic information for estimating exposure concentrations of 1,2-DCP and DCM. Plant II had two printing rooms. The volume and ventilation rate of Room 1 were 170 m^3 and 1,790 m^3/h, respectively, and those of Room 2 were 180 m^3 and 1,100 m^3/h, respectively. Local exhaust ventilation was not installed in the printing machines.

1,2-DCP and DCM were used to remove ink from blankets from 1991 to 1992; 1,2-DCP, DCM, and mineral spirit (MS) were used from 1993 to 1995; and 1,2-DCP, DCM, 1,1-dichloro-1-fluoroethane (DCFE), and MS were used from 1996 to 1997. Kerosene and mineral oil (MO) were used to remove ink from ink

Table 2. Information for estimating exposure concentrations of 1,2-dichloropropane and dichloromethane

Subject	Plant	Calendar year of engagement in printing or coating	Printing or coating room						Removal operation					Chemicals used for removal of ink or dirt	
			No.	Volume (m^3)	Ventilation rate (m^3/h)	Number of ventilation (h^{-1})	Amount of 1,2-DCP (g/h)	Amount of DCM (g/h)	Printing or coating machine	r (m)	β (m^3/h)	Amount of 1,2-DCP (g/h)	Amount of DCM (g/h)	For removing from blankets	For removing from ink rolls
N	II	1991–1992	1	170	1,790	10.5	230	270	Flatbed offset (proof-printing)	0.5	570	330	400	1,2-DCP, DCM	Kerosene, MO
		1993–1995					230–270	270–310				330–430	400–500	1,2-DCP, DCM, MS	
		1996–1997	2	180	1,100	6.1	280–400	56				430–630	100	1,2-DCP, DCM, DCFE, MS	
O	XII	1982–1983	3	210	210*	1.0	NI	NI	Flatbed offset (proof-printing)	0.5	570	NI	NI	NI	NI
	XIII	1983–1984	4	240	240*	1.0									
		1985–1986					180	210				260	300	1,2-DCP, DCM	Kerosene, MO
	XIV	1986–1994	5	130	130*	1.0	90	110				260	300		
P	XV	1991–1997	6	350	350*	1.0	210	240	Flatbed offset (proof-printing)	0.5	570	210	240	1,2-DCP, DCM	Kerosene, MO
		1997–2002					0	450				0	450	DCM, MO	
		2003–2005					210	300				210	300	1,2-DCP, DCM, MO, Nonane	
		2005–2007					140	200				280	400		
		2007–2011					0	370				0	720	DCM, MO, Nonane	
		2011–2013					0	320				0	480	DCM	
Q	XVI	1999–2010	7	250	600	2.4	36–120	42–130	Flatbed offset (proof-printing)	0.5	570	210–260	250–300	1,2-DCP, DCM, DCFE, TCE	1,2-DCP, DCM, DCFE, TCE
		2010–2010					19–57	21–67				110–130	130–150	1,2-DCP, DCM, DCFE, TCE, Toluene, Xylene, Hexane, Kerosene	1,2-DCP, DCM, DCFE, TCE, Toluene, Xylene, Hexane, Kerosene
		2010–2012	8	290	290*	1.0	19–57	21–67				110–130	130–150		
		2012–2013					—	—		—	—	—	—	Toluene, Xylene, Hexane, Kerosene Cyclohexane, PGEE	Toluene, Xylene, Hexane, Kerosene Cyclohexane, PGEE
R	XVII	1981–1988	9	440	12,000	27.3	—	—	Rotary offset	—	—	—	—	TCE	Petroleum solvent
		1989–1993	10	510	9,540	18.7								1,1,1-TCE	
		1993–1999					0	940–1,880		0.5	570	0	1,200–1,600	DCM	
		1999–2000	11	910	910*	1.0	0	310				0	1,200		
		2000–2001	12	710	1,620	2.3	0	310				0	1,200		
		2002–2003					96	200				370	780	1,2-DCP, DCM	
S	XVIII	1996–2001	13	510	300	0.6	16	0	Coating machine	0.5	570	140	0	1,2-DCP, DCFE (removing dirt from cleaning roll)	Toluene (removing dirt from press machines)
	XIX	2001–2003	14	160	1,650	10.3	8	0				140	0		

NI, no information; r, radius of near field; β, air exchange rate between near field and far field=wind velocity × 3,600 × $2\pi r^2$, where wind velocity=0.1 (m/sec); DCFE, 1,1-dichloro-1-fluoroethane; DCM, dichloromethane; 1,2-DCP, 1,2-dichloropropane; IPA, iso-propyl alcohol; MO, mineral oil; MS, mineral spirit; PGEE, polyethylene glycol monoethyl ether; 1,1,1-TCE, 1,1,1-trichloroethane; TCE, trichloroethylene. *: Only with natural ventilation

Table 3. Estimated working environment concentrations of 1,2-dichloropropane and dichloromethane in printing and coating rooms, and exposure concentrations during removal of ink or dirt and shift time-weighted averages (TWAs)

Subject	Plant	Calendar year of engagement in printing or coating	Printing or coating room			Removal operation				Shift TWAs		
			No.	1,2-DCP (ppm)	DCM (ppm)	Printing or coating machine	Duration (h)	1,2-DCP (ppm)	DCM (ppm)	Working hours (h)	1,2-DCP (ppm)	DCM (ppm)
N	II	1991–1992	1	28	43	Flatbed offset (proof-printing)	3	170	270	9	74	120
		1993–1995		29–32	45–50			170–220	270–330		75–94	120–140
		1996–1997	2	55–78	15			250–370	77		120–170	35
O	XII	1982–1984	3	—	—	Flatbed offset (proof-printing)	1.8	—	—	10	—	—
	XIII	1983–1984	4									
		1985–1986		170	250			340	520		200	300
	XIV	1986–1994	5	160	240			560	850		230	350
P	XV	1991–1997	6	130	200	Flatbed offset (proof-printing)	3.3	210	320	10	160	240
		1997–2002		0	370			0	600		0	440
		2003–2005		130	240			210	400		160	300
		2005–2007		86	160			280	530		130	250
		2007–2011		0	300		2.5	0	950		0	470
		2011–2013		0	260			0	850		0	410
Q	XVI	1999–2010	7	13–42	20–64	Flatbed offset (proof-printing)	0.8–1.3	160–190	250–300	9–9.5	25–65	39–98
		2010–2010		7–20	10–32			83–94	120–150		13–31	20–49
		2010–2012	8	14–42	21–66			130–140	190–220		24–57	35–90
		2012–2013		—	—			—	—		—	—
R	XVII	1981–1988	9	—	—	Rotary offset	—	—	—	—	—	—
		1989–1993	10									
		1993–1999		0	28–57		2.5–3.75	0	640–850	9.5	0	190–370
		1999–2000	11	0	99		2.5	0	980		0	330
		2000–2001	12	0	56		2.5	0	820		0	260
		2002–2003		13	36		2.5	190	530		59	170
S	XVIII	1996–2001	13	11	0	Coating machine	0.5	150	0	9	19	0
	XIX	2001–2003	14	1	0		0.5	72	0		5	0

NI, no information; 1,2-DCP, 1,2-dichloropropane; DCM, dichloromethane.

rolls. The amounts used in the printing rooms were 230–400 g/h for 1,2-DCP and 56–310 g/h for DCM. The amounts used during ink removal were 330–630 g/h for 1,2-DCP and 100–500 g/h for DCM.

Table 3 presents the estimated concentrations of 1,2-DCP and DCM. The working environment concentrations in the printing room were estimated to be 28–78 ppm for 1,2-DCP and 15–50 ppm for DCM. The exposure concentrations during ink removal were estimated to be 170–370 ppm for 1,2-DCP and 77–330 ppm for DCM. The shift TWAs (9-h TWAs) of the exposure concentrations were estimated to be 74–170 ppm for 1,2-DCP and 35–140 ppm for DCM. Subject N did not use respiratory protection.

Subject O

Subject O was a male born in 1949 (Table 1). He was employed at a printing company from 1977 to 1979 and used DCM for the ink removal operation. Thereafter, he was also employed at another small printing company from 1982 to 1994 and engaged in offset proof printing at Plants XII, XIII, and XIV throughout his employment. He had no other occupational history of chemical handling. He was diag-

nosed with cholangiocarcinoma in 1993.

Each of Plants XII, XIII, and XIV had one printing room. The volumes of Rooms 3, 4, and 5 were 210, 240, and 130 m^3, respectively (Table 2). Although ventilation fans were installed in these rooms, the fans did not run during working hours. Consequently, the number of ventilation was assumed to be 1 h^{-1} by natural ventilation, which led to ventilation rates of 210, 240, and 130 m^3/h, respectively (Table 2). Local exhaust ventilation was not installed in either of the printing machines.

1,2-DCP and DCM were used to remove ink from blankets from 1985 to 1994, and kerosene and MO were used to remove ink from ink rolls. The amounts of chemicals used in the printing rooms were 90–180 g/h for 1,2-DCP and 110–210 g/h for DCM. The amounts of chemicals used during ink removal were 260 g/h for 1,2-DCP and 300 g/h for DCM.

The working environment concentrations in the printing room were estimated to be 160–170 ppm for 1,2-DCP and 240–250 ppm for DCM (Table 3). The exposure concentrations during ink removal were estimated to be 340–560 ppm for 1,2-DCP and 520–850 ppm for DCM. The shift TWAs (10-h TWAs) of the exposure concentrations were estimated to be 200–230 ppm for 1,2-DCP and 300–350 ppm for DCM. Subject O did not use respiratory protection.

Subject P

Subject P was a male born in 1971 (Table 1). He was employed at the same printing company as Subject O from 1991 to 2014 and engaged in offset proof printing at Plant XV from 1991 to 2013. He had no other occupational history of chemical handling. He was diagnosed with cholangiocarcinoma in 2013.

Plant XV had one printing room, which had a volume of 350 m^3 (Table 2). Although ventilation fans were installed in the room, the fans did not run during working hours. Consequently, the ventilation rate was assumed to be 350 m^3/h.

1,2-DCP and DCM were used to remove ink from blankets from 1991 to 1997; DCM and MO were used from 1997 to 2002; 1,2-DCP, DCM, MO, and nonane were used from 2003 to 2007; DCM, MO, and nonane were used from 2007 to 2011; and DCM was used from 2011 to 2013. Kerosene and MO were used to remove ink from ink rolls. The amounts of chemicals used in the printing rooms were 0–210 g/h for 1,2-DCP and 240–450 g/h for DCM. The amounts used during ink removal were 0–280 g/h for 1,2-DCP and 240–720 g/h for DCM.

The working environment concentrations in the printing room were estimated to be 0–130 ppm for 1,2-DCP and 160–370 ppm for DCM (Table 3). The exposure concentrations during ink removal were estimated to be 0–280 ppm for 1,2-DCP and 320–950 ppm for DCM. The shift TWAs (10-h TWAs) of the exposure concentrations were estimated to be 0–160 ppm for 1,2-DCP and 240–470 ppm for DCM. Subject P did not use respiratory protection.

Subject Q

Subject Q was a male born in 1970 (Table 1). He was employed at a printing company from 1994 to 1998 and used a small amount of DCM for blanket repair for one year. Thereafter, he was employed at another small printing company from 1998 to 2013 and engaged in offset proof printing at Plant XVI from 1999 to 2013. He had no other occupational history of chemical handling. He was diagnosed with cholangiocarcinoma in 2012.

Plant XVI had two printing rooms. The volume and ventilation rate of Room 7 were 250 m^3 and 600 m^3/h, respectively (Table 2). The volume of Room 8 was 290 m^3. Because a ventilation fan was not installed in Room 8, the ventilation rate was assumed to be 290 m^3/h by natural ventilation. Local exhaust ventilation was not installed in the printing machines.

1,2-DCP, DCM, DCFE, and trichloroethylene were used to remove ink from blankets and ink rolls from 1999 to 2010; 1,2-DCP, DCM, DCFE, trichloroethylene, toluene, xylene, hexane, and kerosene were used from 2010 to 2012; and thereafter, 1,2-DCP and DCM were not used. The amounts of chemicals used in the printing rooms were 19–120 g/h for 1,2-DCP and 21–130 g/h for DCM. The amounts used during ink removal were 110–260 g/h for 1,2-DCP and 130–300 g/h for DCM.

The working environment concentrations in the printing room were estimated to be 7–42 ppm for 1,2-DCP and 10–66 ppm for DCM (Table 3). The exposure concentrations during ink removal were estimated to be 83–190 ppm for 1,2-DCP and 120–300 ppm for DCM. The shift TWAs (9 or 9.5-h TWAs) of the exposure concentrations were estimated to be 13–65 ppm for 1,2-DCP and 20–98 ppm for DCM. Subject Q did not use respiratory protection.

Subject R

Subject R was a male born in 1956. He was employed at a jewelry goods production company from 1972 to 1977 and used sulfuric acid to remove contamination from gold. Thereafter, he was employed at a middle-scale company from 1981 to 2011 and was engaged in offset printing at Plant XVII from 1981 to 2003. He had no other occupational history of chemical handling. He was diagnosed with cholangiocarcinoma in 2011 (Table 1).

Plant XVII had five printing rooms, and Subject R worked in four of the rooms. The volumes and ventilation rate of Room 9 were 440 m^3 and 12,000 m^3/h, respectively; those of Room 10 were 510 m^3 and 9,540 m^3/h, respectively; and those of Room 12 were 710 m^3 and 1,620 m^3/h, respectively (Table 2). The volume of Room 11 was 910 m^3. Because a ventilation fan was not installed in Room 11, the ventilation rate was assumed to be 910 m^3/h by natural ventilation. Local exhaust ventilation was not installed in the printing machines.

Before 1993, 1,2-DCP or DCM were not used in any of the rooms. DCM was used from 1993 to 2001, and 1,2-DCP and DCM were used from 2002 to 2003. Petroleum solvent was used to remove ink from ink rolls. The amounts of chemicals used in the printing rooms were 0–96 g/h for 1,2-DCP and 200–1,880 g/h for DCM. The amounts used during ink removal were 0–370 g/h for 1,2-DCP and 780–1,600 g/h for DCM.

The working environment concentrations in the printing room were estimated to be 0–13 ppm for 1,2-DCP and 28–99 ppm for DCM (Table 3). The exposure concentrations during ink removal were estimated to be 0–190 ppm for 1,2-DCP and 530–980 ppm for DCM. The shift TWAs (9.5-h TWAs) of the exposure concentrations were estimated to be 0–59 ppm for 1,2-DCP and 170–370 ppm for DCM. Subject R did not use respiratory protection.

Subject S

Subject S was a male born in 1958 (Table 1). He was employed at a gas station for about half a year in 1986. Thereafter, he was employed at a small company manufacturing IC cards from 1996 to 2005 and engaged in coating plastic plates with an adhesive compound and antistatic additive at Plants XVIII and XIX from 1996 to 2003. He had no other occupational history of chemical handling. He was diagnosed with cholangiocarcinoma in 2008.

Each of Plants XVIII and XIX had one coating room. The volume and ventilation rate of Room 13 were 510 m^3 and 300 m^3/h, respectively, and those of Room 14 were 160 m^3 and 1,650 m^3/h, respectively (Table 2). Local exhaust ventilation was not installed in the coating machines.

1,2-DCP and DCFE were used to remove dirt from the cleaning roll of the coating machines from 1996 to 2003. Toluene was used to remove dirt from press machines. The adhesive compound contained ethyl acetate and toluene, and the antistatic additive contained methanol and *iso*-propyl alcohol. The amount of 1,2-DCP used in the coating rooms was 8–16 g/h, and the amount of 1,2-DCP used during the dirt removal operation was 140 g/h.

The working environment concentration of 1,2-DCP in the printing room was estimated to be 1–11 ppm (Table 3). The exposure concentration of 1,2-DCP during dirt removal was estimated to be 72–150 ppm. The shift TWA (9-h TWA) of the exposure concentration was estimated to be 5–19 ppm. Subject S did not use respiratory protection.

Discussion

We used two models to estimate working environment concentrations and exposure concentrations during the ink removal operation. However, because these models cannot completely express the actual exposure conditions, the values reported herein should be considered crude estimates.

Subjects N, O, P, Q, and R were exposed to both 1,2-DCP and DCM during offset printing. The estimated maximum exposure concentrations for each of these five printing workers were 190 to 560 ppm for 1,2-DCP and 300 to 980 ppm for DCM, which were similar to those reported for eight printing workers exposed to both 1,2-DCP and DCM (Subjects C, D, E, F, G, H, I, and J) in our previous reports[5,6] (230 to 620 ppm for 1,2-DCP; 58 to 720 ppm for DCM). The estimated shift average exposure concentrations were 0 to 230 ppm for 1,2-DCP and 20 to 470 ppm for DCM, which were also similar to those reported previously[5,6] (0 to 240 ppm for 1,2-DCP; 0 to 270 ppm for DCM) but lower than those reported for workers from the Osaka offset proof-printing plant[2] (70 to 670 ppm for 1,2-DCP; 0 to 540 ppm for DCM).

Subject S was engaged in coating plastic plates with an adhesive compound and antistatic additive and was exposed to 1,2-DCP when removing dirt from the cleaning roll of the coating machines. The estimated maximum exposure concentration of 1,2-DCP (150 ppm) was similar to those of the above printing workers with cholangiocarcinoma, but the estimated shift average exposure concentration (5–19 ppm) was lower, which is an important finding for determining the occupational exposure limit as an 8-hr time-weighted average. Also noteworthy is that Subject S did not use ink. Because all other workers had used ink, we could not make a definitive statement that pigments included in the ink did not contribute. However, this finding would suggest that pigments are not causative agents of cholangiocarcinoma.

Conclusion

Five of the six subjects analyzed in this study were exposed to both 1,2-DCP and DCM in offset printing, and the estimated exposure concentrations were similar to those in previous reports. Our findings support the notion that 1,2-DCP contributes to the development of cholangiocarcinoma and the notion that DCM

may also be a contributing factor. The other subject was exposed to 1,2-DCP in IC card manufacturing, but his estimated shift average exposure concentration was lower than those of the printing workers, which is an important finding for determining the occupational exposure limit. Furthermore, the subject did not use ink, which suggests that ink did not contribute to the development of cholangiocarcinoma.

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Ⅱ　職業性胆管癌症例の臨床的特徴

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はじめに

大阪のＳ印刷事業場従業員に多発した胆管癌事例[1,2]を踏まえて、2013年10月に「ジクロロメタン（dichloromethane;DCM）や1,2-ジクロロプロパン（1,2-dichloropropane;DCP）にさらされる業務による胆管癌」が業務上疾病に分類されるようになり、この胆管癌が職業性胆管癌として認識されるようになった。2015年６月の時点で、上記Ｓ事業場の17例と他地域の印刷事業場での19例の計36例が職業性胆管癌として労災認定されている。

本稿では、胆管癌全般の動向と職業性胆管癌の臨床的特徴について述べる。

1　胆管癌の動向、危険因子と職業性胆管癌

胆管癌は、肝内胆管癌と肝外胆管癌に大きく分類される。ヨーロッパ、米国及びオーストラリアにおいては、肝内胆管癌の罹患率が上昇しているが、肝外胆管癌の罹患率はむしろ低下傾向にある[3]。疫学的研究によると、本邦においても同様の傾向であると考えられる[4]。

胆管癌の危険因子として、従来より肝内結石症、膵･胆管合流異常、原発性硬化性胆管炎、肝吸虫やニトロソアミンなどの化学物質が報告されてきた[3]。

そのうち、肝内結石症は、その７～10%に胆管癌がみられ[5]、原発性硬化性胆管炎からの胆管癌発症率は、１年当たり0.6～1.5%と報告されている。肝吸虫による胆管癌は、タイ北西部が好発地域であり、同地区のタイ男性での胆管癌発症率は、10万人当たり100人前後と極めて高率であることが報告されている。

肝内結石症における胆管癌は、胆管炎を繰り返す結石存在部位に発生することが多く、膵・胆管合流異常では胆嚢癌及び拡張胆管に胆管癌が発生する。

一方、C型肝炎やB型肝炎では、末梢型肝内胆管癌が多くみられる[6,7]。また、胆管癌の危険因子として糖尿病、肥満、飲酒、喫煙、炎症性腸疾患が報告されている。International Agency for Research on Cancer（IARC）[8]からは、アフラトキシン、経口避妊薬、プルトニウムやトロトラストが胆管癌の危険因子であると報告されている。さらに、IARC monographにおいて、印刷工程やCarbon blackは喉頭・咽頭癌、膀胱癌、腎癌、白血病などのリスクとなり、group 2

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A（possibly carcinogen）に分類されてきたが、これまで胆管癌の報告はみられなかった[8]。

大阪のＳ印刷事業場のオフセット校正印刷部門の元及び現従業員に多発した胆管癌事例を踏まえ、2013年３月に厚生労働省による「印刷事業場で発生した胆管がんの業務上外に関する検討会」の報告[9]において、（1）胆管癌は、DCMまたはDCPに長期間、高濃度曝露することにより発症し得ると医学的に推定でき、（2）本件事業場で発生した胆管癌は、DCPに長期間、高濃度曝露したことが原因で発症した蓋然性が極めて高いことが報告された。さらに、昨年、開催されたIARCの会議において、本邦からの報告を受けて、DCPがgroup 3（分類不能）からgroup 1（carcinogenic to humans）に、DCMがgroup 2Bからgroup 2A（probably carcinogenic to humans）に変更された[10]。

2　職業性胆管癌症例の臨床所見

現在までに大阪のＳ事業場のオフセット校正印刷部門での従業員17例及び全国の印刷事業場での19例が、職業性胆管癌と労災認定されている[2,11]。

Ｓ事業場の17例の胆管癌診断時年齢は、25歳から45歳で全例男性であった（表１）。これは、同部門の従業員のほとんどが比較的若い男性で、50歳以上の従業員や女性従業員が少なかったためと考えられる。その他の事業場の19例では、31歳から62歳であり、全例男性であった。

表１　職業性胆管癌症例の臨床的所見

項目	大阪Ｓ事業場（17例）	その他（19例）
年齢（歳）	25－45	31-62
性（男：女）	17:0	19:0
受診のきっかけ		
有症状	5	15
健診での異常所見	12	4
臨床検査値異常		
総ビリルビン	8	11/18
AST	13	13/18
ALT	14	15/18
γ-GTP	17	18/18
CEA	11	7/18
CA19-9	13	10/18
HBs抗原陽性	0	0
HCV抗体陽性	0	0
限局性肝内胆管拡張像	5/11	3/5

Ｓ事業場の17例における就業中の急性症状には、嘔気、眩暈、頭痛などの全身症状や皮膚が荒れるなどの皮膚症状がみられた。喫煙歴は13例に、アルコール多飲歴は３例にみられた。17

例中５例では腹痛や黄疸などの症状が、11例では検診時の臨床検査値異常や肝腫瘤像の指摘が、１例では副鼻腔炎治療時の肝機能異常が、胆管癌診断のきっかけとなった。その検診時の検査成績の推移をみると、胆管癌診断の数年前よりγ-GTP値が上昇し、それと同時あるいは遅れてAST、ALTが上昇する症例が多くみられた[12,13]。他の事業場でも同様の所見がみられた症例があり、職業性胆管癌症例における重要な所見と考えられる。

S事業場17例の胆管癌診断時の臨床検査値をみると、総ビリルビン値は８例で、ASTやALT値はそれぞれ13例と14例で、γ-GTP値は全例で高値であった。また、CEA値は11例で、CA19-９値は13例で高値であった。その他の事業場の症例でも同様の所見であった。その中でγ-GTP値が全例で高値であったが、これは後述する病理所見における慢性胆管傷害や前癌病変と関連していると考えられる。なお、HBs抗原及びHCV抗体は全例で陰性であった。

S事業場17例での腹部超音波検査、CTやMRIにおいて、主腫瘍は肝内腫瘍像（図１A）、胆管内腫瘤像（図１B）、胆管内乳頭状病変として描出された。

また、腫瘍による胆管閉塞を伴う末梢側胆管拡張像（図１C）が11例にみられた。さらに、主腫瘍による胆管閉塞を伴わない限局性肝内胆管拡張像（図１D）が５例にみられたが、この画像所見は、原発性硬化性胆管炎の画像所見と類似しており、職業性胆管癌症例の特徴と考えられた。他の事業場の症例においても限局性肝内胆管拡張像が確認できた症例があった。さらに、少数例ではあるが、胆管癌診断の数年前からの腹部超音波検査、CTやMRI画像所見の推移を検討したところ、限局性肝内胆管拡張像が数年前から徐々に進行していくことが確認された[12,13]。

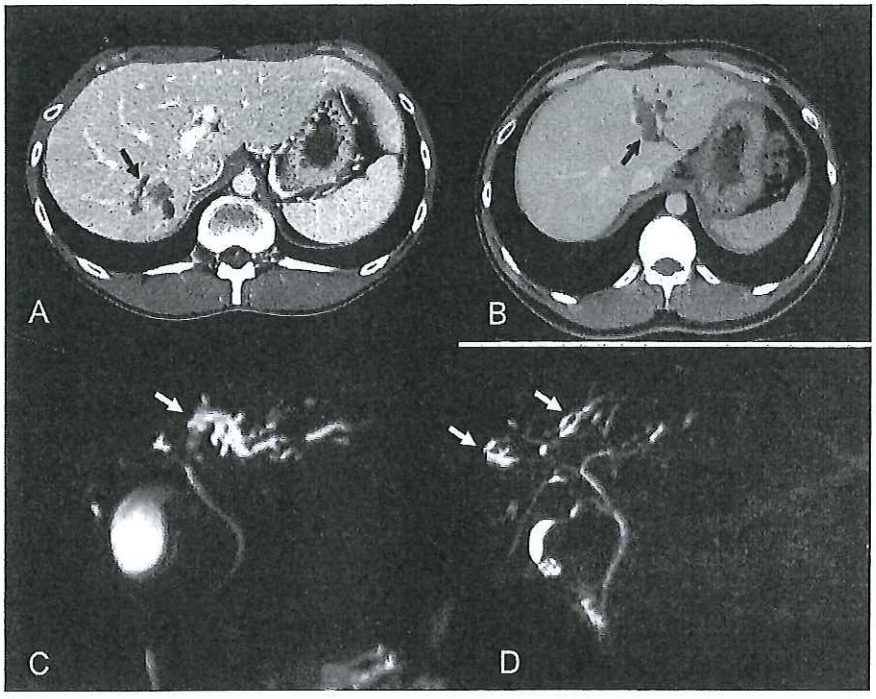

図１　職業性胆管癌の画像診断所見（参考文献２より引用）

A、肝内腫瘤性病変（矢印）。B、肝内胆管内腫瘤像（矢印）。C、胆管狭窄およびその末梢側胆管拡張像（矢印）。D、限局性肝内胆管拡張像（矢印）。

これら職業性胆管癌症例の臨床検査値や画像診断所見の特徴や推移は、健康診断の重要性を示すものと考えられる。現在、1,2-ジクロロプロパンの高濃度、長期間曝露を受けた従業員に対し健康管理手帳が交付され、年２回の健康診断が行われるようになったが、著者らの成績を参考に、AST、ALT、γ-GTP、CA19-９に加えて、腹部超音波検査、CTあるいはMRIが必要に応じて行うこととなっている。

胆管癌の新分類法[14]によると、S事業場の17例中、肝内胆管癌が10例、肝門部領域癌が４例、遠位胆管癌が１例、肝内および肝門部領域癌が１例、肝内および遠位胆管癌が１例であった（表2）。その他の事業場の19例では、肝内胆管癌が７例、肝門部領域癌が８例、遠位胆管癌が４例であった。

表２　職業性胆管癌症例の病理学的所見

所見	大阪Ｓ事業場（17例）	その他（19例）
主腫瘍の部位		
肝内胆管	10	7
肝門部領域	4	8
遠位胆管	1	4
肝内＋肝門部領域	1	0
肝内＋遠位胆管	1	0
BilIN 病変	8/8	4/4
IPNB 病変	7/7	1/4
慢性胆管傷害像	8/8	4/4

BilIN, biliary intraepithelial neoplasia; IPNB, intraductal neoplasm of the bile duct

しかし、特にＳ事業場の症例では、後述するように広範囲伸展例、多発例、前癌病変や早期癌病変であるBiliary intraepithelial neoplasia（BilIN）やIntraducatal papillary neoplasm of the bile duct（IPNB）が広範囲に観察された症例が多く、通常の進行度分類では分類できない症例が多くみられた[2,11]。

当初、Ｓ事業場の症例の解析から胆管癌の原発部位が総肝管から肝内第３次分枝までの比較的大型の胆管が多いと考えていたが、Ｓ事業場の症例の中で肝内胆管癌の治療後、総胆管に癌が再発した例や遠位胆管癌症例（職業性胆管癌として申請中）を経験したこと、さらにＳ事業場以外の症例で遠位胆管癌が４例みられたことから、肝内の比較的大型胆管から下部胆管に至る胆管全体に発癌する可能性があると考えられる。

病理学的検討が可能であったＳ事業場の８例において、主腫瘍をみると、高分化から低分化を示す腫瘤形成型胆管癌（図２A、図３A）に加えて、通常の胆管癌では頻度が低い浸潤性IPNB

を示す胆管内発育型胆管癌（図２B）あるいは乳頭状肝外胆管癌（図２C）が比較的多くみられたことが特徴であった[2)]。

また、全例において広範囲の胆管に、前癌病変であるBilIN-２/３病変（図３B）やIPNB（図３C）がみられた（**表２**）。さらに、胆管付属腺を含めた広範囲の胆管に、炎症性細胞浸潤を伴う胆管硬化像、胆管消失を伴う胆管傷害像や増殖性病変、胆管周囲の線維化がみられた（図３D)。これらの胆管傷害像を示す胆管、BilIN-２/３、IPNB病変及び胆管癌病変では、免疫組織学的にγ-H２AX陽性であり、広範囲の胆管にDNA損傷が起きていることが判明した[15)]。その他の事業場の４例でも、BilINが４例、IPNBが１例および慢性胆管傷害が４例にみられた。一方、背景肝には胆管閉塞に伴う胆汁うっ滞像や非特異的な反応がみられるのみで、肝硬変や進行性肝実質病変はみられなかった。

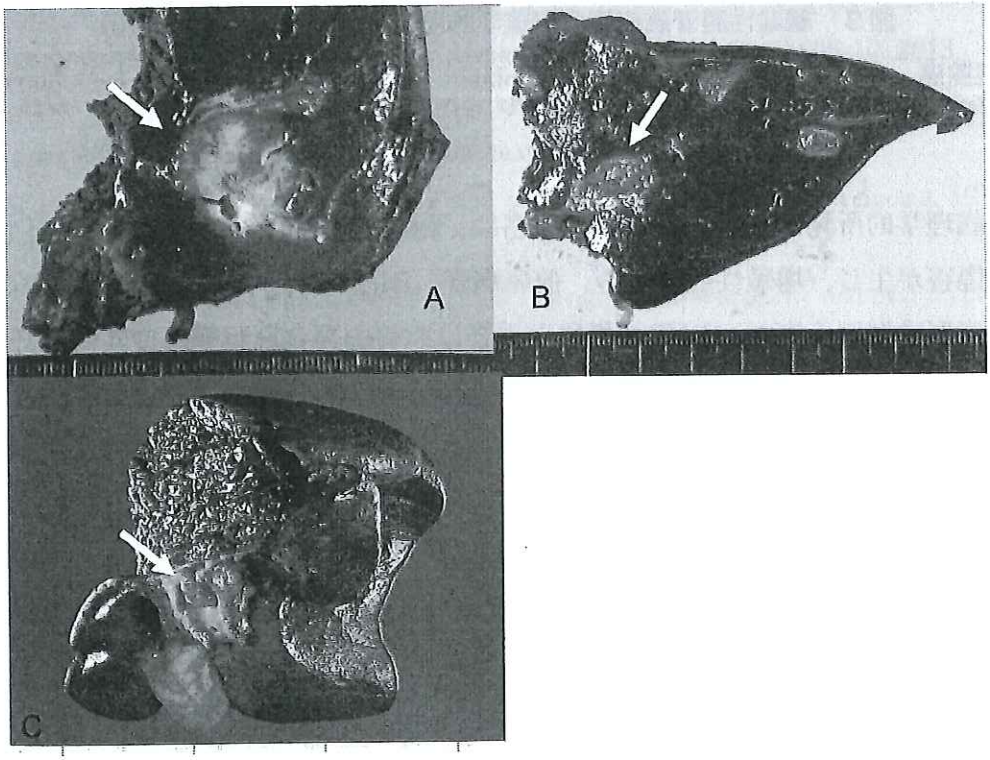

図２　職業性胆管癌の切除標本の肉眼所見（参考文献２より引用）

A、腫瘤形成型肝内胆管癌（矢印)。B、胆管内発育型肝内胆管癌（矢印)。C、乳頭型肝外胆管癌（矢印)。

シンポジウム7—2

職業性胆管癌の臨床的特徴

久保　正二[1)]，竹村　茂一[1)]，田中　肖吾[1)]，新川　寛二[1)]
西岡　孝芳[1)]，木下　正彦[1)]，濱野　玄弥[1)]，伊藤　得路[1)]
中沼　安二[2)]，圓藤　吟史[3)]

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（平成27年4月3日受付）

要旨：1,2-ジクロロプロパンやジクロロメタンの長期間，高濃度曝露を受けた印刷労働者に胆管癌が多発した事例を受けて，厚生労働省はこの胆管癌を業務上疾病，すなわち職業性胆管癌と認定した．胆管癌診断のきっかけは，腹痛や黄疸などの症状，γ-GTP高値などの肝機能異常，CA19-9などの腫瘍マーカー上昇や超音波検査での異常所見であった．画像診断上，腫瘤像，胆管狭窄像，主腫瘍による末梢側胆管拡張像に加えて，主腫瘍による胆管狭窄を伴わない限局性肝内胆管拡張像がみられた．主腫瘍は腫瘤形成型肝内胆管癌や胆管内発育型肝内胆管癌や乳頭型肝外胆管癌であった．また，広範囲の胆管に前癌病変であるbiliary intraepithelial neoplasia（BilIN）やintraductal papillary neoplasm of the bile duct（IPNB）がみられ，さらに慢性胆管傷害像やDNA傷害を示すγ-H2AH陽性胆管上皮がみられた．前述の限局性肝内胆管拡張像を示す胆管には，胆管傷害，前癌病変や浸潤癌がみられた．したがって，本病態は広範囲のDNA傷害を伴う胆管傷害，BilINやIPNB病変を経て浸潤性胆管癌に至る多段階発育を示すと考えられた．また，そのなかで乳頭状増殖を示す胆管癌（浸潤性IPNB）が多くみられることが特徴的であった．なお，従来から指摘されている胆管癌の危険因子はみられなかった．

（日職災医誌，64：1—5，2016）

—キーワード—
職業性胆管癌，ジクロロメタン，1,2-ジクロロプロパン

はじめに

2012年，大阪の印刷事業場S社のオフセット校正印刷部門の元および現従業員において，胆管癌が多発していることが報告された[1)]．2013年3月，厚生労働省による「印刷事業場で発生した胆管がんの業務上外に関する検討会」によって，(1) 胆管癌は，ジクロロメタン（dichloromethane；DCM）または1,2-ジクロロプロパン（1,2-dichloropropane；DCP）に長期間，高濃度曝露することにより発症し得ると医学的に推定でき，(2) 本件事業場で発生した胆管癌は，DCPに長期間，高濃度曝露したことが原因で発症した蓋然性が極めて高いことが報告された[2)]．2013年10月1日には，DCMやDCPにさらされる業務による胆管癌が業務上疾病に分類され，この胆管癌が職業性胆管癌と認識されることとなった．さらに，2014年，Intetnational Agency for Research on Cancer（IARC）によって，DCPがgroup 1（carcinogenic to humans）に，DCMがgroup 2A（probably carcinogenic to humans）に変更された[3)]．2015年2月末までに，大阪の印刷事業場の17例と他地域の印刷事業場の19例の計36例が職業性胆管癌と認定されている．本稿ではこの職業性胆管癌の臨床的特徴について述べる．

1．大阪の印刷事業場における職業性胆管癌症例

大阪の印刷事業場S社における元あるいは現従業員にみられた職業性胆管癌は17例で，全員が男性，診断時年齢は25歳から45歳（中央値36歳）であった[4)]．曝露期間と推定されるDCMあるいはDCP使用中の勤務期間は，6年1カ月から16年1カ月であった．また，退職9年7カ月後に胆管癌と診断された症例があり，曝露終了後長期間の経過観察が必要と考えられる．就業中の急性症状には嘔気，眩暈，頭痛などの全身症状や皮膚症状

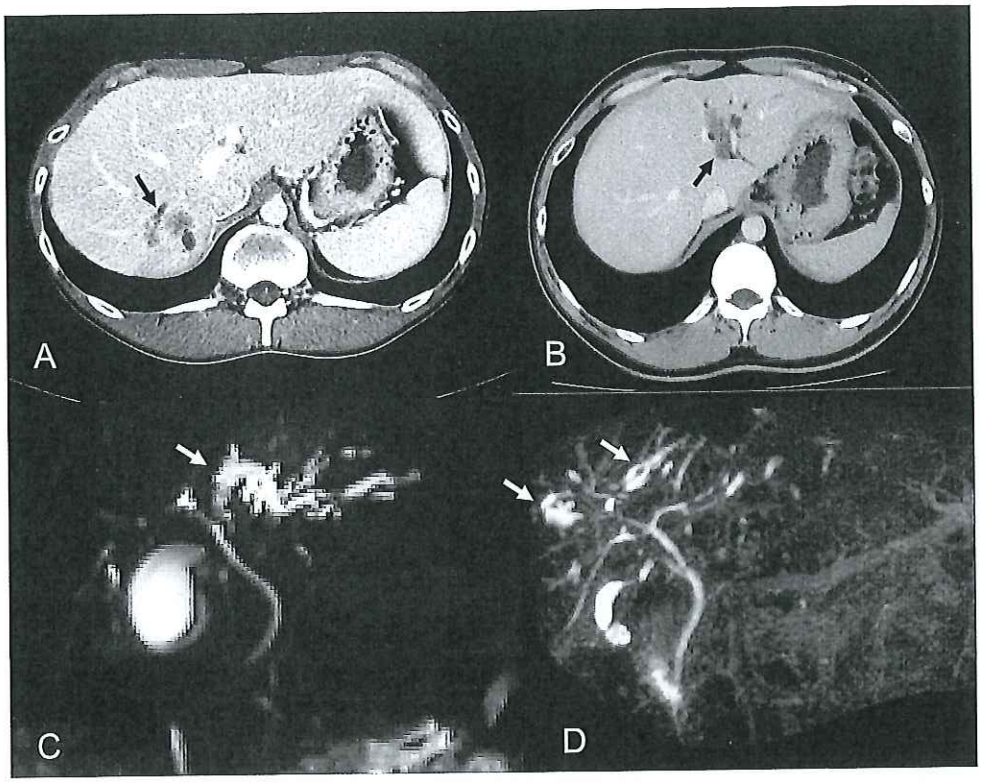

図1 職業性胆管癌症例の画像診断所見
A，肝内腫瘤性病変（矢印）．B，肝内胆管内腫瘤像（矢印）．C，胆管狭窄およびその末梢側胆管拡張像（矢印）．D，限局性肝内胆管拡張像（矢印）．

がみられた．喫煙歴は13例に，アルコール多飲歴は3例にみられた．17例中5例は腹痛や黄疸などの症状が，11例では検診時の臨床検査値異常や肝腫瘤像の指摘が，1例では他疾患加療中の肝機能検査値異常が，胆管癌診断のきっかけとなった．胆管癌診断時において，総ビリルビン高値が8例に，ASTやALT高値はそれぞれ13例と14例にみられ，全例でγ-GTPが高値であった．CEAは11例で，CA19-9は13例で高値であった．HBs抗原およびHCV抗体は全例で陰性であり，その他に自己免疫性肝炎などを疑わせる所見はみられなかった．胆管癌診断の数年前よりγ-GTP値が上昇し，同時期あるいはそれに遅れてASTやALTが上昇する症例が多くみられた[5)6)]．また，画像診断においても，胆管癌診断の数年前より肝内胆管の拡張像が検出され，その後徐々に増悪し，最終的に腫瘤像が確認された症例がみられた．胆管癌診断時の画像所見では，肝内あるいは胆管内腫瘤像，胆管狭窄像，主腫瘍による胆管閉塞に伴う末梢側胆管拡張像に加えて，17例中5例では主腫瘍による胆管閉塞を伴わない限局性の肝内胆管拡張像がみられ，この画像所見が職業性胆管癌症例の特徴であると考えられた（図1）．また，この限局性胆管拡張像は原発性硬化性胆管炎の画像所見と類似している点があり，注意を要すると考えられた．術中，胆道内視鏡が行われた症例では，胆管内腔の不整像や胆管内乳頭状腫瘍（Intraductal papillary neoplasm of the bile duct；IPNB）を示唆する所見が観察された．

17例は肝内胆管癌や肝外胆管癌であったが，両者が混在する症例，広範囲進展例や後述の前癌病変や早期癌病変が広範囲にみられる症例が多く，肝内外の分類や進行度分類（Stage分類）の決定が困難な症例が少なくなかった．肝内胆管癌では腫瘤形成型胆管癌が多かったが，胆管内発育型胆管癌が4例にみられた．また，肝外胆管癌においては乳頭型が多かった（図2）．診断時にすでに進行癌であったため，原発部位の同定が困難であった2例を除くと，他の15例での原発部位は総胆管から肝内胆管第3次分枝までの比較的大型の胆管であった．病理組織学的検討が可能であった症例での主腫瘍は低分化腺癌から高分化腺癌を示す腫瘤形成型肝内胆管癌，浸潤型IPNBを示す胆管内発育型肝内胆管癌や乳頭型肝外胆管癌であった（図3）．一方，胆管癌の前癌病変と考えられているBiliary intraepithelial neoplasia（BilIN）-2/3やIPNBが主腫瘍以外の広範囲の胆管にみられた．さらに，広範囲の胆管や付属腺に，炎症性細胞浸潤を伴う胆管硬化像，胆管消失を伴う胆管傷害像や増殖性病変がみられた（図4）．γ-H2AXを用いた免疫染色によってDNA傷害を検討したところ，癌部，前癌病変部や正常にみえる胆管においてもDNA傷害が広範囲に起きていることが判明した[7)]．なお，前述の限局性肝内胆管拡張像を示す胆管には，胆管傷害，前癌病変や浸潤癌がみられた．したがって，本病態は広範囲の胆管傷害，BilIN病変やIPNBを経

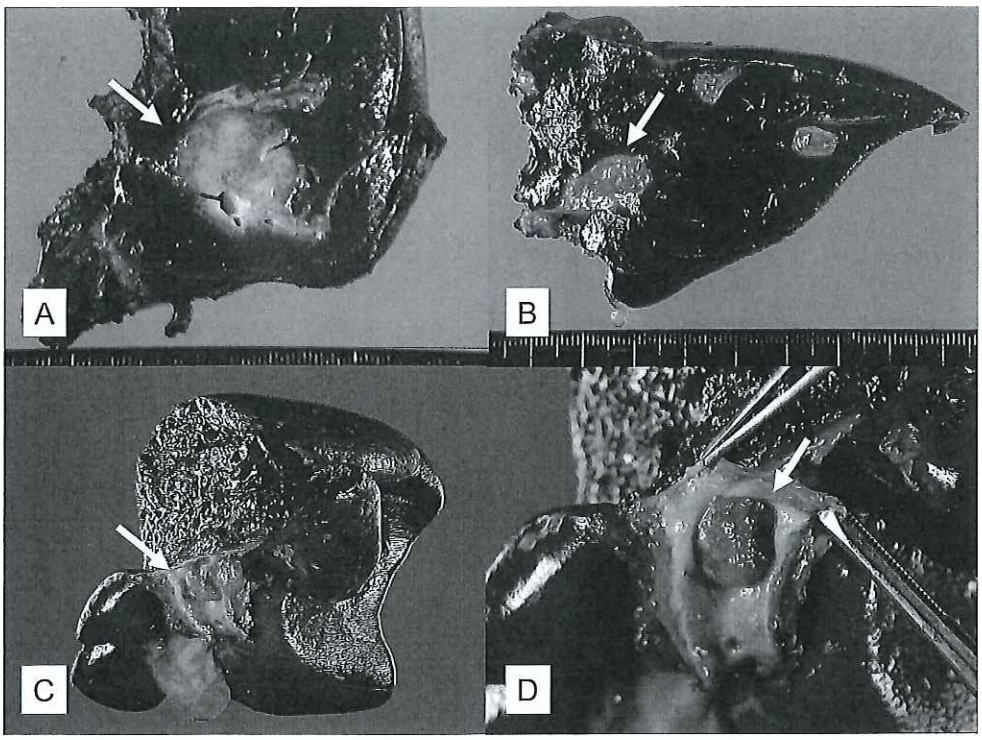

図2　職業性胆管癌症例の切除標本
A，腫瘤形成型肝内胆管癌（矢印）．B，胆管内発育型肝内胆管癌（矢印）．C，乳頭型肝外胆管癌（矢印）．D，Cの拡大像．矢印は胆管癌．

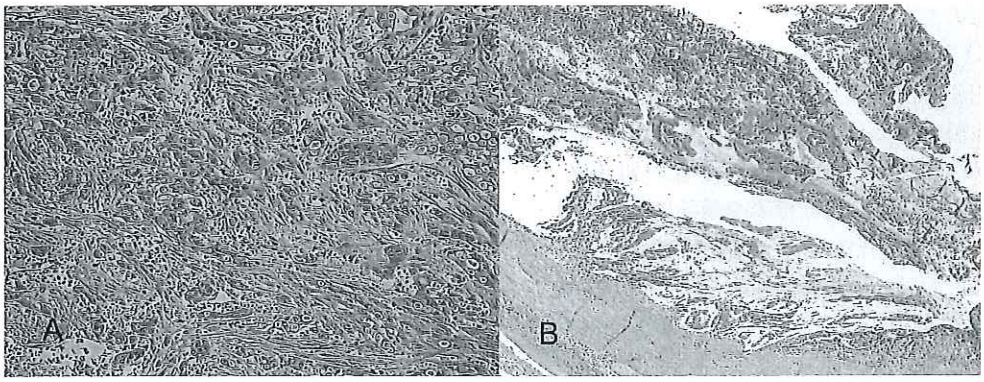

図3　職業性胆管癌症例における主腫瘍の病理学的所見
A，腺癌像を示す腫瘤形成型肝内胆管癌．B，浸潤性 Intraductal papillary neoplasm of the bile duct 像を示す胆管内発育型肝内胆管癌．

て浸潤性胆管癌に至る多段階発育を示すと考えられ，そのなかで乳頭状増殖を示す胆管癌（浸潤性 IPNB）が多くみられることが特徴的であった．また，傷害された胆管の複数部位から癌が発生したと考えられる症例がみられ，胆管癌が多発する点も特徴的であった．なお，肝硬変や進行性肝実質病変などはみられなかった．また，膵・胆管合流異常，原発性硬化性胆管炎，胆石症，肝吸虫など従来から胆管癌の危険因子と報告されている所見はみられなかった．

17例中5例では，胆管癌診断時すでに進行癌の状態であったため，化学療法やステント挿入などが行われた．12例には外科的治療が行われた．8例ではS-1やゲムシタビンなどによる術後補助療法が，2例では胆管断端が癌陽性であったため，放射線治療が行われた．現在までに切除12例中4例と非切除5例中4例が癌死し，切除例のうち1例では癌再発はみられなかったものの，肝線維化の進展と肝不全の進行により死亡した[8]．

2. 全国での職業性胆管癌症例

2015年2月末までに，上記の大阪の印刷事業場での症例を除いて，19例が職業性胆管癌と認定されている．このうち詳細な臨床像が判明している当初の9例をみると，年齢は31歳から57歳，全例男性であった[7]．DCMのみの暴露歴を有する症例が2例，DCMとDCP両者の

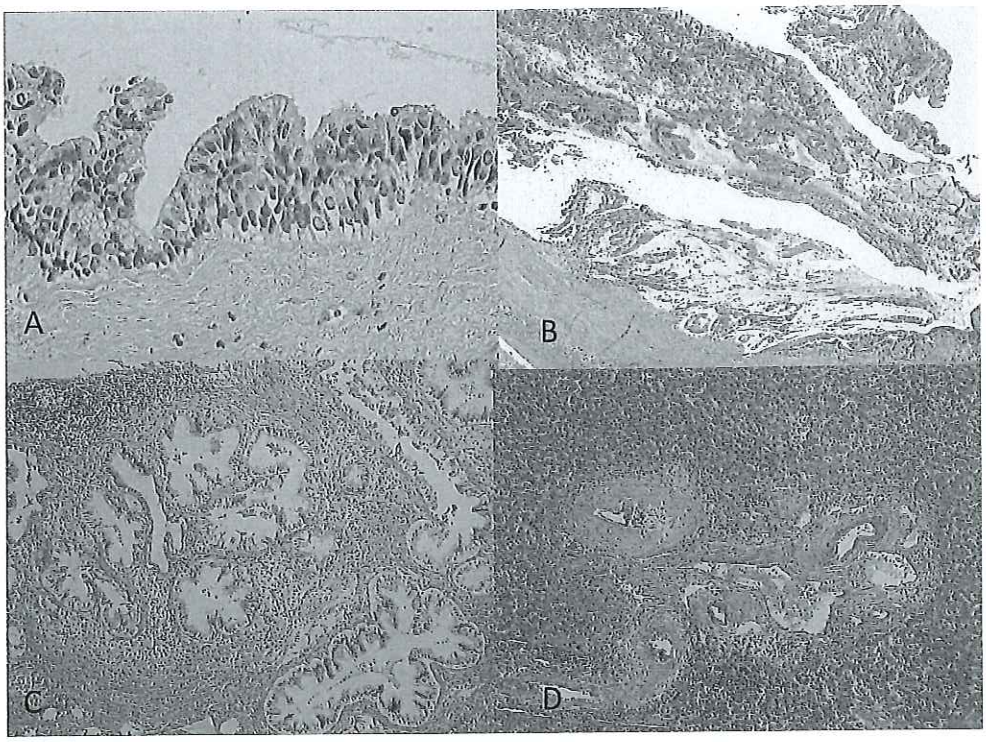

図4 職業性胆管癌症例における主腫瘍以外の胆管の病理学的所見
A, Biliary intraepithelial neoplasia. B, Intraductal papillary neoplasm of the bile duct. C, 胆管および付属腺の腫瘍性増殖. D, 胆管の硬化像と上皮傷害

暴露歴を有するのが7例であった. 全例でγ-GTPが高値で, CA19-9値は6例で高値であった. 主腫瘍による胆道閉塞のため肝内胆管が拡張している症例を除く4例中2例において, 主腫瘍による胆管閉塞を伴わない限局性肝内胆管拡張像がみられた. 9例中4例が肝内胆管癌, 5例が肝外胆管癌であった. 切除標本や剖検標本の検討が可能であった4例では, BilIN-2/3病変やIPNBがみられた. したがって, 全国の職業性胆管癌症例においても, 大阪の印刷事業場S社の胆管癌症例の臨床的特徴を有する症例が多くみられた.

おわりに

印刷労働者にみられた職業性胆管癌の臨床像は, 通常の胆管癌のそれらと異なる特徴を有する. この臨床的特徴の詳細を検討することが, 本病態や発癌メカニズムの解明につながると考えられる. 2015年2月に大阪市立大学医学部附属病院に「職業性胆管癌臨床・解析センター」が設立された. 同センターを中心に, 今後も引き続いて職業性胆管癌症例やDCPの長期間曝露を受けた従業員の健診などのデータを集積し, 解析を進めていきたいと考えている.

利益相反：利益相反基準に該当無し

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Clinical Characteristics of Occupational Cholangiocarcinoma

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The cholangiocarcinoma developed in workers who are exposed to high concentrations of 1,2-dichloropropane and/or dichloromethane for a long term in the printing company is now recognized as "occupational cholangiocarcinoma" by the Ministry of Health, Labour and Welfare of Japan. Some patients with the occupational cholangiocarcinoma visited hospitals because of complaints such as abdominal pain or jaundice and other patients were diagnosed on further examination after regular health examination revealing abnormal findings on either laboratory tests or ultrasonography. On the diagnostic imaging, space-occupying lesions, stenosis or obstruction of the bile ducts, dilatation of the peripheral bile ducts due to tumor-induced stenosis, and regional dilatation of the intrahepatic bile ducts without tumor-induced stenosis were observed. The main tumors were mass-forming type intrahepatic cholangiocarcinoma, intraductal growth type intrahepatic cholangiocarcinoma, and papillary-type extrahepatic cholangiocarcinoma. At the various sites of the bile duct, chronic bile duct injury and precancerous or early cancerous lesions such as biliary intraepithelial neoplasia and intraductal papillary neoplasm of the bile duct were often immunohistochemically positive for γ-H2AH. These findings indicate that the cholangiocarcioma might have developed from the chronic bile duct injuries with DNA injuries into precancerous or early cancerous lesions, and eventually developing into invasive cholangiocarcinoma. Known risk factors for cholangiocarcinoma were not found in the patients with occupational cholangiocarcinoma.

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—Key words—
Occupational cholangiocarcinoma, dichloromethane, 1,2-dichloropropane

職業性胆管癌の病態とその特徴

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索引用語：職業性胆管癌，γ-glutamyl transpeptidase，Biliary intraepithelial neoplasia，intraductal papillary neoplasm of the bile duct，多中心性発癌

1 はじめに

2012年に，大阪のS印刷事業場従業員に胆管癌が多発している事例[1,2]が報告され，その後の厚生労働省による報告[3]をふまえて，2013年10月に「ジクロロメタン（dichloromethane：DCM）や1,2-ジクロロプロパン（1,2-dichloropropane：DCP）にさらされる業務による胆管癌」が業務上疾病に分類されるようになった．これにより，全国での職業性胆管癌としての労災申請が進み，2015年6月の時点で，上記のS印刷事業場の17例と他地域の事業場での19例の計36例が職業性胆管癌として労災認定されている．その一部の症例は，すでに報告されている[2,4]．

本稿では胆管腫瘍の観点から，職業性胆管癌の発癌過程に関連する病態とその特徴について述べる．

2 推定されている原因物質

S印刷事業場では多くの化学物質が使用されていたが，そのなかにDCM，DCP，1,1,1-trichloroethane（TCE），trichloroethyleneなどの塩素系有機溶剤が含まれていた[1-7]．17例全例が高濃度のDCPに長期間曝露されており，11例が高濃度のDCMに長期間曝露されていた．これらの結果，DCPやDCMが胆管癌発癌に重要な役割を果たしたと考えられている．S事業場を含め，いくつかの事業場での曝露状況は，これまでに報告されてきている[1,3,5,6]．

従来より，Globally Harmonized System of Classification and Lebelling of Chemicals（GHS）[8]ではDCM，DCPおよびTCEがcategory 2（suspected human carcinogens）に分類され，International Agency for Research on Cancer（IARC）[9]ではDCMがgroup 2B（possibly

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表1　実験動物を用いたジクロロメタンと1,2-ジクロロプロパンの発癌性試験

著者	発表年	動物	投与法	結果
ジクロロメタン				
Burek[11]	1984	ハムスター，ラット	吸入	有意な発生なし
NTP[12]	1986	マウス	吸入	肝細胞腺腫，肝細胞癌，肺腫瘍
NTP[12]	1986	ラット	吸入	乳腺腺腫，乳腺線維腺腫，肝臓の腫瘍結節，肝細胞癌
Aiso[13]	2014	ラット	吸入	皮膚線維腫，乳腺線維腺腫，胸膜中皮腫
		マウス	吸入	気管支肺胞腺腫，肺癌，肝細胞腺腫，肝細胞癌
1,2-ジクロロプロパン				
NTP[14]	1986	マウス	経口	肝細胞腺腫，肝癌
NTP[14]	1986	ラット	経口	乳癌
Uemeda[15]	2010	ラット	吸入	鼻腔扁平上皮乳頭腫
Matsumoto[16]	2014	マウス	吸入	ハーダー腺腺腫，肺腫瘍
Gi[17]	2015	ハムスター，マウス	経口	有意な発生なし

carcinogenic to humans)に分類されていた．また，2012年10月には，trichloroethyleneがgroup 1(carcinogenic to humans)に変更された．しかし，これらの報告では咽頭・喉頭癌，白血病，腎癌，膀胱癌などがみられるものの，胆管癌についての報告はみられていない．

塩素系有機溶剤と胆管癌についての疫学的研究に関しては，米国の三酢酸セルロース繊維工場における高濃度DCMに曝露した労働者のコホート研究がみられる．その研究では，当初，肝癌と胆管癌のリスクが高かったことが報告されたものの，その後の追跡調査の結果では肝癌と胆管癌発生の有意性は消失している[10]．

一方，これまでにDCMやDCPを用いた多くの動物実験が行われてきたが，胆管癌の発癌は確認されていない(表1)[11-17]．

昨年，大阪の印刷事業場の報告[1,2]を受けて，IARCの会議において，DCPがgroup 1 (carcinogenic to humans)に，DCMはgroup 2 (probable carcinogenic to humans)に変更されることとなった[18]．

このように，DCMやDCPが胆管癌発癌の中心的役割を果たしたと考えられるものの，使用されたほかの化学物質やDCMやDCP製剤に含まれた不純物の影響を完全には否定できず，さらに詳細な検討が必要である[19]．

3　臨床検査値や画像診断の特徴

一般的に胆管癌症例では，黄疸などの症状出現後に医療機関を受診する症例が多いため，胆管癌診断以前の長期間の肝機能検査値の変動を知ることは容易ではない．臨床検査における胆道系酵素の上昇が胆管癌発見のきっかけとなる症例がみられるものの，比較的稀である．また，慢性肝胆道疾患以外の疾患で加療中に胆管癌が発見された症例の臨床検査値の推移をみても，胆管癌診断の数年前より肝機能検査値異常が持続している症例はほとんど経験していない．ところが，職業性胆管癌症例のうち，定期健診や他疾患での受診時の臨床検査値などによって連続した臨床検査値を評価しえた9例(3事業場)では，全例において当初，正常あるいはほぼ正常であったγ-GTP値が胆管癌診断の数年前より徐々に上昇していた(図1，2)[20]．さらに，同時あるいは遅れてASTやALT値が上昇していた．飲酒歴を有する従業員には飲酒制限な

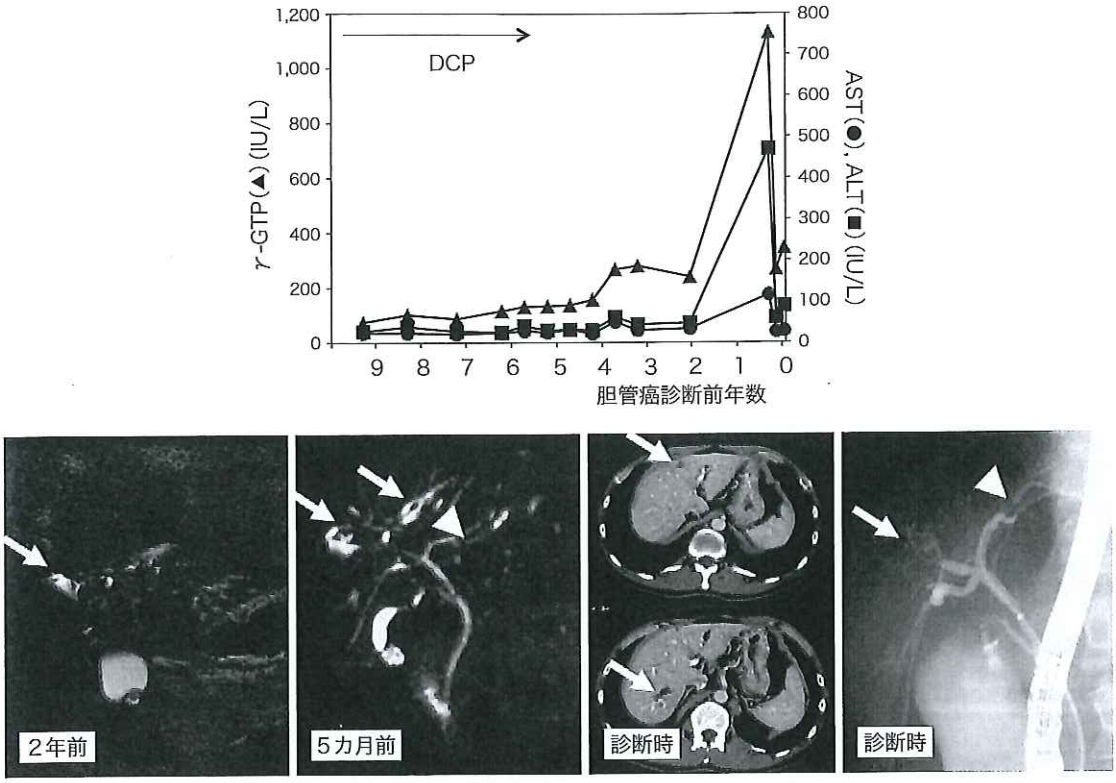

図1　職業性胆管癌症例における胆管癌診断までの臨床検査値と画像所見
矢印：限局性肝内胆管拡張像．矢頭：胆管狭窄像．

どの指導が行われていたが，禁酒や減量によっても肝機能検査値が改善しなかった．また，連続ではないものの数回の臨床検査値を評価しえた症例においても，多くの症例で肝機能異常を確認しえた[21]．さらに，異なる事業場(3事業場)の従業員において，同様の所見が得られたことは，これらの塩素系有機溶剤の長期曝露が原因となって，慢性的な胆管傷害や肝障害が惹起されたことを示している．また，これらの変動の特徴は，塩素系有機溶剤の曝露終了後(退職後，大阪の印刷事業場では2006年10月でのDCP使用中止後)も，継続して上昇していた症例がみられたことである．このことは曝露終了後も胆管傷害やその影響が長期間にわたって持続していることを示している．

職業性胆管癌2例では，肝機能障害のため医療機関を受診し，経過観察されていたため，胆管癌診断以前の画像診断所見を検討することが可能であった．いずれの症例においても，限局性の肝内胆管拡張像や狭窄像がみられ，それらが経時的に進行していた(図1，2)[20,22]．これら症例のMRCP像をみると，原発性硬化性胆管炎(PSC)の画像所見と類似している点があり，実際，これらの症例ではPSCの診断のもとに経過観察されていた．経過中1例では肝内占拠性病変が出現し，腫瘤形成性胆管癌と診断された(図1)．他の1例では，胆管の狭窄像や拡張像が強くなったため，ERCPを施行したところ，胆管癌と診断された(図2)．以上の所見は，DCMやDCPに曝露された従業員に対するγ-GTP，ASTやALT

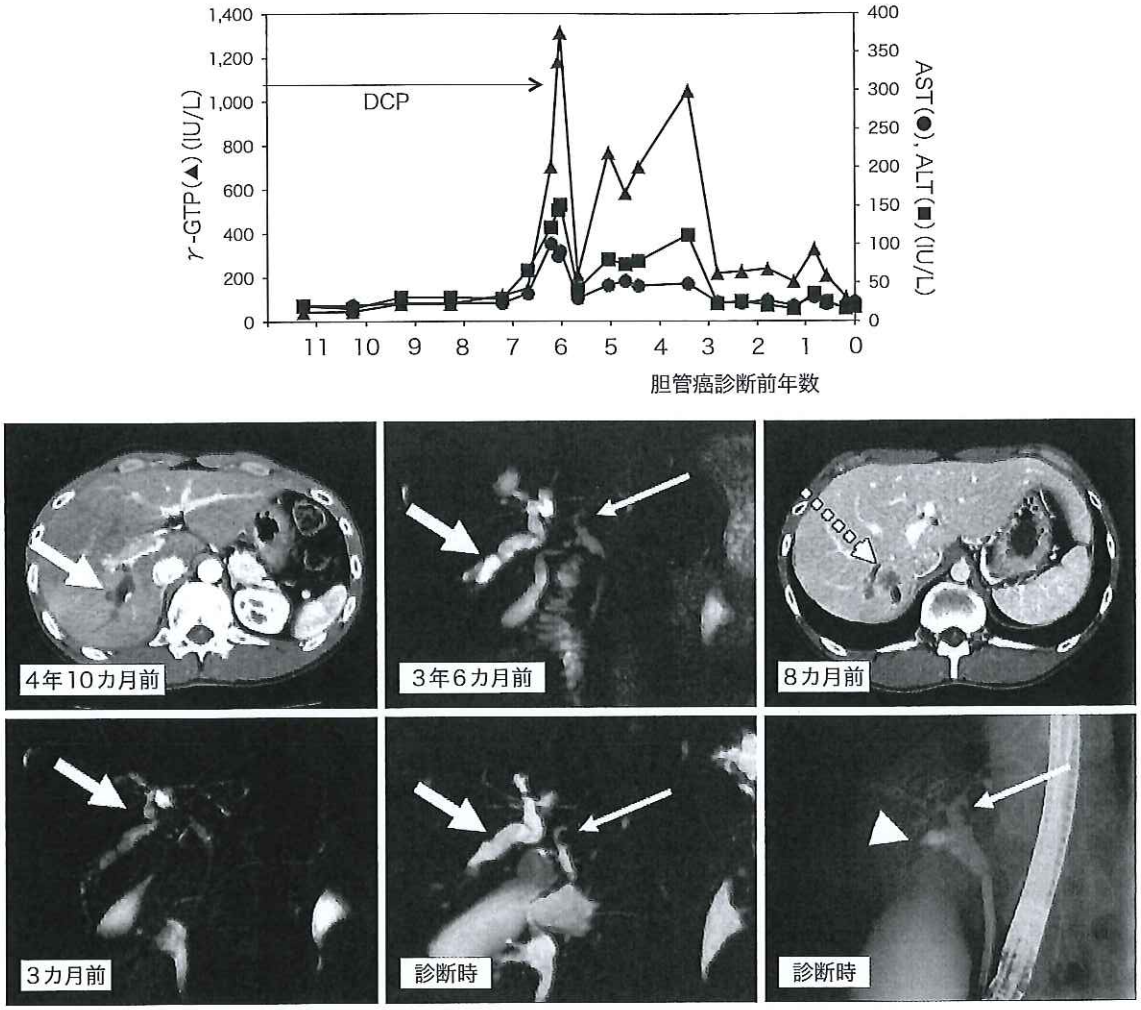

図2　職業性胆管癌症例における胆管癌診断までの臨床検査値と画像所見
矢印：限局性肝内胆管拡張像．矢頭：胆管狭窄像．
長矢印：胆管内乳頭状病変．点線矢印：腫瘤形成型肝内胆管癌．

を含む肝機能検査や画像診断による定期健診の重要性を示している[20]．

胆管癌診断時の臨床検査値では，全例でγ-GTP値は上昇しており，多くの症例でAST，ALT値が上昇していた[2,4]．また，画像診断では，肝内腫瘤像，胆管内乳頭状腫瘤像，胆管狭窄や閉塞像がみられた(図1，2)．さらに，胆管癌による肝内末梢側胆管拡張像に加えて，胆管閉塞像を伴わない限局性の胆管拡張像がみられたことが，職業性胆管癌症例特有の画像所見と考えられた(図1，2)[2,4]．また，これらの画像所見は，前述のようにPSCの画像所見と類似点がみられた．

4　病理学的特徴

職業性胆管癌症例のほとんどが，腫瘤形成型肝内胆管癌，胆管内発育型肝内胆管癌，乳頭型肝外胆管癌であり(図3)，それらは高分化型から低分化型を示す腺癌や浸潤性intraductal papillary neoplasm of the bile duct

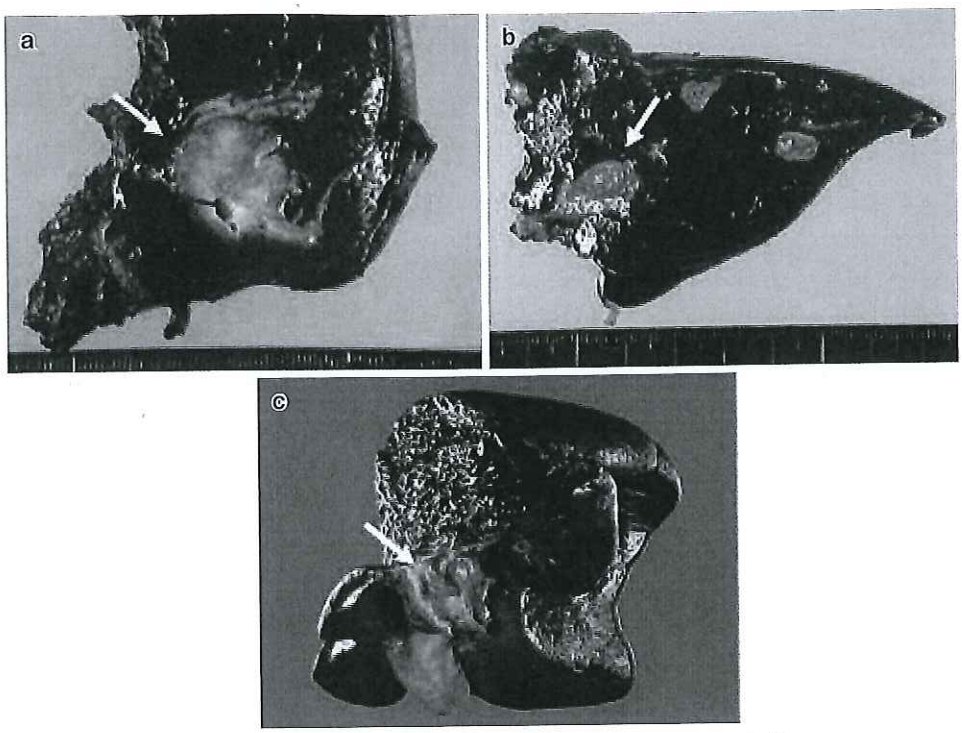

図3　職業性胆管癌の切除標本の肉眼所見(文献2より引用)
a：腫瘤形成型肝内胆管癌(矢印)．b：胆管内発育型肝内胆管癌(矢印)．c：乳頭型肝外胆管癌(矢印)．

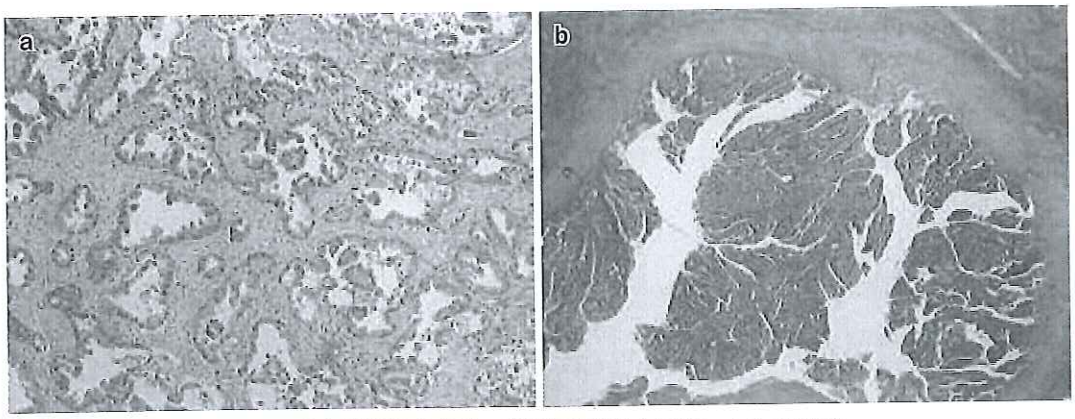

図4　職業性胆管癌症例の病理学的所見(文献2，4より引用)
a：高分化型腺癌．b：浸潤性intraductal papillary neoplasm of the bile duct.

(IPNB)を呈した(図4)[2,4]．また，多くの症例で，比較的大型胆管や胆管周囲付属腺にBiliary intraepithelial neoplasia (BilIN)-2/3病変，IPNB病変や浸潤部を伴う同病変が認められた(図4，5)．特に，肝内結石症に合併する胆管癌症例に比べIPNBが高率にみられた[23,24]．さらに，広範囲の胆管に胆管周囲線維化や胆管上皮傷害像がみられたことが特徴的であった(図6)．なかには，胆管癌再発はみられなかったものの，線維化の進行により

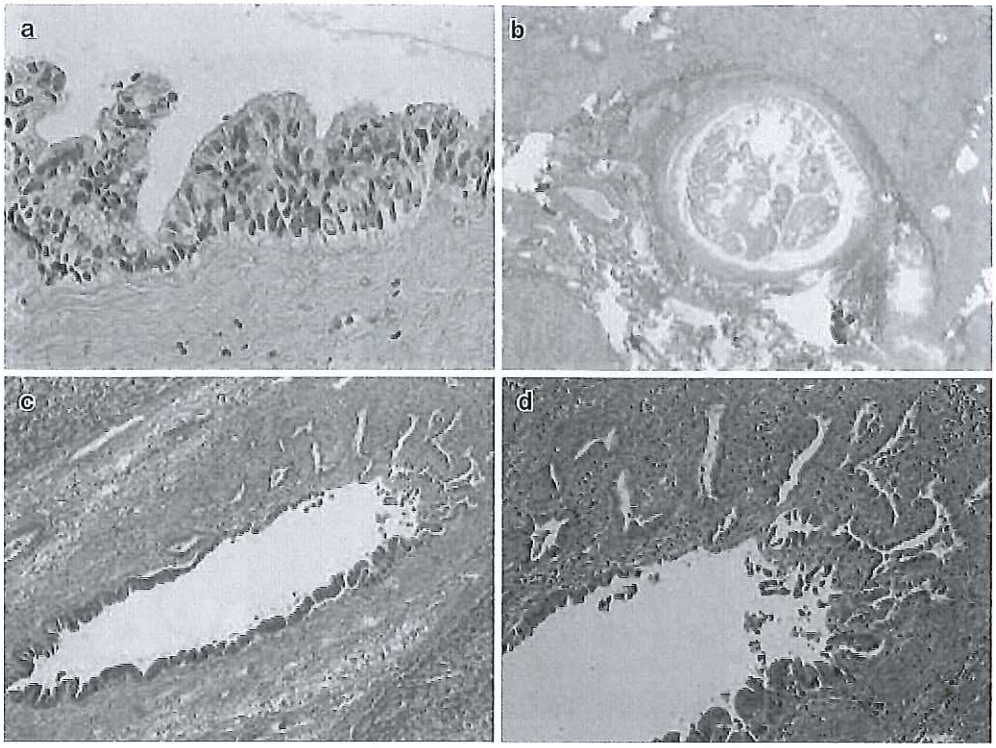

図5　職業性胆管癌症例の病理学的所見（文献2，4より引用）
a：Biliary intraepithelial neoplasia病変．b：intraductal papillary neoplasm of the bile duct病変．
c：浸潤を伴うBiliary intraepithelial neoplasia病変．d：cの浸潤部の拡大像．

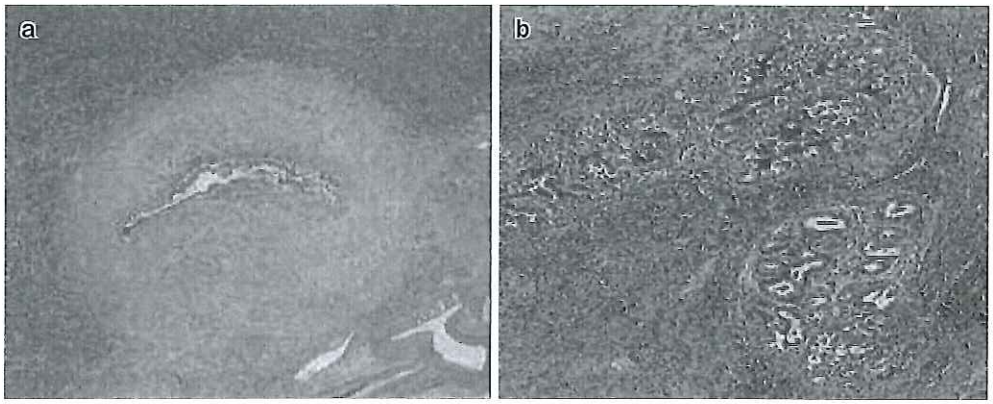

図6　職業性胆管癌症例の病理所見
a：胆管の硬化像．b：胆管と付属腺の増殖性病変．

肝不全死した症例がみられた[25]．切除標本全体を観察しえた症例では，広範囲の胆管でこれらの慢性胆管傷害や前癌病変が観察された．このような胆管癌，前癌病変および胆管の硬化像が限局性肝内胆管拡張像や胆管狭窄像などPSCに類似した画像所見を呈したと考えられた．

また，BilIN-2/3病変やIPNB病変から進行

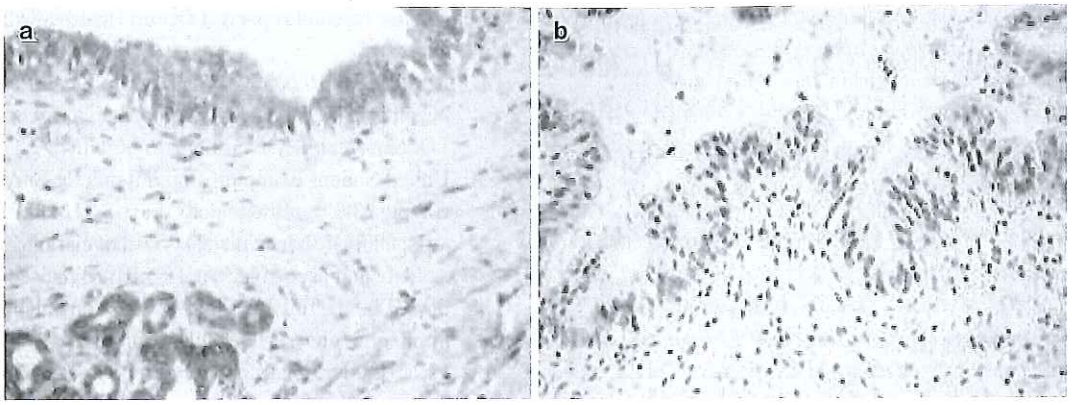

図7　職業性胆管癌症例の免疫組織学的検討（文献24より引用）
a：GST T-1による免疫染色．肝内大型胆管および付属腺にGST T-1の発現がみられる．
b：γ-H2AXによる免疫染色．Biliary intraepithelial neoplasia病変にγ-H2AXの発現がみられる．

癌への進展の過程で，BilIN病変が優位の場合，腫瘤形成性肝内胆管癌が，IPNB病変が優位の場合，胆管内発育型肝内胆管癌や乳頭型肝外胆管癌の像を呈するようになったと推測される．特に，IPNBが高率にみられ，その結果，胆管内発育型肝内胆管癌や乳頭型肝外胆管癌が多くみられたことが通常の胆管癌と異なった点であり，職業性胆管癌の特徴と考えられる．これらの，病理学的所見全体を勘案すると，職業性胆管癌では，慢性胆管傷害から前癌病変を経て浸潤癌に至る多段階発癌が推定される．

5　推定される発癌過程

ヒトにおいて，肝細胞にCYPが豊富に存在し，GST T1-1も存在している．一方，胆管上皮にはCYPはほとんど存在せず，逆にGST T1-1が存在する（図7）[23,24]．DCMはcytochrome P450（CYP）経路およびglutathione S-transferase（GST）T1-1経路によって代謝される．通常ではまず，CYP経路で代謝されるが，高濃度曝露になるとCYP経路が飽和され，GST経路で代謝されることとなる[26]．大阪のS事業場従業員のインタビューにおいて，同社に勤務開始後，飲酒できなくなったとか，飲酒するとすぐに赤くなり，時には発疹がみられるようになったとの話が多く聞かれたことは，CYP経路が飽和されていたことと関連があるかもしれない．主に胆管上皮でみられるGST経路では反応性の高い中間代謝物質であるホルムアルデヒドやS-クロロメチルグルタチオンが産生され，これらによって胆管の慢性炎症や遺伝子障害が惹起された可能性がある．一方，DCPの代謝に関しては，完全には解明されておらず，今後の検討が必要である．

最近の職業性胆管癌症例の癌部の遺伝子解析によると，通常の胆管癌に比較して，職業性胆管癌症例では極めて高頻度の遺伝子異常がみられたことが判明している．病理組織学的には，大阪のS印刷事業場の症例をはじめ多くの職業性胆管癌症例において，広範囲に胆管周囲線維化や胆管上皮傷害像がみられたが，これらの傷害を受けた胆管，前癌病変および胆管癌部はγ-H2AX陽性であり（図7）[23,24]，遺伝子解析でみられた結果と合致するものであった．

職業性胆管癌症例では，肝内胆管癌と肝外

胆管癌の併存例や初発部位と異なった部位での胆管癌再発例がみられた．これらは広範囲の胆管が傷害されていることや高頻度の遺伝子異常を伴っていることと関連し，その結果，広範囲の胆管の発癌ポテンシャルが極めて高く，いわゆる多中心性発癌を示すことも職業性胆管癌の特徴である．

6 おわりに

塩素系有機溶剤が原因となった職業性胆管癌では，塩素系有機溶剤が原因となって広範囲の胆管が傷害された結果，高い発癌ポテンシャルを有するようになったと考えられる．今回の事例によって，化学物質が原因となって胆管癌が発症することが示された．今後，頻度は少ないと考えられるものの，このような事例を念頭におく必要がある．

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1．肝内胆管癌の疫学的動向と危険因子*

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〔要旨〕肝内胆管癌は世界的に増加傾向にある．肝内結石症，原発性硬化性胆管炎，膵・胆管合流異常，肝吸虫，B型肝炎，C型肝炎が発癌危険因子となる．さらに糖尿病，肥満などの生活習慣病などとの関連も報告されている．印刷労働者に胆管癌が多発した事例をふまえて，最近，職業性胆管癌として労働災害認定され，国際癌研究機関(IARC)において，1, 2-ジクロロプロパンがgroup 1 (carcinogenic to humans)に，ジクロロメタンがgroup 2A (probably carcinogenic to humans)に改訂された．

はじめに

肝内胆管癌は，原発性肝癌の中で肝細胞癌に次いで多く，近年，増加傾向にある．臨床例の検討や疫学的調査から，種々の危険因子が明らかとなってきている．本稿では肝内胆管癌の疫学的動向と危険因子について述べる．

Ⅰ．肝内胆管癌の動向

胆管癌の罹患率は地域によって異なるが，2000年代になり肝内胆管癌(ICC)罹患率および死亡率が上昇していると報告される一方，肝外胆管癌(ECC)の罹患率はむしろ低下傾向にあると報告されている[1~5]．すなわち英国では，ICCによる10万人あたりの年齢調整死亡率が1968年からの30年間で0.1以下から約1.0へと著明に上昇し，1993年以降は原発性肝癌におけるICCの割合がもっとも高率になっている[2,4]．米国およびオーストラリアにおいても1980年から20年間にICCによる年齢調整死亡率が200％以上，上昇している[3~5]．この原因として，ICD分類における肝門部胆管癌の取り扱いの変更，内視鏡技術や画像診断の進歩による診断能の向上，原発性硬化性胆管炎(PSC)やウイルス性肝炎の罹患率の増加などが考えられているが，明確な要因は不明である．

本邦においても，厚生労働省による人口動態統計[6]によると，男性におけるICCによる人口10万人対死亡率は増加している一方，ECCは1991年を境に低下傾向にあると報告されている(図1, 2)．宮城，山形，福井，長崎の4県における癌登録[7]や大阪府における癌登録[8]による集計においてもICCは増加傾向にあるが，ECCはむしろ低下傾向にある(図3, 4)．なお，厚生労働省の統計および大阪府癌登録の集計を含めて，一般的にICCの発症年齢は60～70歳代であり，50歳未満の発症はきわめて少ない．

キーワード：肝内胆管癌，1, 2-ジクロロプロパン，ジクロロメタン，職業性胆管癌

* Epidemiology of and risk factors for intrahepatic cholangiocarcinoma

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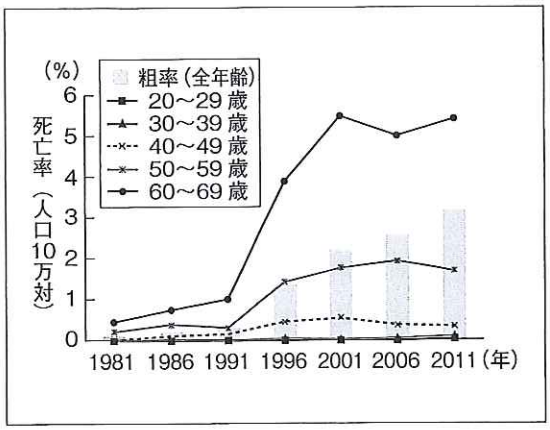

図1. 肝内胆管癌の年齢階級別死亡率の推移（男性）

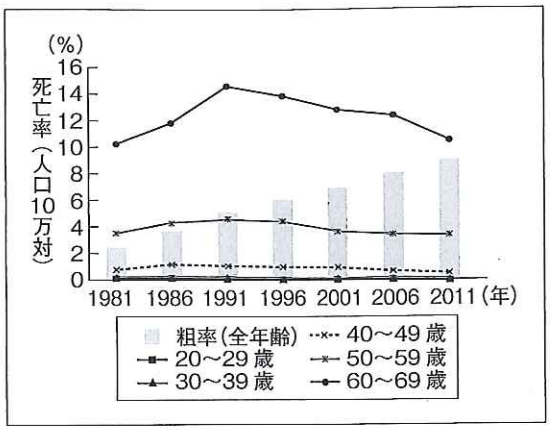

図2. 肝外胆管癌の年齢階級別死亡率の推移（男性）

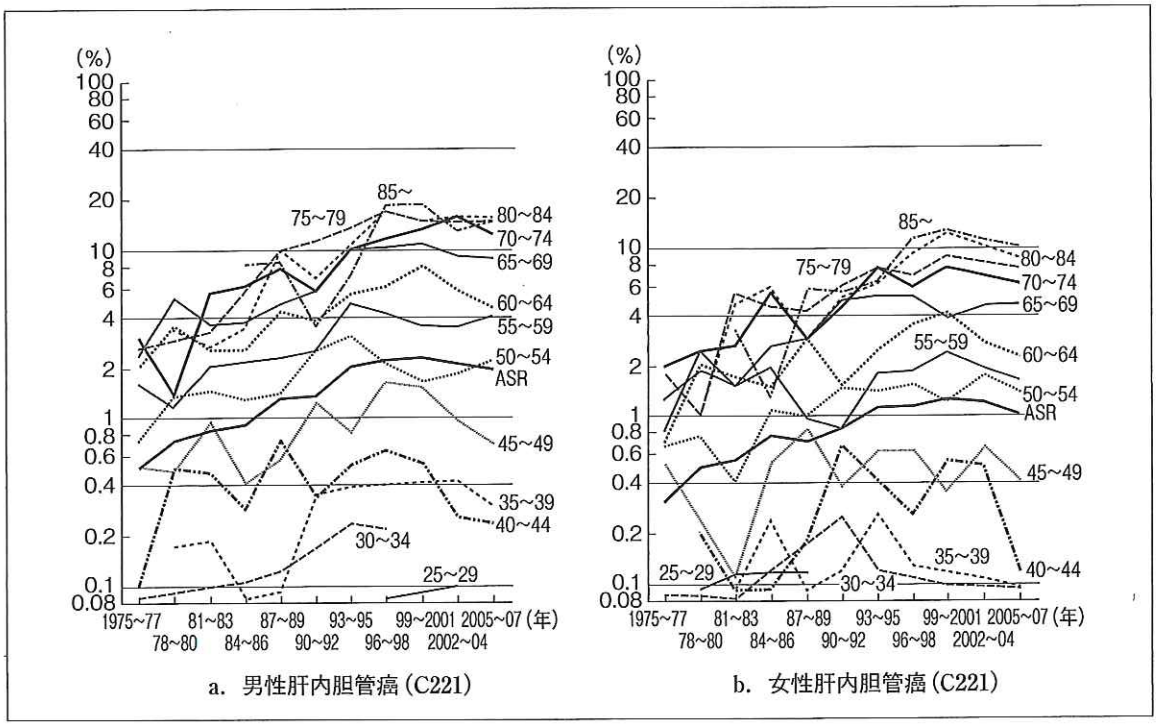

図3. 大阪府における肝内胆管癌の推移（文献8より引用）
ASR：年齢標準化発生率

Ⅱ．胆管癌の危険因子

ICCを含めた胆管癌の危険因子として，従来より肝内結石症，PSC，肝吸虫，膵・胆管合流異常やニトロソアミンなどの化学物質が報告されてきた[1, 9～11]．肝内結石症ではその1.3～8.8％に胆管癌がみられ[12, 13]，胆管癌が肝内結石症の予後規定因子であると報告されている[14]．肝内結石症における胆管癌は，胆管炎を繰り返す結石の存在する胆管に発生することが知られている．PSCからの胆管癌発生率は1年あたり0.6～1.5％とされ，PSC患者における胆管癌合併率は10～20％と報告されている[15, 16]．本邦での集計ではPSC患者のうち約4％に胆管癌がみられたと報告されており，欧米ほど高くない[17]．肝吸虫による胆管癌は世界的にみられるが，タイ北西部は*Opisthorchis viverrini*

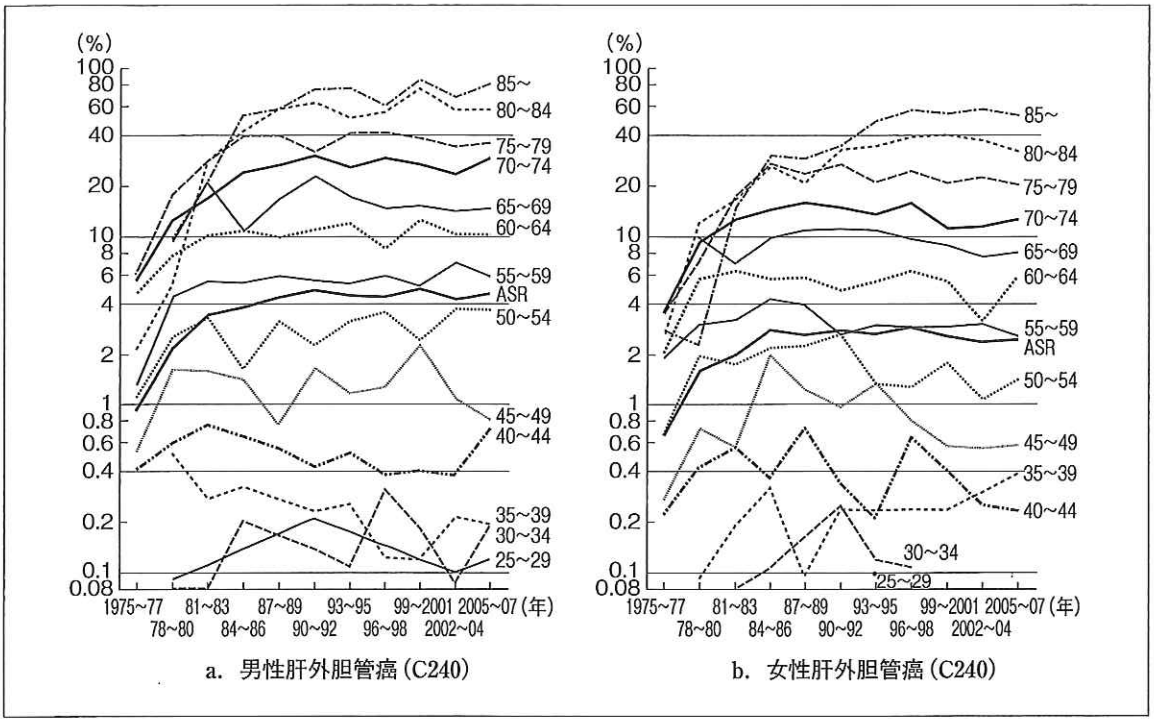

図4. 大阪府における肝外胆管癌の推移 (文献8より引用)

表1. 肝内胆管癌の危険因子 (文献20より引用改変)

因　子	単変量解析オッズ比 (95 % CI)	多変量解析 adjusted オッズ比 (95 % CI)	*p*値
C型肝炎	16.87 (5.69 ～ 50.0)	6.02 (1.51 ～ 24.1)	0.01
B型肝炎	1.84 (0.34 ～ 10.11)		
糖尿病	2.19 (0.98 ～ 4.90)	1.95 (0.65 ～ 5.85)	0.23
胆石症	0.41 (0.12 ～ 1.48)		
肝硬変	18.72 (3.43 ～ 102.05)	5.03 (0.45 ～ 56.82)	0.19
ALT (IU/*l*)	5.26 (2.62 ～ 10.58)	2.89 (1.13 ～ 7.38)	0.03
Alb (g/d*l*)	3.56 (1.72 ～ 7.35)	2.85 (1.04 ～ 7.79)	0.04
血小板 ($\times 10^4$/mm^3)	3.18 (1.61 ～ 6.28)	2.39 (0.96 ～ 5.95)	0.06

による肝吸虫症の好発地域であり，同地区のタイ人男性での胆管癌発症率は10万人あたり100人前後ときわめて高率であることが報告されている[9～11]．本邦では *Clonorchis sinensis* が原因である肝吸虫症により生じることが多く，肝吸虫感染患者にみられた胆管癌がこれまで12例報告されている[18]．膵・胆管合流異常では胆嚢癌および拡張胆管に胆管癌が発生する．

ウイルス性肝炎がICCの原因となることも，多くの研究によって明らかにされてきた．すなわちC型肝炎ウイルス (HCV) 感染に関しては，Kobayashiら[19]がHCV関連肝硬変600例を経過観察したところ，ICCの発生率が5年で1.6 %，10年で3.5 %であり，本邦における一般的なICC発生リスクの1,000倍であったと報告している．筆者らのhospital based case-control study[20]においては，HCV感染率の高い大阪地区であるためか，ICC 50例中18例 (36 %) にHCV感染が認められ，調整オッズ比は6.0 (95 % CI 1.5 ～ 24.1) であった (表1)．ちなみに日本肝癌研究会の追跡調査では，

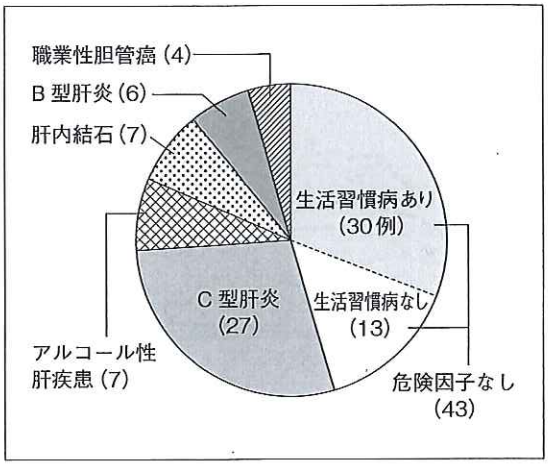

図5. 当科における切除肝内胆管癌例の要因（文献28より引用改変）

ICC患者828例中156例（18.8％）がHCV抗体陽性であったと報告されている[21]．Welzelら[22]もHCVはECCの危険因子ではなかったが，ICCの危険因子であったと報告している．一方，ICCとB型肝炎ウイルス（HBV）感染の関連については，慢性B型肝炎の有病率が高い韓国や台湾などから報告されている．すなわち，韓国からは対照2,488例のHBs抗原陽性率5.0％に対し，ICC 622例では13.5％がHBs抗原陽性であり，HBVは独立した危険因子であったと報告されている[23]．台湾におけるcase-control studyでは，HBV感染およびHCV感染ともにICCの危険因子であったと報告されている[24]．このようにICCとウイルス性肝炎の関連については地域によって若干異なるものの，近年のメタアナライシスにおいてHBV感染（オッズ比3.2，95％CI 1.9～5.3）およびHCV感染（オッズ比3.4，95％CI 2.0～6.0）ともにICCの危険因子であることが報告されている[25]．ところで最近，ウイルス性肝炎を背景にした肝内胆管癌と肝細胞癌の遺伝子異常が類似していることが報告された[27]．実際，肝内胆管癌と肝細胞癌による重複癌を発症するC型肝炎患者を経験する．

上記以外に肝硬変，糖尿病，肥満などの生活習慣病，飲酒，喫煙，炎症性腸疾患が胆管癌と関連していることも報告されてきた[11, 22, 26]．当科でのICC切除例の背景因子の検討では，従来より報告されているICCの危険因子を有さない症例の約60％に生活習慣病がみられた（図5）[28]．

さらに，国際がん研究機関（IARC）からは，アフラトキシン，経口避妊薬，プルトニウム，トロトラストが，「liver and bile duct」における「carcinogenic agents with sufficient evidence in humans」として報告されている[29]．一方，IARC monographにおいて，印刷工程やcarbon blackはgroup 2A（possibly carcinogen）に分類されているが，その際に指摘されている癌腫は喉頭・咽頭癌，膀胱癌，腎癌，白血病などであり，胆管癌の報告はみられなった[30]．しかし最近，後述する1, 2-ジクロロプロパン（1, 2-dichloropropane：DCP）やジクロロメタン（dichloromethane：DCM）の長期間，高濃度曝露を受けた印刷事業場従業員での胆管癌多発事例が報告され[31, 32]，これらの塩素系有機溶剤が胆管癌の原因となることが強く推定されることから，2014年のIARCの会議において，DCPがgroup 1（carcinogenic to humans）に，DCMがgroup 2A（probably carcinogenic to humans）に改訂された[33]．

Ⅲ．職業性胆管癌の疫学と対策

前述のように最近，大阪のS印刷事業場のオフセット校正印刷部門の元および現従業員において，胆管癌が多発していることが報告された[31, 32]．その職業性胆管癌症例の臨床的および病理学的特徴については，ほかの論文を参照していただきたい[32, 34～36]．

過去の就業状態調査の追加，変更などがあったが，2015年3月時点の調査結果によると，この印刷事業場でオフセット校正印刷業務に従事した元・現従業員は106名（男性86名，女性20名）である．この時点で17名に胆管癌が認められ，その標準化罹患比（SIR）は1132.5（95％信頼区間659.7～1813.2）であった[37]．DCMとDCPともに曝露していた従業員，DCPのみの曝露があった従業員に限ると，SIRはさらに高値を示した．一方，2012年末の標準化死亡比は633と高値であった[38]．また，ほかの部門勤務者では胆管癌の発症はみられなかったことから，この胆管癌発症は業務特異性があると考えられる．17例の胆管癌患者は診断時25～45歳と若年であり，全例男性であった．これは，オフセット校正印刷部門のほとんどの従業員が若く，長期間曝露された50歳以上の従業員数がもともと少なかったことが関連し

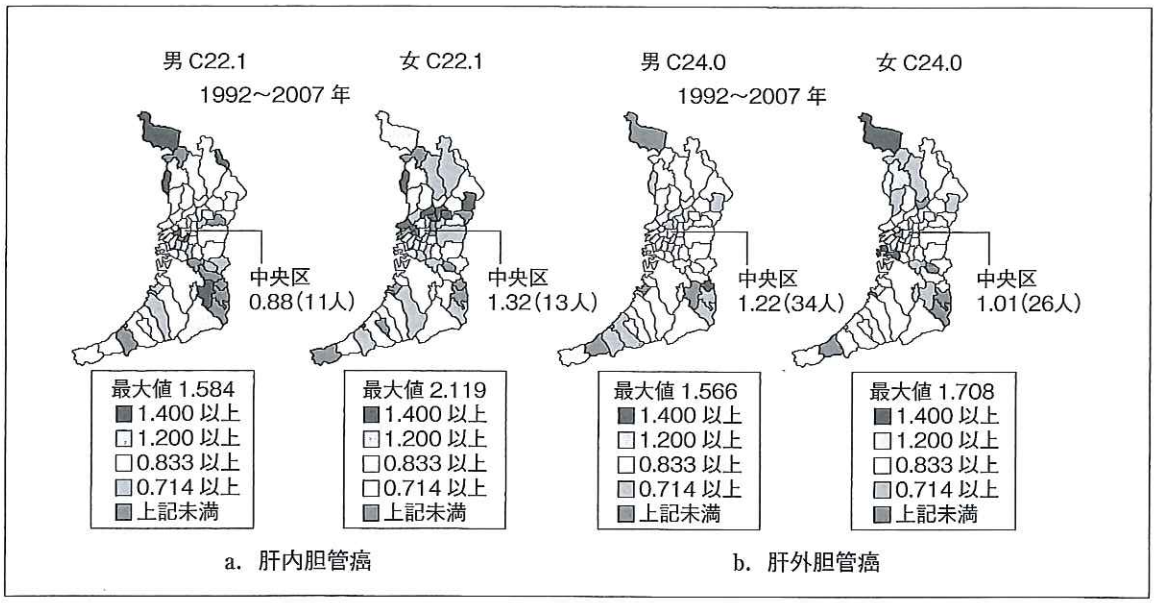

図6. 大阪府における胆管癌罹患の地理的集積性（1992～2007年大阪府がん登録資料より）

ている．また，女性従業員に胆管癌発症がみられないのは，同部門で塩素系有機溶剤に高濃度，長期間曝露した女性従業員がわずかであったためと考えられる．なお本事業場では，胆管癌患者以外に胃癌，腎癌，皮膚Bowen病患者がみられたが，これらの疾患の集積はみられなかった．

全国の中小企業を網羅する全国健康保険協会の2009年4月～2012年3月までのレセプトデータを用いた胆管癌受療率の検討によると，印刷業事業所と全業態での胆管癌受療率に有意差はみられず，中小の印刷業での胆管癌の多発は認められなかった[39]．全国印刷健康保険組合の2009年7月～2011年3月請求レセプトデータと包括医療費支払い制度（DPC）の全国データの胆管癌患者の比較によると，21～60歳男性本人では，期待退院数7.79に対し，観察された退院数は17で，標準化受療率比は2.18，その95％信頼区間は0.93～5.09で，高値であったものの有意な増加は認められなかった[40]．したがって，印刷業全体に胆管癌発症が高いわけではなく，各事業場の就業環境が大きく影響していると考えられた．

一方，大阪府がん登録資料に基づいたICD-10のC22.1（肝内胆管癌）およびC24.0（肝外胆管癌）の地理的分布の検討によると，大阪府および府内市町村レベル，S印刷事業場の5 km圏，2 km圏，1 km圏において胆管癌に関連する罹患率の上昇や罹患リスクの上昇はみられず，地域集積性は確認されなかった（図6）．

最近，北欧4ヵ国の職業別疾患登録による解析から，男性印刷関係労働者における胆管癌の発症率は，ほかの職業の約2倍であったと報告されている[41]．しかし，この印刷関係労働者にはタイピストなども含まれるなど就業内容がさまざまであることや，長期間のデータであり，同期間における胆管癌診断能などの医療状況の変化も考慮したうえで，慎重な評価が必要と考えられる．

2013年3月に本事例に関する「印刷事業場で発生した胆管がんの業務上外に関する検討会」報告書である「化学物質ばく露と胆管がん発症との因果関係について―大阪の印刷事業場の症例からの検討」[42]において，①胆管癌は，DCMまたはDCPに長期間，高濃度曝露することにより発症しうると医学的に推定でき，②本件事業場で発生した胆管癌は，DCPに長期間，高濃度曝露したことが原因で発症した蓋然性がきわめて高いことが報告された．この報告を受けて2013年10月に厚生労働省は，DCMやDCPにさらされる業務による胆管癌が労働基準法施行規則別表第1の2に規程する業務上の疾病に分類され，いわゆる労働災害認定されることとなった．現在，全国でDCM，DCPあるいはその両者の曝露を受けた36例の胆管癌患者が労働災害認定を受けている．

大阪のS印刷事業場では，厚生労働省による調査後，開放式プッシュプル型換気装置が設置されるなど作業環境が改善されている[43]．一方，大阪市立大学医学部附属病院では，2012年から元あるいは現従業員に対して胆管癌検診を行ってきた．2013年10月には特定化学物質障害予防規則改正に伴い，DCPを長期間取り扱った従業員に対して健康管理手帳に基づく健康診断が行われることとなった．大阪のS印刷事業場の胆管癌患者では，発症数年前よりγ-GTP，ASTやALT値の上昇と画像診断において限局性胆管拡張像などの所見がみられたこと[44,45]をふまえて，この健診においてはγ-GTP，AST，ALT値やCA19-9値に加え，必要に応じて腹部超音波検査などの画像診断を実施することとなっている．2015年2月には大阪市立大学医学部附属病院に「職業性胆管癌臨床・解析センター」[46]が開設され，特殊健康診断，健康管理手帳交付者の健康診断，職業性胆管癌患者の治療にあたるとともに，それらの成績の集積や解析から職業性胆管癌の病態解明と，適切な診断方法や治療法の確立をめざしている．

おわりに

ICCを中心とした胆管癌の疫学的動向と危険因子について概説した．また，職業性胆管癌の疫学的研究結果と現在の取り組みについて報告した．

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日消誌　2015；112：1341―1347

症例報告

印刷業職業性胆管癌に対する化学放射線療法と根治的肝切除の経験

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要旨：症例は42歳，男性．印刷オペレーターとして勤務し，1,2ジクロロプロパンを含む製品を使用していた．尿濃染と全身倦怠・掻痒で近医受診し精査加療目的に当科紹介，肝門部領域胆管癌の診断で術前化学放射線療法後に肝拡大右葉切除を施行した．手術後に職業性胆管癌として労災認定されている．術後30カ月以上経過するが再発を認めず，職業性胆管癌に術前化学放射線療法が奏効した貴重な治療経験として報告する．

索引用語：胆管癌，印刷業，化学放射線療法，1,2ジクロロプロパン，集学的治療

はじめに

2012年3月に，特定の事業所でオフセット印刷業務を担当した労働者に多数の胆管癌発症があったことが，労務災害請求を通して報告された．厚生労働省や日本産業衛生学会からも有機塩素系洗浄剤の被曝の状況や胆管癌の発生症例数が報告され，疫学的に有機塩素系洗浄剤の高濃度被曝が胆管癌を惹起した可能性が示唆された[1)2)]．本誌でも新たな職業癌として詳しく解説されている[3)]．

われわれはこれらの報告以前に，印刷業従事者に若年発症した肝門部領域胆管癌に対して，術前療法として化学放射線療法を施行後に根治的肝切除を施行，33カ月無再発生存している症例を経験している．職業性胆管癌に対する集学的治療の経験を報告する．

I　症　例

症例：42歳，男性．

主訴：黄疸．

家族歴：特記事項なし．

既往歴：30歳頃右手首骨折手術．40歳右膝半月板手術．

生活歴：喫煙20本/日×21年．習慣飲酒（1日1単位以下）．アレルギーなし．

初診時検査所見：黄疸と肝細胞障害・胆道系酵素異常を認めた．CA19-9も高値を示していた（Table 1）．HBs抗原陰性，HCV抗体陰性．

現病歴：1989年4月から印刷企業に勤務し，2011年3月まで同一の現場環境で印刷工程作業に従事していた．2011年12月に尿の濃染と黄疸を自覚し近医を受診した．閉塞性黄疸の診断で，原因検索の精査を必要としたが，出身地の宮城県での加療を希望し当科紹介となった．当院消化器

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Table 1. 当院初診時血液検査所見

WBC	4500 /μl	T-bil	12.4 mg/dl
Hb	16.4 g/dl	D-bil	9.1 mg/dl
Plt	23万 /μl	ALP	1311 U/l
		γGTP	1254 U/l
PT	120 以上 %	AST	502 U/l
	0.85 INR	ALT	839 U/l
APTT	30.2 sec	LDH	355 U/l
		ChE	292 U/l
BUN	15 mg/dl		
Cr	0.8 g/dl	TP	7.6 g/dl
		Alb	4.2 g/dl
Na	138 mEq/l		
K	3.8 mEq/l	CEA	2.5 ng/ml
Cl	101 mEq/l	CA19-9	8870 U/ml

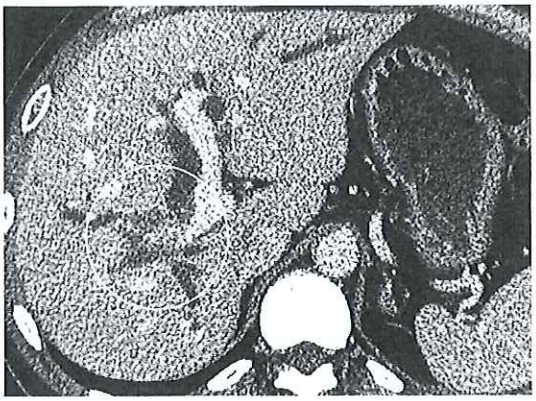

Figure 1. 腹部造影CT所見：左右肝管合流部から三管合流部にかけて，造影効果をともなう壁の不整な肥厚と内腔狭窄を認めた．門脈右枝の狭窄を認めた．

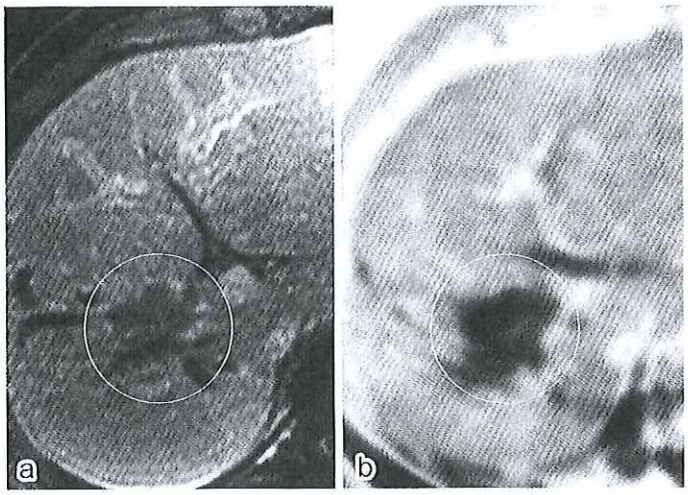

Figure 2. 腹部造影MRI所見 a：門脈右枝分岐部に腫瘤所見．b：拡散強調画像（DWI）では腫瘤が高信号で描出された．

内科で施行した胆管狭窄部の組織生検で腺癌を認め，肝門部領域胆管癌と診断した．

CT・MRI所見（Figure 1，2a，b，3）では三管合流部から左右肝管合流部にわたる領域に造影効果をともなった壁肥厚と胆管狭窄を認め，前後区域分岐部には25mm大の腫瘤性浸潤を認めた．同部で右門脈の閉塞を認め，PETで腫瘍はmaximum standardized uptake value（SUVmax）10.0の集積亢進を呈していた（Figure 4）．Bhp T4b N0 M0 Stage IVA（胆道癌取扱い規約（第6版））(Bismuth IV）と診断した．

Endoscopic retrograde cholangiopancreatography（ERCP）でも同様の狭窄所見であったが，狭窄より末梢側の胆管造影では胆管の不整所見を認めなかった（Figure 5）．Endoscopic ultrasound（EUS）では肝門部胆管から中部胆管にかけての胆管壁肥厚を認めた．Intraductal ultrasonography（IDUS）・peroral cholangioscopy（POCS）は施行しなかった．

全肝容量は1188mlと算出され，減黄後のICG

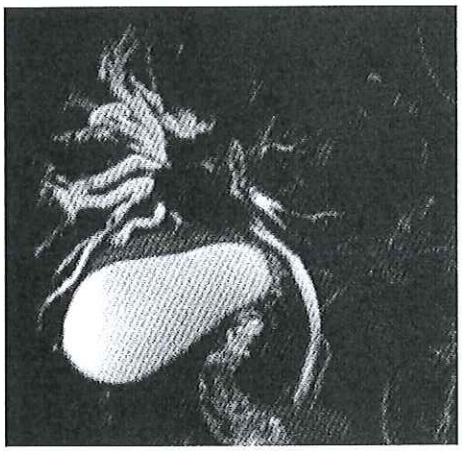

Figure 3. Magnetic resonance cholangiopancreatography（MRCP）所見：三管合流部から肝門までの胆管閉塞所見.

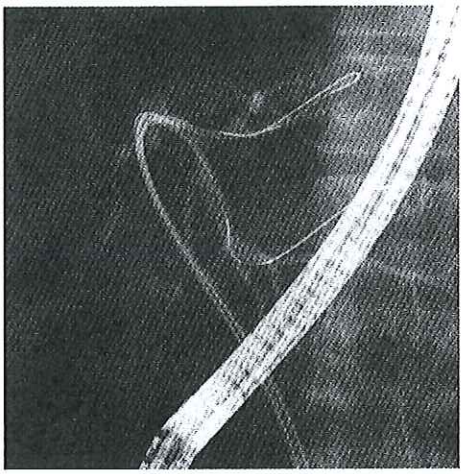

Figure 5. ERCP所見：末梢側胆管の壁不整や狭窄は認められない.

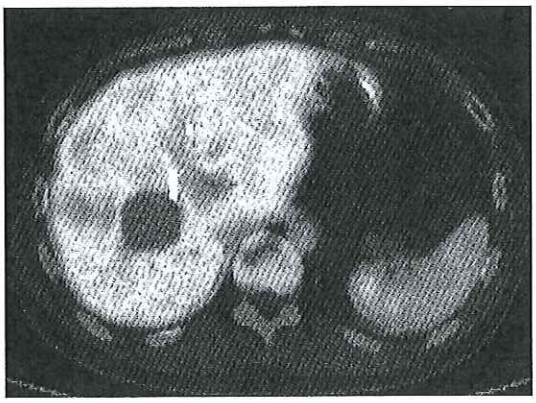

Figure 4. PET所見：腫瘤部位に一致してSUVmax 10.0の集積．遠隔転移を疑う取込みを認めない.

k値は0.168であった．拡大肝右葉切除・尾状葉切除・肝外胆管切除が可能と判断，本術式での予定残肝容量が731m*l*（全肝の62%）で予定残肝ICG消失率（ICG Krem）が0.104であり，予定術式に耐術可能と判断した[4]．当科で施行している切除可能胆管癌に対する術前化学放射線治療の臨床試験について説明，参加の同意を得た（進行胆管癌に対する術前化学放射線療法の有効性と安全性の検討−第II相試験：UMIN000001754）．プロトコールに則って，体外照射45Gy（1.8Gy×25回）とGemcitabine（GEM）600mg/m²（2投1休：4回）を併用した化学放射線療法[5]を施行した（Figure 6）．放射線照射領域はCTで読影した癌病巣に対し呼吸変動を加味した1cmのマージンを設け，さらに肝十二指腸間膜に照射するよう外科医が原案として提案し，残肝や主要臓器の安全性を確保できるよう放射線腫瘍医が設定した（Figure 7）.

手術所見：門脈右枝の狭窄により，右葉の萎縮が顕著であった．門脈右枝は根部で切離し断端を連続縫合した．肝切離施行し術中迅速診断で胆管断端陰性を確認した．切除肝重量は405gで，Bhp sT4b sN0 sM0 sDM0 sHM0 sEM0 sPV1 sA1 sR0 Stage IVAの手術診断であった．手術時間は778分，出血量は1600m*l*で自己血貯血の返血以外に輸血を必要としなかった.

病理組織学的検査所見：Bhp, circ, adenocarcinoma(tub2>tub3), pT2b(se), sci, INFγ, ly1, v2, ne3, pN1（1/18）, pDM0, pHM0, pEM0, pPV0, pA0, R0, cM0, Stage IIIB（胆道癌取扱い規約（第6版））.

術前化学放射線療法の効果判定は，大星・下里分類Grade IIB（残存する腫瘍細胞が1/3未満）であった（Figure 8a）．神経浸潤部の癌が消失し痕跡となった病理所見も得られた．術前に癌の浸潤による閉塞が疑われた門脈右枝は，中膜まで線

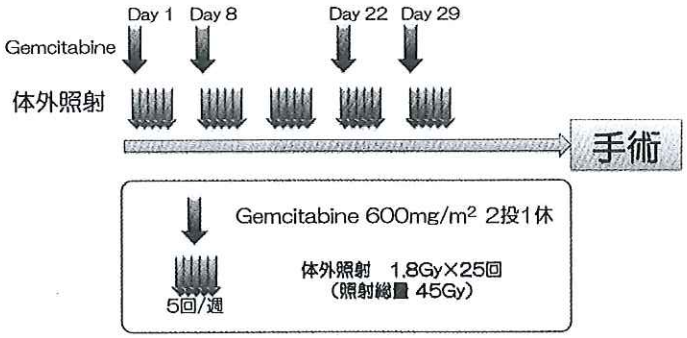

Figure 6. 術前化学放射線療法の regimen.

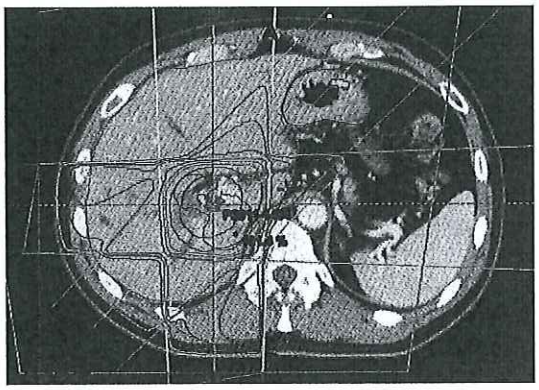

Figure 7. 放射線照射領域.

維化を認め，腫瘍細胞の存在は同定されなかった(Figure 8b)．初診時に腫瘍進展が高度であった肝浸潤部に化学放射線療法が良好な奏効を示したと考えている．

術後経過：非感染性の腹水貯留を認め加療を要した．胆管炎も呈したが抗菌薬加療にて改善し術後49日で退院となった（Clavien-Dindo Grade II)．

外来にてGEM 1000mg/m²の補助療法を開始したが，好中球減少により，減量しても継続困難となり，5回投与（術後投与量7820mg）で中止した．以降S-1 100mg/bodyの隔日投与を導入．皮疹・好中球減少が出現したがコントロール可能で，本人の希望を考慮して3年間の継続を予定とし，S-1 100mg/bodyの2週投薬・1週休薬を施行している（Figure 9)．

II 考 察

1991年から2012年までに，当科で診療した病理学的に胆管癌と診断された症例は625例であり，年齢は15歳から88歳（中央値69歳）で男性が401例，女性224例である．このうち50歳未満は本症例を含んで28症例(4%)のみであり，日本国内では50歳未満の胆管癌の発症・死亡は極めて少ないと報告されているのと同様である．

本症例は1989年4月から印刷オペレーターとして印刷事業所に勤務し，オフセット印刷の校正を担当していた．就業時間は1日16時間程度で300から800の校正工程作業を行った．職場には4台の印刷機が設置されており，エアコンはあるが換気装置のない環境下で2011年3月の震災まで勤務していた．半袖もしくは長袖の作業着に帽子を着用していたが，マスクはしていなかった．オフセット印刷のローラー洗浄などを担当し，インク色換え工程の洗浄剤として1,2ジクロロプロパン（1,2-dichloropropane；DCP）を90%以上含む製品が使用されていた．当該の事業所では本症例以外に，同じ職場で作業していた30歳代男性にも胆管癌発症を認めている．他院にて加療中であったが2014年2月に永眠されている[6]．

DCPは，化学物質審査規制法第二種監視化学物質および化学物質排出把握管理促進法第一種指定化学物質として指定されている．消化管および肺から速やかにほとんど完全に吸収され，肝臓に多く取り込まれ，65%は尿から排泄される．マウスの経口投与において特に雄で肝細胞癌の発生

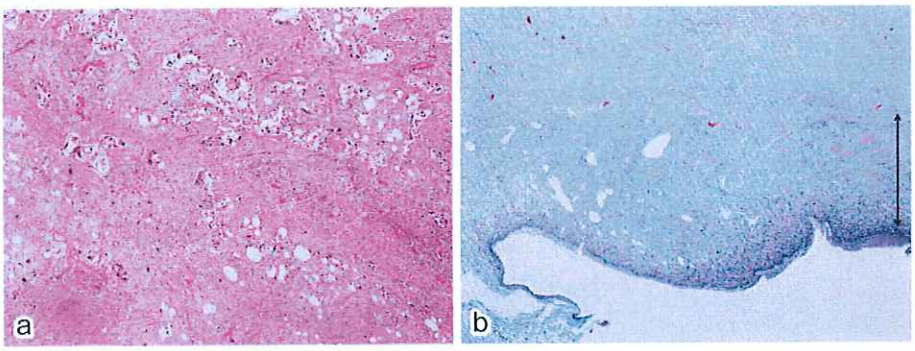

Figure 8. 病理所見　a：HE 染色（×40）．腫瘍の胞巣は崩れ，腫瘍細胞には高度の変性所見を認める．b：弾性線維染色（×40）．右門脈枝は中膜（↔）まで線維化を認めた．

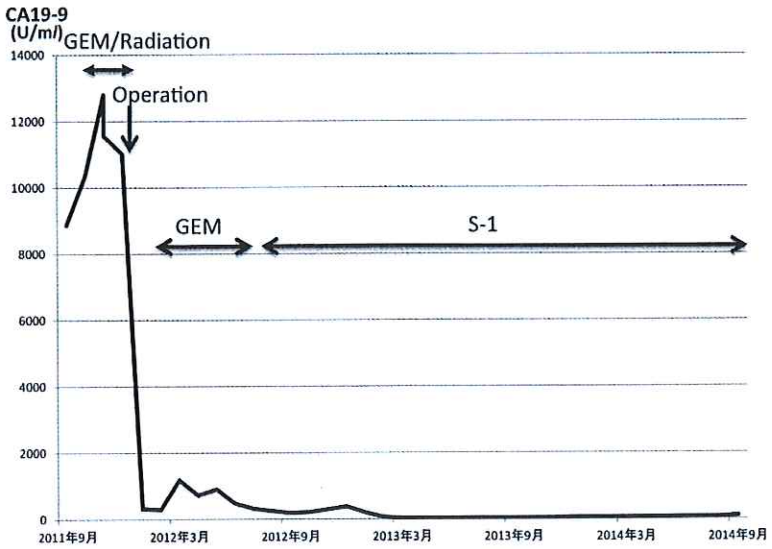

Figure 9. 治療経過と CA19-9 の推移．

率が上がると報告されていたが[7]，DCP の代謝と発癌の詳細な関係は明らかとなっておらず[8]，国際的にこれまでヒトの発癌性物質として証拠は不十分（Not classified as to its carcinogenicity to humans（group 3）；International Agency for Research on Cancer（IARC）の分類）とされてきた．しかし，本邦の環境曝露と胆管癌発症の報告から[9]，発癌性物質として（Carcinogenic to humans(group 1)）認識されるようになった[10]．DCP を含む化学物質に曝露した印刷業従事者から生じた胆管癌として，本症例を含め本邦では 2014 年 10 月の時点で 34 人が労災認定を受けている．

印刷事業所で発生した胆管癌の厚生労働省報告書では[2]，13 例全例で浸潤性病変の腺癌が認められ，高分化から低分化であった．さらに，背景肝に肝硬変や進行性肝実質病変を認めず，主腫瘤以外の特に肝門部で胆管内上皮内もしくは胆管周囲付属腺に腫瘤性病変を認め，胆管上皮には変性と消失をともなう硬化性病変が認められたと報告されている．本症例の病理学的特徴の検証においては，術前化学放射線治療を施行した当科経験症例と同様，高度の肝細胞の出血と変性が切除肝のほ

とんどの領域に見受けられており，上記の特徴すべての照合は困難であった．病変が肝門中心で，胆管障害像として小葉間胆管が消失した門脈域や細胆管増生の目立つ門脈域を認めており，環境被曝からの発癌として矛盾しない所見を得ている．本症例では，biliary intraepithelial neoplasia (BilIN) や intraductal papillary neoplasm of bile duct (IPN-B) の所見はなく，切除標本内には多中心性発癌を示唆する所見は認められなかった．

当科では，切除可能胆管癌に臨床研究として術前化学放射線療法を施行しており，良好なR0切除率を獲得し，胆管癌局所再発の制御が期待される[11)~13)]．本症例では術前CTで明らかな右門脈浸潤所見を呈していたにもかかわらず，切除標本で同門脈枝周囲には線維化を認めるのみで癌細胞の残存が確認されなかった．

当科での通常の胆管癌に対する化学放射線療法後の切除標本では，ほとんどすべての症例で細胞障害・原病巣パターンの破壊が認められている(大星・下里の病理組織学的効果判定基準[14)]・Grade II）が，本症例でも化学放射線療法により2/3以上の癌組織が変性している（Grade IIB）．

これまで，職業性胆管癌に放射線治療や陽子線治療を施行した症例も報告されている[6)15)]が，手術切除を前提とした化学放射線療法の施行により，化学放射線治療直後に切除標本から病理組織評価が可能であった本症例は，環境因子による職業性胆管癌に化学放射線療法が有効であることが病理学的に検証できる貴重な経験である．

結　語

若年発症の肝門部領域胆管癌に化学放射線療法後に根治手術を施行した．後に職業性胆管癌症例であることが判明し，結果として職業性胆管癌症例に対する化学放射線療法の奏効を病理学的に確認する貴重な治療経験を得た．

現在，切除後33カ月無再発生存中であるが，再発だけでなく異時性発癌の可能性を含め，今後も注意深く経過を確認していきたい．

本論文内容に関連する著者の利益相反
：なし

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受理，2015年2月8日

Occupational cholangiocarcinoma in a printer that responded to neoadjuvant chemoradiotherapy

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A 42-year-old man working at a printing company was referred to our hospital for examination and treatment of icterus. We diagnosed resectable hilar cholangiocarcinoma and provided neoadjuvant chemoradiotherapy, extended right hepatectomy, and extrahepatic bile duct resection. A detailed history revealed that he had used 1,2-dichloropropane as part of his work as an offset colour proof-printer, and he has subsequently been recognized as having occupational cholangiocarcinoma. He has survived without recurrence for more than 2 and half years since the liver resection. In the present report, we describe our valuable experience of neoadjuvant chemoradiotherapy for occupational cholangiocarcinoma.

胆　道　29巻5号　972～977（2015）

＜症例報告＞

印刷業勤務を背景に発症した肝内胆管癌の1例

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要旨：47歳，男性．12年間印刷業に従事していた．心窩部痛の増悪のため前医を受診した際に肝内腫瘤を認め，当科紹介となった．精査にて脈管浸潤や複数のリンパ節転移を認める手術不適応な肝内胆管癌と診断された．CDDP＋5-FU療法を施行したが発熱，嘔気の出現にて継続困難となった為，症状緩和目的に肝右三区域切除術が施行されたが癌の進行により死亡した．切除標本ではS6とS8/4の2カ所に胆管癌を認め，その2つの腫瘍間の胆管にBiliary intraepithelial neoplasiaが認められた．今回我々は，20年前に経験した肝内胆管癌症例に印刷業勤務の背景が存在していたため文献的考察を加えて報告する．

索引用語：　肝内胆管癌　　印刷業

緒　　言

職業や生活環境などで高用量曝露により発症頻度が増大する発癌因子が報告されている[1)2)]．最近，校正印刷業務従事者における胆管癌の発症例が報告され[3)4)]，ジクロロメタン(DCM)や1,2-ジクロロプロパン(DCP)の長期間，高濃度曝露が原因と推定されている[5)]．現在では，厚生労働省によって，これらの胆管癌は職業性胆管癌と認定されている[6)]．著者らは，約20年前に経験した若年者の肝内胆管癌症例の背景に印刷業業務歴が存在していたことが明らかになったので，その詳細の報告とともに文献的考察を行った．

症　　例

症例は47歳，男性．生来健康であった．心窩部痛が出現したため市販の内服薬を服用したが改善せず，徐々に痛みが背部へ進展したため前医を受診した．腹部超音波検査および腹部CTにおいて肝S6/8に5cm大の腫瘤が認められ，肝内胆管癌が疑われたため精査目的に当科紹介となった．家族歴では，母が脳出血のため死亡．悪性腫瘍罹患者はいなかった．生活歴としては喫煙20本/日を28年間行い，飲酒は機会飲酒程度であった．職業歴として前医受診の1カ月前までの計12年間校正印刷業に従事した．来院時は休職中であった．後日の調査により，その印刷事業場ではDCMやDCPが使用され，患者はそれらの高濃度曝露を受けていたことが推定されている[7)]．

検査所見

• 入院時血液検査：ALP：1164U/L，γGTP：346U/Lと胆道系酵素の上昇が認められた．CRP：5.1mg/dLと炎症反応の上昇がみられた．ICG15分値は5.5％と正常値であった．CEAは11.2ng/mlと上昇していたが，CA19-9は3.3U/mLと正常値であった（表1）．HBs抗原およびHCV抗体は陰性であった．

• 腹部超音波所見：肝S8/4にhaloを伴う3cm大の辺縁分葉状の腫瘤が（図1A），S6に境界不明瞭な低エコー腫瘤が認められた（図1B）．後下亜区域の門脈枝(P6)は描出されなかった．また，肝門部に多数の腫大したリンパ節が認められた．

• 腹部CT検査所見：腹水なし．S8/4の肝表面に2-3cm大の低吸収な腫瘤が認められた（図2A）．肝S6にも径5-6cmの低吸収性腫瘤がみられ，腫瘍辺縁のみが造影された（図2B）．P6への腫瘍の浸潤所見と拡張した腫瘍末梢側胆管が認められた．＃8，9，12，13領域のリンパ節腫大が認められた．

• 血管造影検査所見：腹腔動脈造影では肝動脈後区

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[4)] 九州大学大学院形態機能病理学
受付日：平成26年12月11日
採用日：平成27年5月1日

表1　入院時血液検査所見

WBC	9520 /μL	T-Bil	0.6 mg/dL
RBC	425×10⁴ /μL	AST	26 U/L
Hb	12.4 g/dL	ALT	36 U/L
Ht	39.1 %	LDH	384 U/L
PLT	26.3×10⁴ /μL	ALP	1164 U/L
TP	7.7 g/dL	γGTP	346 U/L
Alb	4.4 g/dL	CEA	11.2 ng/mL
BUN	18 mg/dL	CA19-9	3.3 U/mL
Creat	0.6 mg/dL	AFP	2.7 ng/mL
CRP	5.1 mg/dL	PIVKA Ⅱ	<0.016 AU/mL

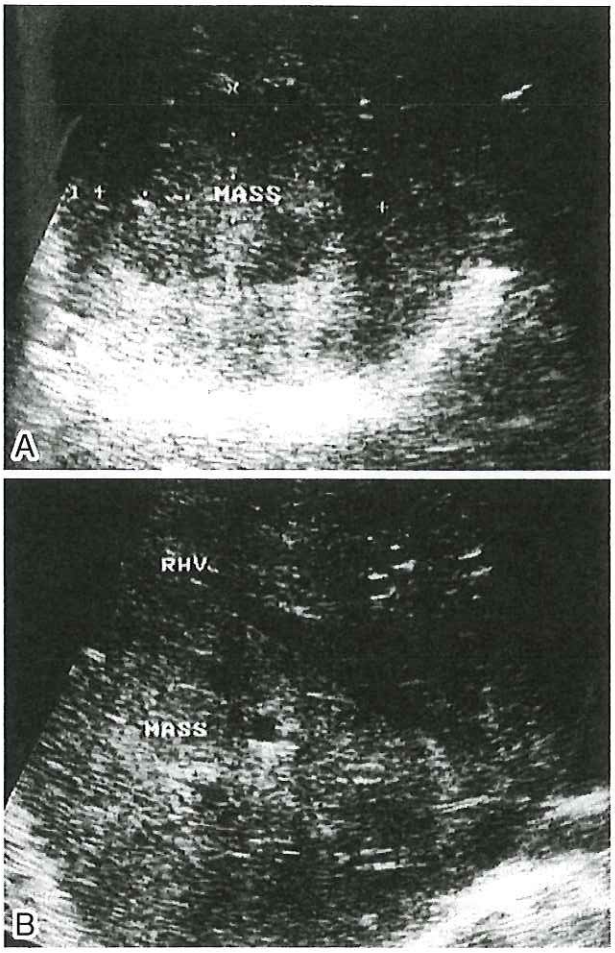

図1　腹部超音波画像所見.
A：S4 腫瘤　B：S6 腫瘤

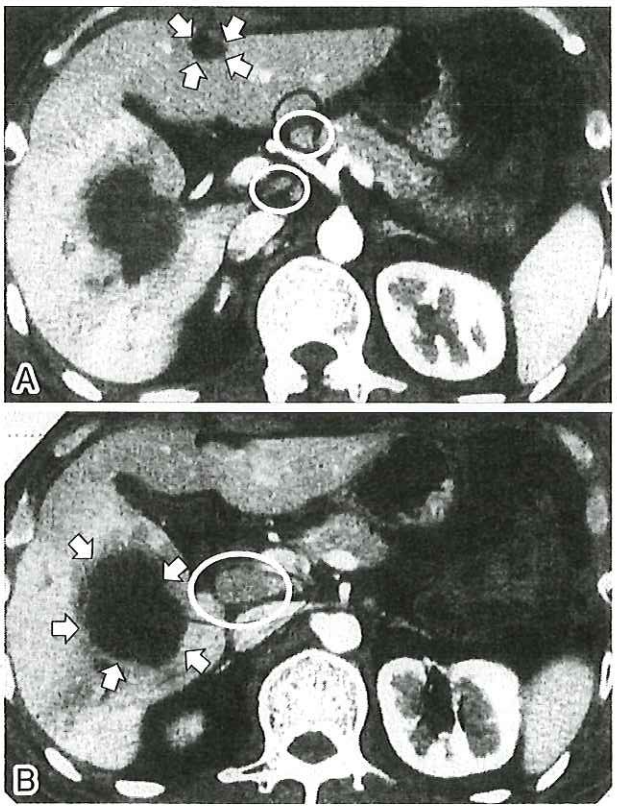

図2　腹部 CT 画像所見
矢印：腫瘤　白丸：腫大したリンパ節
A：S4 頭側肝表面の 2-3cm 大の低吸収腫瘤
B：S6 の径 5-6cm 大の低吸収性腫瘤

域枝に途絶像が，上腸間膜動脈性門脈造影では門脈後区域枝の狭窄像がみられた.

経過①

上記の画像所見から肝内胆管癌が強く疑われた．複数の腫瘍の存在，腫瘍径 5cm 以上，脈管浸潤陽性，大動脈周囲におよぶ複数のリンパ節転移が認められたため，cStage IVB の診断となった．そこで，手術不適応と判断し，化学療法目的に他院へ転院した．その後 CDDP＋5-FU 療法が施行されたが，Grade 2-3 の嘔気と腫瘍熱出現のため化学療法の継続が困難であった．そこで，腫瘍による症状の緩和および化学療法の継続を

目標に，化学療法を施行した施設において肝十二指腸間膜内および膵頭部リンパ節（#12p，#12a，#12c，#13a）廓清を伴う肝右三区域切除が施行された．肝外胆管および尾状葉切除は行わなかった．

病理組織所見

摘出標本内には2つの腫瘤性病変がみられた．S6の腫瘍では内部の多くが凝固壊死で占められ，腫瘍辺縁部に大型核と不整形の細胞塊からなる低分化型腺癌を認めた（図3A）．S8/4の腫瘍は粘液産生性が目立つ高分化（図3B）から中分化型（図3C）の管状腺癌が増殖し，リンパ管侵襲や神経周囲浸潤を伴っていた．胆道癌取り扱い規約第5版に沿った診断ではtublar or papillary adenocarcinoma，tubular adenocarcinoma，int，INFc，ly1，v1，ne2，pN1であり，T4bN1M0 Stage IVAであった．

両腫瘍間に明らかな肉眼的連続性は認めなかったが，摘出組織内の前区域枝および後区域枝の胆管上皮には，様々な異型度の呈するBilIN病変を広範に認めており（図3D-F），BilINを介してS6とS8/4の腫瘤は組織学的に連続した腫瘍であると考えられた．BilIN以外にも大型肝内胆管には壁内腺の増生を認めた（図3G）．肝内の隔壁胆管以下の末梢小型胆管には異型上皮の拡がりは認めず，背景肝実質はほぼ正常構造を保っていた（図4）．

経過②

術後嘔気と発熱は改善し化学療法を継続することができたが，術後20カ月で肝内再発を呈し，癌が進行するとともに全身状態も徐々に増悪し，肝内胆管癌診断から約2年後に死亡した．

考　察

肝内胆管癌は肝腫瘍の5–15％を占め，予後不良な悪性腫瘍である[8)9)]．胆管癌の危険因子として，従来から原発性硬化性胆管炎，膵・胆管合流異常，肝内結石症，肝吸虫に加えてニトロソアミンなどの化学物質が報告されてきた[10)11)]．近年，オフセット印刷業従業員から高率に胆管癌を発生した事例が報告され[3)11)12)]，そのなかで印刷業やDCMやDCPなどの有機溶剤への曝露が注目されるようになった．今回著者らが経験した症例も，12年間の印刷業従事の際に，DCMおよびDCPの曝露を受けていたことが推定されており[7)]，現在この症例は職業性胆管癌として認定されている[13)]．しかし，本症例では治療にあたっていた当時は印刷業や有機溶剤への曝露歴の重要性が理解されておらず，詳細な職場環境や曝露状況は聴取できていなかった．後日，職業性胆管癌事例の報告を契機に本症例も印刷業やDCMやDCPの曝露歴が明らかとなった．このことから，今後はこれらの職業歴の詳細な聴取が必要であると考えている．

印刷業に関連した胆管癌の最初の報告はデンマークにおける報告[14)]である．本邦では，大阪の印刷事業場での17名の胆管癌症例の検討で，標準化罹患比が1226倍，標準化死亡比が633倍と極めて高値であったことが報告されている[4)]．また，本邦における胆道癌の好発年齢は50歳から80歳であり60歳代にピークを迎える[15)]が，この報告における胆管癌発症年齢は25歳から45歳で，その中央値は36歳であった．その後の全国を対象とした報告でも発症年齢は31歳から57歳であり[12)]，本症例も47歳と若年発症であった．

職業性胆管癌症例では特徴的な病理組織学的所見がみられることが報告されている．すなわち，主腫瘤以外の肝組織において，慢性胆管傷害や胆管の増殖性変化，BilINやIntraductal papillary neoplasm of bile duct（IPNB）などの前癌病変・早期癌が広範囲胆管にみられること，背景肝に肝硬変や進行性の肝実質病変が見られないこと等が特徴として挙げられる[2)4)16)]．膵癌とpancreatic intraepithelial neoplasia（PanIN）には共通してkirsten rat sarcoma viral oncogene homolog（KRAS）の遺伝子変異が見られるという報告が散見される[17)18)]が，近年BilINにもKRAS遺伝子変異が見られるという報告があり，肝内胆管癌の一部が膵癌と同様に早期段階でKRAS遺伝子変異を生じる経路を介して発癌する可能性が示唆された[19)]．本症例においても，これまでに報告された症例[4)13)16)20)]と同様に，非癌部の肝細胞に有意な変化は認められなかった一方で，広範囲の大型胆管にBilINがみられた．本症例では2つの腫瘍間に連続した大型胆管内にBilINが認められたが，腫瘍より末梢側の胆管にはBilINが見られなかった．また，腫瘍性変化をきたしていない胆管も一部残存していた．このようなBilINの分布が生じていた点は本症例の特徴であると考えられる．

職業性胆管癌においてはDCMやDCPの長期間高濃度曝露が原因であると推定されている[2)5)7)16)]．その発癌メカニズムはDCPと分子構造が類似している1，2-ジクロロエタンや1，2-ジクロロブロモエタンの研究から，DCPの代謝産物によってDNA損傷が引き起こされることが，胆管癌発症の契機となりうると考えられ

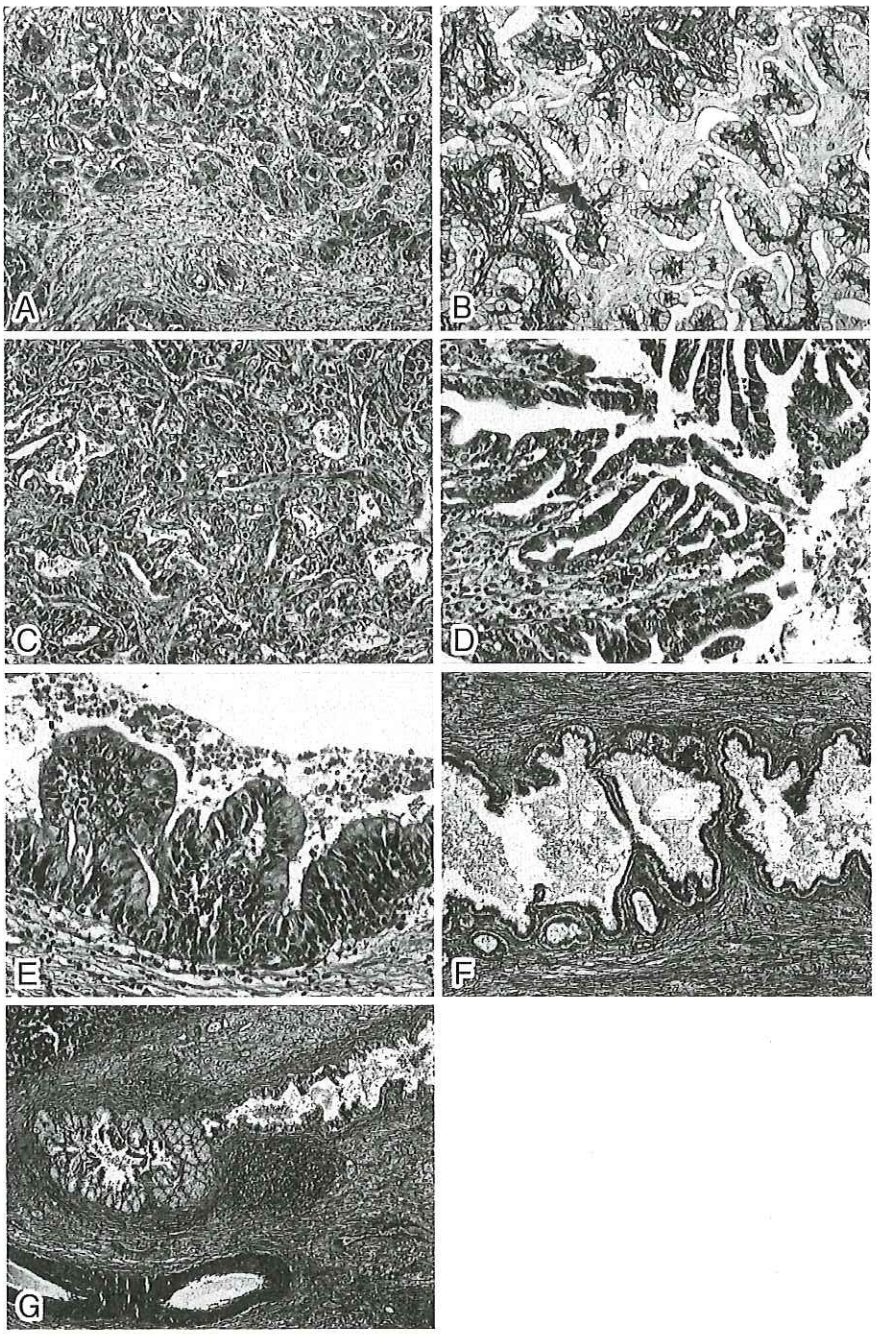

図3　病理組織所見

A：S6の腫瘍は低分化腺癌を示す（×100）.

B：S8/4の腫瘍は粘液産生に富む高分化型管状腺癌（×100）.

C：S8/4の腫瘍は構造が乱れた中分化型管状腺癌部分もみられる（×100）.

D：核異型と極性の乱れが目立ち，乳頭状の増殖を示すBilIN-3であり，腫瘤近傍の大型胆管内の随所にみられる（×200）.

E：炎症細胞浸潤を伴うものの核腫大と極性の乱れを伴うBilIN-2に相当する異型上皮（×200）.

F：クロマチンに富む棍棒状の核を有する異型腺上皮が密に配列するBilIN-1病変（×100）.

G：胆管内では壁内腺の増生と異型腺上皮が密に配列するBilIN-1病変がみられる（×40）.

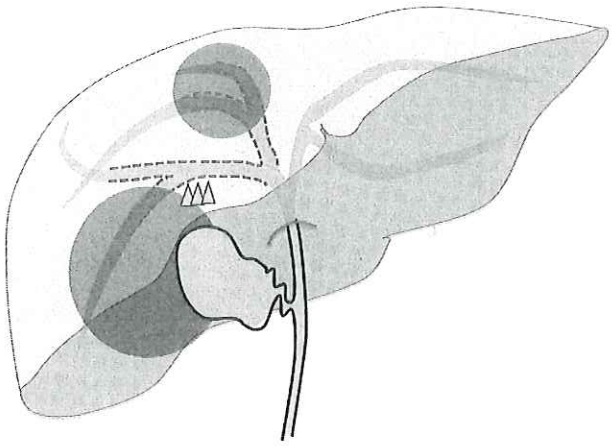

図4　赤点線部にBilINが認められた．青の円は腫瘍を示す．
比較的末梢側の微小な胆管にBilINは見られなかった．青三角部に附属腺の増生が認められた．

る[2)5)]．実際，マウスを用いた動物実験でも，DCMやDCPによるDNA損傷が著明であったという報告[21)]や，DCM吸入曝露試験によって肺胞・気管支腺癌，肝細胞癌，肝細胞腺腫が用量依存的に増加したとされる報告[22)]がみられる．しかし実際には，動物実験や臨床データからは，危険因子としての毒性濃度を示した基準は得られておらず，今後も更なる検討が必要と考えられる．今回，著者らが経験した職業性胆管癌症例の詳細について報告した．今後の臨床現場においては，特に若年やBilINなどの前癌病変を伴う肝内胆管癌症例では有機溶剤の曝露などの既往歴，職業歴などについても詳細に聴取することにより，その関連性を明らかにしていくことが必要である．

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A case of intrahepatic cholangiocarcinoma in a printing industry worker

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A 47-year-old man who had worked in the printing industry for twelve years presented to the hospital with severe epigastralgia. After clinical evaluations, a tumor was found in his liver. He was then referred to our institution, where he was diagnosed with intrahepatic cholangiocarcinoma and was underwent chemotherapy. Nevertheless, fever and nausea were apperared, he was performed hepatic trisegmentectomy. Despite the treatment, the patient's intrahepatic cholangiocarcinoma progressed, and he died from tumor-related complications. The resected specimen showed two intrahepatic cholangiocarcinomas in S6 and S8/4, and biliary intraepithelial neoplasia extending to the intrahepatic bile ducts between these two tumors. Herein, we summarize the findings of this case experienced 20 years ago and a review of the literature.

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