第 29 回高度医療評価会議	資料1－3
平成 24 年2月3日	

高度医療審査の照会事項（珠玖技術委員）に対する回答（１）

高度医療技術名：切除不能・再発胆道癌を対象としたゲムシタビン+CDDP+WT1 ペプチドワクチン併用化学免疫療法とゲムシタビン+CDDP 治療の第 I/II 相試験

2012/01/12

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１．本臨床試験では、各症例での胆道癌に於ける WT1 抗原の発現の検討は行われないと理解します。

胆道癌に於ける WT1 抗原の発現増強症例の頻度について、これまでの自験例、他験例のデータにつき示して下さい。

その際、既発表のものは、発表文献及び発現検討の手法についても記載して下さい。

胆道がんにおける WT1 抗原の発現状況に関しては、Modern Pathology(2006)19,804-814 に記載されています。この中では、Polyclonal 抗体（C-19）並びに単クローン抗体（6F-H2）を用いて、胆道がんにおける WT1 発現を免疫組織化学的に解析しており、それぞれ 80%と 68%の症例で WT1 の発現が確認されています。（参考文献１）

尚、WT1 の免疫染色方法・評価方法に関しては、標準化のための国際共同研究が行われています。染色法・診断法の標準化が終われば、多施設間での客観的評価が可能となると思われます。

２．胆道癌における HLA クラス１分子の発現消失及び減弱の頻度についての検討及び報告につき、データと共に示して下さい。

胆道がんにおける HLA-ClassI の発現状態に関しては、Journal of Experimental & Clinical Cancer Research 2011,30:2 に記載されております。この論文では、肝内胆管がんの 42.7%において HLA-ClassI の発現低下が認められたと報告されています。（参考文献２）

尚、この文献内の実験では、抗 HLA-ClassI 抗体として EMR8-5 が使用されています。この抗体は、札幌医科大学にて開発されたものです。私どもは、同抗体を用いた HLA-ClassI 免疫染色の標準化作業を進めており、ご指摘いただきました質問に対する私ども独自の解析は、この標準化作業の中で実施する予定にしていることを申し添えます。

第 29 回高度医療評価会議	資料1－3
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３．本臨床試験で用いられる WT1 ペプチド特異的なキラーT 細胞の胆道癌細胞に対する反応性（細胞障害性を含む）の検討結果を示して下さい。

胆道がん細胞に対する WT1 ペプチド特異的なキラーT 細胞の反応性（細胞障害活性）のデータは持ち合わせておりません。

同ペプチドを認識する、WT1 特異的 T 細胞からクローニングした TCR 遺伝子を導入したリンパ球が、WT1 陽性膵がん細胞株に対する抗腫瘍効果をしめす結果は報告されています。（参考文献３）

また、今回使用する WT1 ペプチドにて培養した T リンパ球が、HLA 一致 WT1 陽性造血器腫瘍株並びに肺がん細胞株に対して殺細胞効果を示すことも報告されております。（参考文献４、５、６）

ペプチド特異的リンパ球が、悪性腫瘍細胞を傷害するメカニズムは、基本的には HLA-ClassI と抗原の発現状況によると考えられていることから、HLA-ClassI を発現し WT1 を発現する胆道癌においても、同様の殺細胞効果が得られると考えています。

４．WT1 抗原は、腫瘍化した腫瘍細胞に加えて、中皮細胞、腎蛸足細胞等の各種正常細胞に強く発現されています。

ワクチン実施に伴うこれらの正常細胞に対する免疫的反応性を考慮した安全性への配慮につき記載して下さい。

WT1 抗原発現に関しては、ご指摘の通り中皮細胞や腎蛸足細胞等での発現が報告されています。

マウスを用いた動物実験では、WT1 ペプチドワクチン接種によって、WT1 発現正常組織において自己免疫疾患を疑わせる病理像は見られておりません。（参考文献７）また、WT1 ペプチドワクチンにより、正常造血が影響を受けていないことも報告されています。（参考文献８）WT1 ペプチドワクチン療法は、企業治験を含め多くの臨床研究がおこなわれていますが、現時点で、骨髄異形成症候群症例における汎血球減少症を除き、WT1 発現正常組織に関連した重篤な有害事象は報告されておりません。

しかし、因果関係は不明であるものの、WT1 ワクチン接種患者において Grade1 の尿潜血・尿タンパクが見られた例もあることから、臨床試験実施に際しては、腎機能等の検査データを注意深く観察し、異常な変化を見逃さないよう細心の注意を払うことを申し添えます。

文献 1

様々な癌細胞の WT1 蛋白質の免疫組織化学的検出について

WT1 は、当初、ウィルムス腫瘍の発育に携わる腫瘍抑制因子として同定されたが、最近になって、様々な造血器腫瘍および固形腫瘍において、腫瘍遺伝子としての機能を持つことが報告されている。WT1 は、がん免疫療法における分子標的として認識されているため、腫瘍細胞における WT1 の免疫組織化学的検出は、実地医療においても重要になってきている。本研究では、消化管や胆膵系、尿路、男性と女性の生殖器、乳房、肺、脳、皮膚、軟部組織および骨の腫瘍を含む 494 例のヒト由来がんにおいて、WT1 蛋白質に対するポリクローナル（C- 19）とモノクローナル（6F- H2）抗体を使用した WT1 免疫染色を試みた。C - 19 と 6F- H による WT1 陽性率は、各腫瘍で 35~100%と 5～8%であった。WT1 陽性腫瘍には、胃、前立腺、および胆管や尿システム由来がん、および悪性黒色腫が含まれていた。卵巣腫瘍および線維形成性小円形細胞腫瘍の多くが核が染まっているのに対し、陽性例の大半は、細胞質にびまん性または顆粒状染色を示した。他の腫瘍と比較して神経膠芽腫、いくつかの軟部組織肉腫、骨肉腫、および皮膚の悪性黒色腫の一部が非常に強い細胞質染色を示した。２例の肺腺癌細胞にて行ったウェスタンブロット解析の結果は、免疫組織染色によって、WT1 が主として細胞質内で染まることを支持している。通常用いる病理切片を用いた免疫組織化学的検出は、癌細胞における WT1 の発現に有意義な情報を提供する。

Modern Pathology (2006) 19, 804–814

www.modernpathology.org

Immunohistochemical detection of WT1 protein in a variety of cancer cells

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WT1 was first identified as a tumor suppressor involved in the development of Wilms' tumor. Recently, oncogenic properties of WT1 have been demonstrated in various hematological malignancies and solid tumors. Because WT1 has been identified as a molecular target for cancer immunotherapy, immunohistochemical detection of WT1 in tumor cells has become an essential part of routine practice. In the present study, the expression of WT1 was examined in 494 cases of human cancers, including tumors of the gastrointestinal and pancreatobiliary system, urinary tract, male and female genital organs, breast, lung, brain, skin, soft tissues and bone by immunohistochemistry using polyclonal (C-19) and monoclonal (6F-H2) antibodies against WT1 protein. Staining for C-19 and 6F-H2 was found in 35–100 and 5–88% of the cases of each kind of tumor, respectively. WT1-positive tumors included tumor of the stomach, prostate, and biliary and urinary systems, and malignant melanomas. A majority of the positive cases showed diffuse or granular staining in the cytoplasm, whereas ovarian tumors and desmoplastic small round cell tumors frequently showed nuclear staining. Glioblastomas, some of soft tissue sarcomas, osteosarcomas, and malignant melanomas of the skin showed extremely strong cytoplasmic staining as compared with other tumors. Western blot analysis showed that WT1 protein was predominantly expressed in the cytoplasm of the tumor cells in two cases of lung adenocarcinoma, supporting the intracytoplasmic staining for WT1 using immunohistochemistry. Immunohistochemical detection with routinely processed histologic sections could provide meaningful information on the expression of WT1 in cancer cells.
Modern Pathology (2006) **19,** 804–814. doi:10.1038/modpathol.3800588; published online 17 March 2006

Keywords: WT1; immunohistochemistry; overexpression; oncogenesis

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Received 14 November 2005; revised 21 February 2006; accepted 23 February 2006; published online 17 March 2006

The *WT1* gene, identified as a tumor suppressor gene located at 11p13, is involved in the development of Wilms' tumor.[1,2] Germline mutations of *WT1* have been described in Denys-Drash[3] and WAGR[4] syndromes, which predispose individuals to the development of Wilms' tumor. Somatic mutations[5] and loss of heterozygosity[6] of *WT1* can

be detected in 10% of sporadic Wilms' tumors. The *WT1* gene encodes a transcription factor with four DNA-binding zinc fingers at the C terminus.[1,2,7] *In vitro* studies showed that WT1 suppresses or activates a number of genes, including those for *PDGF-A chain*, *EGF receptor*, *CSF-1*, *IGF-II*, *IGF-I receptor*, *RAR-α*, *c-myc*, *bcl-2*, and *WT1* itself.[7,8] In embryonic life, WT1 plays a critical role in the development of the genitourinary tract, spleen, and mesothelial structures.[3,4,9] In normal adult tissue, it is expressed in mesothelium, glomerular podocytes and mesangial cells of the kidney, CD34-positive hematopoietic stem cells, Sertoli cells of the testis, stromal cells, surface epithelium and granulosa cells of the ovary, and myometrium and endometrial stromal cells of the uterus.[7]

The *WT1* gene was originally recognized as a tumor suppressor gene, but evidence of the oncogenic properties of the gene has been accumulating. WT1 mRNA is overexpressed in several kinds of hematological malignancies, and quantitative detection of its expression could be useful for monitoring minimal residual disease in case of leukemia.[10–12] Furthermore, recent studies showed the overexpression of WT1 mRNA in various kinds of solid tumors,[13] the growth inhibition of WT1-expressing cells by WT1 antisense oligomers,[13,14] and a correlation between a high level of WT1 and a poor prognosis in patients with certain kinds of tumors.[12,15] These findings suggest that WT1 plays an oncogenic rather than tumor-suppressive role in human cancers. In such cases, sequencing revealed an absence of mutations in the *WT1* gene in tumors, therefore wild-type *WT1* could be oncogenic.

Immunohistochemically, WT1 is detected in the nucleus of tumor cells of Wilms' tumor and mesothelioma; therefore, WT1 has traditionally been used as a diagnostic marker for these tumors.[16–20] Recent reports showed that other types of cancers, such as ovarian serous cancers and rhabdomyosarcomas[17,19–21] also express WT1. However, immunohistochemical data on WT1 expression in other types of cancers are either lacking or conflicting.[17,18,21–24] As for staining patterns, cytoplasmic staining has been regarded as nonspecific and not been counted as positive in most previous reports. Therefore, the cytoplasmic staining of WT1 had not been evaluated in most types of tumors until now. However, recent reports have shown evidence that WT1 is involved not only in transcriptional regulation in the nucleus but also in RNA metabolism and translational regulation in the cytoplasm. The binding of WT1 to splicing factors[25] and murine IGF-II mRNA[26] *in vitro* was demonstrated. Furthermore, nucleocytoplasmic shuttling of WT1 and the association of WT1 with actively translating polysomes were reported.[27] Therefore, aberrant cytoplasmic localization of WT1 might alter the properties of tumor cells through the expressional regulation of variable genes.

An appropriate evaluation of WT1 expression in tumor cells is important at present, because WT1 is now regarded as a molecular target of immunotherapy for various malignant tumors. The clinical trial of a WT1 peptide-based cancer immunotherapy is on-going: WT1 vaccination was safe in all cases and clearly effective against several kinds of malignancies.[28,29] In routine practice, immunohistochemical analysis for WT1 expression using routinely processed histologic sections is essential to judge the eligibility of a patient for this immunotherapy. The present study was conducted to examine the availability of the immunohistochemical detection of WT1 in various human cancer cells with the use of a polyclonal and/or monoclonal antibody. Because intracytoplasmic staining was the predominant pattern detected with immunohistochemistry, the subcellular distribution of WT1 protein was examined by Western blotting.

Materials and methods

Specimens

Formalin-fixed and paraffin-embedded tissues from 494 tumors were retrieved with informed consent from archive sources at Osaka University Hospital and affiliated hospitals. The histologic diagnosis of each tumor was confirmed on the hematoxylin and eosin-stained sections: there were 13 esophageal cancers, 52 gastric cancers, 53 colorectal cancers, 26 pancreatic cancers, 23 biliary cancers, 65 lung cancers, 25 prostate cancers, 15 renal cancers, 39 urothelial cancers, 32 breast cancers, 22 uterine

Table 1 Age and sex for each type of malignancy

Tumor types	*Case number*	*Age (median)*	*Sex (M:F)*
Gastrointestinal and pancreatobiliary tumors			
Esophageal cancer	13	54–85 (63)	5.5
Gastric cancer	52	34–90 (69)	1.4
Colorectal cancer	53	43–81 (58)	2.1
Pancreatic cancer	26	50–79 (65)	0.9
Biliary cancer	23	41–86 (70)	1.5
Lung cancer	65	52–79 (66)	1.7
Urinary and male genital tumors			
Prostate cancer	25	53–75 (68)	—
Renal cancer	15	45–77 (61)	2.3
Urothelial cancer	39	53–90 (73.5)	6.2
Breast and female genital tumors			
Breast cancer	32	31–65 (50.5)	—
Cervical cancer	22	21–74 (50)	—
Endometrial cancer	24	32–78 (55)	—
Ovarian cancer	33	42–84 (53.5)	—
Brain tumor	27	2–82 (43.5)	0.9
Soft tissue sarcoma	32	2–64 (17)	2.0
Osteosarcoma	6	5–31 (16)	2.0
Malignant melanoma (skin)	7	41–73 (68)	1.3
Total	494		

cervical cancers, 24 uterine endometrial cancers, 33 ovarian cancers, 27 brain tumors, 6 osteosarcomas, 32 soft tissue sarcomas, and 7 malignant melanomas of the skin. The demographic features of these cases are listed in Table 1.

Immunohistochemistry

Anti-WT1 antibodies used were a rabbit polyclonal antibody (clone C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against the C terminus (amino acids 431–450) of WT1 protein and a mouse monoclonal antibody (clone 6F-H2; Dako cytomation, Carpinteria, CA, USA) against the N terminus amino acids (1–181). After dewaxing and rehydration, 3-μm-thick sections were subjected to heat-induced epitope retrieval by microwaving them for 15 min in 1 mM citrate buffer (pH 6.0), followed by incubation with anti-WT1 antibody diluted 1:100 at 4°C overnight. For 6F-H2, a positive signal was detected using the ENVISION+ kit (Dako cytomation). For C-19, after incubation with biotinylated anti-rabbit or anti-mouse secondary antibody, sections were treated with a 3% H_2O_2 solution to reduce endogenous peroxidase activity. Visualization was performed by a standard avidin–biotin complex method using a Vectastain ABC elite kit (Vector Labs., Burlingame, CA, USA). For malignant melanoma, a positive signal was detected by the alkaline phosphatase system using a ENVISION labeled polymer-AP kit (Dako cytomation). As positive controls, sections from Wilms' tumor or mesothelioma were stained in parallel.

Subcellular Fractionation

Cancerous tissues (approx. 0.5 ml) were obtained from two patients with lung adenocarcinoma with informed consent. After a wash with PBS, the tissues were cut into small pieces and homogenized in 9 ml of 0.25 M sucrose using a Potter-Elvehjem homogenizer. The suspension was filtered with gauze and centrifuged in microcentrifuge tubes at 3300 r.p.m. for 10 min at 4°C. The pellet was solved in 240 μl of SDS sample buffer and stored as a nuclear fraction. The supernatant was centrifuged at 15 000 r.p.m. for 10 min at 4°C. The proteins in the supernatant were precipitated with acetone, solved in 240 μl of SDS sample buffer, and stored as a cytoplasmic fraction.

Western Blot Analysis

Nuclear and cytoplasmic proteins from the cancerous tissues were loaded onto each well of the gel, separated by SDS-PAGE, and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). After the blocking of nonspecific binding, the membrane was immunoblotted with the anti-WT1 mouse monoclonal antibody 6F-H2, anti-lamin B goat polyclonal antibody, or anti-α-tubulin mouse monoclonal antibody (Santa Cruz Biotechnology), followed by incubation with the appropriate secondary antibody conjugated with alkaline phosphatase. Antibody binding was detected using a BCIP/NBT kit (Nacalai Tesque, Kyoto, Japan).

Statistical Analysis

Statistical analyses of the differences in positive rates for WT1 immunostaining among subtypes and grades of each tumor were performed with Fisher's exact probability test.

Results

In the positive controls, Wilms' tumor and mesothelioma, immunohistochemistry with either C-19 or 6F-H2 showed positive staining in the nucleus and/or cytoplasm of the tumor cells (Figure 1). The

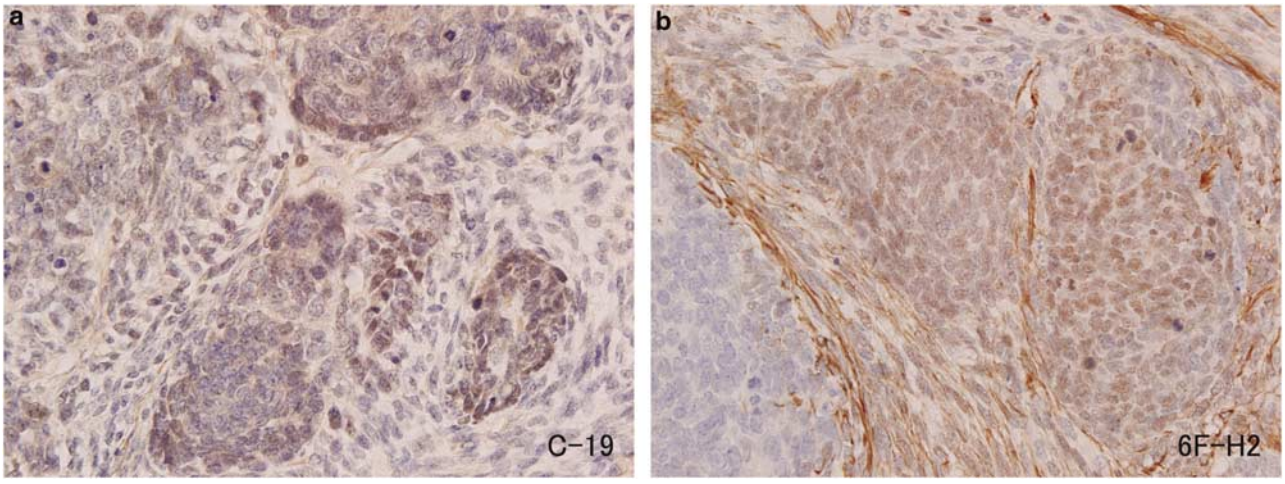

Figure 1 Immunohistochemical detection of WT1 in Wilms' tumor with C-19 (**a**) and 6F-H2 (**b**). Epithelial and mesenchymal components showed nuclear and/or cytoplasmic immunoreactivity for either antibody. Original magnification $\times$ 400.

Table 2 Results of immunohistochemistry for WT1 according to tumor type

Tumor types	*Polyclonal (C-19)*		*Monoclonal (6F-H2)*		
	No. of positive cases	*Ratio (%)*	*No. of positive cases*	*Ratio (%)*	
Esophageal cancer	**9/13**	**69**	**5/11**	**45**	
Squamous cell carcinoma					
Moderately differentiated	7/11	64	4/9	44	
Poorly differentiated	2/2	100	1/2	50	
Gastric cancer	**17/32 [1]**[a]	**53**	**21/50 [1]**	**42**	
Adenocarcinoma					
Well to moderately differentiated	7/14	50	15/23	65	]*
Poorly differentiated	10/18 [1]	56	5/24 [1]	21	]*
Mucinous adenocarcinoma			1/3	33	
Colorectal cancer	**33/48**	**69**	**31/45**	**69**	
Adenocarcinoma					
Well to moderately differentiated	27/41	66	28/37	76	]**
Poorly differentiated	6/7	86	3/8	38	]**
Pancreatic cancer	**7/20**	**35**	**11/17**	**65**	
Ductal adenocarcinoma					
Well to moderately differentiated	7/16	44	10/14	71	
Poorly differentiated	0/1	0	0/1	0	
Mucinous noncystic carcinoma	0/2	0	1/1	100	
Islet cell tumor	0/1	0	0/1	0	
Biliary cancer	**12/15**	**80**	**15/22**	**68**	
Adenocarcinoma					
Well to moderately differentiated	9/11	82	12/17	71	
Poorly differentiated	3/4	75	3/5	60	
Lung cancer	**47/65 [1]**	**72**	**13/43 [1]**	**30**	
Adenocarcinoma					
Well differentiated	8/9	89	3/6	50	
Moderately differentiated	17/25	68	6/17	35	
Poorly differentiated	12/15 [1]	80	2/10 [1]	20	
Squamous cell carcinoma					
Well differentiated	1/1	100	0/1	0	
Moderately differentiated	7/9	78	2/4	50	
Small cell carcinoma	1/3	33	0/3	0	
Mucoepidermoid carcinoma	0/2	0	0/1	0	
Adenoid cystic carcinoma	1/1	100	0/1	0	
Prostate cancer	**8/15 [1]**	**53**	**6/24**	**25**	
Adenocarcinoma					
Well to moderately differentiated	6/11	55	4/15	27	
Poorly differentiated	2/4	50	2/9	22	
Renal cancer	**7/15**	**47**	**5/14**	**36**	
Clear cell carcinoma					
Grade 1	4/8	50	2/8	25	
Grade 2	2/4	50	1/3	33	
Grade 3	1/1	100	1/1	100	
Sarcomatous carcinoma	0/1	0	0/1	0	
Papillary carcinoma	0/1	0	1/1	100	
Urothelial cancer	**11/17 [3]**	**65**	**13/39**	**33**	
Urotheilal carcinoma					
Grade 1+Grade 2	8/12 [3]	67	6/30	20	]*
Grade 3	3/5	60	7/9	78	]*
Breast cancer	**24/32**	**75**	**13/25 [2]**	**52**	
Intraductal carcinoma	0/1	0	0/1	0	
Invasive ductal carcinoma					
Papillotubular type	9/12	75	7/11	64	
Solid tubular type	6/8	75	2/6	33	
Scirrhous type	9/10	90	4/6 [2]	67	
Paget's disease	0/1	0	0/1	0	

Table 2 Continued

Tumor types	*Polyclonal (C-19)*		*Monoclonal (6F-H2)*	
	No. of positive cases	*Ratio (%)*	*No. of positive cases*	*Ratio (%)*
Nuclear grade				
Grade 1	1/1	100	1/1 [1]	100
Grade 2	13/18	72	7/13	54
Grade 3	8/11	73	5/9 [1]	56
Histologic grade				
Grade 1	2/3	67	2/3 [1]	67
Grade 2	14/18	78	8/13	62
Grade 3	6/9	67	3/7 [1]	43
Cervical cancer	**9/16**	**56**	**1/19**	**5**
Squamous cell carcinoma	7/13	54	1/18	6
Adenocarcinoma	2/3	67	0/1	0
Endometrial cancer	**7/14**	**50**	**17/21 [2]**	**81**
Endometrioid adenocarcinoma				
Grade 1	4/6	67	8/10	80
Grade 2	0/1	0	2/2 [1]	100
Grade 3	1/3	33	3/4	75
Serous adenocarcinoma	1/3	33	2/3 [1]	67
Carcinosarcoma	1/1	100	1/1	100
Endometrial stromal tumor, high grade			1/1	100
Ovarian cancer	**18/29 [4]**	**62**	**21/32 [11]**	**66**
Serous adenocarcinoma	11/17 [2]	65	12/17 [7]	71
Serous borderline tumor	0/1	0	0/1	0
Mucinous adenocarcinoma	1/2	50	1/2	50
Mucinous borderline tumor			0/1	0
Transitional cell carcinoma	1/1	100	1/1 [1]	100
Endometrioid adenocarcinoma	0/2	0	1/2	50
Clear cell carcinoma	3/4	75	3/5	60
Sex cord stromal cell tumor	2/2 [2]	100	2/2 [2]	100
Carcinosarcoma			1/1 [1][b]	100
Brain tumor	**17/27**	**63**	**23/26**	**88**
Astrocytoma	3/3	100	3/3	100
Ependymoma	0/1	0	1/1	100
Central neurocytoma	1/1	100	1/1	100
Anaplastic astrocytoma	3/4	75	3/4	75
Anaplastic oligodendroglioma	1/1	100	1/1	100
Anaplastic ependymoma	1/1	100	1/1	100
Glioblastoma	8/15	53	12/14	86
Gliosarcoma	0/1	0	1/1	100
Histological grade				
Grade 2	4/5	80	5/5	100
Grade 3	5/6	83	5/6	83
Grade 4	8/16	50	13/15	87
Soft tissue sarcoma	**21/30 [3]**	**70**	**19/27 [1]**	**70**
PNET[c]/Ewing's sarcoma	5/8	63	3/7	43
Rhabdomyosarcoma	3/7	43	6/6	100
Leiomyosarcoma	4/4 [1]	100	2/3 [1]	67
Malignant fibrous histiocytoma	2/3	67	2/2	100
Liposarcoma	1/1	100	1/2	50
Malignant peripheral nerve sheath tumor	1/1	100	1/1	100
Desmoplastic small round cell tumor	2/2 [2]	100	1/2	50
Angiosarcoma	2/3	67	2/3	67
Clear cell sarcoma	1/1	100	1/1	100
Osteosarcoma	**6/6 [1]**	**100**	**3/6**	**50**
Malignant melanoma (skin)	**4/7**	**57**	**6/7**	**86**

[a]Number of cases showing nuclear staining of tumor cells is shown in square brackets.

[b]Nuclear staining was found in the epithelial element and cytoplasmic staining in the mesenchymal element.

[c]PNET, primitive neuroectodermal tumor.

$^{*}P<0.01$; $^{**}P<0.05$.

Bold values indicate subtotal number of each type of tumor.

vascular endothelium and peripheral nerve fibers in the sections also showed cytoplasmic staining; therefore, the positive staining of these cells could be used as an inner control.

Results of staining are summarized in Table 2. Immunohistochemistry with C-19 revealed that substantial proportion (35–100%) of the cases of each kind of cancer showed a positive reaction. In contrast, positive rates for 6F-H2 varied with the type of tumor; relatively high rates in cases of rhabdomyosarcoma (100%), brain tumors (88%), malignant melanoma of the skin (86%), uterine endometrial cancer (81%), and ovarian serous adenocarcinoma (71%). A majority of positive cases showed a diffuse or granular staining in the cytoplasm of the tumor cells (Figure 2). A majority

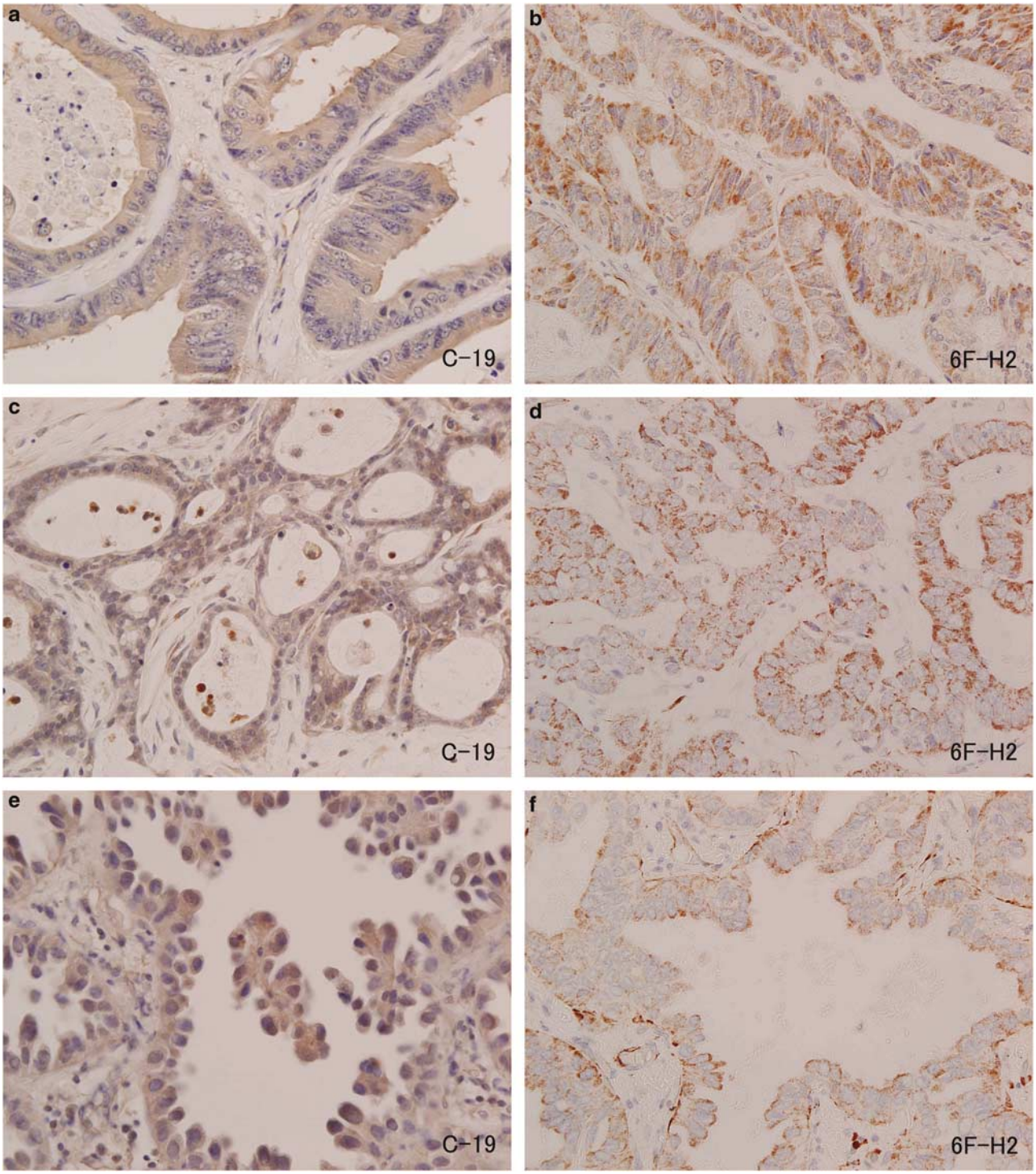

Figure 2 Colon cancer (**a**, **b**), uterine endometrioid cancer (**c**, **d**), and lung adenocarcinoma (**e**, **f**) showed diffuse cytoplasmic staining for C-19 (**a**, **c**, **e**), and granular cytoplasmic staining (**b**, **d**, **f**) for 6F-H2. Original magnification ×400.

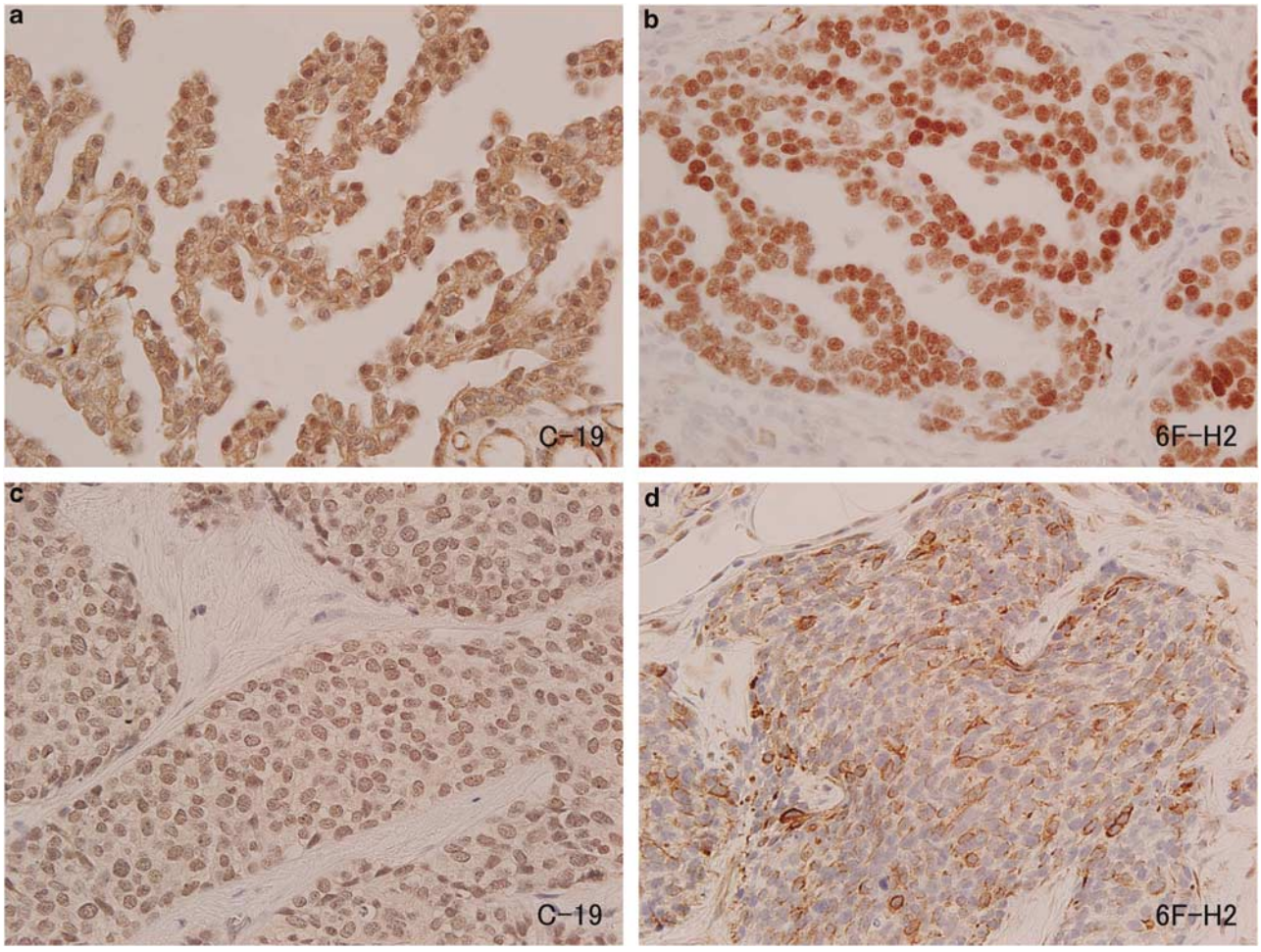

Figure 3 WT1 expression in ovarian serous adenocarcinoma (**a**, **b**) and desmoplastic small round cell tumor (**c**, **d**). Serous adenocarcinoma of the ovary showed nuclear and cytoplasmic staining for both antibodies (**a**, **b**). Desmoplastic small round cell tumor showed nuclear staining for C-19 (**c**), but also cytoplasmic staining for 6F-H2 in one of two cases (**d**). Original magnification ×400.

of serous adenocarcinomas and sex cord stromal tumors of the ovary showed positive nuclear staining for C-19 and/or 6F-H2 (Figure 3). Both cases of desmoplastic small round cell tumor showed positive nuclear staining for C-19, and one of them also showed cytoplasmic staining for 6F-H2 (Figure 3). Nuclear staining was also found in a small number of the tumors from the stomach, lung, urinary tract, prostate, breast, endometrium, and soft tissue. Extremely strong cytoplasmic staining for 6F-H2 was observed in glioblastomas, some soft tissue sarcomas (clear cell sarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor, Ewing's sarcomas and rhabdomyosarcomas), osteosarcomas, and malignant melanomas of the skin as compared to other types of tumors (Figure 4). Most of these tumors showed a distinct diffuse or granular pattern of staining. One case of carcinosarcoma of the ovary showed nuclear staining in the epithelial element and cytoplasmic staining in the mesenchymal element for 6F-H2. Another case of carcinosarcoma of the uterus showed strong cytoplasmic staining exclusively in rhabdoid cells.

Immunohistochemistry using the immunoglobulin fraction of non-immune rabbit serum and mouse monoclonal immunoglobulin to fungal antigen as primary antibodies showed no positive staining in tumor cells.

Discrepancies in the immunohistochemical results obtained with polyclonal *vs* monoclonal antibodies were observed in 129 cases (38%) (Table 3). Among 338 cases examined, 84 (25%) were C-19 (+)/6F-H2 (−) and 45 (13%) were C19 (−)/6F-H2 (+). The frequency of C-19 (+)/6F-H2 (−) was relatively high in lung cancers (21 of 43 cases) and cervical cancers (six of 13 cases), whereas that of C19 (−)/6F-H2 (+) was high in glioblastomas (six of 14 cases).

No significant difference in the rate of WT1 expression was found among the genders and age groups for each type of tumor (data not shown). There was no significant correlation between histologic or cytologic grade and WT1 staining for C-19 in any of the tumors. However, the positive rate for 6F-H2 was significantly lower in undifferentiated adenocarcinoma than differentiated adenocarcinoma of stomach (21 *vs* 65%, $P=0.0032$) and colorectum

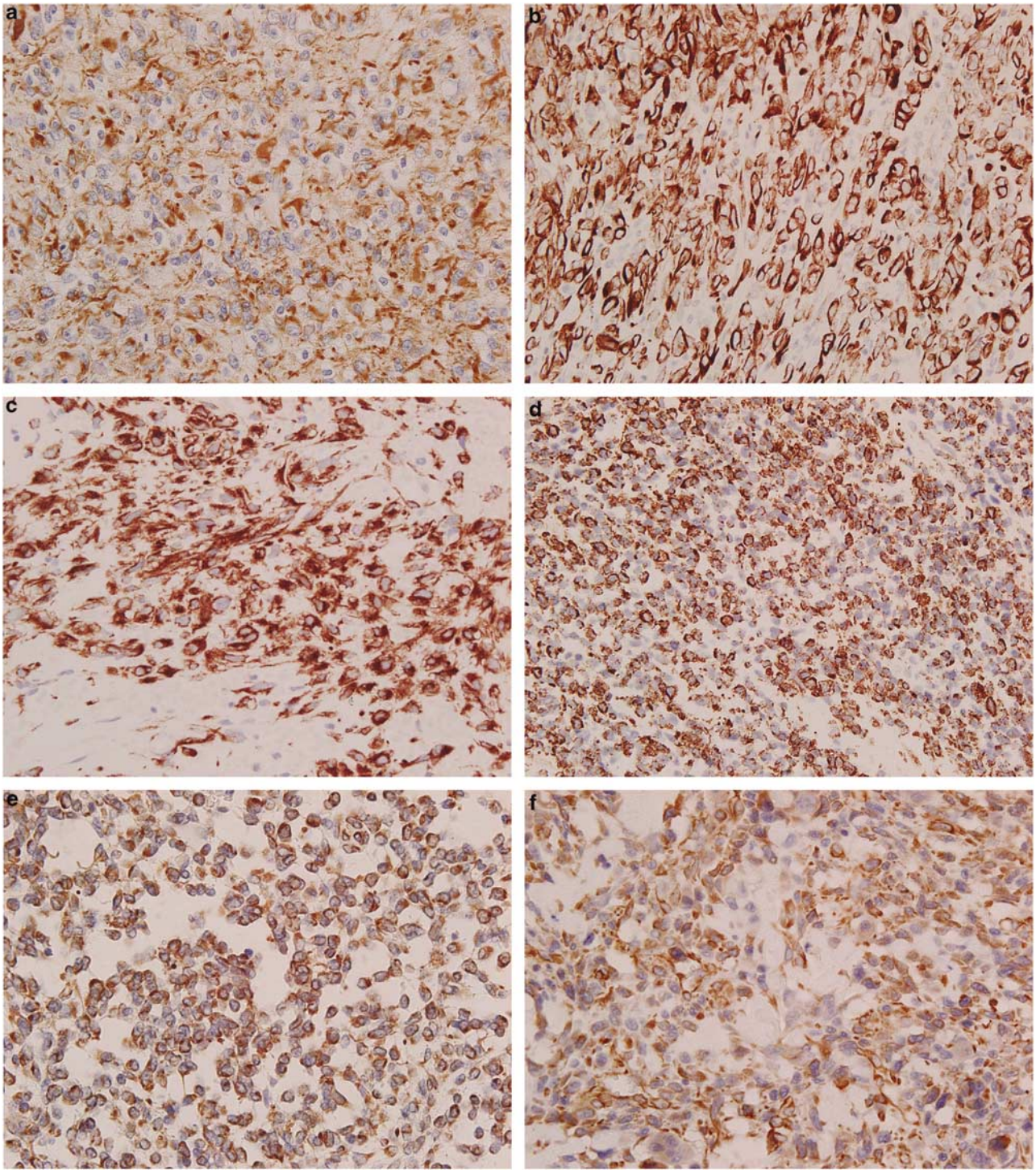

Figure 4 Glioblastoma (**a**), malignant melanoma of the skin (**b**), malignant peripheral nerve sheath tumor (**c**), rhabdomyosarcoma (**d**), Ewing's sarcoma (**e**), and osteosarcoma (**f**) showed extremely strong cytoplasmic staining. Original magnification $\times 400$.

(38 *vs* 76%, $P=0.0487$). Urothelial carcinoma of grade 3 showed a significantly higher positive rate for 6F-H2 than did carcinomas of grades 1 and 2 (78 *vs* 20%, $P=0.0027$). No significant correlation was found between tumor stage and WT1 staining with either antibody in any kind of tumor.

Western Blot Analysis

To determine the subcellular distribution of WT1 protein in the lung cancer cells from clinical samples, cellular proteins were separated into nuclear and cytoplasmic fractions. Western blot

Table 3 Correlation between immunohistochemical detection of WT1 using C-19 and 6F-H2

	6F-H2		*Total*
	Positive cases	*Negative cases*	
C-19			
Positive cases	134	84	218
Negative cases	45	75	120
Total	179	159	338

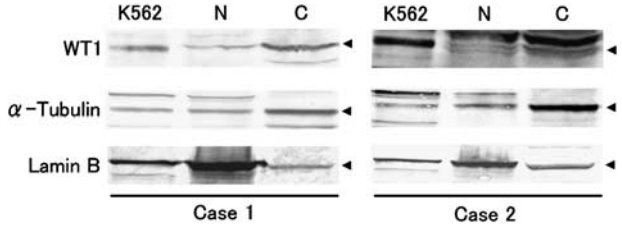

Figure 5 Western blot analysis revealed predominant intracytoplasmic expression of WT1 protein in lung cancer cells. N and C show the nuclear and cytoplasmic fractions of the tumor tissues, respectively. K562 shows whole cell lysate from leukemic cell line K562 which expresses WT1.

analysis revealed that WT1 protein was predominantly located in the cytoplasm (Figure 5).

Discussion

With the use of anti-WT1 polyclonal (C-19) and monoclonal (6F-H2) antibodies, positive staining in the tumor cells was observed in 35–100 and 5–88% of the cases, respectively. The relatively high rates of positivity for WT1 in the present study contrast with some previous reports. Hwang *et al*[21] reported that only a small number of breast cancers, and no colon cancers or lung cancers, expressed WT1. Ordonez *et al*[17] also found that lung, breast, colon, and renal cancers did not express WT1. The discrepancy between our findings and previous results could be explained by the different criteria employed to judge WT1 positivity: they regarded nuclear but not cytoplasmic staining in the tumor cells as positive, because WT1 is principally a DNA binding transcription factor mainly distributed in the nucleus. In the present study, granular or diffuse cytoplasmic staining in the tumor cells was judged as positive, for reasons explained below.

The Western blot analysis revealed the intracytoplasmic localization of WT1 protein in the lung cancer cells. In addition, we[30] and other investigators[31] showed the cytoplasmic expression of WT1 protein in cell lines derived from glioblastoma and lymphoma. Recent studies have revealed that phospholylation in the DNA-binding domain of WT1 alters the affinity for DNA and subcellular distribution of WT1.[32] Post-translational phosphorylation at zinc fingers inhibits the ability to bind DNA, resulting in the cytoplasmic retention of WT1, and also inhibits transcriptional regulatory activity. Another study suggested that WT1 along with p53 can be sequestered in the cytoplasm of adenovirus-transformed kidney cells.[33] There is an interesting report that WT1 shuttles between the nucleus and cytoplasm and might be involved in the regulation of translation through its association with actively translating polysomes.[27] Finally, particular kinds of tumors, such as glioblastomas, a subset of soft tissue sarcomas, osteosarcomas, and malignant melanomas of the skin frequently showed strong cytoplasmic staining, suggesting that WT1 may be involved in the development of these tumors. These findings are generally consistent with recent reports; Nakahara *et al*[34] and Oji *et al*[30] found that most glioblastomas showed cytoplasmic staining for WT1 and the overexpression of WT1 mRNA in the same glioblastoma tissues. Carpentieri *et al*[19] and Sebire *et al*[20] reported that all cases of rhabdomyosarcoma showed strong cytoplasmic staining.

The present immunohistochemical study revealed that WT1 is expressed in a wide variety of human malignancies, including those of the gastrointestinal and pancreatobiliary, urogenital and respiratory tracts, neuronal system and mesenchymal tissues. As far as we know, the present paper is the first report on the overexpression of WT1 in primary tumor tissues of the stomach, prostate, and biliary and urinary systems, and in malignant melanomas, newly adding these tumors to the list of WT1-expressing cancers. Oji *et al* showed overexpression of WT1 mRNA in cell lines derived from various cancers and their primary tumors.[22,24,30,35] They also demonstrated that the growth of WT1-expressing cancer cells was inhibited by treatment with WT1 antisense oligomers.[13,14] These findings suggest that WT1 plays an important role in the carcinogenesis of various cancers.

The sensitivity of the staining for C-19 and 6F-H2 differed greatly between some kinds of tumors, that is, about half of all cases of lung cancer and cervical cancer showed C19 (+)/6F-H2 (−), while 43% of glioblastomas were C19 (−)/6F-H2 (+). The difference in immunoreactivity between C-19 and 6F-H2 might be due to aberrant or dysregulated splicing and alterations of the WT1 gene. The WT1 gene encodes at least 24 isoforms produced by a combination of alternative splicing, RNA editing, and alternative usage of translation initiation sites.[7] The initiation of translation at upstream or downstream of the original initiation site generates WT1 proteins extended or shortened at the N terminus, resulting in possible alteration of immunoreactivity to 6F-H2, which recognizes the N terminus of WT1. An aberrant transcript lacking the N terminal domain of WT1 in cell lines of prostate cancer, breast cancer, and leukemia were described.[36]

Previous reports showed that the level of WT1 mRNA correlated with tumor stage in testicular germ-cell tumors[37] and head and neck squamous cell carcinomas,[35] that is, higher levels in more advanced tumors. However, there was no correlation between WT1 expression and tumor stage in gastric and colorectal cancers in the present study (data not shown). To date, there has been no report showing the relationship between the expression of WT1 examined with immunohistochemistry and prognosis. Miyoshi *et al*[15] reported that the disease-free survival rate was significantly lower in breast cancer patients with high levels of WT1 mRNA than those with low levels. Inoue *et al*[10] showed that leukemia with strong WT1 mRNA expression showed a significantly lower rate of complete remission and significantly worse overall survival than that with weak expression.

WT1 could be a novel tumor rejection antigen in immunotherapy for various kinds of WT1-expressing cancers. WT1-specific cytotoxic T-lymphocytes induce the lysis of endogenously WT-1-expressing tumor cells *in vitro*, but do not damage physiologically normal WT1-expressing cells. It was shown that mice immunized with an MHC class I-restricted WT1 peptide rejected WT1-expressing tumor cells, whereas the cytotoxic T-lymphocytes did not affect normal healthy tissues. Clinical trials of WT1 peptide-based cancer immunotherapy showed that WT1 vaccination induced a reduction in tumor size or decrease in tumor marker levels in breast cancer, lung cancer, and leukemia.[28,29] The results of the present study provide a rationale for immunotherapy targeting WT1 as a new treatment strategy for various kinds of tumors resistant to conventional surgery or chemoradiotherapy.

In conclusion, immunohistochemical study showed the cytoplasmic expression of WT1 in a large proportion of various kinds of human cancers. Immunohistochemical detection using routinely processed histologic sections could provide meaningful information on the expression of WT1 in cancer cells.

Acknowledgements

We thank Ms M Sugano and K Fujikawa (Osaka University Graduate School of Medicine), Mrs K Tanaka, Mr K Miyamoto, Y Fujita, and K Wakabayashi (Osaka Minami Medical Center) for technical assistance. We also thank Ms T Umeda, M Chatani, and S Watanabe (Osaka University Graduate School of Medicine) for assistance with inter-institutional communication.

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文献２

肝内胆管癌のおける癌精巣抗原の発現と予後因子としての意義

背景：癌 - 精巣抗原（CTAs）は、癌特異的免疫療法に適した標的抗原である。本研究の目的は、肝内胆管癌（Intrahepatic cholagiocarcinoma: IHCC）における CTAs の発現並びに、治療法開発におけるその意義の解析である。

方法: 89 人の IHCC の患者を対象として、各種 CTAs と HLA クラス I の発現を、MAGE- A1 を認識する MA454 、MAGE-A（MAGE-A3/A4）群を認識する 57B 、NY- ESO-1 を認識する E978、HLA クラス I を認識する EMR8-5 を用いた免疫組織化学染色により評価した。それに加えて、各々の CTAs あるいはその組み合わせの臨床病理学的及び予後因子としての意義を検討した。結果 : MAGE- A1、MAGE- A3/4、NY- ESO-1 の発現率は、それぞれ 29.2%、27.0%および22.5%であった。CTAs と HLA クラス I 抗原の同時発現は、IHCC 腫瘍の 33.7%に観察された。我々は、MAGE- 3 /4 の発現は、腫瘍の大きさ（≥5 センチ）、腫瘍の再発や予後不良と相関していることを発見した。

更に、少なくとも 1 つの CTA が発現している IHCC 患者 52 例（58.4%）では、他の患者群と比較して、腫瘍サイズがより大きいことと、生存期間が短いことを確認した。また少なくとも 1 つの CTA のマーカーの発現が、独立した予後因子であることも確認した。結論：我々のデータは CTAs を標的とする特異的免疫療法は、IHCC 患者のための新たな治療選択になりうることを示唆している。

Journal of Experimental & Clinical Cancer Research

RESEARCH Open Access

Expression and prognostic significance of cancer-testis antigens (CTA) in intrahepatic cholagiocarcinoma

Jin-xue Zhou[1†], Yin Li[2†], Sun-xiao Chen[3*], An-mei Deng[3*]

Abstract

Background: Cancer-testis antigens (CTAs) are suitable targets for cancer-specific immunotherapy. The aim of the study is to investigate the expression of CTAs in intrahepatic cholagiocarcinoma (IHCC) and evaluate their potential therapeutic values.

Methods: Eighty-nine IHCC patients were retrospectively assessed for their expression of CTAs and HLA Class I by immunohistochemistry using the following antibodies: MA454 recognizing MAGE-A1, 57B recognizing multiple MAGE-A (MAGE-A3/A4), E978 recognizing NY-ESO-1, and EMR8-5 recognizing HLA class I. The clinicopathological and prognostic significance of individual CTA markers and their combination were further evaluated.

Results: The expression rates of MAGE-A1, MAGE-A3/4 and NY-ESO-1 were 29.2%, 27.0% and 22.5%, respectively. The concomitant expression of CTAs and HLA class I antigen was observed in 33.7% of the IHCC tumors. We found that positive MAGE-3/4 expression correlated with larger tumor size (≥ 5 cm), tumor recurrence and poor prognosis. Moreover, we identified 52 cases (58.4%) of IHCC patients with at least one CTA marker expression, and this subgroup displayed a higher frequency of larger tumor size and a shorter survival than the other cases. Furthermore, expression of at least one CTA marker was also an independent prognostic factor in patients with IHCC.

Conclusion: Our data suggest that specific immunotherapy targeted CTAs might be a novel treatment option for IHCC patients.

Introduction

Intrahepatic cholagiocarcinoma (IHCC) is a relatively uncommon malignancy, comprising approximately 5%-10% of the liver cancers, and both its incidence and mortality have increased in recent years in China and other countries [1,2]. IHCC is not sensitive to radiation therapy and chemotherapy. Even the patients undergoing a radical surgical resection is still at a high risk for early recurrence, and the patients' survival is thus unsatisfactory. Therefore, there is a great need to identify molecular targets for developing novel therapeutic approaches for patients with IHCC.

Cancer testis antigens (CTAs) comprise a group of non-mutated self-antigens selectively expressed in various tumors and normal testis tissues, but not in other normal tissues [3]. Several studies have shown that if presented with human leukocyte antigen (HLA) class I molecules, these tumor-associated antigens could induce effective anti-tumor cytotoxic T lymphocytes (CTLs) response in vitro and in vivo [4]. Because of these unique characteristics, CTAs are regarded as promising targets for cancer-specific immunotherapy [5]. However, the possibility that IHCC patients might benefit from CTA-targeted therapies has not been evaluated.

Given their potential therapeutic significance, it may have significance for exploring the presence of CTAs in IHCC. However, to our knowledge, until now, only two studies examined the mRNA and protein expression of CTAs in small number of IHCC cases [6,7]. The CTAs expression at protein level and their clinicopathological and prognostic significance in a larger cohort have not been investigated.

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The aims of the current study were to analyze the expression of MAGE-A1, MAGE-A3/4 and NY-ESO-1 CTAs in IHCC tissues by immunohistochemistry, and to investigate correlations between their expression with HLA class I expression, clinicopathologic parameters and survival in patients with IHCC.

Materials and methods

Patients

The study was approved by the research ethics committee of our institutions, and informed consent was obtained from each patient. A total of consecutive 102 patients with IHCC who underwent curative resection at Department of Hepatobiliary and Pancreatic Surgery, Henan Tumor Hospital (Zhengzhou, China) and Changzheng Hospital (Shanghai, China) from 1999 to 2006 were retrospectively reviewed. Patients with lymphnode-positive metastasis routinely received 5-fluorouracil-based chemotherapy, and Gemcitabone chemotherapy was given when recurrence occurred. Patients were followed up every two month during the first postoperative year and at every four month afterward. Follow-up was finished on May 2008. The median follow-up was 24 month (range, 4-61 month). Overall survival (OS) time was defined as the time from operation to cancer-related death only.

Cases were included according to the following inclusion criteria: having archived formalin-fixed, paraffin-embedded specimens available; having complete clinicopathological and followed-up data; receiving no anticancer treatment before operation. Patients who died of unrelated diseases and within one month after operation were excluded, leaving 89 patients eligible for this analysis. The clinical and pathological details of these patients were summarized in Additional file 1.

Immunohistochemical analysis

Immunohistochemical analysis was performed on archived tissue blocks containing a representative fraction of the tumors. Briefly, 5-μm-thick paraffin-embedded tissue sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with methanol and 0.3% H_2O_2 for 20 min. Antigen retrieval was performed with microwave treatment in 0.1 M sodium citrate buffer (pH 6.0) for 10 min. Expression of CTAs was detected with the monoclonal antibody against MAGE-A1 (clone MA454), MAGE-A3/4 (clone 57B) and NY-ESO-1 (clone E978), as described previously [8-10]. Clone 57B was originally raised against MAGE-A3, and later has been reported to primarily recognize the MAGE-A4 antigen [11,12]. Currently, 57B is considered to be anti-pan-MAGE-A (MAGE-A3/4). Expression of HLA class I was detected with an anti-pan HLA class I monoclonal antibody EMR8-5, as described previously [13]. Detection was performed with the Dako Envision system using diaminobenzidine (DAB) as the chromogen. Non-specific mouse IgG was used as negative control and normal human testis tissues were used as positive controls for CTA expression. Immunochemical results were evaluated and scored by two and independent observers according to the previous criteria [14]. Positive CTA expression was assigned to any extent of immunostaining in sections and further graded into four groups: + : < 5% of tumor cells stained; ++ : 5-25% of tumor cells stained; +++ : > 25-50% of tumor cells stained; ++++ : > 50% of tumor cells stained. A patient was considered CTA-positive if at least one of three markers demonstrated positive immunoactivity. HLA class I expression was classified as positive and down-regulated compared with stromal lymphocytes as an internal control as previously described [13].

Statistical analysis

The associations between CTAs expression and clinicopathological parameters were evaluated using Chi-square or Fisher's exact test, as appropriate. Overall survival of patients were estimated by the Kaplan-Meier method, differences between groups were compared were by the log-rank test. Multivariate analysis was performed using a Cox proportional hazard model. Statistically significant prognostic factors identified by univariate analysis were entered in the multivariate analysis. All the statistical analyses were performed with SPSS 16.0 software. P value less than or equal to 0.05 was considered statistically significant.

Results

Expression of MAGE-A1, MAGE-A3/4, NY-ESO-1 and HLA class I proteins in IHCC patients by immunohistochemistry

MAGE-A1, MAGE-A3/4 and NY-ESO-1 showed a predominantly, although not exclusively, cytoplasmic staining (Figure 1). The frequency and grade of various CTA expressions in tumors is shown in Table 1. Figure 2 showed a Venn diagram dipicting the overlap of three CTAs expression. When the CTA combinations were tested, 52 from 89 IHCC cases (58.4%) showed expression of at least one marker, 14 cases (15.7%) demonstrated co-expression of two CTAs, and only three cases (3.3%) were positive for all the three antigens. As seen in table 2, down-regulated HLA class I expression was found in 42.7% of all tumors (n = 38). Comparing the relationship between individual or combined CTAs expression and HLA-class I expression, no correlation was observed. And 30 IHCC cases (33.7%) demonstrated concomitant expression of CTAs and HLA class I antigen.

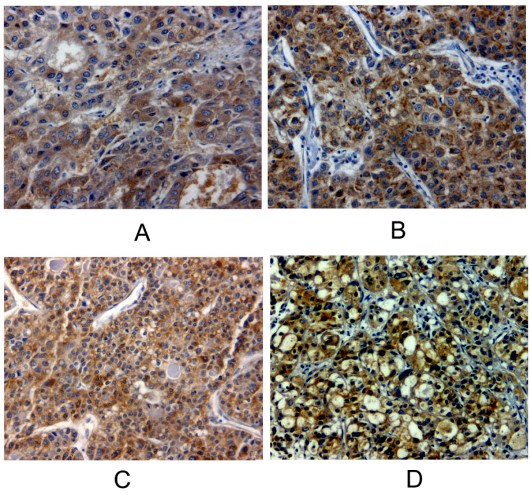

Figure 1 Immunohistochemical analysis of MAGE-A1, MAGEA3/4, NY-ESO-1 and HLA Class I in intrahepatic cholagiocarcinoma. Sections were stained with antibody against (A) MAGE-A1 (MA454); (B) MAGE-A3/A4 (57B); (C) NY-ESO-1 (E978); (D) HLA Class I (EMR8-5).

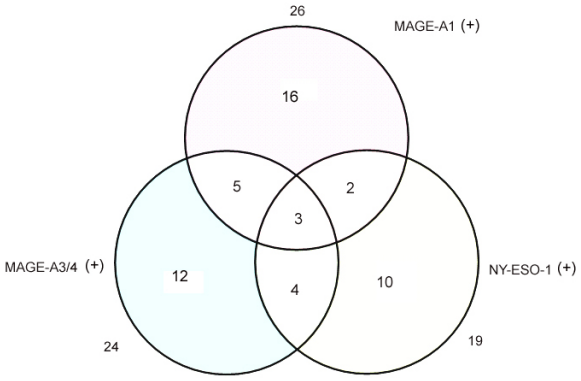

Figure 2 Venn diagram depicting the overlap in the expression of cancer-testis antigens in intrahepatic cholagiocarcinoma.

Correlation between CTAs expression with HLA-class I expression and clinicopathological parameters

We found that positive MAGE-A3/4 and one CTA marker expression were detected more frequently in tumors with bigger size (≥ 5 cm) (20/24, 38/52), than in smaller tumors (P = 0.011, P = 0.009). In addition, MAGE-A3/4 positive IHCC had a higher recurrence rate (17/24) than negative subgroup (30/65, P = 0.038). There was no statistically significant correlation found between individual or combined CTA expression and any other clinicopathological traits.

Correlation between CTAs expression and overall survival

The correlation of clinicopathological parameters and individual or combined CTA expression with overall survival was further investigated. As shown in Table 3, univariate analysis showed that overall survival significantly correlated with TNM stage, lymphnode metastasis, resection margin, differentiation and tumor recurrence but not with gender, age, tumor size and number, vascular invasion and perineural invasion.

Table 1 Expression of cancer-testis antigens in intrahepatic cholanglocarcinoma

	MAGE-A1 N (%)	MAGE-A3/4 N (%)	NY-ESO-1 N (%)
Negative	63 (70.8)	65 (73.0)	70 (78.7)
Positive	26 (29.2)	24 (27.1)	19 (21.3)
+	2 (2.2)	1 (1.1)	1 (1.1)
++	3 (3.4)	4 (4.4)	1 (1.1)
+++	12 (13.5)	14 (15.7)	7 (7.9)
++++	9 (10.1)	5 (5.6)	10 (11.2)

Patients with MAGE-A3/4 positive tumors had a significantly poorer outcome compared to those without MAGE-A3/4 expression. MAGE-A1 and NY-ESO-1 also demonstrated the same trend but did not reach statistical significance. Interestingly, negative expression in all CTAs correlated with a better prognosis than at least one CTAs expression, meanwhile, two or three CTAs expression had no impact on survival (Figure 3, Table 3). COX proportional hazard model analysis showed that at least one CTA expression was an independent prognostic indicator for IHCC, whereas the association of MAGE-A3/4

Table 2 Correlation between CTA expression pattern and HLA class I expression

CTA expression pattern	HLA class I expression		P value
	Positive (n = 51)	Down-regulated (n = 38)	
MAGE-A1			
Positive	18	8	0.144
Negative	33	30	
MAGE-A3/4			
Positive	11	13	0.184
Negative	40	25	
NY-ESO-1			
Positive	11	8	0.953
Negative	40	30	
1 CTA positive			
With	30	22	0.930
Without	21	16	
2 CTA positive			
With	9	5	0.565
Without	42	33	
3 CTA positive			
With	1	2	0.795
Without	50	36	

Table 3 Univariate analyses of prognostic factors associated with overall survival (OS)

Variable	Category	No. of patients	P
Gender	female vs. male	31 vs. 58	0.587
Age	< 60 vs. ≥60, years	19 vs. 70	0.532
TNM stage	1/2 vs. 3/4	34 vs. 55	0.007
Tumor size	≥5 cm vs. < 5 cm	55 vs. 34	0.690
Differentiation	well or mod vs. poor	26 vs. 63	0.008
Resection margin	R0 vs. R1/2	56 vs. 33	0.008
Tumor number	single vs. multiple	58 vs. 31	0.385
Vascular invasion	with vs. without	42 vs. 47	0.227
Perineural invasion	with vs. without	33 vs. 56	0.736
Lymph node metastasis	with vs. without	38 vs. 51	0.001
Tumor recurrence	with vs. without	47 vs. 42	0.022
MAGE-A1	Positive vs. negative	26 vs. 63	0.116
MAGE-A3/4	Positive vs. negative	24 vs. 65	0.009
NY-ESO-1	Positive vs. negative	19 vs. 70	0.068
1 CTA positive	with vs. without	52 vs. 37	0.001
2 CTA positive	with vs. without	14 vs. 75	0.078
3 CTA positive	with vs. without	3 vs. 86	0.372

with a shorter survival failed to persist in the multivariate analysis (Table 4).

Table 4 Multivariate analyses of factors associated with overall survival (OS)

Variable	HR	95% Confidence Interval		P value
		Lower	Upper	
1 CTA positive	0.524	0.298	0.920	0.024
MAGE-A3/4	0.897	0.505	1.594	0.711
Differentiation	0.447	0.263	0.758	0.003
TNM stage	1.122	0.597	2.110	0.721
Lymph node metastasis	0.389	0.207	0.732	0.003
Tumor recurrence	0.706	0.386	1.291	0.258
Resection margin	1.138	0.574	2.258	0.711

Discussion

In this study, expression of three CTAs at protein level was investigated by immunohistochemistry. MAGE-A1, MAGE-A3/4 and NY-ESO-1 were selected considering that these antigens have been well-accredited and are being applied for clinical trials of vaccine immunotherapy [15-18]. The expression frequency of CTAs varies greatly in different tumors type [19,20]. Our results showed that expression rates of MAGE-A1, MAGE-A3/4 and NY-ESO-1 in IHCC were less than 30%. According to the established criteria [21], IHCC should be classified to be low "CTA expressors". In a previous study, the expression rates of MAGE-A1, MAGE-A3 and NY-ESO-I in IHCC were 20.0% (4/20), 20.0% (4/20) and 10.0% (2/20) detected by RT-PCR [6]. However, in the immunohistochemical study by Tsuneyama et al. [7], 32 of 68 IHCC cases (47.1%) demonstrated positive MAGE-A3 expression using a polyclonal antibody.

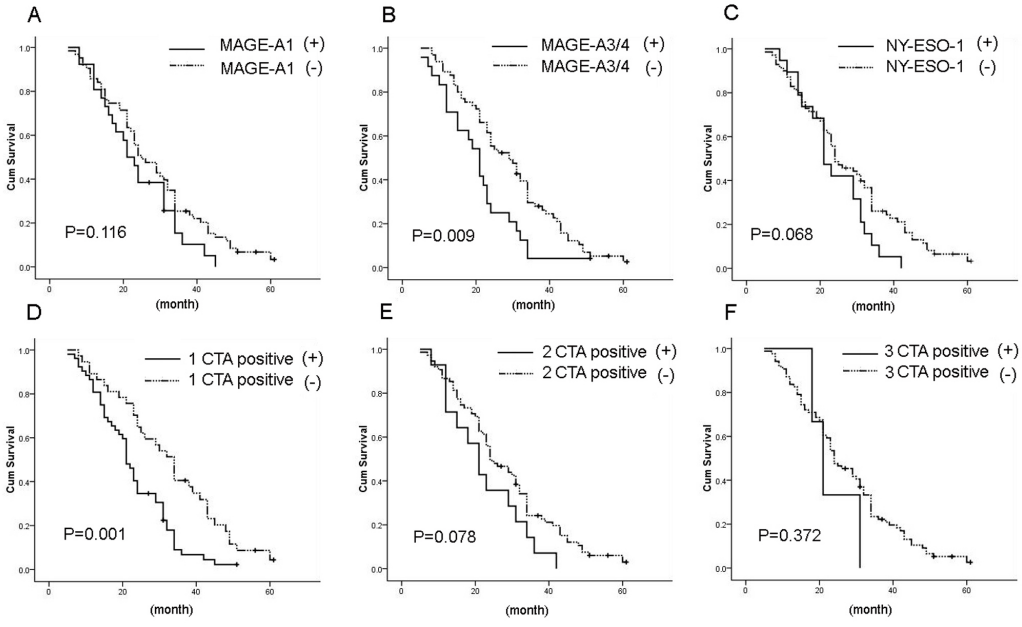

Figure 3 Correlation between individual or combined CTA expression and survival. Kaplan-Meier survival curves performed according to CTAs expression.(A) MAGE-A1; (B) MAGE-A3/4; (C) NY-ESO-1; (D) at least one CTA positive; (E) two CTAs expression; (F) with three CTAs expression.

These discrepancies between our and previous studies may be related to the difference in the method of detection, the antibodies adopted and patient populations.

In this study, we also identified that only MAGE-3/4 and at least one positive CTA expression correlated aggressive phenotypes including bigger tumor size and higher recurrence rate. There was no other association observed between CTA markers (either individual or combined) with HLA class I expression and clinicopathological parameters of IHCC patients.

Curves of patients with positive for the individual or multiple CTAs (with two or three CTA positive) markers leaned towards a poorer outcome, however, only MAGE-A3/4 reach statistical significance. We speculated that such statistically insignificant trends were likely to be due to the fact that only a small number of IHCC cases presented with positive CTA expression (either individual or co-expressed) in this study. Considering that combination of CTAs makers may reinforce the predictive value for prognosis and malignant phonotype by one single CTA alone, we next asked whether at least one CTA expression had n significant impact on outcome. We found that at least one CTA expression did indeed correlate with a significantly poorer survival. Furthermore, at least one positive CTA expression was also an independent prognostic factor for patients with IHCC.

Interestingly, in this study, MAGE-A1 and NY-ESO-1 positive IHCC tumors seem to have a relatively higher frequency of positive expression of HLA class I than MAGE-A3/4 positive cases. Recently, Kikuchi et al. [22] indicated that co-expression of CTA (XAGE-1b) and HLA class I expression may elicit a CD8+ T-cell response against minimal residual disease after surgery and resulted in prolonged survival of NSCLC patients, while expression of CTA combined with down-regulated HLA class I expression correlated with poor survival. Therefore, we speculated that a relatively high proportion of HLA Class I-negative cases in MAGE-A3/4 positive group may partly account for its association with significantly poor survival.

MAGE-A1, MAGE-A3/4 and NY-ESO-1 have been applied for clinical trials of vaccine immunotherapy for multiple cancer patients, but the utility of CTA immunotherapy against patients with IHCC remains investigated. In this study, using three CTA markers MAGE-A1, MAGE-A3/4 and NY-ESO-1, we identified a subgroup (58.4%) of IHCC patients with at least one CTA expression having a poor prognosis. Moreover, high levels of expression of these antigens were observed in most positive cases. In our study, the concomitant expression of CTAs and HLA class I antigen was observed in 33.7% of the IHCC tumors, which indicating that it may be possible to immunise a significant proportion of IHCC patients with tumor-specific CTLs. Based on our data, we suggest that a considerable number of IHCC patients at high-risk might benefit from specific immunotherapy targeted MAGE-A and NY-ESO-1.

This is the first study demonstrating a correlation between CTA and prognosis in IHCC. Furthermore, this present retrospective cohort study is limited to relatively small case series (although more than previous studies); therefore, further validation will be required before these antigens can be tested for targeted immunotherapy.

Conclusion

In conclusion, our data suggest that the cancer-testis antigens identified in this study might be novel biomarkers and therapeutic targets for patients with IHCC.

Additional material

Additional file 1: Table S1 Clinicopathological characteristics of patients included in this study. a table for the clinicaopathological characteristics of 89 IHCC patients.

Acknowledgements

This research was supported by grants from National Science Foundation of China (30772017, 30972730), Shanghai Municipal Commission for Science and Technology (08QH14001, 09JC1405400).

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Authors' contributions

JXZ and YL contributed to clinical data, samples collection, immunohistochemistry analysis and manuscript writing. SXC and AMD were responsible for the study design and manuscript writing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 8 November 2010 Accepted: 6 January 2011
Published: 6 January 2011

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doi:10.1186/1756-9966-30-2
Cite this article as: Zhou *et al.*: Expression and prognostic significance of cancer-testis antigens (CTA) in intrahepatic cholagiocarcinoma. *Journal of Experimental & Clinical Cancer Research* 2011 **30**:2.

文献３

ゲムシタビンは、ウィルムス腫瘍遺伝子 WT1 の発現を増強し、WT1 特異的 T 細胞のヒト膵がん細胞に対する免疫応答を増強する

ヒト膵臓癌（PC）で発現するウィルムス腫瘍遺伝子（WT1）は、T 細胞による抗腫瘍免疫応答時に認識される腫瘍特異抗原である。本研究では、PC の標準的な治療薬であるゲムシタビン（GEM）が、膵がん細胞における WT1 発現調節と WT1 特異的 T リンパ球の感受性に与える影響について検討した。 WT1 の発現は、定量 PCR、免疫ブロット分析、および共焦点顕微鏡にて解析した。 HLA クラス I 分子上に提示される WT1 抗原ペプチドは、質量分析法で同定した。WT1 特異的 T 細胞受容体遺伝子を導入したヒト T 細胞を、細胞傷害活性解析のためのエフェクターT 細胞として使用した。
ヒト MIAPaCa2 PC 細胞を GEM で処理すると、WT1 mRNA 発現レベルが増強し、これは核内因子カッパー β の活性増強と関連していた。MIAPaCa2 細胞を移植した SCID マウスに GEM を投与すると、腫瘍組織における WT1 mRNA 発現が増加した。MIAPaCa2 以外の複数のヒト PC 細胞株においても、GEM 治療によって WT1 mRNA 発現レベルが増加した。GEM 治療によって、WT1 蛋白質が核内から細胞質に移行し、プロテアソームによって切断され、抗原ペプチドの形成が促進されたのかもしれない。実際、HLA- A * 2402 拘束性抗原ペプチドである CMTWNQMNL ペプチドの提示量は、GEM 未処理細胞に比べて GEM 処理 MIAPaCa2 細胞で増加していた。WT1 特異的細胞傷害性 T 細胞は、未処理の MIAPaCa2 細胞と比較し、GEM の至的濃度処理 MIAPaCa2 細胞をより効果的に傷害した。GEM はヒト PC 細胞において WT1 の発現を増強し、 WT1 特異的 T 細胞性による高腫瘍免疫応答を増強した。

Cancer Immunol Immunother (2011) 60:1289–1297
DOI 10.1007/s00262-011-1033-3

ORIGINAL ARTICLE

Gemcitabine enhances Wilms' tumor gene WT1 expression and sensitizes human pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune response

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Received: 27 December 2010 / Accepted: 9 May 2011 / Published online: 24 May 2011

Abstract Wilms' tumor gene (WT1), which is expressed in human pancreatic cancer (PC), is a unique tumor antigen recognized by T-cell-mediated antitumor immune response. Gemcitabine (GEM), a standard therapeutic drug for PC, was examined for the regulation of WT1 expression and the sensitizing effect on PC cells with WT1-specific antitumor immune response. Expression of WT1 was examined by quantitative PCR, immunoblot analysis, and confocal microscopy. Antigenic peptide of WT1 presented on HLA class I molecules was detected by mass spectrometry. WT1-specific T-cell receptor gene–transduced human T cells were used as effecter T cells for the analysis of cytotoxic activity. GEM treatment of human MIAPaCa2 PC cells enhanced WT1 mRNA levels, and this increase is associated with nuclear factor kappa B activation. Tumor tissue from GEM-treated MIAPaCa2-bearing SCID mice also showed an increase in WT1 mRNA. Some human PC cell lines other than MIAPaCa2 showed up-regulation of WT1 mRNA levels following GEM treatment. GEM treatment shifted WT1 protein from the nucleus to the cytoplasm, which may promote proteasomal processing of WT1 protein and generation of antigenic peptide. In fact, presentation of HLA-A*2402-restricted antigenic peptide of WT1 (CMTWNQMNL) increased in GEM-treated MIAPaCa2 cells relative to untreated cells. WT1-specific cytotoxic T cells killed MIAPaCa2 cells treated with an optimal dose of GEM more efficiently than untreated MIAPaCa2 cells. GEM enhanced WT1 expression in human PC cells and sensitized PC cells with WT1-specific T-cell-mediated antitumor immune response.

Keywords Pancreatic cancer · WT1 · Gemcitabine · NF kappa B · T-cell response

Electronic supplementary material The online version of this article (doi:10.1007/s00262-011-1033-3) contains supplementary material, which is available to authorized users.

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Introduction

Pancreatic cancer (PC) is a devastating disease with a 5% overall 5-year survival rate [1, 2]. This high mortality rate is due to a combination of factors that include a high incidence of metastatic disease at initial diagnosis, an aggressive clinical course, and the failure of systemic therapies used for treatment. Despite the fact that advanced loco-regional disease is found in 40% of patients [3], only 5–25% of patients with pancreatic cancer are treated surgically [4]. Even in cases where pancreatic cancer is discovered at a resectable stage, only 10–20% of patients are expected to survive for more than 5 years after curative resection [5].

Gemcitabine (GEM) is currently the most commonly used therapeutic drug prescribed in cases of advanced PC [6, 7]. Numerous phase III trials testing gemcitabine in combination with other cytotoxic drugs have failed to reveal any additional benefit compared with gemcitabine alone [8]. Erlotinib, a small molecule inhibitor of the epidermal growth factor receptor tyrosine kinase, is a notable exception in that it is the only drug reported to confer a significant improvement in survival over gemcitabine alone [9]. Recently, Folfirinox was reported to be a more efficient, but more toxic, regimen for pancreatic cancer and might be promising for the patients with good performance status [10]. Ultimately, improved treatment of advanced PC will likely require additional selected and targeted agents that provide the benefit of prolonged survival with minimum risk.

The Wilms' tumor gene WT1 encodes a zinc finger transcription factor. Although the WT1 gene was originally defined as a tumor suppressor gene [11–13], additional reports demonstrate that it is highly expressed in leukemia and various types of malignant tumors [14] and can confer oncogenic functions [15]. WT1-specific cytotoxic T lymphocytes (CTLs) and WT1 antibodies have both been shown to be induced spontaneously in tumor-bearing leukemia patients [16]. These results indicate that WT1 protein is highly immunogenic and establish it as a promising tumor antigen for recognition by specific CTLs [17]. The safety and clinical efficacy of major histocompatibility complex (MHC) class I-restricted WT1 epitope peptides against various malignancies have been confirmed in clinical immunotherapy trials [14, 15].

Reports indicate that WT1 is frequently overexpressed in human pancreatic cancer cells [18]. Recent clinical reports on treatments combining GEM drug therapy with peptide vaccine immunotherapy have demonstrated safe and promising results in cases of advanced PC [19, 20]. In our recent phase I clinical trial that tested a combination of WT1 peptide vaccine and GEM in treatment of advanced PC, several cases showed marked tumor regression (manuscript in preparation). These results suggest that the actions of WT1-targeted antitumor immunity and GEM can function synergistically against PC cells. In the present study, we demonstrate that GEM treatment up-regulates WT1 expression in PC cell lines, and that antitumor immune activity against PC cells via a WT1-specific T-cell response is augmented by GEM treatment.

Materials and methods

Cell lines, antibodies, and mice

Human pancreatic cancer cell lines (MIAPaCa2, PANC-1, AsPC-1, BxPC-3, Capan-1 and Capan-2) were obtained from the American Type Culture Collection (Manassas, VA, USA) [21]. A rabbit polyclonal antibody against WT1 (C-19) and a goat polyclonal antibody against Lamin B (C-20) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Eight- to ten-week-old SCID mice were supplied by Nihon SCL Co., Ltd. (Hamamatsu, Japan) and were maintained in our specific pathogen-free facilities. Mice received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication 86-23 revised 1985).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Tissue or cell samples were lysed directly in Buffer RLT Plus (Qiagen, Hilden, Germany) and homogenized. Reverse transcription (RT) was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan primers and non-fluorescent quencher probes complementary to WT1 (Assay ID:Hs00240913_m1) and 18S ribosomal RNA (rRNA, Assay ID:Hs99999901_s1) genes were purchased from Applied Biosystems. qRT-PCR was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). WT1 expression levels were normalized relative to those of 18S rRNA.

Inhibition of nuclear factor kappa B (NF-kB)

Inhibition of NF-kB activity in human PC cells was achieved using an NF-kB p65 (Ser276) inhibitory peptide kit (IMGENEX, San Diego, CA, USA). Briefly, MIAPaCa2 cells (6×10^4/well) were seeded in 24-well culture plates and incubated for 24 h. Growth medium was then changed to medium containing GEM (0 or 30 ng/ml) with NF-kB blocking peptide (50 μM) or control peptide (50 μM). After 24-h incubation, cellular expression of NK-kB was determined using qRT-PCR.

Immunoblot analysis

The nuclear fraction of MIAPaCa2 cells used for the detection of WT1 protein was isolated using an Active Motif extraction kit (Carlsbad, CA, USA). Protein samples (30 μg/well) separated by electrophoresis in 15% sodium dodecyl sulfate-polyacrylamide gels were then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). After blocking in 5% nonfat milk for 1 h, membranes were exposed to antibodies specific to WT1 (1:100) and beta-actin (1:10,000; Sigma–Aldrich, St. Louis, MO, USA) and then to horseradish peroxidase-conjugated secondary antibodies. The ECL-PLUS Detection System (GE Healthcare, Buckinghamshire, UK) was used for chemiluminescent detection of secondary antibodies.

Confocal microscopy

MIAPaCa2 cells cultured on glass coverslips were incubated with or without GEM (30 ng/ml) for 24 h. Cells were then washed and fixed in 4% paraformaldehyde. Immunofluorescent visualization of cells expressing WT-1 was achieved by incubating slides in rabbit anti-WT1 antibody (1/200), followed by Amaxa488-conjugated donkey anti-rabbit IgG antibody (Molecular probes, Eugene, OR, USA). Cell nuclei were stained with TO-PRO-3 iodide (Molecular Probes), and a laser scanning confocal microscope (LSM510, CarlZeiss, Thornwood, NY, USA) was used to obtain fluorescence images.

Positive ion ESI LC–MS/MS analysis of MHC class I binding peptides from MIAPaCa2 cells

MIAPaCa2-bearing mice were injected intraperitoneally with PBS or GEM (3.75 mg/mouse). After 48 h, tumors were resected and digested using collagenase to obtain single cells. MHC class I binding peptides were isolated from 10^8 cells using the method described by Storkus et al. [22]. Isolated peptides were dissolved in 50% methanol and analyzed via electrospray ionization (ESI) liquid chromatography (LC)-tandem mass spectrometry (MS/MS) using a triple quadrupole mass spectrometer (Q TRAP) (Applied Biosystems, Foster City, CA, USA). The mass spectrometer interfaced with an Agilent 1100 liquid chromatography (Agilent Technologies, Wilmington, DE, USA) was employed. The WT1 antigenic peptide (aa 235–243 CMTWNQMNL; MW = 1,139.5 Da) in 50% methanol was easily produced m/z 1171.5 as a methanol adduct ion $(M + MeOH)^+$. The multiple reaction monitoring (MRM) transition monitored for the detection of this peptide was m/z 1,171.5/1,154.5. This peptide was eluted at a flow rate 0.2 mL/min from an Intersil C8-3 column [50 × 2.1 mm, 3 μm particle size] (GL Science Inc., Tokyo Japan) using a linear gradient of 9.5% min^{-1} of 5–100% acetonitrile containing 1% formic acid. To estimate cellular peptide concentrations, a standard curve was prepared by increasing concentrations (0–1,000 pmol) with chemically synthesized WT-1 antigenic peptide. The response was considered to be linear if the correlation coefficient (r^2) was greater than 0.99, calculated by least-squares linear regression analysis.

Cytotoxicity assay

WT1-specific cytotoxic effector cells were generated as described below. Full-length WT1-specific T-cell receptor (TCR) a/b genes (Va20/J33/Ca for TCR-a and Vb5.1/J2.1/Cb2 for TCR-b, respectively) isolated from the HLA-A*2402-restricted $WT1_{235–243}$-specific $CD8^+$ CTL clone TAK-1 [23] were cloned into a pMEI-5 retroviral vector (Takara Bio, Shiga, Japan). WT1-specific TCR genes were then transduced into normal $CD8^+$ lymphocytes as described previously [24]. Cytotoxicity assays were performed using a standard 4-h culture ^{51}chromium (Cr) release assay described elsewhere [25].

Statistical analysis

The significance of differences between groups was analyzed using Student's *t* test for two independent groups and with Tukey's test for multiple-group comparisons. Values that did not fit a Gaussian distribution were analyzed with the Bonferroni method for multiple-group comparisons.

Results

Up-regulation of WT1 mRNA in human PC cells by in vitro treatment with GEM

Proliferation of MIAPaCa2 cells was inhibited for 48 h with stable numbers of viable cells following treatment with 30 and 100 ng/ml of GEM (Fig. 1a). Growth of MIAPaCa2 cells was also impaired by treatment with 10 ng/ml of GEM for 72 h. Levels of WT1 mRNA were enhanced significantly by treatment of MIAPaCa2 cells with 10, 30, and 100 ng/ml of GEM for 24, 48 and, 72 h, respectively (Fig. 1b). Enhancement of WT1 mRNA was also observed after 2-h treatment with GEM (100 ng/ml) in following 72 h (Fig. 1c). This GEM-mediated enhancement was suppressed by the addition of NF-kB blocking peptide in the culture (Fig. 1d).

GEM-mediated up-regulation of WT1 mRNA expression was examined in various human pancreatic cancer cell lines. GEM-treated Capan-2 cells showed a significant enhancement of WT1 mRNA expression (Fig. 2a). Low steady-state levels of WT1 mRNA expression in AsPC-1 and BxPC-3 cells were also enhanced by GEM treatment (Fig. 2b). In contrast, expression of WT1 mRNA in Capan-1 and PANC-1 cells was not up-regulated by GEM treatment (Fig. 2b, c).

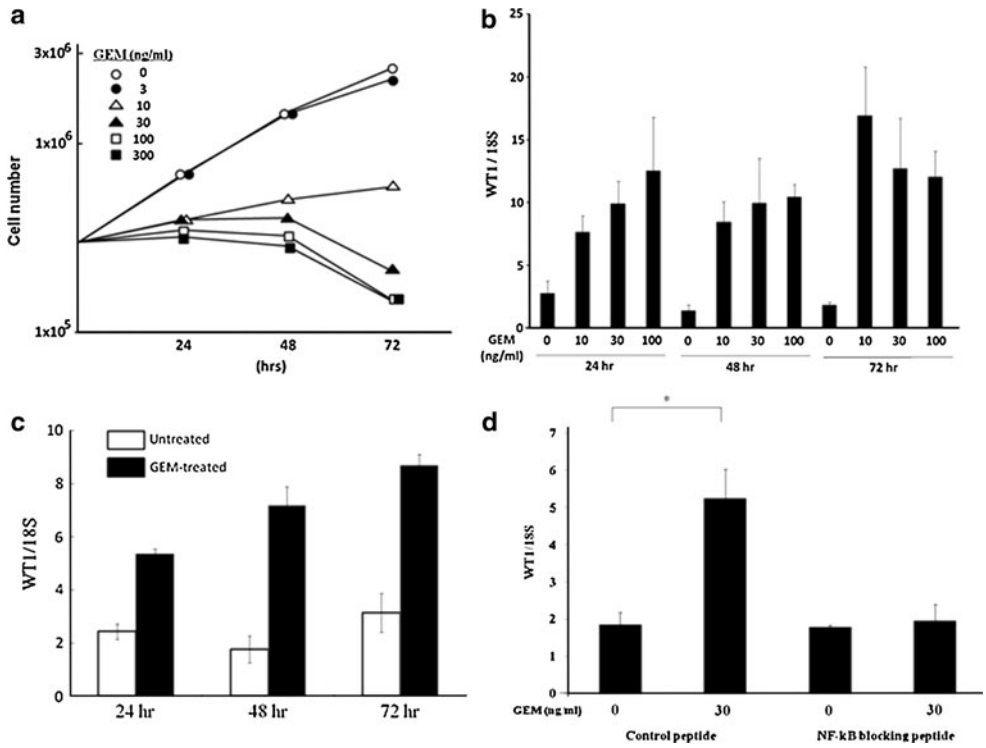

Fig. 1 **a** Proliferation of MIAPaCa2 cells in medium containing various concentrations of GEM. MIAPaCa2 cells (3×10^5/well) were seeded in 6-well culture plates in regular culture medium, which was then exchanged for GEM-containing medium after 24 h. At 24-h intervals, cells were detached using trypsin, and cell numbers were counted using a hemocytometer ($n = 3$). **b** Up-regulation of WT1 mRNA in MIAPaCa2 cells by GEM treatment. Twenty-four hours after plating, culture medium was exchanged to media containing GEM at indicated concentrations (0, 10, 30 and 100 ng/ml). MIAPaCa2 cells were harvested at 24-h intervals, and WT1 mRNA in cell homogenates was analyzed using qRT-PCR. WT1mRNA levels were normalized relative to those of 18S ribosomal RNA (18S). **c** Up-regulation of WT1 mRNA in MIAPaCa2 cells after short treatment with GEM. Twenty-four hours after plating, MIAPaCa2 cells were untreated or treated with 100 ng/ml of GEM for 2 h. MIAPaCa2 cells did not proliferate but kept alive for following 72 h by this treatment with GEM. After GEM treatment, cells were washed well, cultured in regular culture medium, and harvested at 24-h intervals. WT1 mRNA in cell homogenates was analyzed using qRT-PCR, and WT1mRNA levels were normalized relative to those of 18S ribosomal RNA (18S). **d** NF-kB suppresses GEM-induced up-regulation of WT1 mRNA. MIAPaCa2 cells (6×10^4/well) were seeded in 24-well culture plates. After 24 h, medium was exchanged for media containing GEM (0 or 30 ng/ml) and/or NF-kB blocking peptide (50 μM) or control peptide (50 μM). WT1 mRNA levels were quantified after 24-h incubation using qRT-PCR. $^*P < 0.01$

Changes in WT1 mRNA expression levels were also examined in MIAPaCa2 cells following in vitro treatment with various other chemotherapeutic agents. Oxaliplatin, Doxorubicin, and five-fluorouracil showed significant enhancement of WT1 mRNA expression, but cisplatin and irinotecan did not (Suppl. 1). Because GEM is the standard drug used to treat human PC, its effect on human PC cells was studied thereafter.

In vivo up-regulation of WT1 mRNA in tumor tissue by treatment of MIAPaCa2-bearing SCID mice with GEM

In order to clarify whether in vivo treatment of tumor cells with GEM induces an enhancement of WT1 mRNA expression, SCID mice implanted subcutaneously with MIAPaCa2 cells were treated with a clinical dosage of GEM. We observed a significant increase in the levels of WT1 mRNA 48 h after injection of GEM (Fig. 3).

GEM treatment shifts localization of WT1 from the nucleus to the cytoplasm

We used immunoblot analysis to examine the levels of WT1 protein in MIAPaCa2 cells cultured in the absence or presence of GEM. Relative to untreated cells, WT1 protein levels in GEM-treated MIAPaCa2 cells were augmented; however, after 36 h of cell culture, levels of WT1 protein diminished in both untreated and GEM-treated cells (Fig. 4a). This decline in WT1 protein levels was rescued by treatment with the proteasome inhibitor MG-132, indicating that WT1 protein is susceptible to proteasomal degradation (Fig. 4b).

Confocal microscopy images demonstrate that WT1 protein is primarily located in nuclei of untreated cells (Fig. 5a). However, in MIAPaCA2 cells treated with GEM, localization of WT1 protein shifted to the cytoplasm and the intensity of WT1 immunofluorescence in the nucleus decreased

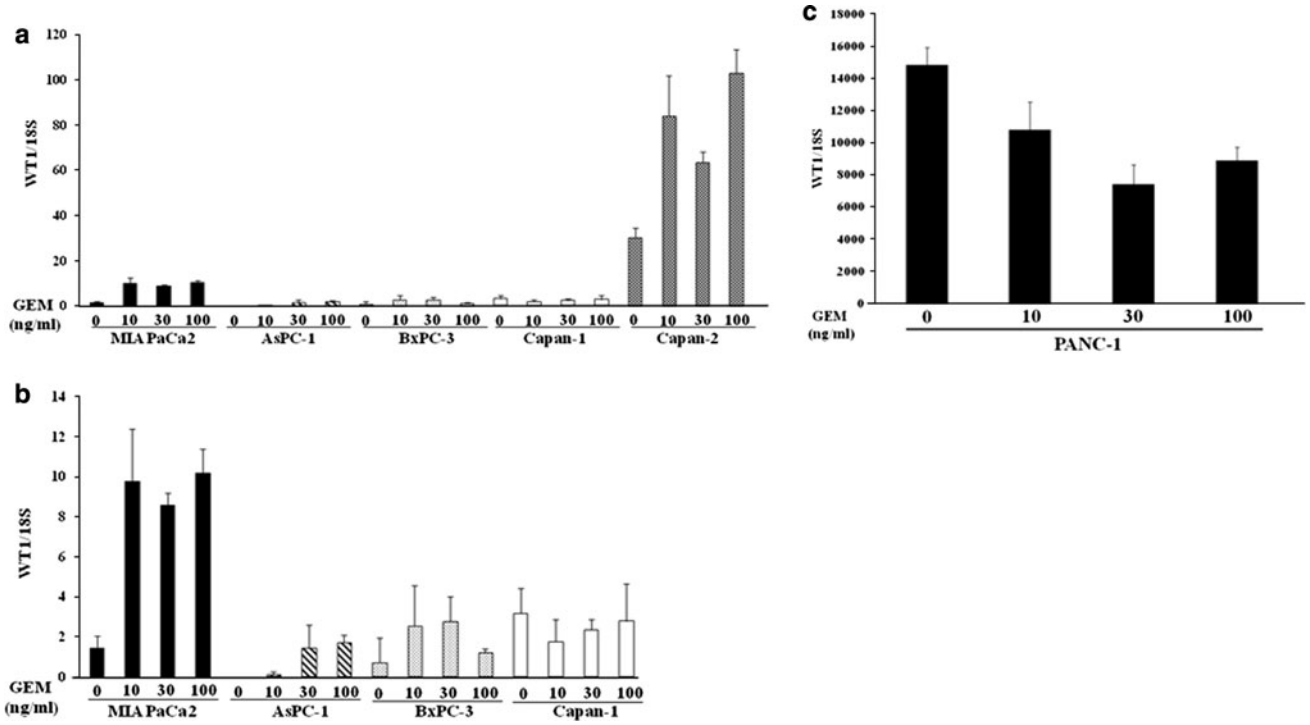

Fig. 2 **a** Up-regulation of WT1 mRNA levels in various human PC cell lines following GEM treatment. Human PC cells (1×10^6 MIAPaCa2, AsPC-1, BxPC-3, Capan-1 or Capan-2) were seeded in 10-cm culture plates. After 24-h incubation, medium was changed to media containing GEM (10, 30 or 100 ng/ml). After 48 h, we used qRT-PCR to quantify the relative ratio of WT1 to 18S mRNA levels in each cell line ($n = 3$). **b** GEM-induced up-regulation of WT1 mRNA in human PC cells with low basal levels of WT1 mRNA (MIAPaCa2, AsPC-1, BxPC-3 and Capan-1). To illustrate these results, we replotted data from (**a**) to represent a considerably narrower range of mRNA level ratios (0–14) on the *y*-axis. (**c**) Expression of WT1 mRNA in human PC cells with high basal levels of WT1 mRNA (PANC-1). To illustrate the results, we plotted data to represent a considerably wider range of mRNA level ratios (0–18,000) on the *y*-axis

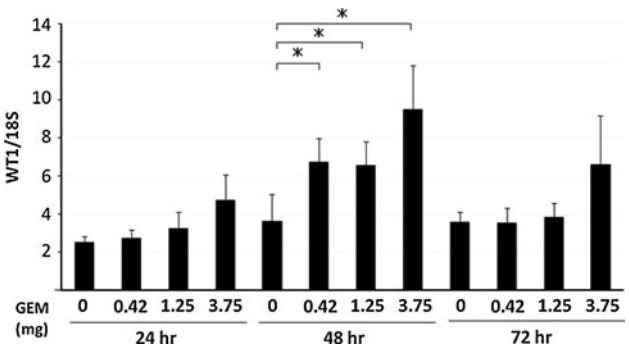

Fig. 3 Tumors in PC-bearing SCID mice treated with GEM show increased WT1 mRNA levels. Ten days after subcutaneous inoculation of SCID mice with 5×10^6 MIAPaCa2 cells (formation of approximately 1-cm diameter tumors), mice were injected intraperitoneally with GEM (0, 0.42, 1.25 and 3.75 mg/mouse). Tumors were resected every 24 h thereafter, and relative levels of WT1 mRNA were quantified using qRT-PCR ($n = 3$). Duplicate trials of the same protocol showed similar results. $^*P < 0.01$

(Fig. 5a). Decline in WT1 protein levels following GEM treatment was also observed in immunoblot analyses of the nuclear fraction of treated MIAPaCa2 cells (Fig. 5b).

Enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide following GEM treatment

Figure 6a shows typical standard curve obtained with increasing quantities of WT1 antigenic peptide. The data indicate a linear relation over a wide range (0–1,000 pmol) of analyte amount with correlation coefficients greater than 0.99. The data in the Fig. 6b demonstrate the sensitivity as well as the noise background of the LC–MS/MS. The noise background is less than 1 cps. The signal from injection of 10 pmol of this peptide spiked to MIAPaCa2 cells is approximately 16 cps, giving an S/N ratio of approximately 16. The low noise background and signal of 10 pmol of this peptide indicated the extrapolated limit of detection is less than 0.8 pmol on column under S/N = 2.

The level of the WT1 antigenic peptide was estimated among MHC class I binding peptides from MIAPaCa2 cells treated with either PBS or GEM to 6.49 pmol/10^8cell or 8.78 pmol/10^8cell, respectively. GEM treatment increased the presentation of HLA-A*2402-restricted WT1 antigenic peptide on MIAPaCa2 cells.

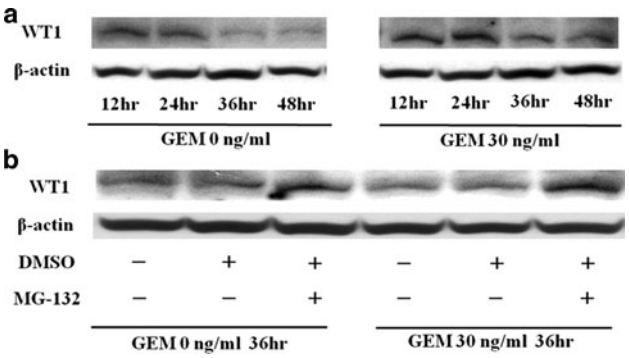

Fig. 4 **a** WT1 protein is degraded by proteasomal enzymes. Twenty-four hours after 3×10^5 MIAPaCa2 cells/well were seeded in 6-well culture plates, medium was exchanged from untreated to media containing GEM (0 or 30 ng/ml). Expression of WT1 protein in the cells was analyzed every 12 h thereafter from immunoblots described in Sect. "Materials and methods". **b** Protease inhibitors block WT1 degradation. Twenty-four hours after incubating MIAPaCa2 cells with GEM (0 or 30 ng/ml), MG-132 in DMSO or DMSO alone was added to each well at a concentration of 5 μM and 0.05%, respectively. Treated and control cells (in 0.05% DMSO alone) were incubated for 12 h before harvesting cells for immunoblot analysis of WT1 and beta-actin proteins

GEM-treated PC cells are killed efficiently by effector cells transduced with genes encoding a WT1-specific T-cell receptor

The susceptibilities of untreated and GEM-treated MIAPaCa2 cells to WT1-specific cytotoxic effector T cells were compared. The cytotoxic effect of WT1-specific effector cells on MIAPaCa2 cells was enhanced significantly when PC cells were treated with either 10 or 30 ng/ml of GEM for 48 h (Fig. 7). Notably, effector cell cytotoxicity was not enhanced by treatment of PC cells with 100 ng/ml of GEM, although this high dose of GEM was more toxic to PC cells than 10 or 30 ng/ml. Up-regulation of MHC class I in MIAPaCa2 cells by GEM treatment that possibly provides the similar results was not observed (data not shown).

Discussion

In the present study, we demonstrate that expression of WT1 mRNA in human PC cells is enhanced by treatment

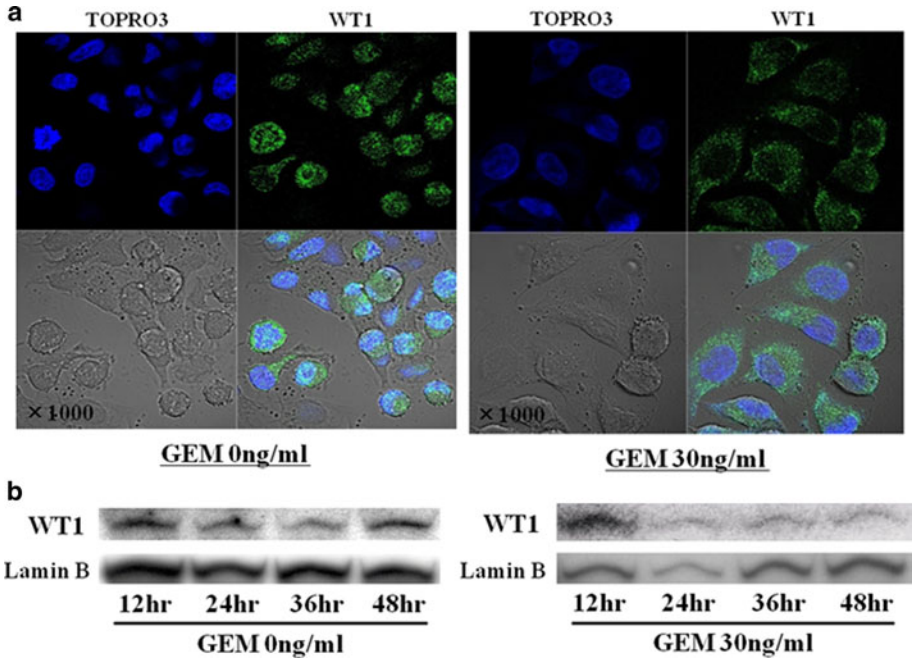

Fig. 5 **a** GEM treatment shifts WT1 protein localization from nucleus to cytoplasm. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, untreated medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). After 24-h incubation, cells were fixed with paraformaldehyde, followed by nuclear staining with TO-PRO-3 iodide (*blue color*) and detection of WT1 with rabbit anti-WT1 polyclonal antibody and anti-rabbit IgG conjugated with fluorescein isothiocyanate (*green color*). Stained cells were observed using confocal microscopy (original magnification ×1,000). **b** GEM treatment diminishes nuclear localization of WT1 protein. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). At 12-hour intervals thereafter, nuclei were isolated and WT1 protein levels of nuclear extracts were analyzed on immunoblots as described in Sect. "Materials and methods"

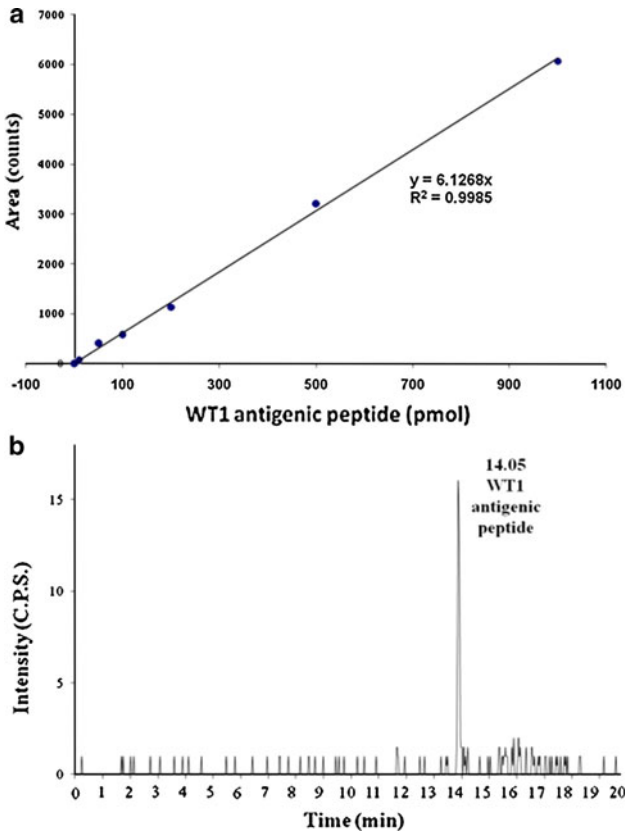

Fig. 6 **a** Standard curve for HLA-A*2402 restricted WT1 antigenic peptide. **b** Trace of MRM signal during LC–MS/MS analysis of spiked HLA-A*2402-restricted WT-1 antigenic standard peptide (10 pmol) in MIAPaCa2 cells

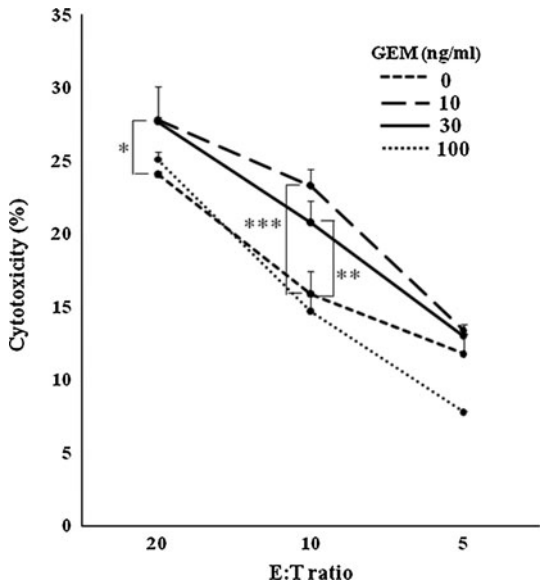

Fig. 7 WT1-specific CTLs kill GEM-treated MIAPaCa2 cells efficiently. MIAPaCa2 cells pretreated with 0, 10, 30, or 100 ng/ml GEM for 48 h were labeled with ^{51}Cr. ^{51}Cr release assays were used to measure the cytotoxic activity of WT1-specific effector cells against untreated or GEM-pretreated MIAPaCa2 cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

with GEM. MIAPaCa2 cells demonstrating GEM-mediated enhancement of WT1 mRNA levels did not proliferate but maintained stable numbers of viable cells with impaired growth by continuous treatment with low-dose GEM as well as short treatment with high-dose GEM. WT1 is a transcription factor with oncogenic potential, in that it can induce malignant cellular phenotypes, suppress apoptosis, and promote cell proliferation [15]. We hypothesize that up-regulation of WT1 levels in PC cells aids cell survival by conferring chemoresistance against GEM's toxic effects.

Based on the fact that GEM-mediated augmentation of WT1 mRNA expression was attenuated by addition of an NF-kB blocking peptide in the culture, activation of NF-kB also appears to play a significant role in WT1 enhancement. NF-kB is known to be active in many malignant tumors and has been implicated in cellular resistance to cytotoxic agents and escape from apoptosis [26]. Previous reports demonstrate that GEM activates NF-kB [27] and that the ensuing regulatory cascade activates the WT1 gene downstream [28]. Human PC cell lines with high NF-kB activity are resistant to GEM [27], and that silencing or suppression of NF-kB increases the sensitivity of PC cells to GEM and induces apoptosis [29–31].

It is of note and interest that some chemotherapeutic agents other than GEM showed capability on up-regulation of WT1mRNA expression. Especially, treatment with oxaliplatin (L-OHP) induced marked enhancement of WT1mRNA expression. Folfirinox including L-OHP was recently reported to be a more efficient regimen for metastatic pancreatic cancer (10). However, combined treatment with Folfirinox and WT1 targeting immunotherapy might be unsuccessful because of severe leukopenia by Folfirinox. GEM has relatively low hematologic toxicity and thus seems to be preferable for combination therapy with WT1 targeting immunotherapy.

We also observed up-regulation of WT1 mRNA by GEM treatment in vivo. Within 48 h of treating MIAPaCa2-bearing SCID mice with a clinical dose of GEM, steady-state levels of WT1 mRNA in the tumor increased. Despite its rapid disappearance after intraperitoneal injection, the enhancement of WT1 mRNA expression in tumor tissue was significant. Enhancement of WT1 mRNA expression was also observed after in vitro short treatment with GEM. These results suggest strongly that GEM treatment of human PC in a clinical setting might induce up-regulation of WT1 in PC cells.

In the present study, we found that the localization of WT1 protein shifted from nucleus to cytoplasm following GEM treatment. WT1 protein has been shown to undergo nucleocytoplasmic shuttling [32], and the function of WT1 has been suggested to correlate with its cellular location: Siberstein et al. [33] described that WT1 was localized to

the cytoplasm and not to nuclei in some human breast cancers and suggested that such localization may be regulated by alternative splicing of WT1 mRNA. On the other hand, immunohistochemical studies of Nakatsuka et al. [34] demonstrate a majority of WT1-positive tumors with diffuse or granular staining in the cytoplasm. Ye et al. [35] report that phosphorylation of WT1 protein resulted in cytoplasmic retention of WT1, thereby inhibiting DNA binding and altering transcriptional activity. Through the activation of NF-kB, GEM treatment may mediate a similar phosphorylation and translocation of WT1 protein from nucleus to cytoplasm.

In order for MHC class I-restricted antigen to be presented and recognized by antigen-specific CTLs, tumor antigen must be degraded by proteasomal enzymes located in the cytoplasm [36]. Retention of an intra-nuclear tumor antigen such as WT1 in the cytoplasm should favor tumor antigen processing, and in fact, we observed enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide using ESI LC–MS/MS analyses. GEM-treated MIAPaCa2 cells showed greater susceptibility than untreated cells to the cytotoxic effects of WT1-specific CTLs generated by transduction of a gene encoding a WT1-specific T-cell receptor. Importantly, treatment with 10–30 ng/ml of GEM enhanced the susceptibility of MIAPaCa2 cells to CTL, but treatment with 100 ng/ml did not. This phenomenon indicates that the enhanced susceptibility of GEM-treated MIAPaCa2 cells to CTLs is not due to GEM toxicity, but to augmented expression of the WT1 target antigen.

GEM is a nucleoside analog with clinical relevance to the treatment of several solid tumors, including PC; nonetheless, its antitumor effect is limited. We observed significant clinical response in a phase I clinical study of combined treatment against advanced PC using a WT1 peptide vaccine and GEM (manuscript in preparation). The presumed actions of GEM up-regulating WT1 expression in vivo and WT1-specific CTLs killing GEM-treated tumor cells efficiently may prove valuable for the treatment of human PC. It has been reported that GEM may suppress the activity of myeloid-derived suppressor cells that inhibit antitumor immunity [37]. In addition, GEM has been shown to increase the number of dendritic cells in blood without affecting T-cell activity in patients with PC [38]. We propose that combining GEM's proven role as an immunopotentiator with its ability to up-regulate target WT1 expression of PC cells will enhance the susceptibility of PC cells to WT1-specific CTLs. Furthermore, PC cells already acquired GEM resistance by the activation of NF-kB might be injured by WT1-specific CTLs. Assessment of the clinical response to combined therapy with WT1 peptide vaccine and GEM is presently underway.

Acknowledgments This work has been supported by Foundation for Promotion of Cancer Research, Mitsui Life Social Welfare Foundation, Grants-in-Aid for Scientific Research (B) and (C) from the Ministry of Education, Cultures, Sports, Science and Technology of Japan, Grantin-Aid of the Japan Medical Association, Takeda Science Foundation, and Pancreas Research Foundation of Japan.

Conflict of interest There are no financial disclosures of any of the authors to declare.

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文献４

WT1 特異的細胞傷害性 T リンパ球の抗腫瘍効果について

近年、我々を含む幾つかのグループは、WT1 由来ペプチドを認識する特異的リンパ球（CTL）が、HLA クラス I 拘束性に白血病細胞を傷害することを報告している。WT1 は、白血病細胞だけでなく、様々な固形腫瘍で発現している。今回我々は、WT1 特異的 CTL が肺癌の増殖を抑制できるか否かを、in vitro における肺がん細胞株に対する細胞傷害試験と、ヌードマウス移植ヒト肺癌細胞に対する増殖阻害実験により検討した。WT1 遺伝子の転写は、検討したほとんどの肺癌細胞株で検出された。WT1 特異的、HLA- A24 拘束性 CTL クローン（TAK- 1）は、HLA- A24 陽性肺癌細胞株に対する細胞傷害活性を示したが、この HLA をもたない肺がん細胞株は傷害しなかった。このことは、TAK-1 が認識する HLA-A24 陽性肺がん細胞上の標的抗原が、自然に処理された WT1 由来抗原であることを示唆している。HLA- A24 陽性肺癌細胞株を移植したヌードマウスに対する TAK- 1 の養子免疫療法は、癌細胞の増殖抑制効果や生存期間の延長をもたらした。これらの知見は、WT1 が普遍的な腫瘍関連抗原であることと、WT1 を標的とする免疫療法が、白血病と同様に肺癌に対する治療の選択肢となることを強く示唆している。

Antilung Cancer Effect of WT1-specific Cytotoxic T Lymphocytes

Masanori Makita, Akio Hiraki, Taichi Azuma, et al.

Clin Cancer Res 2002;8:2626-2631.

Antilung Cancer Effect of WT1-specific Cytotoxic T Lymphocytes[1]

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ABSTRACT

We and other groups have recently reported that CTLs that specifically recognize a peptide derived from WT1 lyse leukemia cells in a HLA class I-restricted manner. Because WT1 is expressed in various solid tumors as well as in leukemic cells, we investigated whether WT1-specific CTLs can also inhibit the growth of lung cancer by examining their cytotoxic activity against lung cancer cell lines *in vitro* and their inhibitory effect on the growth of human lung cancer cells engrafted into nude mice. The *WT1* transcript was detected in most of the lung cancer cell lines examined. A WT1-specific, HLA-A24-restricted CTL clone (designated TAK-1) exhibited cytotoxicity against lung cancer cell lines bearing HLA-A24 but did not lyse cells lacking this HLA. This suggests that the target antigen for TAK-1 on HLA-A24-positive lung cancer cells is the naturally processed WT1 peptide. Adoptive transfer of TAK-1 into nude mice that had been engrafted with a HLA-A24-positive lung cancer cell line resulted in inhibition of cancer cell growth and prolonged survival. These findings strongly suggest that WT1 is a universal tumor-associated antigen and that WT1-targeting immunotherapy offers a potentially effective treatment option for lung cancer as well as leukemia.

Received 2/01/02; revised 4/17/02; accepted 4/19/02.
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[1] Supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Welfide Medicinal Research Foundation, the Sagawa Cancer Research Foundation, the Cancer Research Foundation, the Yamanouchi Foundation for Research on Metabolic Disorders, the Uehara Memorial Foundation, and the Takeda Science Foundation.
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INTRODUCTION

Despite recent progress in conventional chemotherapeutic, radiotherapeutic, and surgical approaches to anticancer treatment, the 5-year survival rate for most patients with lung cancer is still low, especially in those with advanced disease. New therapeutic strategies are therefore required. One recent development in this field is immunotherapy targeted against lung cancer-associated antigens. The identification of tumor-associated antigens is essential to the development of efficacious immunotherapy; however, to date, only a limited number of human lung cancer-associated antigens have been identified.

The *WT1* gene encodes a zinc finger transcription factor (1), and WT1 binds to the early growth response-1 DNA consensus sequence present in various growth factor gene promoters (2). Although WT1 was initially shown to act as a transcriptional repressor, its specific functions in normal and neoplastic tissues remain to be fully elucidated. During normal ontogenesis, the *WT1* gene is expressed in a time- and tissue-dependent manner, mainly in the fetal kidney, testis, ovary, and supportive structures of mesodermal origin (3, 4). In contrast, in adults, *WT1* gene expression is limited to very few tissues, including the splenic capsule and stroma, the Sertoli cells of the testis, and the granulosa cells of the ovary (5, 6). With regard to malignant cells, it has been reported that most patients with leukemia aberrantly overexpress WT1, regardless of their leukemia subtype (7–10). WT1 has also recently been reported to be expressed in various solid tumors, including lung cancer (11, 12). These findings suggest that WT1 would be an attractive target for immunotherapy against various solid tumors as well as leukemia.

Recently, we have succeeded in establishing $CD8^+$ CTL clones that recognize a 9-mer peptide derived from WT1 (13, 14). These WT1-specific CTLs efficiently lyse HLA-A24-positive but not HLA-A24-negative leukemic cells and do not lyse normal cells, regardless of their HLA-A24 expression status. Because WT1 is expressed in most types of lung cancer, we investigated whether WT1-specific CTLs can inhibit the growth of lung cancer cells by examining the cytotoxic activity of our WT1-specific CTL clone against lung cancer cells *in vitro* and the inhibitory effect of adoptive transfer of this clone on the growth of human lung cancer cells engrafted into nude mice. Our results strongly suggest that cell-mediated WT1-targeting immunotherapy will be effective against lung cancer as well as leukemia.

MATERIALS AND METHODS

Lung Cancer Cell Lines. Ten human lung cancer cell lines were used in the study. Of these, OU-LC-A2 was established in our laboratory; the others were kindly provided by Dr. E. Nakayama (Okayama University Graduate Schools, Okayama, Japan). All of the cell lines were cultured in RPMI 1640 supplemented with 10% FCS. The HLA class I genotypes of the cell lines were determined as described previously (15). Their HLA-A24 expression status was examined by flow cy-

tometry using an anti-HLA-A24 mAb[3] (One Lambda, Canoga Park, CA) with mouse IgG as the control.

RT-PCR for WT1 Gene Expression Analysis. A quantitative RT-PCR procedure for determining *WT1* gene expression in lung cancer cells was performed as described previously (8), with some modifications. Briefly, 2 μg of total RNA were isolated from each sample and converted into cDNA in 30 μl of reaction buffer. PCR was performed for 22–35 cycles for quantification of *WT1* mRNA and for 16 cycles for quantification of β-*actin* mRNA. All analyses were performed in duplicate. To normalize differences in RNA degradation between the individual samples and in RNA loading for the RT-PCR procedure, the *WT1* expression level for a particular sample was defined as its *WT1* gene expression level divided by its β-*actin* gene expression level. The *WT1* gene expression level of K562 leukemia cells, which strongly express *WT1*, was designated 1.0, and the levels for the experimental samples were calculated relative to this value.

Generation of the WT1 Peptide-specific $CD8^+$ CTL Clone. A CTL clone that specifically recognizes a peptide derived from WT1, designated TAK-1, was generated as described previously (13). Briefly, WT1-derived peptides containing the binding motifs for HLA-A24 were synthesized. The peptide sequences of these synthetic peptides, designated WT1-T1, WT1-T2, WT1-T3, and WT1-T4, were as follows: (*a*) WT1-T1, QMTSQLECM (residues 228-236); (*b*) WT1-T2, CMTWNQMNL (residues 235-243); (*c*) WT1-T3, DFKDCERRF (residues 356-364); and (*d*) WT1-T4, RWPSCQKKF (residues 417-425). DCs were generated from peripheral blood monocytes as described previously (16) and treated with MMC (Kyowa Hakko, Tokyo, Japan). One million $CD8^+$ T lymphocytes were isolated from the peripheral blood lymphocytes of the same donor and cultured with 1×10^5 MMC-treated DCs in RPMI 1640, supplemented with 10% human AB-type serum and 5 ng/ml recombinant human IL-7 (Genzyme, Boston, MA) and containing 10 μM of one of the WT1 synthetic peptides, in a 16-mm well. After culturing for 7 days, half of the medium was exchanged for fresh IL-7-supplemented medium, and the cells were restimulated by adding 1×10^5 autologous MMC-treated DCs and 10 μM of the WT1 peptide. After an additional 7 days of culture, the cells were restimulated in the same way, except that no IL-7 was added. Four days later, recombinant human IL-2 (10 units/ml; Boehringer Mannheim, Mannheim, Germany) was added to each well. The cytotoxicity of the growing cells was then examined, and cells that exerted a cytotoxic effect on a WT1 peptide-loaded autologous B-LCL were cloned using a limiting dilution method as described previously (17).

Cytotoxicity Assays. Chromium-51 release assays were performed as described previously (18). Briefly, 1×10^4 ^{51}Cr ($Na_2{}^{51}CrO_4$; New England Nuclear, Boston, MA)-labeled target cells, suspended in 100 μl of RPMI 1640 supplemented with 10% FCS (assay medium), were seeded into round-bottomed microtiter wells and incubated with or without synthetic peptide for 2 h. In some experiments, the target cells were incubated with an anti-HLA class I framework mAb (w6/32; American Type Culture Collection, Manassas, VA) or an anti-HLA-DR mAb (L243; American Type Culture Collection) at an optimal concentration (10 μg/ml) for 30 min to determine whether cytotoxicity was restricted by HLA class I. Various numbers of effector cells, suspended in 100 μl of assay medium, were added to the well and incubated for 4 h, and then 100 μl of supernatant were collected from each well.

To determine whether WT1-specific CTLs lyse lung cancer cells via recognition of the WT1 peptide, which is naturally processed in lung cancer cells and expressed in the presence of HLA-A24, cold target inhibition assays were performed as follows. Autologous LCL cells were incubated with one of the WT1-derived peptides at a concentration of 10 μM for 2 h. After extensive washing, the peptide-loaded cells were used as cold target cells. Various numbers of these cells were incubated with 5×10^4 cytotoxic effector cells for 1 h, and then 5×10^3 ^{51}Cr-labeled lung cancer cells were added to the wells. Cytotoxicity assays were then performed as described above. The percentage of specific lysis was calculated as follows: (experimental release cpm − spontaneous release cpm)/(maximal release cpm − spontaneous release cpm).

Adoptive Immunotherapy Model. Six-week-old BALB/c-*nu/nu* female mice were purchased from Nippon Clea (Tokyo, Japan) and maintained at the Animal Center of the Ehime University School of Medicine. For xenografting, 5×10^6 human lung cancer cells were injected s.c. into the right midabdomen of each mouse. Four days later, 5×10^6 WT1 peptide-specific CTL clone cells, suspended in PBS, were injected i.v. via the orbital vein. Control mice received an equal volume of PBS alone i.v. Each CTL-treated and control group contained five mice. Each week, the mice were injected with an additional dose of 5×10^6 CTL clone cells or PBS alone, and the groups were monitored for tumor growth until all of the mice in the control group had died. The tumors were measured at 10-day intervals, and tumor volumes were calculated using the ellipsoid formula (length × width × height).

Statistical Analysis. The significance of differences between the mean values for the CTL-treated and control groups was analyzed using the Mann-Whitney exact test. Differences were considered significant at $P < 0.05$.

RESULTS

WT1 Expression in Lung Cancer Cell Lines. WT1 expression levels in the human lung cancer cell lines were determined by quantitative RT-PCR and calculated relative to the *WT1* expression level in the human leukemia cell line K562. Because relative *WT1* expression levels in most normal tissues are $<10^{-6}$, levels of $>10^{-5}$ were considered positive. As shown in Table 1, 2 of the 10 lung cancer cell lines (LC99A and Sq-1) expressed high levels of *WT1*, whereas 4 (RERF-LC-AI, LK79, LK87, and QG56) expressed intermediate levels of WT1 (10^{-1} to 10^{-3}), and 3 (11-18, LC65A, and OU-LC-A2) expressed low levels of WT1 (10^{-3} to 10^{-5}). *WT1* expression in the remaining cell line (PC-9) was considered negative ($<10^{-6}$).

[3] The abbreviations used are: mAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; DC, dendritic cell; MMC, mitomycin C; IL, interleukin; LCL, lymphoblastoid cell line.

Table 1 Cytotoxicity of TAK-1 against various lung cancer cell lines

Target cells	*WT1* expression level	Origin[a]	HLA-A24	% Specific lysis[b] E:T ratio 20:1	10:1	5:1
LC99A	2.0×10^{-1}	lc	+	90.1	65.5	48.0
LK79	7.9×10^{-2}	sc	+	73.8	54.7	35.1
RERF-LC-AI	8.0×10^{-2}	sq	+	68.6	50.0	31.4
11-18	7.0×10^{-4}	ad	+	50.1	34.5	25.0
PC-9	5.0×10^{-7}	ad	+	9.6	4.8	3.2
Sq-1	1.9×10^{-1}	sq	−	5.8	4.9	5.7
LC65A	1.0×10^{-4}	sc	−	6.1	3.6	5.4
QG56	2.0×10^{-2}	sq	−	7.8	8.0	6.6
LK87	5.0×10^{-2}	ad	−	6.8	2.9	3.8
OU-LC-A2	4.0×10^{-5}	ad	−	0.0	0.3	0.1

[a] ad, adenocarcinoma; lc, large cell carcinoma; sc, small cell carcinoma; sq, squamous cell carcinoma.
[b] The cytotoxicity of TAK-1 against the various lung cancer cell lines in the absence of the WT1 peptide was determined by 4-h ^{51}Cr release assays at E:T ratios of 20:1, 10:1, and 5:1.

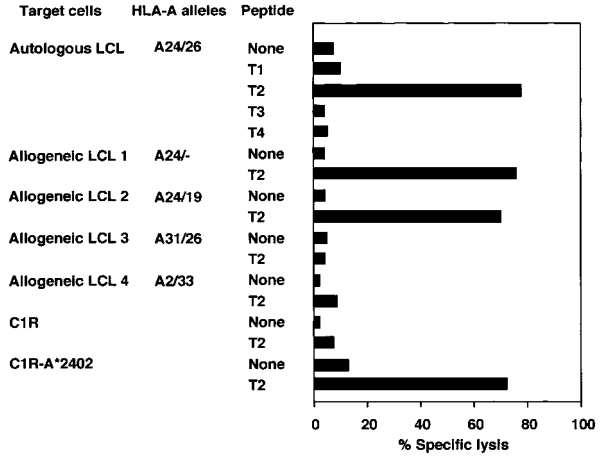

Fig. 1 HLA-A24-restricted and WT1-T2 peptide-specific cytotoxicity of TAK-1. The cytotoxicity of TAK-1 against unloaded LCLs and LCLs loaded with the WT1 peptide or control peptides was determined by 4-h ^{51}Cr release assays at an E:T ratio of 10:1. The results shown represent the mean of triplicate experiments.

Cytotoxic Activity of the WT1 Peptide-specific CTL Clone. We previously established a WT1 peptide-specific, HLA-A24-restricted CTL clone, designated TAK-1. Flow cytometric analysis demonstrated that >99% of TAK-1 cells were $CD3^+$, $CD4^-$, $CD8^+$, and $CD56^-$. The TAK-1 clone cells had been stored frozen in liquid nitrogen and were thawed for use in the present study. To confirm that the freezing and thawing procedures had not affected the antigen specificity and HLA restriction of the TAK-1 cells, we first investigated their cytotoxic activity against peptide-loaded and unloaded cells. As shown in Fig. 1, TAK-1 lysed autologous LCLs that had been loaded with the WT1-T2 peptide but was not cytotoxic to unloaded LCLs or to those loaded with WT1-T1, WT1-T3, or WT1-T4. TAK-1 appeared to be cytotoxic only to HLA-A 24-positive allogeneic LCLs and the HLA-A*2402 transfectant cell line C1R-A*2402 (but not its parent cell line, C1R) in the presence of WT1-T2 peptide, as demonstrated previously (13). These data findings confirmed that TAK-1-mediated cytotoxicity is WT1-T2 peptide specific and restricted by HLA-A24.

Cytotoxicity of TAK-1 against Lung Cancer Cell Lines. HLA-A24 expression in the lung cancer cell lines was examined by flow cytometry and genotyping. Among the 10 cell lines examined, 5 cell lines appeared to be positive for HLA-A24 (HLA-A*2402).

The cytotoxicity of TAK-1 against the lung cancer cell lines is shown in Table 1. TAK-1 exhibited cytotoxicity only against HLA-A24-positive lung cancer cell lines and not against HLA-A24-negative cells. Interestingly, the degrees of TAK-1-mediated cytotoxicity against the lung cancer cell lines reflected their WT1 expression levels. That is, TAK-1 lysed LC99A, in which WT1 is expressed at the highest WT1 level, most efficiently. The LK79, RERF-LC-AI, and 11-18 cell lines, which expressed WT1 at intermediate or low levels, were also efficiently lysed by TAK-1, but the degrees of cytotoxicity against these cell lines were not as great as the degree of cytotoxicity against LC99A. In contrast, PC-9, in which WT1 expression was undetectable by quantitative RT-PCR, was hardly lysed by TAK-1. These results strongly suggest that WT1-specific CTLs can lyse lung cancer cells via recognition of their WT1-derived peptide in the context of HLA-A24.

Inhibition of TAK-1-mediated Cytotoxicity against Lung Cancer Cells by an Anti-HLA Class I mAb. To confirm that the cytotoxicity of TAK-1 against lung cancer cells is restricted by HLA-A24 status, inhibition assays using mAbs were performed. As shown in Fig. 2, the addition of an anti-HLA class I framework mAb, but not a control HLA-DR mAb, to the assay medium inhibited the cytotoxic effect of TAK-1 on HLA-A24-positive lung cancer cells. Taken together with the data shown in Table 1, these findings demonstrate that the cytotoxicity of TAK-1 against lung cancer cells is restricted by HLA-A24.

Cold Target Inhibition Assays. To further confirm that the cytotoxicity of TAK-1 against lung cancer cells was mediated by specific recognition of endogenously processed WT1, we performed cold target inhibition experiments. As shown in Fig. 3, the addition of WT1-T2-loaded autologous LCLs decreased the cytotoxicity of TAK-1 against LC99A and RERF-

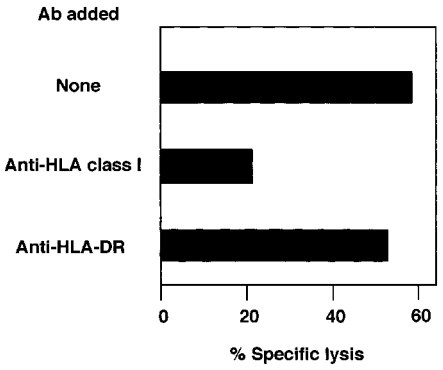

Fig. 2 Inhibition of TAK-1-mediated cytotoxicity against lung cancer cells by a HLA class I mAb. The cytotoxic effect of TAK-1 on the HLA-A24-positive lung cancer cell line LC99A was inhibited by adding an anti-HLA class I framework mAb, but not an anti-HLA-DR mAb, to the culture medium. The results shown represent the mean of triplicate experiments.

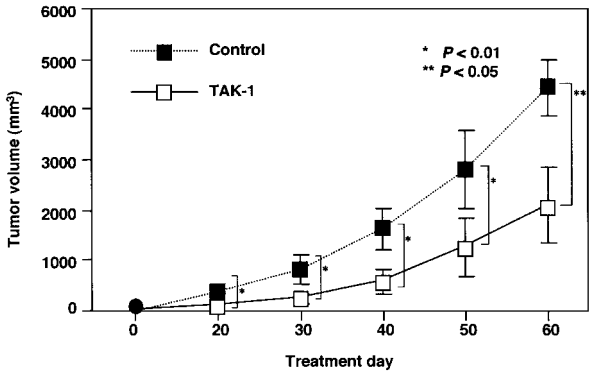

Fig. 4 Effect of adoptive transfer of TAK-1 on the growth of human lung cancer cells engrafted into nude mice. Mean tumor volumes in nude mice receiving weekly treatment with TAK-1 or vehicle (PBS) alone are shown. Mean tumor volumes ± SD are expressed in cubic millimeters.

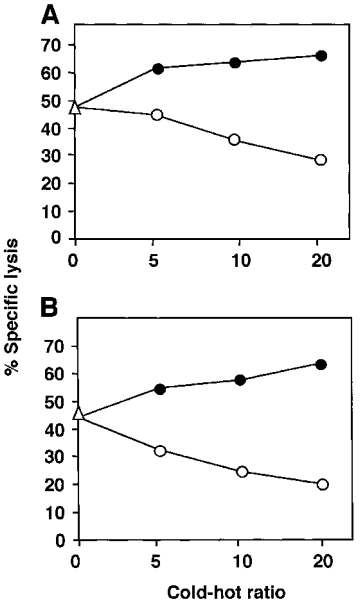

Fig. 3 Cold target inhibition assays. ^{51}Cr-labeled LC99A (*A*) or ^{51}Cr-labeled RERF-LC-AI (*B*) cells (5×10^3 cells) were mixed with various numbers of unlabeled autologous LCLs (○) or HLA-A24-negative allogeneic LCLs (●) that had been loaded with the WT1-T2 peptide. The cytotoxicity of TAK-1 against the mixture of ^{51}Cr-labeled and unlabeled target cells was determined by 4-h ^{51}Cr release assays at an effector: ^{51}Cr-labeled target cell ratio of 10:1. The results shown represent the mean of triplicate experiments.

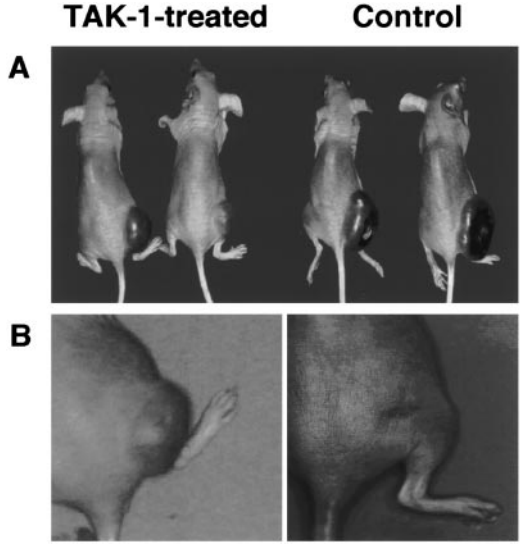

Fig. 5 Inhibition of human lung cancer cell growth in nude mice after adoptive transfer of TAK-1. *A*, representative illustrations of tumors in mice with human lung cancer cell xenografts treated with TAK-1 (*left*) or PBS alone (*right*). *B*, complete tumor regression was achieved in one mouse treated with TAK-1. The photographs were taken on day 55 (*left*) and day 115 (*right*).

LC-AI, whereas the addition of WT1-T2-loaded HLA-A24-negative LCLs had no effect on cytotoxicity. These findings strongly suggest that WT1 is naturally processed in lung cancer cells, expressed in the context of HLA-A24, and recognized by WT1-specific CD8$^+$ CTLs.

Inhibition of Lung Cancer Cell Growth in Nude Mice by TAK-1. Because TAK-1 showed highly WT1-specific cytolytic activity against lung cancer cells *in vitro*, its therapeutic efficacy was assessed in an experimental lung cancer xenograft model. After engrafting nude mice with a s.c. dose of a human lung cancer cell line, we performed adoptive transfer experiments in which TAK-1 or PBS alone was administered. The resulting lung cancer cell line growth curves are shown in Fig. 4. Adoptive transfer of TAK-1 resulted in significant inhibition of tumor growth. Representative examples of tumor formations in TAK-1- and PBS-treated mice are shown in Fig. 5. The tumors in the mice treated with TAK-1 were significantly smaller than those in control mice (Fig. 5*A*), and complete tumor regression occurred in one mouse that received TAK-1 (Fig. 5*B*).

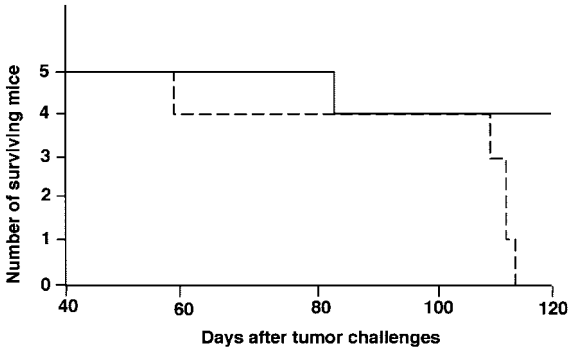

Fig. 6 Effect of adoptive transfer of TAK-1 on the survival of mice engrafted with human lung cancer cells. The mice received weekly i.v. injections of TAK-1 (*solid line*) or PBS alone (*dashed line*).

Survival of Mice Engrafted with Human Lung Cancer Cells and Treated with or without TAK-1. Survival curves for nude mice that had been engrafted with a human lung cancer cell line and received adoptive transfer of TAK-1 or control treatment with PBS alone are shown in Fig. 6. A significant difference was observed between the two groups in that all of the mice in the control group died within 113 days, whereas only one mouse in the TAK-1-treated group died within the observation period. Because the growth rate of the TAK-1 cells in the *in vitro* culture slowed as time progressed and sufficient numbers of TAK-1 cells to continue transfer could not be obtained, the adoptive transfer procedure was discontinued on day 120.

DISCUSSION

In the present study, we demonstrated that WT1-specific CTLs exert a strong cytotoxic effect against human lung cancer cells in a HLA class I-restricted manner. The cytotoxicity of WT1-specific CTLs against lung cancer cells was shown to be mediated by recognition of the WT1-derived peptide in the context of HLA-A24 in several ways. Firstly, the WT1-specific CTL clone TAK-1 lysed HLA-A24-positive but not HLA-A24-negative lung cancer cells, and its cytotoxicity was inhibited by an anti-HLA class I mAb. Secondly, the degree of cytotoxic activity exhibited by TAK-1 against various lung cancer cell lines reflected the WT1 expression level in the particular cell line. Thirdly, cold target inhibition assays demonstrated that the addition of WT1 peptide-loaded autologous cells but not HLA-mismatched allogeneic cells inhibited the cytotoxic effect of TAK-1 against lung cancer cells. Although previous studies by us and other groups have demonstrated that WT1-specific CTLs exert a cytotoxic effect against leukemic cells in a HLA class I-restricted manner (13, 19, 20), the present study is the first to demonstrate that WT1 protein is naturally processed in human lung cancer cell lines, becomes apparent in the context of HLA class I molecules, and is recognized by $CD8^+$ CTLs.

It has previously been shown that WT1 is essential for the formation of the urogenital system during fetal development (21); however, in adults, WT1 expression is extremely limited, occurring in only a few tissues and at a low level. To ensure that WT1-targeting immunotherapy is safe, it is essential to demonstrate that WT1-specific CTLs are not cytotoxic to normal tissues. In this respect, Oka *et al.* (22) generated WT1-specific CTLs by vaccinating mice with a WT1-derived peptide. The mice remained quite healthy, and histopathological investigations demonstrated no adverse effects on any of the organs examined, including the kidney and bone marrow. These data strongly suggest that WT1 is a tumor-specific antigen and that WT1-targeting immunotherapy for lung cancer can be performed safely.

There are several methods of delivering cell-mediated cancer immunotherapy, including peptide vaccination (23), immunization with DCs that have been pulsed with a peptide or tumor cell lysate (24, 25), immunization with DC/tumor cell hybrids (26), and adoptive transfer of tumor-specific CTLs (27). One of the most important factors governing the success of adoptive transfer is the effectiveness of CTL migration toward the tumor cells. In the present study, we examined the distribution of transferred WT1-specific CTLs in nude mice that had been engrafted with human lung cancer cells and sacrificed 6–12 h after subsequent CTL transfer. Immunohistochemistry demonstrated that only a few human T lymphocytes were detectable in the lung cancer lesions (data not shown). This might have been due to the relatively small number of CTLs transferred. Interactions between species-specific adhesion molecules and chemokine systems may also be important for effective migration of CTLs toward tumor cells. It is therefore possible that, during the present study, the human CTLs could not accumulate to effective levels in the human lung cancer lesions because the environment surrounding the lung cancer cells was not human but murine. Gene therapy targeted against adhesion molecules and chemokines might be able to overcome this problem and increase the efficacy of adoptive immunotherapy using CTLs. On the other hand, our findings also suggest that transfer of a large number of CTLs will not necessarily be needed to exert an antitumor effect *in vivo* because the CTLs may be actively recruited to tumor lesions. The most effective number of antitumor CTLs for transfer to cancer patients will need to be determined in future studies.

In conclusion, we have demonstrated that WT1-specific CTLs can efficiently lyse human lung cancer cells in a HLA class I-restricted manner. We also found that adoptive transfer of WT1-specific CTLs inhibits the growth of human lung cancer cells engrafted into nude mice. To the best of our knowledge, this is the first report to describe the efficacy of WT1-specific CTLs against human solid tumors. The present findings may contribute to the development of novel immunotherapeutic methods for lung cancer and suggest that vaccination with a WT1-derived peptide or with WT1-coding DNA (28) and adoptive immunotherapy using WT1-specific CTLs may provide an effective treatment option for solid tumors as well as leukemia.

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文献 5

HLA-A*2402 結合残基改変型 9-mer WT1 ペプチドによるヒト WT1 特異的細胞傷害性 T リンパ球の誘導増強

Wilms 腫瘍遺伝子 WT1 は、白血病や肺がん、乳がんなどの様々な固形腫瘍など、多くの悪性腫瘍に高発現しており、それらの白血病化や腫瘍化にかかわっている。WT1 タンパクは、マウスやヒトにおける有望な腫瘍抗原候補と報告されている。本研究では、天然型の HLA-A*2402 拘束性 9 mer WT1 ペプチド（CMTWNQMNL;a.a. 235-343）内の、上流のアンカーモチーフとなる 2 番目のアミノ酸をメチオニン（M）からチロシン（Y）に置換した（CYTWNQMNL）。その結果、9-mer WT1 ペプチドの HLA-A*2402 分子への結合親和性は $1.82X10^{-5}$ から $6.40X10^{-7}$M に増強した。この結合親和性増強から予想されたことであるが、改変型 9-mer WT1 ペプチド（CYTWNQMNL）は、天然型 9-mer WT1 ペプチド（CMTWNQMNL）と比較して、HLA-A*2402 陽性健常ボランティア末梢単核球から、WT1 特異的細胞傷害性 T リンパ球（CTL）をより効率的に誘導した。この改変型 9-mer WT1 ペプチドで誘導した CTL は、天然型 9-mer WT1 ペプチドをパルスした CIR-A*2402 細胞や、WT1 を内在性に発現する白血病細胞、肺がん細胞株を、WT1 特異的並びに HLA-A*2402 拘束性に傷害した。これらの結果は、改変型 9-mer WT1 ペプチドが、天然型 9-mer WT1 ペプチドと比較し免疫原性が強く、効率的に WT1 特異的 CTL を誘導することができること、さらに、改変型 9-mer WT1 ペプチドによって誘導された CTL は WT1 を内在性に発現する腫瘍細胞を効率的に認識し殺していることを示している。したがって、改変型 9-mer WT1 ペプチドを用いた腫瘍免疫治療は、HLA-A-2402 陽性の白血病や固形腫瘍患者に対する効果的な治療となることが期待される。

Cancer Immunol Immunother (2002) 51: 614–620
DOI 10.1007/s00262-002-0328-9

ORIGINAL ARTICLE

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Enhanced induction of human WT1-specific cytotoxic T lymphocytes with a 9-mer WT1 peptide modified at HLA-A*2402-binding residues

Received: 14 February 2002 / Accepted: 14 July 2002 / Published online: 18 October 2002

Abstract The Wilms' tumor gene WT1 is overexpressed in most types of leukemias and various kinds of solid tumors, including lung and breast cancer, and participates in leukemogenesis and tumorigenesis. WT1 protein has been reported to be a promising tumor antigen in mouse and human. In the present study, a single amino-acid substitution, M→Y, was introduced into the first anchor motif at position 2 of the natural immunogenic HLA-A*2402-restricted 9-mer WT1 peptide (CMTWNQMNL; a.a. 235–243). This substitution increased the binding affinity of the 9-mer WT1 peptide to HLA-A*2402 molecules from 1.82×10^{-5} to 6.40×10^{-7} M. As expected from the increased binding affinity, the modified 9-mer WT1 peptide (CYTWNQMNL) elicited WT1-specific cytotoxic T lymphocytes (CTL) more effectively than the natural 9-mer WT1 peptide from peripheral blood mononuclear cells (PBMC) of HLA-A*2402-positive healthy volunteers. CTL induced by the modified 9-mer WT1 peptide killed the natural 9-mer WT1 peptide-pulsed CIR-A*2402 cells, primary leukemia cells with endogenous WT1 expression and lung cancer cell lines in a WT1-specific HLA-A*2402-restricted manner. These results showed that this modified 9-mer WT1 peptide was more immunogenic for the induction of WT1-specific CTL than the natural 9-mer WT1 peptide, and that CTL induced by the modified 9-mer WT1 peptide could effectively recognize and kill tumor cells with endogenous WT1 expression. Therefore, cancer immunotherapy using this modified 9-mer WT1 peptide should provide efficacious treatment for HLA–A*2402-positive patients with leukemias and solid tumors.

Keywords Cancer immunotherapy · Cytotoxic T lymphocyte · Wilms' tumor gene · WT1

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Introduction

Peptides presented with major histocompatibility complex (MHC) class I molecules on the cell surface and derived from self-proteins that are expressed at elevated levels by cells in a wide variety of human malignancies theoretically provide potential targets for cytotoxic T lymphocyte (CTL)-based immunotherapy of cancer. However, since the proteins are self-antigens, their specific CTL repertoire may be tolerated, as recently demonstrated for p53 protein [1, 2]. This tolerance principally concerns immunodominant epitopes with high MHC affinity, but not cryptic epitopes with low MHC affinity [3]. Therefore, the antitumor CTL repertoire that remains available for recruitment by

peptide-based vaccination must be specific for the tumor-associated antigen (TAA)-derived cryptic epitopes with low to intermediate MHC affinity, which can be considered as the best candidates for vaccination. However, a major drawback to using epitopes with low to intermediate MHC affinity for immunotherapy is their poor immunogenicity. In fact, a correlation between immunogenicity and MHC binding affinity and/or stability of MHC/peptide complexes for class I epitopes has been demonstrated [1, 2, 4]. Previous reports have demonstrated that peptides from viral and tumor-derived proteins that bound with higher affinity to HLA class I molecules elicited stronger CTL immune responses than those that bound with lower affinity to these molecules [5, 6]. Enhancement of the immunogenicity of epitopes with low to intermediate MHC affinity may therefore be achieved by improvement of their binding affinity and/or MHC/peptide complex stability, resulting in the induction of CTL specific for the epitopes.

The finding that the wild-type WT1 gene was overexpressed in various kinds of human malignancies and exerted an oncogenic function led us to consider WT1 protein as a promising tumor antigen for cancer immunotherapy. Indeed, mice immunized with WT1 peptides with anchor motifs needed for binding to MHC class I molecules [7, 8], or vaccinated with WT1 plasmid DNA elicited WT1-specific CTL and rejected challenge from WT1-expressing tumor cells [9]. Furthermore, in vitro stimulation of peripheral blood mononuclear cells (PBMC) with 9-mer WT1 peptides could induce WT1-specific HLA-A*0201- or HLA-A*2402-restricted CTL [10, 11, 12]. Since HLA-A*2402 is the major type of HLA-A allele that is present in approximately 60% of the Japanese population, identification of WT1 epitopes for HLA-A*2402 is important for the clinical application of WT1 peptide-based immunotherapy for Japanese patients.

In the present study, we have reported that an amino-acid substitution, M→Y, at HLA-A*2402-binding anchor position 2 into an immunogenic, natural 9-mer WT1 peptide, CMTWNQMNL [12], significantly increased the binding affinity of the modified 9-mer WT1 peptide to HLA-A*2402 molecules, and that the modified 9-mer WT1 peptide could thus more efficiently induce WT1-specific HLA-A*2402-restricted CTL than the natural 9-mer WT1 peptide.

Materials and methods

Cell lines

CIR, a B cell-lymphoblastic cell line with the loss of expression of HLA-A and -B molecules and CIR-A*2402, CIR transfected with the HLA-A*2402 gene, were a kind gift from M. Takiguchi (Kumamoto University, Kumamoto, Japan). TAK-1 was a WT1 peptide (CMTWNQMNL)-specific, HLA-A*2402-restricted CTL clone that could recognize and kill tumor cells with endogenous WT1 expression [12]. Four human lung cancer cell lines, RERF-LCAI (HLA-A*2402, WT1-expressing), LC1sq (HLA-A*2402/1101, WT1-expressing), 11–18 (HLA-A*0201/2402, WT1-non-expressing), and LK87 (HLA-A*0207/1101, WT1-expressing) were established as described previously [13]. All cell lines apart from CIR-A*2402 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, N.Y.). CIR-A*2402 was cultured in RPMI 1640 medium supplemented with 10% FBS and 500 μg/ml hygromycin B (Calbiochem, La Jolla, Calif.).

Synthetic peptides

Peptides were synthesized by Fmoc chemistry and purified by HPLC with a C18 Microbondasphere column (Waters, Milford, Mass.). Peptide concentrations were determined by Micro BCA assay using bovine serum albumin (BSA) as standard.

Binding affinity of WT1 peptides to HLA-A*2402 molecules

Binding affinity of peptides to HLA-A*2402 molecules was measured by an acid stripping method as described previously [14] with a minor modification. Briefly, CIR-A*2402 cells were exposed to acid buffer (131 mM citric acid, 66 mM sodium phosphate; 290 m osmol, pH 3.3) for 1 min, and then acid buffer containing these cells was neutralized by adding Dulbecco's modified Eagle's medium (DMEM) containing 0.5% BSA. Cells were then washed and resuspended at a cell density of 2×10^6 cells/ml in DMEM containing 0.5% BSA and 200 nM human β_2-microglobulin (Sigma, St. Louis, Mo.). Fifty microliters of cell suspension were added to microwells with 50 μl of the medium containing grading concentrations of WT1 peptides, and the mixture was incubated at room temperature for 4 h. Cells were then washed, stained with FITC-conjugated anti-HLA class I monoclonal antibody (mAb), 7A12 (a kind gift from Dr. U. Haemmerling) [15] that specifically reacted to HLA-A24 molecules, and was analyzed by FACS (Becton Dickinson). Kilodalton values were calculated by using a melanoma antigen, pmel15 peptide (AYGLDFYIL) [16] that could bind to HLA-A*2402 molecules as standard, as described previously [17].

In vitro induction of WT1-specific CTL

WT1 peptide-specific CTL were generated as described previously with a minor modification [18]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated with informed consent from HLA-A*2402-positive healthy volunteers by centrifugation on Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) gradient. CTL cultures were initially established by plating PBMC in 24-well plates (2×10^6 cells/ml; 2 ml/well) in complete medium containing 45% RPMI 1640 medium, 45% AIM-V medium, 10% heat-inactivated human AB serum, 1×non-essential amino acid (Gibco), 25 ng/ml 2-mercaptoethanol, 50 IU/ml penicillin, and 50 mg/ml streptomycin. Five days later, recombinant interleukin-2 (rIL-2; kindly donated by Shionogi, Osaka, Japan) was added to the culture at a concentration of 30 IU/ml, and the cultured cells were stimulated by using 10 μM WT1 peptide-pulsed autologous PBMC as antigen-presenting cells (APC). After stimulation (3 times at weekly intervals), cells were harvested and replated as responder cells in new 24-well plates at a concentration of 1×10^6 cells/well. Then WT1 peptide-pulsed autologous PBMC were added to the wells as stimulator cells at responder/stimulator ratios of 1/2 in the presence of 30 IU/ml rIL-2. After culture for 6 days, the cells were assayed for cytotoxic activity as effector cells.

^{51}Cr release cytotoxicity assay

Target cells (1×10^4 cells in 100 μl) labeled with ^{51}Cr were added to wells containing varying numbers of effector cells (100 μl) using U-bottomed 96-well plates. After 4 h incubation at 37°C, cells were centrifuged and 100 μl supernatant was collected and measured for radioactivity. Percentage of specific lysis (% specific lysis) was calculated as follows: percentage lysis = (cpm experimental

release–cpm spontaneous release)/(cpm maximal release–cpm spontaneous release)×100. In order to confirm WT1-specific killing, cytotoxicity of the modified 9-mer WT1 peptide-induced CTL against HLA-A*2402-positive WT1-non-expressing 11–18 cells pulsed with either 1 μM natural WT1 peptide or an irrelevant peptide, pmel15, was assayed at an effector:target (E/T) ratio of 10. Cold inhibition of modified 9-mer WT1 peptide-induced CTL cytotoxicity was determined by measuring cytotoxicity against ^{51}Cr-labeled HLA-A*2402-positive WT1-expressing RELF-LCAI cells in the presence of non-labeled CIR-A*2402 cells (the same number as that of the hot target cells) pulsed with either the natural 9-mer WT1 peptide or an irrelevant peptide, pmel15, at an E/T ratio of 10. For the cytotoxicity blocking assay using mAb, a mixture of 2×10^5 E cells and 1×10^4 ^{51}Cr-labeled T cells (100 μl/well) was incubated with serially diluted mAb at 37°C for 4 h, and then the percentage specific lysis was measured.

Results

Binding affinity of modified WT1 peptide to HLA-A*2402 molecules

To enhance the immunogenicity of WT1 peptides by increasing their binding affinity to HLA-A*2402 molecules, an amino-acid substitution was introduced into a natural immunogenic 9-mer WT1 peptide, CMTWNQMNL (a.a. 235–243), which was the only peptide with immunogenicity among the 4 9-mer WT1 peptides (a.a. 228–236, 235–243, 356–364, and 417–425) with anchor motifs for binding to HLA-A*2402 molecules [12]. This natural immunogenic HLA-A*2402-restricted 9-mer WT1 peptide had only one HLA-A*2402-binding anchor motif, L, at position 9, but did not have another anchor motif at position 2. Therefore, an amino-acid substitution, M→Y at position 2, was introduced into this WT1 epitope, and the binding affinity of this modified 9-mer WT1 peptide to HLA-A*2402 molecules was then evaluated (Table 1). The binding affinity was 1.82×10^{-5} M for the natural 9-mer WT1 peptide and 6.40×10^{-7} M for the modified 9-mer WT1 peptide; thus the modified 9-mer WT1 peptide had a much higher HLA-A*2402-binding affinity than the natural 9-mer WT1 peptide.

In vitro induction of WT1-specific CTL with the modified 9-mer WT1 peptide

PBMC were isolated from 3 healthy volunteers and stimulated 3 times at weekly intervals with either the natural or modified 9-mer WT1 peptide-pulsed autologous PBMC. Six days after the last stimulation, the cultured cells were tested for cytotoxic activity against either the natural 9-mer WT1 or the irrelevant (pmel15) peptide-pulsed CIR-A*2402 (Fig. 1). Although both the natural and modified 9-mer WT1 peptides induced natural 9-mer WT1 peptide-specific CTL from the PBMC of all 3 healthy volunteers, the modified 9-mer WT1 peptide more strongly elicited natural 9-mer WT1 peptide-specific CTL than the natural 9-mer WT1 peptide.

Table 1 Binding affinity of a modified 9-mer WT1 peptide to HLA-A*2402 molecules

Peptides	Amino acid	Sequence	kd (M)
Natural	a.a. 235–243	C<u>M</u>TWNQMN<u>L</u>	1.82×10^{-5}
Modified	a.a. 235–243	C**<u>Y</u>**TWNQMN<u>L</u>	6.40×10^{-7}

Underlined capital letters indicate anchor positions [20]; *bold capital letter* indicates a substituted amino acid

WT1-specific HLA-A*2402-restricted killing of tumor cells with endogenous WT1 expression by CTL induced by the modified 9-mer WT1 peptide

Bulk WT1-specific CTL induced by in vitro stimulation with either the natural or modified 9-mer WT1 peptide from the PBMC of healthy volunteer no. 1 were examined for cytotoxicity against tumor cells with endogenous WT1 expression (Figs. 2 and 3). CTL induced by the modified 9-mer WT1 peptide more strongly exerted cytotoxic activity against primary leukemia cells with endogenous WT1 expression (Fig. 2) and lung cancer cell lines (Fig. 3) than that induced by the natural WT1 peptide in a WT1-specific HLA-A*2402-restricted manner. To confirm that the cytotoxic activity of the CTL induced by the modified 9-mer WT1 peptide was WT1-specific, the cytotoxicity of the CTL against HLA-A*2402-positive WT1-non-expressing 11–18 cells pulsed with either the natural 9-mer WT1 peptide or the irrelevant peptide pmel15 was assayed (Fig. 4A). The CTL could kill the 11–18 cells pulsed with the natural 9-mer WT1 peptide, but could not kill the cells pulsed with the irrelevant peptide. Furthermore, a cold target inhibition cytotoxicity assay was performed by measuring the cytotoxicity of the modified 9-mer WT1 peptide-induced

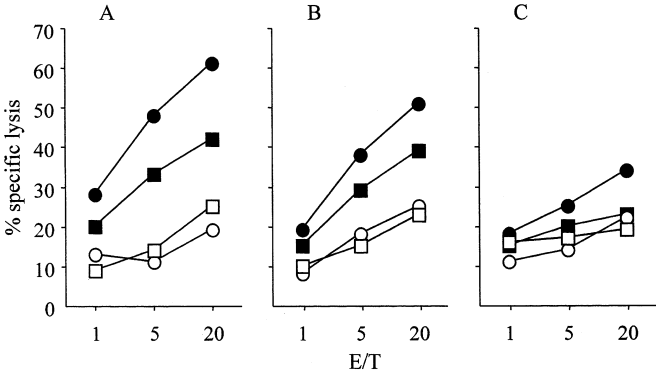

Fig. 1 In vitro induction of WT1-specific CTL. CTL were induced from PBMC by stimulation with either natural (*open squares, closed squares*) or modified (*open circles, closed circles*) 9-mer WT1 peptide and assayed for cytotoxic activity against ^{51}Cr-labeled CIR-A*2402 cells pulsed or not pulsed with 10 μM natural 9-mer WT1 peptide at the indicated E/T ratios in triplicate. *Closed* and *open symbols* indicate that CIR-A*2402 target cells were pulsed or not pulsed with the natural 9-mer WT1 peptide, respectively. **A**, **B** and **C** represent healthy volunteers nos. 1, 2 and 3, respectively

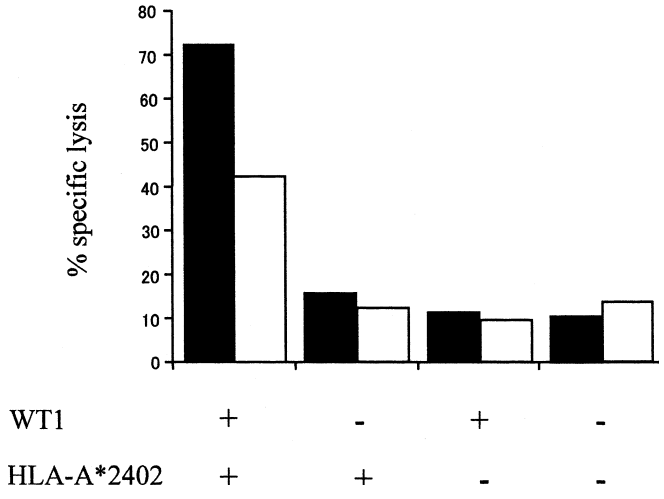

Fig. 2 WT1-specific HLA-A*2402-restricted killing of primary leukemic cells with endogenous WT1 expression by 9-mer WT1 peptide-induced CTL. The cyototoxicity of 9-mer WT1 peptide-induced CTL from healthy volunteer no. 1 against 4 different types of leukemia cells freshly isolated from 4 leukemia patients was determined using ^{51}Cr release assay at an E/T ratio of 20. *Closed* and *open columns* indicate CTL cytotoxicity induced by the modified or natural 9-mer WT1 peptide, respectively

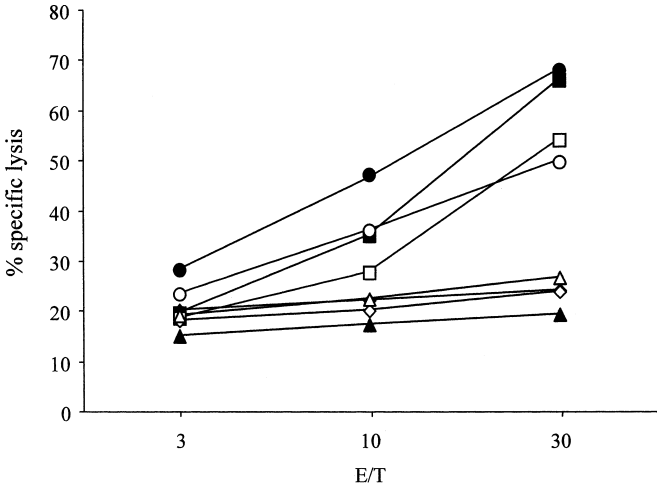

Fig. 3 WT1-specific HLA-A*2402-restricted killing of lung cancer cell lines with endogenous WT1 expression by 9-mer WT1 peptide-induced CTL. Cytotoxicity of modified (*closed symbols*) or natural (*open symbols*) 9-mer WT1 peptide-induced CTL against RERF-LCAI (WT1$^+$, HLA-A*2402$^+$; *closed* and *open squares*), LC1sq (WT1$^+$, HLA-A*2402$^+$, *closed* and *open circles*), 11–18 (WT1$^-$, HLA-A*2402$^+$, *closed* and *open triangles*), and LK87 (WT1$^+$, HLA-A*2402$^-$, *closed* and *open diamonds*) were determined by ^{51}Cr release assays at the indicated E/T ratios in triplicate

CTL against ^{51}Cr-labeled HLA-A*2402-positive WT1-expressing RELF-LCAI cells in the presence of non-labeled HLA-A*2402-positive WT1-non-expressing CIR-A*2402 cells pulsed with either the natural 9-mer WT1 peptide or the irrelevant peptide pmel15 (Fig. 4B). CTL cytotoxicity was inhibited in the presence of CIR-A*2402 cells pulsed with the natural 9-mer WT1 peptide. These results showed that the CTL cytotoxic activity was WT1-specific. To confirm that the CTL activity was carried by CD8-positive cells and restricted to HLA-A*2402, a blocking assay was performed using a mAb against HLA class I, II or CD8 molecules. The CTL cytotoxic activity was blocked in the presence of either anti-HLA class I or anti-CD8 mAb, but not in the presence of anti-HLA class II mAb (Fig. 5). These results confirmed that CD8$^+$ CTL specifically recognized the WT1 peptide/HLA-A*2402 complex and killed target cells.

The modified 9-mer WT1 peptide/HLA-A*2402 complex can be effectively recognized by a natural 9-mer WT1 peptide-specific CTL clone

Whether the modified 9-mer WT1 peptide/HLA-A*2402 complex could be recognized by a natural WT1 peptide (CMTWNQMNL)-specfic CTL clone, TAK1, was tested (Fig. 6). CIR-A*2402 pulsed with various concentrations of either the natural or modified 9-mer WT1 peptide was tested for killing by TAK1 at an E/T ratio of 10. TAK1 had a stronger killing effect on the modified 9-mer WT1 peptide-pulsed CIR-A*2402 than the natural 9-mer WT1 peptide-pulsed CIR-A*2402 in a peptide concentration-dependent manner. Peptide concentrations for 50% specific lysis were 100 μM for the natural 9-mer WT1 peptide and 10 μm for the modified 9-mer WT1 peptide respectively. These results showed that the modified 9-mer WT1 peptide/HLA-A*2402 complex could be more effectively recognized by the natural WT1 epitope-specific CTL clone than the natural 9-mer WT1 peptide/HLA-A*2402 complex.

Discussion

In the present study, the novel modified HLA-A*2402-restricted 9-mer WT1 peptide that could more effectively elicit WT1-specific CTL than the natural 9-mer WT1 peptide was identified. It is well known that the immunogenicity of antigenic peptides is dependent both upon their binding affinity to MHC class I molecules and on the stability of peptide/MHC class I complexes [5, 19]. Previously identified natural HLA-A*2402-restricted 9-mer WT1 peptide CMTWNQMNL did not contain a tyrosine residue, considered to be a favorable amino acid, as the first anchor motif at position 2 [20], although the WT1 peptide did contain a favorable amino acid, L, as the second anchor motif at position 9. As expected from the above findings, the binding affinity of this natural 9-mer WT1 peptide to HLA-A*2402 molecules was as low as 1.82×10^{-5} M. The most straightforward way to increase HLA class I-binding affinity of peptides not having an anchor motif at the primary anchor position is to introduce amino acids favorable for binding to MHC class I molecules in this position. This straightforward method was successfully used to enhance the binding affinity of two gp100 (a.a. 154–162

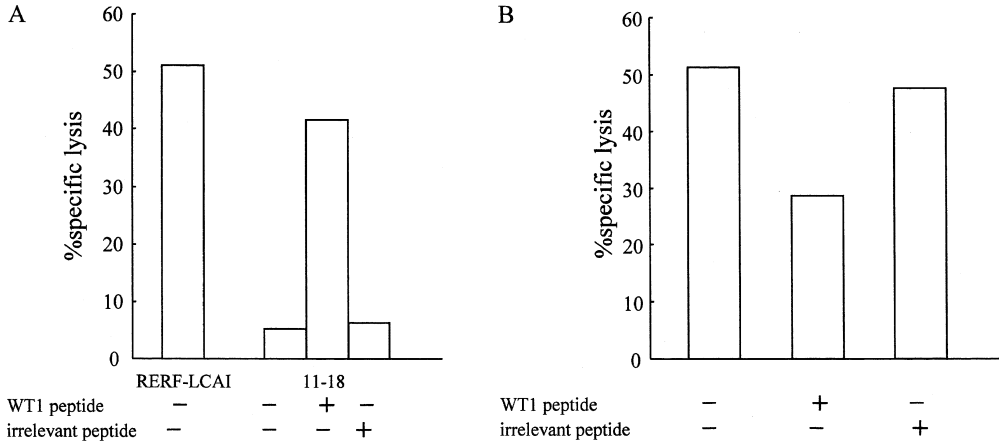

Fig. 4 Natural 9-mer WT1-peptide-specific cytotoxic activity of modified 9-mer WT1 peptide-induced CTL. **A** Cytotoxicity of modified 9-mer WT1 peptide-induced CTL against RERF-LCAI cells (HLA-A*2402$^+$,WT1$^+$), 11–18 cells (HLA-A*2402$^+$,WT1$^-$), or 11–18 cells pulsed with either 1 μM natural 9-mer WT1 peptide or 9-mer irrelevant peptide, pmel15, was determined by ^{51}Cr release assays at an E/T ratio of 10 in triplicate.**B** Cold target inhibition assay. Cytotoxicity of the modified 9-mer WT1 peptide-induced CTL against ^{51}Cr-labeled HLA-A*2402-positive WT1-expressing RERF-LCAI cells was assayed in the presence of non-labeled HLA-A*2402-positive WT1-non-expressing CIR-A*2402 cells pulsed with either natural 9-mer WT1 peptide or 9-mer irrelevant peptide, pmel15, at an E/T ratio of 10 in triplicate

and a.a. 209–217) and one Mart-1 (a.a. 26–34) peptides to HLA-A*2402 molecules [18, 21, 22, 23, 24]. In our present study, this rule was applied to enhance the binding affinity to HLA-A*2402 molecules of the natural 9-mer WT1 peptide that did not contain an anchor motif at position 2. Since the anchor motif at position 2 was Y for HLA-A*2402 molecules [20], a single amino-acid substitution, M→Y, was introduced into position 2. As expected, the binding affinity of the modified 9-mer WT1 peptide to HLA-A*2402 molecules was increased from as low as 1.82×10^{-5} M to as high as 6.40×10^{-7} M. These results demonstrated that the existence of an anchor motif at position 2 is important for the acquisition of sufficient affinity to bind to HLA-A*2402 molecules.

The modified 9-mer WT1 peptides with higher binding affinity to HLA-A*2402 molecules compared to the natural 9-mer WT1 peptide more effectively induced CTL against both the natural 9-mer WT1 peptide-pulsed cells and tumor cells with endogenous WT1 expression than the natural 9-mer WT1 peptide. These results reinforce the hypothesis that an increase in binding affinity of peptides to MHC class I molecules promotes immunogenicity of the peptides. The modified 9-mer WT1 peptide should constitute a promising tumor antigen for WT1 peptide-based cancer immunotherapy.

TAK1, a CTL clone induced by the natural 9-mer WT1 peptide, could recognize and more effectively kill the modified 9-mer WT1 peptide-pulsed target cells than the natural 9-mer WT1 peptide-pulsed target cells. This indicated that CTL precursors against the WT1 protein, if present in patients with WT1-expressing tumor cells could effectively recognize the modified 9-mer WT1 peptide/HLA-A*2402 complex present on APC, and be stimulated to expand sufficiently to kill the WT1-

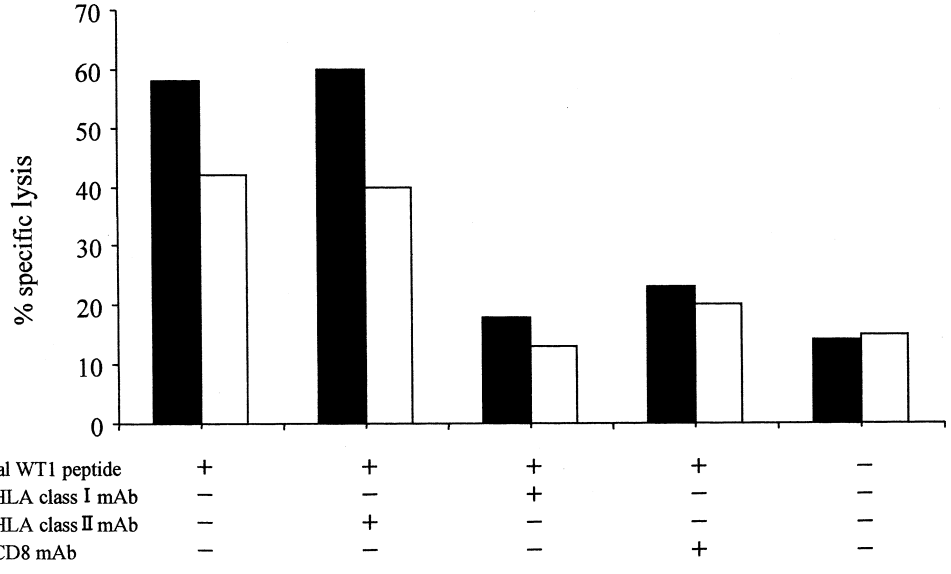

Fig. 5 Blocking of cytotoxicity of WT1-specific CTL by mAb against HLA class I or CD8. The 9-mer WT1 peptide-induced CTL were preincubated with mAb against HLA class I, II, or CD8, and cytotoxicity against natural 9-mer WT1 peptide-pulsed CIR-A*2402 cells was examined by ^{51}Cr release assay at an E/T ratio of 20. *Closed* and *open columns* indicate the CTL cytotoxicity induced by the modified or natural 9-mer WT1 peptide, respectively

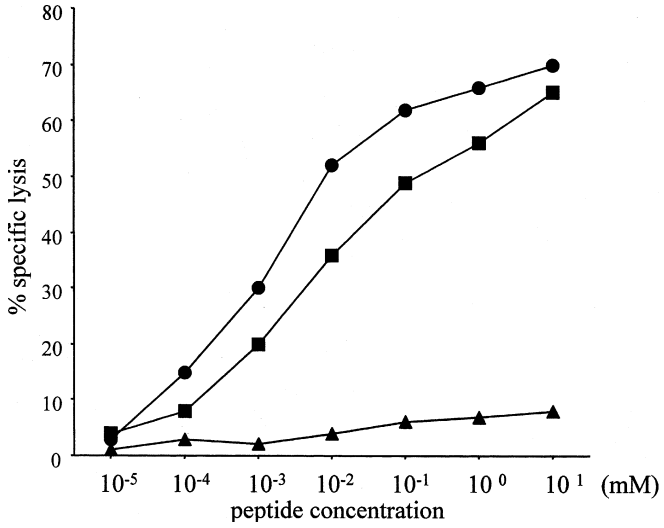

Fig. 6 WT1 peptide concentration-dependent cytotoxicity of TAK1. TAK1 cytotoxicity against CIR-A*2402 cells pulsed with the indicated concentrations of the natural (*closed squares*), or modified 9-mer WT1 peptide (*closed circles*), or not pulsed with peptide (*closed triangles*) was determined by ^{51}Cr release assay at an E/T ratio of 10

expressing tumor cells, when the modified 9-mer WT1 peptide was administered to patients for cancer immunotherapy.

In clinical practice, the question arises with WT1 peptide-based cancer immunotherapy of whether patients with WT1-expressing tumor cells have CTL precursors against the WT1 protein. Our preliminary data showed that the modified 9-mer WT1 peptide could more effectively induce WT1-specific CTL than the natural 9-mer WT1 peptide from the PBMC of some leukemia patients (data not shown). Therefore, CTL precursors for the modified 9-mer WT1 peptide should not be abolished, and thus the latter should be one of the most promising candidate peptides for cancer immunotherapy.

The Wilms' tumor gene WT1 encodes a zinc finger protein that functions as a transcription or splicing factor [25, 26]. This gene produces at least 4 isoform proteins (17AA ± /KTS ±). The WT1 gene has been reported to be overexpressed in acute leukemias, chronic myelogeneous leukemia, myelodysplastic syndromes [27, 28] and in various types of solid tumors and tumor cell lines [29]. The WT1 gene was initially defined as a tumor suppressor gene. However, we proposed that the WT1 gene exerted an oncogenic rather than a tumor suppressor function both in leukemias and in solid tumors on the basis of the following findings [30]: (1) overexpression of the wild-type WT1 gene in leukemias and various types of solid tumors; (2) growth inhibition of leukemia and solid tumor cells by treatment with WT1 antisense oligomers [31]; and (3) blockage of differentiation, but promotion of growth in hematopoietic progenitor cells transfected with the wild-type WT1 gene [32]. This oncogenic function of the WT1 gene means that loss of WT1 expression in leukemia and solid tumor cells induces the discontinuation of cell proliferation, and thus that escape of the tumor cells from immune surveillance as a result of the loss of the WT1 antigen is difficult to achieve.

Mice immunized with either the WT1 peptide or DNA elicited WT1-specific CTL, and rejected challenge from WT1-expressing tumor cells [7, 9, 11]. In these immunized mice, bone marrow and kidney, both of which physiologically expressed WT1, were histopathologically normal [7, 11]. Furthermore, the numbers of CFU-GEMM, CFU-GM, CFU-G, CFU-M, and BFU-E in the bone marrow of the immunized mice were similar to those in the nonimmunized mice. In man, WT1-specific HLA-A*0201 or-A*2402-restricted CTL induced by in vitro stimulation of PBMC with immunogenic 9-mer WT1 peptides specifically killed WT1-expressing leukemia clones, but did not influence the colony formation of normal CFU-GM, CFU-G, and CFU-M [11, 12]. These findings strongly indicate that WT1-specific CTL ignored normal healthy WT1-expressing cells, and thus that the WT1 protein is a favorable target for cancer immunotherapy.

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文献 6

Wilms 腫瘍遺伝子(WT1)産物由来天然型ペプチドに特異的なヒト細胞傷害性 T リンパ球反応

Wilms 腫瘍遺伝子産物 WT1 は、ほとんどすべての急性骨髄性白血病、急性リンパ性白血病並びに慢性骨髄性白血病患者のリンパ芽球だけでなく、様々な固形腫瘍細胞において高発現している転写因子である。このことは、WT1 遺伝子が、白血病の発生と腫瘍発生のいずれにおいても重要な役割を果たしていることを示唆している。今回我々は、WT1 が白血病や固形腫瘍に対する免疫療法の標的となり得るかどうかを検討した。HLA-A2.1 結合 anchor motif を有する 4 つの 9-mer WT1 ペプチドを合成した。このうち Dp126 と WH187 の 2 つは、抗原提示能を欠損した T2 細胞に関連するトランスポーターを用いた binding assay により、HLA-A2.1 分子と結合することが確認された。HLA-A2.1 陽性の健常ドナー末梢血単核球を、この 2 つの WT1 ペプチドをパルスした T2 細胞を用いて in vitro にて繰り返し刺激したところ、WT1 ペプチドパルス T2 細胞を HLA-A2.1 特異的に傷害する CTL が誘導された。この CTL は WT1 発現 HLA-A2.1 陽性白血病細胞を特異的に傷害したが、WT1 発現 HLA-A2.1 陰性白血病細胞や WT1 非発現 HLA-A2.1 陽性 B リンパ芽球様細胞は傷害しなかった。これらのデータは、WT1 ペプチドに対する特異的なヒト CTL 反応を初めて証明したものであり、白血病や固形腫瘍に対する WT1 ペプチドを用いた養子 T 細胞療法やワクチン治療の根拠となるものである。

Immunogenetics (2000) 51:99–107

ORIGINAL PAPER

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Human cytotoxic T-lymphocyte responses specific for peptides of the wild-type Wilms' tumor gene (*WT1*) product

Received: 1 March 1999 / Revised: 27 August 1999

Abstract The product of the Wilms' tumor gene *WT1* is a transcription factor overexpressed not only in leukemic blast cells of almost all patients with acute myeloid leukemia, acute lymphoid leukemia, and chronic myeloid leukemia, but also in various types of solid tumor cells. Thus, it is suggested that the *WT1* gene plays an important role in both leukemogenesis and tumorigenesis. Here we tested the potential of WT1 to serve as a target for immunotherapy against leukemia and solid tumors. Four 9-mer WT1 peptides that contain HLA-A2.1-binding anchor motifs were synthesized. Two of them, Db126 and WH187, were determined to bind to HLA-A2.1 molecules in a binding assay using transporter associated with antigen processing-deficient T2 cells. Peripheral blood mononuclear cells from an HLA-A2.1-positive healthy donor were repeatedly sensitized in vitro with T2 cells pulsed with each of these two WT1 peptides, and $CD8^+$ cytotoxic T lymphocytes (CTLs) that specifically lyse WT1 peptide-pulsed T2 cells in an HLA-A2.1-restricted fashion were induced. The CTLs also exerted specific lysis against WT1-expressing, HLA-A2.1-positive leukemia cells, but not against WT1-expressing, HLA-A2.1-negative leukemia cells, or WT1-nonexpressing, HLA-A2.1-positive B-lymphoblastoid cells. These data provide the first evidence of human CTL responses specific for the WT1 peptides, and provide a rationale for developing WT1 peptide-based adoptive T-cell therapy and vaccination against leukemia and solid tumors.

Key words Wilms' tumor gene · *WT1* · Cytotoxic T lymphocytes · Tumor-specific antigen · Immunotherapy

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Introduction

It is well understood that tumor-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) constitute the most important effector to the antitumor response (Melief and Kast 1995). CTLs recognize endogenously processed peptides that were presented on the cell surface in association with major histocompatibility complex (MHC) class I molecules. Peptides that bind to a given MHC class I molecule have been shown to share common amino acid motifs (Rammensee et al. 1995). Hence, tumor-specific CTLs can recognize and select the antigenic peptides by scanning peptide sequences, and then kill the tumor cells in an antigenic peptide-specific fashion.

Tumor cells express a variety of peptide antigens produced by the processing of overexpressed or mutated endogenous proteins in association with MHC class I molecules (Melief and Kast 1995). Peptides from

these overexpressed or mutated proteins may be recognized by CTLs as tumor-specific antigens. A large number of tumor antigens that can induce CTL responses have recently been identified using molecular genetic techniques or acid elution of peptides from peptide-MHC complexes. However, only scores of antigens have been identified to be processed naturally and presented on tumor cells in the manner in which CTLs can recognize the antigens. These include fusion proteins (e.g., bcr/abl and ETV6-AML1) (Yotnda et al. 1998a, 1998b), mutated proteins (e.g., connexin 37, p53, and ras) (Gjertsen et al. 1997; Mandelboim et al. 1994; Theobald et al. 1995), self-proteins that are specifically expressed in tumor or embryonic cells but not in normal differentiated cells (e.g., *MAGE* gene family) (Van der Bruggen et al. 1991), tissue-specific differentiation antigens (e.g., tyrosinase) (Wölfel et al. 1994), or overexpressed normal proteins (e.g., HER-2/neu) (Fisk et al. 1995).

The Wilms' tumor gene, *WT1*, was isolated as a gene responsible for a childhood neoplasm, Wilms' tumor, and categorized as a tumor suppressor gene (Call et al. 1990; Gessler et al. 1990). The *WT1* gene encodes a zinc finger transcription factor and represses transcription of growth factor (Drummong et al. 1992; Gashler et al. 1992; Harrington et al. 1993) and growth factor receptor (Werner et al. 1993) genes. We (Inoue et al. 1994, 1996) and others (Brieger et al. 1994; Menssen et al. 1995; Miwa et al. 1992; Miyagi et al. 1993) have shown that the wild-type *WT1* gene is strongly expressed in fresh leukemic cells regardless of the type of disease and plays an essential role in leukemogenesis. Normal hematopoietic progenitor cells physiologically express the *WT1* gene (Baird and Simmons 1997; Inoue et al. 1997; Maurer et al. 1997; Menssen et al. 1997), but the expression levels are less than one-tenth of those in leukemic cells (Inoue et al. 1997). On the basis of accumulated evidence (Inoue et al. 1994, 1998; Tamaki et al. 1996; Tsuboi et al. 1999; Yamagami et al. 1996), we have proposed that the wild-type *WT1* gene performs an oncogenic rather than a tumor suppressor gene function in leukemic cells. These results suggest that the WT1 protein is a promising target for immunotherapy against leukemia.

Furthermore, we have recently reported that the *WT1* gene is expressed in 28 (82%) of 34 solid tumor cell lines examined, including lung cancer, gastric cancer, colon cancer, and breast cancer cell lines, and that growth of the tumor cells is inhibited by the suppression of WT1 protein by the treatment with WT1 antisense oligomers (Oji et al. 1999). These results show that the *WT1* gene plays an important role in cell growth of solid tumors and that the WT1 protein may be an attractive target for immunotherapy against various types of solid tumors.

In the present study, we describe the identification of two WT1-derived peptides that bind to HLA-A2.1 molecules, and induction of human HLA-A2.1-restricted CTLs specific for these peptides.

Materials and methods

Cell lines

T2 cells, which bear the gene *HLA-A*0201*, but express very low levels of cell surface HLA-A2.1 molecules and are unable to present endogenous antigens due to a deletion of most of the MHC class II region including *TAP* (transporter associated with antigen processing) and proteosome genes, were kindly provided by P. Cresswell (Salter and Cresswell 1986; Zweerink et al. 1993). Peptide-pulsed T2 cells were used as stimulators to generate CTLs against WT1 peptides. WT1-expressing TF-1 (erythroleukemia cell line, HLA-A2.1 positive) (Kitamura et al. 1989) and WT1-nonexpressing JY [Epstein-Barr virus (EBV)-transformed B-cell line, HLA-A2.1 positive] (Parham et al. 1977) were kindly provided by T. Kitamura (DNAX Research Institute, Palo Alto, Calif.) and D. Wiley through H.N. Eisen, respectively. WT1-expressing Molt-4 (T-ALL cell line, HLA-A2.1 negative) was kindly provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan) (Minowada et al. 1972).

Peptide synthesis

Nine amino acid-long peptides derived from WT1 protein, which contain anchor motifs for binding to HLA-A2.1 molecules, were synthesized (Table 1). Peptides were either purchased from Sawady Technology (Tokyo, Japan) or synthesized manually using Fmoc chemistry. Peptides were purified by reverse-phase-high-performance liquid chromatograpy with a MicroBONDASPHERE 5-μm C18 column (Waters Japan, Osaka, Japan). Synthesis of the correct peptides was monitored by API IIIE triple quadrupole mass spectrometer (Sciex, Toronto, Canada). Concentrations of peptides were determined by MicroBCA assay (Pierce, Rockford, Ill.) using bovine serum albumin as standard.

Peptide-binding assay

Peptide binding was measured by means of peptide-dependent stabilization of HLA-A2.1 molecules on TAP-deficient, HLA-A2.1-positive T2 cells as described previously (Udaka et al. 1995). Briefly, T2 cells cultured at 26 °C overnight were incubated with a graded amount of peptides at room temperature for 30 min. The temperature was then raised to 37 °C and the incubation was continued for 2 h. After washing, T2 cells were immediately stained with anti-HLA-A2.1 monoclonal antibody (mAb), BB7.2, followed by staining with fluorescein isothiocyanate (FITC)-labeled $F(ab')_2$ fragments of polyclonal goat anti-mouse IgG. Fluorescence on the T2 cells was measured by FACScan (Becton Dickinson, San Jose, Calif.), and the dissociation constant of HLA-A2.1 peptide complex was calculated on the basis of the fluorescence intensity as described previously (Udaka et al. 1995). Briefly, the total number of peptide-receptive sites of the T2 cells was determined by the differences in mean fluorescence intensity (MFI)

Table 1 Binding of WT1 peptides to HLA-A2.1 molecules (bold letters represent anchor motifs)

Peptide name	Amino acid sequence	K_d (M)
WT1 peptides		
Db126	126–134 R **M** F P N A P Y **L**	1.89×10^{-6}
WH187	187–195 S **L** G E Q Q Y S **V**	7.61×10^{-6}
Db235	235–243 C **M** T W N Q M N **L**	$10^{-4}<$
WH242	242–250 N **L** G A T L K G **V**	4.33×10^{-5}
Known epitope peptide		
HIV-1 RT	476–484 I **L** K E P V H G **V**	3.99×10^{-7}

that were measured at either 26 °C or 37 °C in the absence of WT1 peptides and acquired linearly. Fractional occupancy (y) of HLA-A2.1 molecules by WT1 peptides was calculated from the MFI at varying concentrations of WT1 peptides. The log $y/(1-y)$ values were plotted against log peptide concentrations and their correlation was analyzed by linear regression with the least-squares method. The slope represented their relationship, and the peptide concentration that filled half of the sites, the x-intercept at log $y/(1-y)=0$ was determined as log K_d values.

Generation of WT1 peptide-specific cytotoxic T lymphocytes from healthy human peripheral blood mononuclear cells

After informed consent, peripheral blood mononuclear cells (PBMCs) from a healthy HLA-A2.1-positive donor were separated using Ficoll-Hypaque gradient density centrifugation (Organon Teknika, Durham, N.C.). The PBMCs were stimulated in vitro with Db126 or WH187 peptide using the protocol adapted from previous studies (Houbiers et al. 1993; Molldrem et al. 1996). Briefly, T2 cells were washed three times in serum-free medium and incubated with WT1 peptide at concentrations of 20 μg/ml for 2 h. The peptide-pulsed T2 cells were then irradiated with 7500 cGy, washed once, and added to freshly isolated PBMCs in RPMI 1640 supplemented with 10% heat-inactivated human AB serum. After 7 days of co-culture, the second stimulation was performed, and on the following day, recombinant human interleukin-2 of 100 Japan reference units/ml (kindly provided by Shionogi, Osaka, Japan) was added. The cultured cells were maintained by weekly stimulation with peptide-pulsed T2 cells. After a total of four in vitro stimulations, responder cells were tested for cytotoxic activity against peptide-pulsed or WT1-expressing target cells.

Cytotoxicity assay

The cytotoxicity of CTLs against target cells was tested by a standard Europium-release assay as described previously (Visseren et al. 1995). Briefly, target cells were labeled with Europium, followed by intensive washing, and resuspended in the medium. The target cells pulsed or not pulsed with peptides were plated at 1×10^4 cells per well and then effectors were added at various effector to target (E/T) ratios in a final volume of 200 μl. After 3 h of incubation at 37 °C, culture supernatant was collected and specific cytotoxicity was determined as percent specific lysis: [(release from test sample-spontaneous release)/(maximal release spontaneous release)] × 100. Fluorescence emissions of culture supernatant of target cells alone, or of target cells lysed completely by treatment with 1% Triton X-100 were used as measures of spontaneous and a maximal release, respectively.

Antibodies and flow cytometry

FITC-conjugated anti-human CD3, CD4, and CD19 mAbs, and phycoerythrin (PE)-conjugated anti-human CD8 mAb were purchased from Becton Dickinson (Mountain View, Calif.). PE-conjugated anti-human CD56 mAb was purchased from Pharmingen (San Diego, Calif.). Anti-HLA-A2.1 mAb, BB7.2, and anti-H-2K^b mAb, Y3 (both hybridomas were purchased from ATCC) were purified from ascites using DE52 ion exchange chromatography (Whatman, Maidstone, UK). FITC-labeled F(ab′)$_2$ fragments of affinity-purified goat anti-mouse IgG were purchased from Cappel (Aurora, Ohio). The surface phenotype of the cells were determined by flow cytometry as described previously (Molldrem et al. 1996; Inoue et al. 1998). Briefly, cells were stained with labeled or unlabeled mAbs, washed twice, and then stained with the second labeled antibodies in the same way as the first staining with unlabeled antibodies. The stained cells were then analyzed by FACScan (Becton Dickinson).

Results

Peptide synthesis and binding assay

WT1 amino acid sequences were searched for 9-mer peptides that contain the major anchor motifs essential for binding to HLA-A2.1 molecules (Rammensee et al. 1995). Four 9-mer WT1 peptides and one natural CTL epitope peptide, HIV-1 RT 476–484 (Tsomides et al. 1991) with such anchor motifs were synthesized and tested for binding to HLA-A2.1 molecules (Table 1). The binding affinity of naturally occurring CTL epitope peptide HIV-1 RT 476–484 was approximately 4.4 times higher than that of the Db126 peptide, which has the highest binding affinity among four WT1 peptides. Two peptides (Db126 and WH187) exhibiting stronger binding activity were used to generate peptide-specific CTLs.

Induction of CTL responses to WT1 peptides

PBMCs from an HLA-A2.1-positive healthy donor were stimulated with peptide-pulsed T2 cells. T2 cells were incubated with either Db126 or WH187 peptide at 37 °C for 2 h, irradiated, washed once, and co-cultured with the donor PBMCs to induce WT1 peptide-specific CTLs. After 4 weeks of culture with a weekly stimulation with WT1 peptide-pulsed T2 cells, the cultured cells were tested for cytotoxic activity against Europium-labeled T2 cells pulsed or not pulsed with the peptides.

PBMCs stimulated with Db126- or WH187-pulsed T2 cells exerted cytotoxic activity against T2 cells pulsed with the respective peptide (Fig. 1A). Furthermore, a cytotoxic assay was performed using another target, EBV-transformed cell line JY, which has HLA-A2.1 molecules but does not express WT1 protein. PBMCs stimulated with Db126-pulsed T2 cells killed JY cells pulsed with the Db126 peptide (Fig. 1B). The optimal assay conditions, such as peptide concentrations, E/T ratios, and time of killing assay were determined in preliminary experiments. CTLs against the WT1 peptides could be induced reproducibly with PBMCs from the same donor. Since the effectors were bulk-cultured cell lines, background lysis against peptide-unpulsed target cells was substantial and variable in individual lines. As shown in Fig. 2, specific lysis by the CTL line was assayed for the T2 cells that were pulsed with varying concentrations of the Db126 peptide. Specific lysis increased in parallel with an increase in peptide concentration and reached a plateau at a concentration of 1.0 μg/ml. The half-maximal lysis was obtained at about 30 ng/ml of peptide, indicating that the affinity of the CTL line for Db126 peptide is lower than that of virus-specific CTL lines for viral antigen peptides (Bertoletti et al. 1994; Corundolo et al. 1990; Morrison et al. 1992), but equivalent to that of the CTL

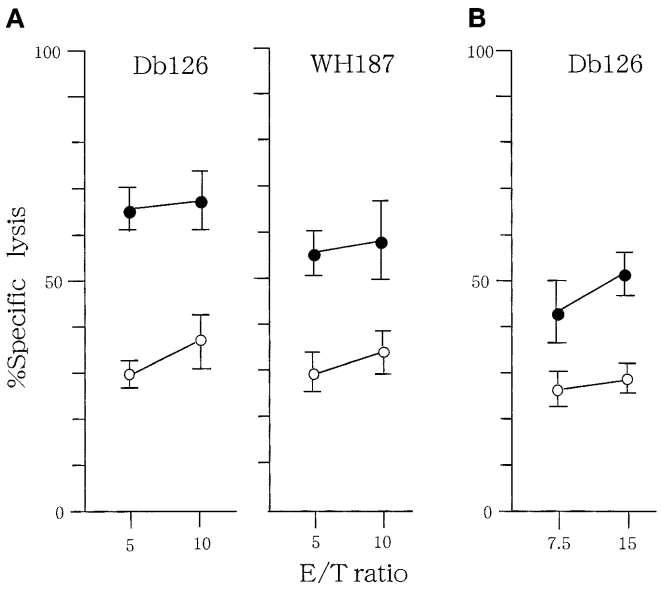

Fig. 1A,B Generation of WT1 peptide-specific cytotoxic lymphocytes (CTLs). Specific lysis was measured by means of Europium release assay at the indicated effector/target (E/T) ratios using **A** T2 or **B** JY target cells pulsed with (*closed circles*) or without (*open circles*) WT1 peptides (5 μg/ml for T2 and 10 μg/ml for JY)

lines against other tumor antigens (Traversari et al. 1992; Van der Bruggen et al. 1994a, 1994b; Wölfel et al. 1994). These results confirmed that the CTL line specifically recognized the Db126 peptide in order to kill the peptide-pulsed T2 cells.

The majority of the CTLs were CD3 and CD8 positive. Neither lymphocytes displaying a B-cell surface phenotype ($CD19^+$) nor those displaying a natural killer cell surface phenotype ($CD56^+CD3^-$) were detected (data not shown).

CTL responses are HLA-A2.1 restricted

Since the Db126-specific CTLs exerted peptide-specific cytotoxicity stronger than WH187-specific CTLs, the Db126-specific CTLs were examined further. To investigate the MHC restriction of CTL responses to Db126 peptide, experiments to block the cytotoxic activity of the CTLs against the peptide-pulsed T2 cells were performed using an anti-HLA-A2.1 mAb. As shown in Fig. 3, the cytotoxic activity was reduced to a background lysis of T2 cells by the addition of 60 μg/ml anti-HLA-A2.1 mAb. An irrelevant isotype-matched mAb, Y3, had no effects on the lysis.

Specific CTL responses to endogenously WT1-expressing, HLA-A2.1-positive leukemia cells

We next examined whether the Db126-specific CTLs could kill endogenously WT1-expressing, HLA-A2.1-positive leukemia cells. The Db126-specific CTLs exerted significant cytotoxicity against endogenously WT1-expressing, HLA-A2.1-positive leukemia cells, TF1, but only background lysis against Molt-4 (WT1-expressing, HLA-A2.1-negative) or JY (WT1-non-expressing, HLA-A2.1-positive) cells (Fig. 4). High levels of *WT1* mRNA expression in both TF1 and Molt-4 have been confirmed by RT-PCR performed under the optimized conditions (Inoue et al. 1994). In TF1, not

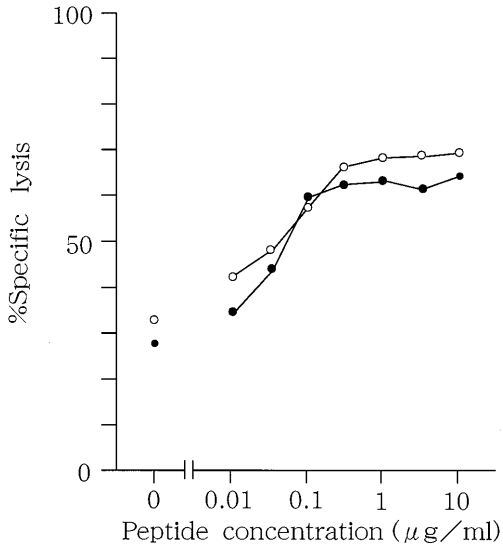

Fig. 2 Peptide-dependent specific lysis of T2 cells. T2 cells were pulsed with a varying concentration of Db126 peptide and assayed for specific lysis by the Db126-specific CTL line. Two different experiments (*open* and *closed circles*) are shown

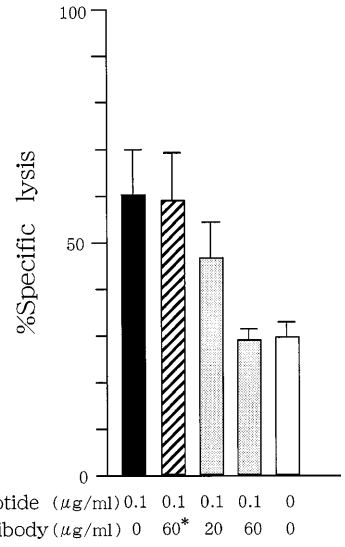

Fig. 3 Inhibition of cytotoxicity of the Db126-specific CTL line by an anti-HLA-A2.1 mAb. Specific lysis of Db126-pused T2 cells was measured at E/T ratios of 5:1 in the presence or absence of a blocking mAb (BB7.2) against HLA-A2.1 molecules. An *asterisk* indicates the use of an anti-H-$2K^b$ mAb (Y3) instead of the anti-HLA-A2.1

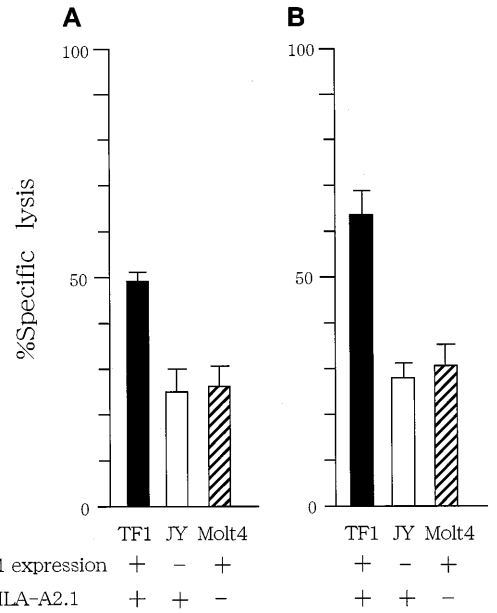

Fig. 4A,B Specific lysis of endogenously WT1-expressing cells by Db126-specific CTLs. Cytotoxicity assays were performed at E:T ratios of **A** 7.5:1 or **B** 15:1 using TF1 (WT1-expressing, HLA-A2.1-positive), JY (WT1-non-expressing, HLA-A2.1-positive), and Molt-4 (WT1-expressing, HLA-A2.1-negative) cells as targets

only surface expression of HLA-A2.1 molecules but also retainment of the *HLA-A*0201* gene were confirmed by FACS analysis using an anti-HLA-A2.1 mAb and by microplate hybridization using *HLA-A2* allele-specific probes (Kawai et al. 1993), respectively (data not shown). Furthermore, no point mutations and deletions of the *HLA-A*0201* gene of TF1 were determined by means of the PCR single-stranded conformation polymorphism method (Maruya et al. 1996) (data not shown). These results strongly suggested that the Db126-specific CTLs killed the WT1-expressing leukemia cells by recognizing WT1 peptides that were presented by HLA-A2.1 molecules on the cell surface.

Discussion

The search for widely expressed tumor antigens as targets for MHC class I-restricted CTLs is of great importance for the development of T cell-mediated immunotherapy for cancer patients. Two WT1 peptides, Db126 (RMFPNAPYL) and WH187 (SLGEQQYSV), among four WT1 peptides that contain anchor motifs required for binding to HLA-A2.1 molecules, were identified to have stronger binding activity for the HLA-A2.1 molecules. Immunization in vitro with these WT1 peptides elicited WT1 peptide-specific CTLs. This suggests that the Wilms' tumor gene WT1 product has a high potential as a tumor antigen.

Patients who develop significant graft-versus-host disease (GVHD, grade ≥ 2) after allogeneic bone-marrow transplantation (BMT) were demonstrated to have a significantly lower rate of relapse than patients with either no GVHD or grade 1 GVHD (Ritz 1994) finding has now been confirmed in many large-scale studies (Ritz 1994). Furthermore, the observation that relapse rates are higher after either syngeneic or autologous BMT despite administration of identical preparative regimens supported the existence of graft-versus-leukemia (GVL) activity (Ritz 1994). Patients who receive T-cell-depleted marrow also have a higher rate of relapse after allogeneic BMT than patients who receive unmodified marrow (Ritz 1994). Intensified immunosuppression raises the risk of relapse after allogeneic BMT (Ritz 1994). Moreover, donor lymphocyte infusion is effective for induction of complete remission of the relapsed leukemia patients after allogeneic BMT (Ritz 1994). The demonstration of a GVL effect after allogeneic BMT provides the most convincing clinical evidence for the effectiveness of tumor immunity and gives rise to the rationale of immunotherapy against cancer.

The *WT1* gene encodes a transcription factor and is classified as a tumor suppressor gene. Expression of the *WT1* gene is restricted to a limited set of tissues including kidney, ovary, testis, and spleen, but is highest in the developing kidney. The *WT1* gene is also expressed in many supportive structures of mesodermal origin. On the other hand, in hematopoietic malignancies such as acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), and chronic myelogenous leukemia (CML), the wild-type *WT1* gene is aberrantly overexpressed (Brieger et al. 1994; Inoue et al. 1994, 1997; Menssen et al. 1995; Miwa et al. 1992; Miyagi et al. 1993). WT1 expression levels in leukemic cells are at least ten times higher than those in normal human hematopoietic progenitor cells, including $CD34^+CD33^-$, $CD34^+CD33^+$, $CD34^+CD38^-$, $CD34^+CD38^+$, $CD34^+$HLA-DR^-, $CD34^+$HLA-DR^+, $CD34^+$ c-kit^{high}, $CD34^+$c-kit^{low}, and $CD34^+$c-kit^- cells (Inoue et al. 1997). Thus, WT1 expression levels differ strikingly between normal hematopoietic progenitor cells and leukemic cells. This difference in WT1 expression levels is the basis for the reasoning that CTLs induced by immunization with the WT1 protein would not cause damage to normal hematopoietic progenitor cells. In fact, in surviving mice that rejected tumor challenges by in vivo immunization with the Db126 peptide [this peptide is antigenic for both humans with HLA-A2.1 molecules and mice (C57BL/6) with H-$2D^b$ molecules], no damage of the main organs by the CTLs was observed (unpublished data). Thus, WT1 protein could become a promising target for immunotherapy against leukemia. The WT1 protein may be generally useful for immunotherapy against leukemia, since it is overexpressed in almost all types of leukemia, including AML, ALL, and CML. Overexpressed, normal proteinase 3 protein has recently been proposed as a candidate antigen for immunotherapy against leukemia (Molldrem et al. 1996). However, since the protein is a differentiation protein

restricted to the myeloid lineage, application of immunotherapy against this protein would be limited to myeloid lineage leukemias.

We have recently reported WT1 expression in three of four gastric cancer cell lines, all of five colon cancer cell lines, 12 of 15 lung cancer cell lines, two of four breast cancer cell lines, one germ cell tumor cell line, two ovary cancer cell lines, one uterine cancer cell line, one thyroid cancer cell line, and one hepatocellular carcinoma cell line (Oji et al. 1999). Thus, of the 34 solid tumor cell lines examined, 28 (82%) expressed WT1. Furthermore, fresh cancer cells resected from lung cancer patients also expressed WT1 at high levels (Oji et al. 1999). Further examination of gastric cancer cell line AZ-521, lung cancer cell line OS3, and ovarian cancer cell line TYK-nu, all of which expressed WT1 at levels comparable to those in leukemic cells, showed that these three cell lines expressed wild-type WT1 without mutations and that cell growth of these cell lines was specifically inhibited by WT1 antisense oligomers in association with a reduction in WT1 protein. These results demonstrate that the *WT1* gene plays an important role in the growth of solid tumor cells as well as leukemic cells and thus that WT1 protein may also be an attractive antigen for immunotherapy against various types of solid tumor.

Considering WT1 expression in normal tissues, levels in normal human hematopoietic progenitor cells are less than one-tenth of those in leukemia cells (Inoue et al. 1994, 1997). Lung cancer cells expressed WT1 at 10- to 1000-fold higher levels compared with normal lung tissues (Oji et al. 1999). WT1 is detectable in kidney and ovary by northern blot analysis or in situ hybridization, but exact quantification of WT1 expression levels remains difficult because only a special type of cell (podocytes in glomeruli) expresses a detectable amount of WT1. However, in surviving mice that had rejected WT1-expressing tumors by in vivo immunization with WT1 peptide, the main organs, including kidney, remained intact (unpublished data), indicating that WT1 expression levels in normal tissues are low enough to be ignored by WT1-specific CTLs.

Five categories of tumor antigen can be identified: (1) proteins expressed during fetal development, but only on limited adult tissues (e.g., MAGE1) (Van der Bruggen et al. 1991), (2) mutated or fused proteins related to malignant transformation (e.g., ras, p53, and bcr-abl) (Bocchia et al. 1996; Chen et al. 1992; Houbiers et al. 1993; Jung and Schluesener 1991; Peace et al. 1991; Yanuck et al. 1993), (3) proteins related to the differentiated function of the involved tissue of malignant origin (e.g., immunoglobulin idiotype and gp100) (Brown et al. 1989; Cox et al. 1994; Kawakami et al. 1994), (4) proteins overexpressed aberrantly in the malignant cells (e.g., c-erbB-2/HER-2/neu) (Coussens et al. 1985; Semba et al. 1985), and (5) antigens derived from oncogene viruses, such as the E7 oncoprotein of human papilloma virus 16 (Ressing et al. 1995). Thus, the WT1 protein can be included in the fourth category.

The c-*erbB-2*/*HER-2*/*neu* oncogene is amplified and overexpressed in a variety of human malignancies including breast and ovarian cancer (Gusterson et al. 1992; Slamon et al. 1987). c-erbB-2/HER-2/neu has therefore been proposed as a target for immunotherapy. $CD8^+$ CTL responses specific for c-erbB-2/HER-2/neu peptides have been demonstrated in patients with ovarian cancer overexpressing c-erbB-2/HER-2/neu protein (Disis et al. 1994; Fisk et al. 1995; Peoples et al. 1995). In murine models, immunization with c-erbB-2/HER-2/neu peptides elicited the peptide-specific $CD4^+$ T-cell immunity and antibody immunity (Disis et al. 1996), or the peptide-specific $CD8^+$ CTLs in a MHC class I-restricted fashion (Nagata et al. 1997). Furthermore, the p53 tumor suppressor gene product is also an attractive target for immunotherapy, because p53 is overexpressed in $\sim$50% of all human malignancies. Immunization with wild-type p53 peptide was recently reported to elicit peptide-specific CTLs, some of which recognized p53-overexpressing tumors in vitro (Bertholet et al. 1997; Fujita et al. 1998; Houbiers et al. 1993; Nijman et al. 1994; Noguchi et al. 1994; Vierboom et al. 1997; Yanuck et al. 1993). Our present data showing that immunization with a self-protein, wild-type WT1 peptide, can elicit the peptide-specific CTL responses in a MHC-restricted fashion are fundamentally compatible with the studies on induction of immunity against the c-erbB-2/HER-2/neu or p53 proteins.

The *WT1* gene is classified as a tumor suppressor gene. However, we have proposed that the *WT1* gene has basically two functional aspects, namely that of a tumor suppressor gene and that of an oncogene, and that in leukemic cells it performs an oncogenic rather than a tumor suppressor gene function on the basis of the following evidence: (1) high expression of wild-type WT1 in almost all leukemic cells (Inoue et al. 1994), (2) an inverse correlation between WT1 expression levels and prognosis (Inoue et al. 1994), (3) increased WT1 expression at relapse compared with that at diagnosis in acute leukemia (Tamaki et al. 1996), (4) inhibition of the growth of leukemic cells by *WT1*-antisense oligomers (Yamagami et al. 1996), and (5) blocking of differentiation but induction of proliferation in response to granulocyte-colony stimulating factor in myeloid progenitor cells that constitutively express WT1 by transfection with the *WT1* gene (Inoue et al. 1998; Tsuboi et al. 1999). Similarly, in solid tumors, the *WT1* gene also exerts an oncogenic function, because suppression of *WT1* gene expression by *WT1*-antisense oligomers inhibits the growth of solid tumor cells (this issue of whether the *WT1* gene is an oncogene or tumor suppressor gene has been reviewed elsewhere: Menke et al. 1998). The loss of tumor-specific antigens followed by the escape from immune surveillance by CTLs is a well-known, very important problem. Since, as mentioned earlier, the WT1 protein is essential for the proliferation of leukemic and solid tumor cells, loss or downregulation of WT1 expression is likely to cause the cessation of proliferation of these tumor cells. Thus, immu-

notherapy directed against the WT1 protein would have little risk of tumor escape from immune surveillance due to the loss of WT1 antigen.

Since WT1 is a self-protein, it is considered to be tolerated in classical immunology. However, increasing evidence illustrates that tolerance is defined not only qualitatively by the peptide sequences but also quantitatively by the number of specific MHC-peptide complexes on antigen-presenting cells (Schild et al. 1990). At the moment, the quantitative threshold that divides self from non-self is not clearly understood, nor is the mechanism that makes quantitative discrimination possible. In our recent observations, mice immunized with the Db126 peptide could reject in vivo-administered WT1-expressing tumor cells without damage to normal tissues, which express WT1 at low levels (unpublished data). These observations might indicate that a quantitative threshold for tolerance could be defined by one or two orders of magnitude of WT1 expression. In addition to quantitative tolerance, some of the self-MHC-peptide complexes are also suggested to be spared from immune attack by simply being ignored (Soldevila et al. 1995), anergy induction (Burkly et al. 1989), clonal deletion in the periphery (Webb et al. 1990), or exhaustion of the effector T cells (Moskophidis et al. 1993). A lack of sufficient helper activity and/or costimulation at the site of antigen recognition by CTL precursors is likely to play a role in this phenomenon (Kitagawa et al. 1990; Servetnick et al. 1990; Soldevila et al. 1995). Altogether, these observations clearly indicate a potential for self-peptide-based immunotherapy for tumors.

In conclusion, the WT1 protein is a new, attractive target for immunotherapy for almost all leukemia patients and for patients with various types of solid tumors.

Acknowledgements We thank Tsuyomi Yajima for preparation of the manuscript and Machiko Mishima for her skilful technical assistance. Experiments performed here comply with the current laws of Japan. This work was supported by PRESTO, JST, and by grants from the Ministry of Education, Science, and Culture.

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文献 7

Wilms 腫瘍遺伝子 WT1 産物を標的とした腫瘍免疫療法

Wilms 腫瘍遺伝子 WT1 は、急性骨髄性白血病、急性リンパ性白血病、慢性骨髄性白血病だけでなく、肺がんなど様々な固形腫瘍においても高頻度に発現している。WT1 タンパクが腫瘍特異的免疫の標的抗原となり得るか否かを検討するために、3 つの 9-mer WT1 ペプチド（Db126、Db221、Db235）を用いた WT1 ペプチドに対する CTL の in vivo 誘導を、C57BL/6 マウスモデルを用いて試みた。この 3 つの 9-mer WT1 ペプチドは $H\text{-}2D^b$ 結合 anchor motif を含んでおり、$H\text{-}2D^b$ 分子に比較的高い親和性を有している。このうち $H\text{-}2D^b$ 分子に最も高い親和性を有する Db126 だけが強い CTL 反応を誘導した。この CTL は Db126 がパルスされた標的細胞を、Db126 濃度依存的に傷害すると共に、$H\text{-}2D^b$ 拘束性に WT1 発現腫瘍細胞を傷害した。ペプチド免疫をして誘導された Db126 ペプチド特異的 CTL 細胞株は、それが認識して殺す WT1 発現腫瘍細胞由来溶解液の分画（逆相高速液体クロマトグラフィーを用いて得られた）のうち、合成 Db126 ペプチドが溶出されるのと同じ溶出時間に溶出される分画に感作活性を示すことから、これら CTL が WT1 を発現する腫瘍細胞を殺す際にも、同一の Db126 ペプチドが抗原として認識されることが示唆された。さらに Dp126 ペプチドで免疫されたマウスは WT1 発現腫瘍細胞を拒絶し、CTL による自己免疫反応を示すことなく長期に生存した。このように、WT1 ペプチドは新規腫瘍抗原と考えられ、種々の腫瘍に対して WT1 タンパクを標的とした免疫療法の臨床的な適応を検討すべきと考えられた。

Cancer Immunotherapy Targeting Wilms' Tumor Gene WT1 Product

Yoshihiro Oka, Keiko Udaka, Akihiro Tsuboi, Olga A. Elisseeva, Hiroyasu Ogawa, Katsuyuki Aozasa, Tadamitsu Kishimoto and Haruo Sugiyama

J Immunol 2000;164;1873-1880

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cancer Immunotherapy Targeting Wilms' Tumor Gene WT1 Product[1]

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The Wilms' tumor gene *WT1* is expressed at high levels not only in acute myelocytic and lymphocytic leukemia and in chronic myelocytic leukemia but also in various types of solid tumors including lung cancers. To determine whether the WT1 protein can serve as a target Ag for tumor-specific immunity, three 9-mer WT1 peptides (Db126, Db221, and Db235), which contain H-2D^b-binding anchor motifs and have a comparatively higher binding affinity for H-2D^b molecules, were tested in mice (C57BL/6, H-2D^b) for in vivo induction of CTLs directed against these WT1 peptides. Only one peptide, Db126, with the highest binding affinity for H-2D^b molecules induced vigorous CTL responses. The CTLs specifically lysed not only Db126-pulsed target cells dependently upon Db126 concentrations but also WT1-expressing tumor cells in an H-2D^b-restricted manner. The sensitizing activity to the Db126-specific CTLs was recovered from the cell extract of WT1-expressing tumor cells targeted by the CTLs in the same retention time as that needed for the synthetic Db126 peptide in RP-HPLC, indicating that the Db126-specific CTLs recognize the Db126 peptide to kill WT1-expressing target cells. Furthermore, mice immunized with the Db126 peptide rejected challenges by WT1-expressing tumor cells and survived for a long time with no signs of autoaggression by the CTLs. Thus, the WT1 protein was identified as a novel tumor Ag. Immunotherapy targeting the WT1 protein should find clinical application for various types of human cancers. ***The Journal of Immunology*, 2000, 164: 1873–1880.**

The Wilms' tumor gene *WT1* was first reported as the gene responsible for Wilms' tumor, a pediatric renal cancer (1, 2). This gene encodes a zinc finger transcription factor involved in tissue development, in cell proliferation and differentiation, and in apoptosis, and is categorized as a tumor suppressor gene (3). The WT1 gene product regulates the expression of various genes either positively or negatively depending upon how it combines with other regulatory proteins in different types of cells.

Tumor Ags can be categorized into five groups: ubiquitous proteins such as mutated ras (4) or p53 (5); tumor-specific shared Ags such as P1A in mice (6) and MAGE in humans (7, 8); differentiation Ags with a good example of tyrosinase (9); overexpressed tumor Ags such as HER-2/neu (10); and Ags derived from oncogenic viruses with the best example of the E7 oncoprotein of human papilloma virus 16 (11).

We (12, 13) and others (14–17) have identified high expression levels of the wild-type *WT1* gene in leukemic cells regardless of the type of disease to clarify the essential role of the *WT1* gene in leukemogenesis. On the basis of accumulated evidence (13, 18–20), we have proposed that the wild-type *WT1* gene performs an oncogenic rather than a tumor suppressor gene function in hematopoietic progenitor cells. Moreover, we found that among 34 solid tumor cell lines examined, 28 (82%), including lung, gastric, colon, and breast cancer cell lines, expressed the wild-type *WT1* gene (21). Cancer cells of lung cancer patients also expressed the *WT* gene at high levels (21). Growth of WT1-overexpressing tumor cells was specifically inhibited by WT1 antisense oligodeoxynucleotides, thus suggesting a close relationship between WT1 overexpression and tumorigenesis. These results indicate that the WT1 gene product could be a promising tumor-specific Ag belonging to the fourth category of tumor Ags not only for leukemia but also for various types of solid tumors including lung cancers.

It is well known that tumor-specific CD8$^+$ CTLs constitute the most important effectors for antitumor responses and recognize peptides derived from endogenous proteins presented on the cell surface in association with MHC class I molecules (22). It had been first demonstrated that patients who develop significant graft-versus-host disease (GVHD;[4] grade $\geq$ 2) after allogeneic bone marrow transplantation (BMT) have a significantly lower rate of relapse than patients with either no GVHD or grade 1 GVHD (23). The demonstration of such a graft-vs-leukemia effect after allogeneic BMT provides the most convincing clinical evidence for the effectiveness of tumor immunity.

Peptides that bind to a given MHC class I molecule have been shown to share common amino acid motifs (24). We (25) and others (26) have previously developed a peptide library-based method for predicting MHC class I-binding peptides. MHC-binding scores can be calculated for all of the peptides of eight or nine amino acids in a given protein sequence by using the experimentally obtained specificity profiles of MHC class I molecules. With few exceptions, the binding scores of naturally occurring MHC class I-binding epitopes for CTLs are as high as twice the SD from the mean score of all of the peptides present in the parental proteins.

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Received for publication June 16, 1999. Accepted for publication December 6, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by PRESTO, JST, and grants from the Ministry of Education, Science, and Culture.

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[4] Abbreviations used in this paper: GVHD, graft-versus-host disease; BMT, bone marrow transplantation.

0022-1767/00/$02.00

The present study shows that WT1-derived peptides, which were predicted to bind to H-2D^b molecules according to the peptide library-based scoring system of MHC class I-binding peptides, actually bind to H-2D^b molecules. Furthermore, one of the WT1 peptides induced peptide-specific CTLs as a result of in vivo immunization with the peptide of mice, which then rejected the challenges by WT1-expressing tumor cells.

Materials and Methods

Synthesis of peptides

Peptides were synthesized manually or with an ABI430A peptide synthesizer (Applied Biosystems, Foster City, CA) using Fmoc chemistry. They were then purified by RP-HPLC with a C_{18} Microbondasphere column (Waters Japan, Osaka, Japan). Synthesis of the correct peptides was confirmed with the aid of an API IIIE triple quadrupole mass spectrometer (Sciex, Thornhill, Toronto, Canada), and concentrations of the peptides were determined by means of a MicroBCA assay (Pierce, Rockford, IL) using BSA as the standard. Some peptides were also custom synthesized (Sawady Technology, Tokyo, Japan).

Cells and Abs

FBL3 is a Friend leukemia virus-induced erythroleukemia cell line originated from C57BL/6 (H-2D^b), and was generously provided by Dr. B. Chesebro (National Institutes of Health, Bethesda, MD) via Dr. M. Miyazawa (Kinki University, Japan). C1498 and EL4 are a WT1-nonexpressing leukemia or lymphoma cell line of C57BL/6 origin, respectively, and was obtained from American Type Culture Collection (ATCC, Rockville, MD). RMA is a Rauscher leukemia virus-induced lymphoma cell line, and RMAS is a TAP-deficient subline of RMA (27). These cell lines were kindly provided by Dr. K. Kärre (Karolinska Institute, Sweden) through Dr. H.-G. Rammensee (University of Tübingen, Germany) and maintained in DMEM containing 5% FBS. P815 is a mastocytoma originated from DBA/2 mice (ATCC). YAC-1 cells that were used as target cells for NK activity were obtained from ATCC. Murine WT1-expressing C1498-mWT1 and EL4-mWT1 were established by transfection of murine WT1 cDNA (a kind gift from Dr. D. Housman, Massachusetts Institute of Technology via Dr. H. Nakagama, National Cancer Center Research Institute, Japan).

mAbs B22.249 (anti-H-2D^b, a kind gift from Dr. J. Klein, Max Planck Institute of Biology, Germany) (28), 28.11.5S (anti-H-2D^b from ATCC), B8.24.3 (anti-H-2K^b from ATCC), 28.13.3S (anti-H-2K^b, a kind gift from Dr. D. Sachs, Massachusetts General Hospital), and MA143 (anti-H-2L^d, a kind gift from Dr. J. H. Stimpfling, McLaughlin Research Institute) were prepared as ascites and purified by DE52 anion exchange chromatography (Whatman, Maidstone, U.K.).

Measurement of binding affinity of WT1 peptides for H-2D^b molecules

Binding of WT1 peptides to MHC class I H-2D^b molecules was measured by a stabilization assay using RMAS cells as described previously (29). Briefly, RMAS cells were incubated at 26°C overnight to accumulate peptide receptive MHC class I molecules on the cell surface and then mixed with varying concentrations of WT1 peptides in 100 μl of DMEM containing 0.25% (w/v) BSA. After incubation for 30 min at room temperature, the temperature was raised to 37°C and the incubation was continued for 1 h. The cells were then washed and stained with FITC-labeled B22.249 mAb and analyzed by FACScan (Becton Dickinson, Mountain View, CA). The relative binding affinity in K_d values was calculated from the mean fluorescence intensities as described previously (25).

Induction of WT1 peptide-specific CTLs

WT1 peptide-specific CTL lines were induced in 4–12-wk-old C57BL/6 mice (H-2D^b) by immunization with LPS (from *Escherichia coli* 055:B5; Sigma, St. Louis, MO)-activated spleen cells pulsed with WT1 peptides as described elsewhere (Kakugawa et al., *Submitted for publication*). Briefly, spleen cells were cultured for 3 days with 10 μg/ml LPS in 40 ml of DMEM containing 10% FBS, followed by pulsing with 1 μM WT1 peptide and 10 μM OVAII peptide (OVA 323-339, as a helper epitope) (30) for 2 h. The cells were then irradiated with 3000 rad and injected i.p. into mice. The immunization with LPS-activated spleen cells pulsed with the WT1 peptides was repeated three times at weekly intervals. After 1 wk from the third immunization, the spleen was resected from the immunized mice and the spleen cells were stimulated in vitro with LPS-activated spleen cells, which were pulsed with the WT1 peptide and then irradiated with 3000 rad. After 5 days of the in vitro stimulation, the cells were tested for their killing activity.

Cytotoxicity assay

Cytotoxic activity was measured by means of ^{51}Cr release assay. RMAS cells were incubated at 26°C overnight, labeled with ^{51}Cr for 1 h, and pulsed with the WT1 peptides at room temperature for 30 min. Effector cells were then added to 1×10^4 target cells at varying E:T ratios to a final volume of 200 μl in DMEM containing 5% FBS. After brief centrifugation at 1000 $\times$ *g*, cells were incubated at 37°C for 5 h. Relative cytotoxicity was calculated as follows from the radioactivity released in the culture supernatant: % specific lysis = (experimental release − spontaneous release)/(total release − spontaneous release) $\times$ 100. For Db126 peptide-dependent lysis assays, 50 μl of varying concentrations of Db126 peptide dissolved in DMEM was added to 50 μl of RMAS target cells in DMEM containing 0.5% BSA. After incubation for 30 min at room temperature, Db126-specific CTL lines were added at an E:T ratio of 4:1.

Purification of endogenously processed WT1 peptides from WT1-expressing tumor cells

A total of 1×10^9 FBL3 cells were harvested and acid extracted with 1% trifluoroacetic acid. The Centricon 10 (Amicon, Beverly, MA)-passed fraction was loaded onto a Nova Pak C_{18} RP-HPLC column (4.6 mm $\times$ 15 cm; Millipore and Waters Japan) and eluted at 1 ml/min with a shallow acetonitrile gradient. One-minute fractions were collected and dried by Speed Vac. An aliquot equivalent to 1×10^8 FBL3 cells of the HPLC fractions was added to the wells of the ^{51}Cr release assay, each of which contained 1×10^4 target cells for screening of the peptides recognized by CTLs.

In vivo tumor challenges

C57BL/6 male mice were used to avoid male Ag (H-Y)-specific immune responses because the sex of C57BL/6 mice from which the FBL3 tumor cell line originated was not known. The inoculated dose of the tumor cells was determined by preliminary experiments and a lethal dose for nonimmune mice was used. To determine the effects of immunization, five to eight mice from each group were injected i.p. with PBS, LPS-activated spleen cells alone, or those pulsed with 1 μM Db126 peptide in combination with 10 μM of OVAII (30) at 37°C for 2 h. After three weekly immunizations, 3×10^7 FBL3 leukemia cells were i.p. inoculated into 4–6-wk-old C57BL/6 mice.

Histology

The main organs, including kidney and lung, were removed from the surviving Db126-immunized mice that had rejected the tumor challenges and fixed in Bouin's solution. Paraffin sections of 8-μm thickness were stained with hematoxylin and eosin by means of standard methods.

Results

Identification of H-2D^b-binding peptides

Most CTL epitope peptides can be predicted by means of a peptide library-based scoring system for MHC class I-binding peptides (25, 26). Amino acid sequences of the murine WT1 protein were scanned for peptides with a potential binding capacity for H-2D^b molecules, and five peptides with comparatively high binding scores for H-2D^b molecules were identified (Table I). All five of these WT1 peptides with higher binding scores also exhibited a relatively higher binding affinity for H-2D^b molecules, and some correlation between binding scores and binding affinity (K_d) was established, thus indicating the utility of binding scores for finding peptides which bind to MHC class I molecules. Db126 peptide demonstrated the same order of binding affinity as that of viral Ags (24), which is the strongest Ags for CTL induction. Three peptides (Db126, Db221, and Db235) with anchor motifs for binding to H-2D^b molecules (24) were actually used for in vivo immunization.

Induction of CTLs against WT1 peptides

Whether specific CTLs against these three WT1 peptides could be induced by in vivo immunization with these peptides was examined (Fig. 1). Mice were immunized with LPS-activated spleen

Table I. *Binding of WT1 peptides to H-2D^b* molecules

Peptide	Amino Acid Sequence	Binding Score	K_d (M)
WT1 peptides			
Db126	aa 126–134 RMFPN[a]APY**L**	1.77	5.7×10^{-7}
Db227	aa 227–235 YQMTSQLEC	1.93	1.0×10^{-6}
Db235	aa 235–243 CMTW**N**QMN**L**	1.20	1.3×10^{-6}
Db221	aa 221–229 YSSD**N**LYQ**M**	1.05	2.6×10^{-6}
Db136	aa 136–144 SCLESQPTI	1.61	3.7×10^{-6}
Known epitope peptides			
SV40 T antigen	aa 223–231 CKGV**N**KEY**L**	1.85	1.9×10^{-7}
Influenza A34 NP	aa 366–374 ASNE**N**MET**M**	1.24	1.9×10^{-7}

[a] Bold letters represent anchor motifs.

cells pulsed with the peptides. The spleen cells of the immunized mice were then assayed for cytotoxic activity against peptide-pulsed RMAS target cells. WT1 peptide-specific CTLs were induced by immunization with the Db126 peptide, whereas no CTLs were induced by immunization with the Db221 or Db235 peptide. Thus, only the Db126 peptide with the highest binding affinity for H-2D^b molecules could elicit CTL responses. Therefore, subsequent investigation focused on the CTLs against the Db126 peptide.

To confirm that the Db126 peptide-induced CTLs specifically recognize the Db126 peptide to kill the target cells, three different CTL lines against the Db126 peptide were assayed for cytotoxic activity against RMAS target cells pulsed with increasing concentrations of the Db126 peptide (Fig. 2). Their cytotoxic activity was found to increase in parallel with an increase in peptide concentrations and to reach a plateau. Half the maximal lysis was observed in the range of nanomolar of the peptide. Lysis by the CTL lines of the RMAS target cells pulsed with naturally occurring H-2D^b-binding peptide influenza A34 NP (Table I) was not observed (data not shown). These results proved that the cytotoxic activity of the CTL lines was specific for the Db126 peptide.

Lysis of endogenously WT1-expressing tumor cells by Db126-specific CTLs

We next investigated whether Db126-specific CTLs could recognize and lyse endogenously WT1-expressing tumor cells. As shown in Fig. 3, a panel of tumor cell lines was tested for lysis by Db126-specific CTL lines. Before the tests, specificity of the CTLs for the Db126 peptide was confirmed by specific lysis of Db126 peptide-pulsed RMAS target cells (Fig. 3*a*). The Db126-specific

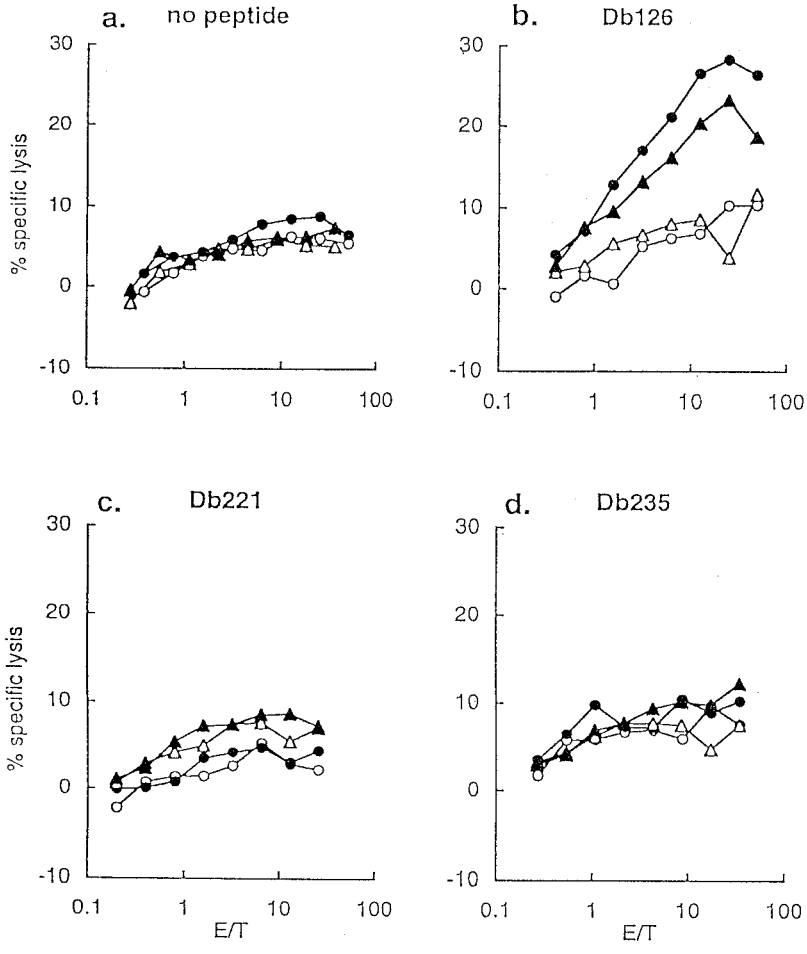

FIGURE 1. Induction of WT1 peptide-specific CTLs. Mice were immunized with LPS-activated spleen cells pulsed with WT1 peptides. Spleen cells of mice not immunized with any peptide (*a*), immunized with Db126 (*b*), Db221 (*c*), or Db235 (*d*) were stimulated in vitro with LPS-activated spleen cells not pulsed with any peptide (*a*) and with LPS-activated spleen cells pulsed with Db126 (*b*), Db221 (*c*), or Db235 (*d*). Their killing activity was tested against RMAS cells pulsed (closed symbols) or unpulsed (open symbols) with the immunized peptides at different E:T ratios. Results from two mice (circles and triangles) are shown. At least three independently performed experiments for each peptide yielded similar results.

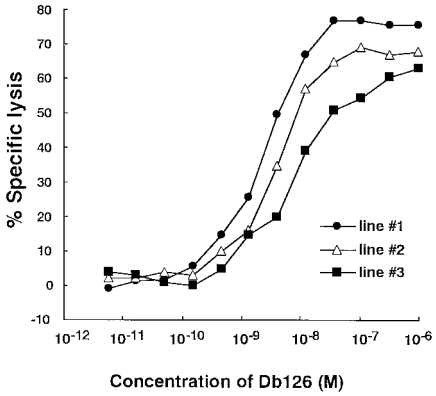

FIGURE 2. Db126 peptide-dependent lysis of RMAS cells by Db126-specific CTL lines. Three CTL lines established from individual mice immunized with the Db126 peptide were tested for cytotoxic activity against RMAS cells pulsed with the indicated concentrations of the Db126 peptide at an E:T ratio of 4:1. Lysis of RMAS cells without the peptide was 2% for line #1, 4% for line #2, and 8% for line #3.

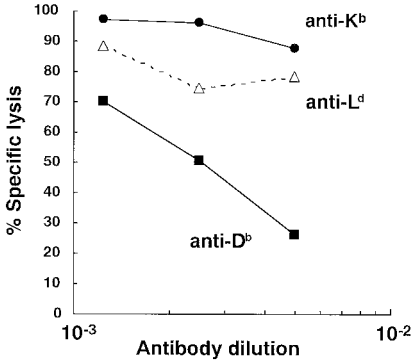

FIGURE 4. H-2D^b-restricted cytotoxic activity of the Db126-specific CTL line. Specific lysis of endogenous WT1-expressing FBL3 cells by the Db126-specific CTL line was tested in the presence of titer adjusted mAbs against H-2K^b (28.13.3S), H-2D^b (28.11.5S), or H-2L^d (MA143). Isotype-matched mAbs were used as control mAbs.

CTLs lysed endogenously WT1-expressing FBL3 cells, but not WT1-nonexpressing RMA cells (Fig. 3*b*). Furthermore, the Db126-specific CTLs killed murine WT1-transfected C1498 cells to a significant extent when compared with parental WT1-nonexpressing C1498 cells, confirming that the molecule targeted for killing by the CTLs is indeed the WT1 peptide (Fig. 3*c*). Similarly, a specific lysis of WT1-nonexpressing EL-4 cells used here (H-2D^b) was obtained as a result of transfection of the WT1 gene, although the lysis was weak because of low expression of H-2D^b molecules on the EL-4 cells used here (Fig. 3*d*). WT1-nonexpressing P815 cells with nonidentical H-2 molecules were not lysed by the CTLs (data not shown). RMAS cells that were common targets for lymphokine-activated killer/NK cells (31) were not killed by the CTLs (Fig. 3*a*). However, when YAC-1 cells were used as target cells for NK activity, the Db126-specific CTLs also lysed the cells. This phenomenon is reasonable, since it has been well known that CTLs frequently display an NK-like cytolytic activity in addition to Ag-specific cytolytic activity (32–34). The Db126-specific CTLs were also 99% positve for CD8 and virtually negative for NK1.1 (data not shown). Since various NK-activating/inhibitory receptors are expressed not only on NK cells but also on CTLs bearing TCR$\alpha\beta$ (31, 35, 36), these receptors may be responsible for the lysis of YAC-1 cells. Taken together, these results suggest that the Db126-specific CTLs can recognize Db126 or related peptides which are naturally produced through intracellular processing of the WT1 protein and are present on H-2D^b molecules in WT1-expressing cells.

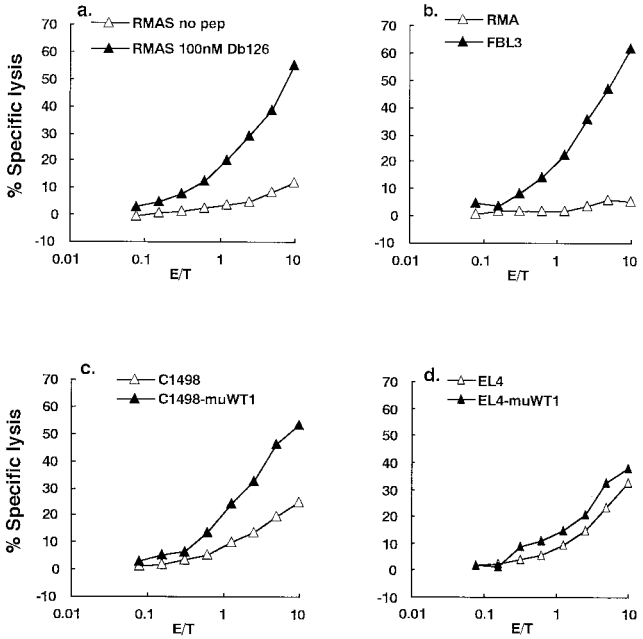

FIGURE 3. Specific lysis of WT1-expressing tumor cells by the Db126-specific CTL line. Specific lysis was tested for Db126-pulsed or -unpulsed RMAS cells (*a*), WT1-expressing (FBL3) or nonexpressing (RMA) tumor cells (*b*), WT1-transfected or -untransfected C1498 cells (*c*), or WT1-transfected or -untransfected EL4 cells (*d*) at the indicated E:T ratios.

Cytotoxic activity is H-2D^b restricted

Furthermore, to demonstrate that the cytotoxic activity of the CTLs is MHC restricted, it was assayed in the presence of Abs against H-2 class I molecules (Fig. 4). The suppression of cytotoxic activity of the CTLs against WT1-expressing FBL3 cells was found to depend upon an increase in the concentration of Abs against H-2D^b, but Abs against H-2K^b or H-2L^d did not show any suppressive effect on the cytotoxic activity of the CTLs. These results showed that the CTLs exert their cytotoxic activity in an H-2D^b-restricted fashion.

Presence of sensitizing activity to Db126-specific CTLs in cell extract of WT1-expressing cells

To confirm that endogenously WT1-expressing tumor cells express the Db126 peptide on their cell surface through intracellular processing of the WT1 protein and that Db126-specific CTL lines recognize this peptide for cell lysis, WT1-expressing FBL3 cells were lysed and peptide fractions were prepared (Fig. 5). The peptide fractions were further fractionated by RP-HPLC, and each fraction was assayed for its sensitizing activity to the Db126-specific CTLs. The sensitizing activity was recovered in the same retention time as that needed for the synthetic Db126 peptide. One additional sensitizing activity peak was detected. Such additional peaks have previously been observed by us and others when naturally processed endogenous peptides were prepared from whole-cell lysate. In such cases, some peaks represented the sensitizing activity of longer peptides harboring the minimal epitope peptide (37, 38), a tissue-specific variant peptide (39), or cross-recognized peptides bearing similar or unrelated amino acid sequences (40, 41). These results showed that the Db126 peptide targeted by the

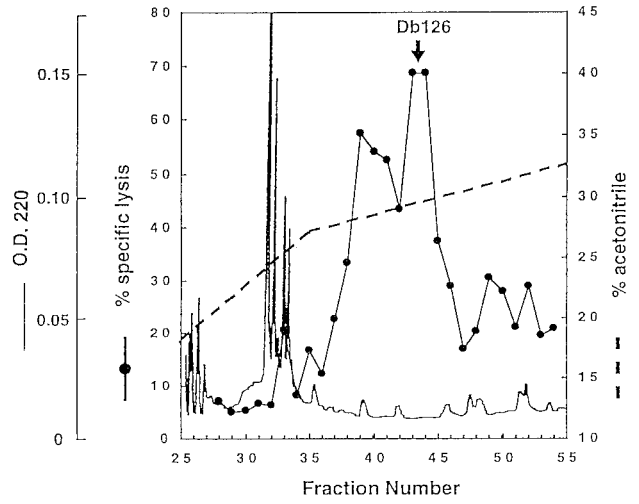

FIGURE 5. Recovery of sensitizing activity from acid extract of FBL3 cells. The acid extract of FBL3 cells was HPLC fractionated, and each fraction was tested for its sensitizing activity for specific lysis of RMAS target cells by the Db126-specific CTL line. The profile of optical absorbance at 220 nm of the eluting peptides is shown in the background.

Db126-specific CTLs is naturally produced by intracellular processing of the WT1 protein in WT1-expressing cells.

Eradication of tumor challenges by preimmunization with the Db126 peptide

We next investigated whether active immunization with the Db126 peptide elicited in vivo tumor immunity. Mice were immunized once a week for 3 wk with LPS-activated spleen cells pulsed with the Db126 peptide and then inoculated i.p. with a lethal number of FBL3 leukemia cells. As shown in Fig. 6, all five mice immunized with LPS-activated spleen cells pulsed with Db126 peptide, none of five mice immunized with LPS-activated spleen cells alone, and one of eight mice inoculated with PBS were alive after tumor challenges. A statistical significance ($p < 0.01$) was found between the group immunized with the WT1 peptide and the group immunized with LPS-activated spleen cells alone, or the group inoculated with PBS alone. This experiment was repeated with similar results. In both the immune and nonimmune mice, ascites was observed 3 days after the i.p. inoculation of the tumor cells. In the nonimmune mice, the ascites continued to increase and the mice died. On the other hand, in the immune mice, the ascites

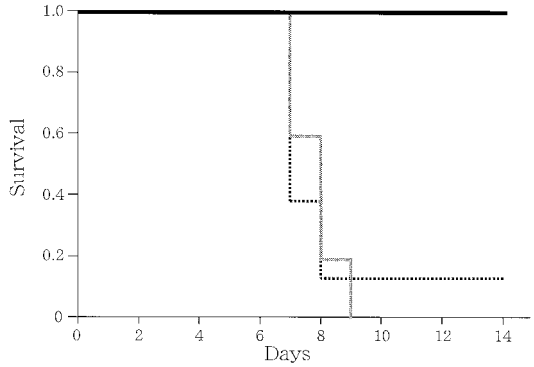

FIGURE 6. Rejection of tumor challenges by immunization with Db126 peptide. Mice were immunized once a week with LPS-activated spleen cells pulsed with Db126 peptide (solid line), LPS-activated spleen cells alone (shaded line), or PBS alone (dashed line). After immunization for 3 wk, 3×10^7 FBL3 leukemia cells were i.p. injected.

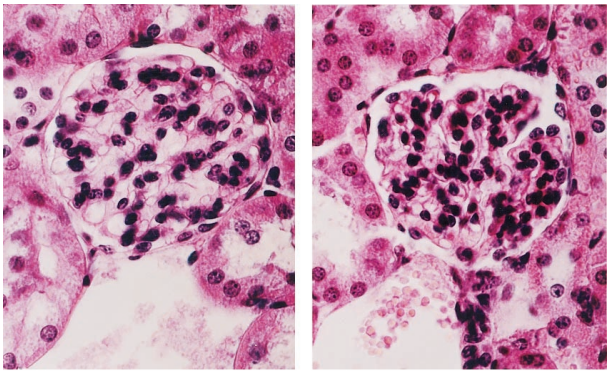

FIGURE 7. No pathological changes of kidney in the immunized mice that rejected tumor challenges. Hematoxylin and eosin staining of the glomeruli of the kidney is shown. No pathological changes such as lymphocyte infiltration or tissue destruction and repair are observed.

gradually decreased afterward, and the mice completely rejected tumor challenges and survived. Spontaneous regression was occasionally observed in nonimmune mice. This regression is presumed to be due to spontaneous induction of CTLs specific for the Friend leukemia virus (FBL3 leukemic cells are transformed by this virus), since such CTL induction was not infrequently observed in C57BL/6 mice (42).

No evidence of autoaggressive reactions in surviving mice that rejected tumor cell challenges

WT1 expression in normal adult mice is limited to a few cell types in several tissues. Moreover, WT1 expression levels in these tissues are considerably lower than those in leukemia (13) and solid tumor (21) cells, suggesting a low risk of normal tissue damage as a result of immune responses to the WT1 protein. To evaluate the risk of autoaggression by immunization against self-WT1 peptide, the tissues of immunized mice were pathologically examined a few weeks after tumor cells had been eradicated. The lung and kidney of three mice were intensively examined because WT1 was mainly expressed in the mesothelial cells of the lung capsule and in the podocytes of the kidney glomeruli (Fig. 7). Both tissues showed normal structure and cellularity in all three mice examined, and no pathological changes caused by immune response, such as lymphocyte infiltration or tissue destruction and repair, were observed. These results showed that the CTLs against the Db126 peptide were ignorant of normal self-cells that express WT1 at physiological levels.

Discussion

The rationale for the efficacy of immunotherapy for cancer patients has been clinically demonstrated by the following findings (23): patients who develop significant GVHD (grade ≥ 2) after allogeneic BMT have a significantly lower rate of relapse than patients with either no GVHD or grade I GVHD; patients who receive T cell-depleted marrow also have a higher rate of relapse after allogeneic BMT than patients who receive unmodified marrow; and donor lymphocyte infusion is effective for complete remission induction of relapsed leukemia patients after allogeneic BMT. This graft-vs-leukemia effect after allogeneic BMT provides the most convincing clinical evidence for the effectiveness of tumor immunity for cancer treatment.

The search for widely expressed tumor Ags as targets for MHC class I-restricted CTLs is of great importance for the development

of T cell-mediated immunotherapy for cancer patients. Reports on such tumor Ags have been increasing exponentially in recent years, and the results indicate that these Ags can be categorized into five groups (43, 44). Ags of the first category correspond to peptides derived from regions of ubiquitous proteins such as mutated ras (4) or p53 (5). Chimeric proteins that result from chromosomal translocation are also unique to tumor cells (45, 46). The second group of tumor Ags consists of tumor-specific shared Ags such as P1A in mice (6) and MAGE in humans (7, 8). The third group of tumor Ags includes differentiation Ags. A good example is tyrosinase, a melanocyte protein that gives rise to different peptides that are presented by either MHC class I or class II molecules (9). The fourth group of tumor Ags is made up of overexpressed tumor Ags. An Ag that is expressed in some normal tissues and overexpressed in tumors is HER-2/neu (10), which is found at high levels in about 30% of breast and ovarian cancers. The last group of tumor Ags includes Ags derived from oncogenic viruses. Thus, the WT1 protein is thought to be a tumor Ag corresponding to the fourth group of tumor Ags.

In the murine models of immunotherapy against WT1-expressing tumors described here, surviving mice that rejected tumor challenges by the immunization of the Db126 peptide did not demonstrate obvious organ damage. These results demonstrate that the CTLs against the WT1 protein can discriminate differences in WT1 expression levels between abnormally WT1-overexpressing tumor cells and physiologically WT1-expressing normal cells, resulting in the killing of tumor cells with no damage to normal tissues. As for the application of immunotherapy with WT1 protein to human cancers, the following evidence suggests that this immunotherapy is promising without damage to normal organs. We (12, 13) and others (14–17) have demonstrated that the wild-type *WT1* gene is aberrantly overexpressed in almost all leukemia cells regardless of type of leukemia: whether it is acute myeloid leukemia, acute lymphoid leukemia, or chronic myeloid leukemia. The WT1 expression levels in leukemic cells are at least 10 times higher than those in normal $CD34^+$ hematopoietic progenitor cells (13). This striking difference in WT1 expression levels between leukemic cells and normal hematopoietic progenitor cells is the basis for the reasoning that CTLs induced against the WT1 protein would not cause damage to normal hematopoietic progenitor cells. Furthermore, we have recently reported WT1 expression in 28 (82%) of 34 various types of solid tumor cell lines, including lung, gastric, colon, breast, and ovary cancer cell lines. High WT1 expression in fresh lung cancer tissues has also been reported (21). Our reports demonstrate that WT1 expression is significantly higher in cancer cell-rich tissues than in tissues appearing to be normal, confirming the abnormal overexpression of the *WT1* gene not only in cultured tumor cells but also in fresh lung cancer cells. As mentioned earlier, the striking difference in WT1 expression levels between tissues appearing to be normal and cancer cell-rich tissues is also the basis for the reasoning that CTLs induced against WT1 protein would not cause damage to normal lung tissue.

The *WT1* gene has been categorized as a tumor suppressor gene (3). However, we have proposed that the *WT1* gene has basically two functional aspects, namely, that of a tumor suppressor gene and that of an oncogene, but that in leukemic cells it performs an oncogenic rather than a tumor suppressor gene function (18–20). The following findings support our proposal: 1) high expression of wild-type WT1 in almost all leukemic cells (12, 13), 2) an inverse correlation between WT1 expression levels and prognosis (12), 3) an increased WT1 expression at relapse compared with that at diagnosis in acute leukemia (47), 4) growth inhibition of leukemic cells by WT1 antisense oligomers (18), and 5) blocking of differentiation but induction of proliferation in response to G-CSF in myeloid progenitor cell line 32D cl3 (19) and normal myeloid progenitor cells (20), both of which constitutively express WT1 by the transfection with the *WT1* gene. Furthermore, it is suggested that the wild-type *WT1* gene also has an oncogenic function in WT1-expressing solid tumors, since the *WT1* gene is overexpressed in various types of solid tumor cells, including lung cancers, and since the suppression of *WT1* gene expression by WT1 antisense oligomers inhibits the growth of solid tumor cells (21).

The loss of tumor-specific Ags followed by the escape from immune surveillance by CTLs is one of the major obstacles of the host's immunological warfare against tumors. Since the WT1 protein plays an essential role in the growth of leukemic and solid tumor cells, loss of the expression of the WT1 protein, i.e., loss of the WT1 Ag, results in cessation of the proliferation of leukemic and solid tumor cells. Thus, immunotherapy directed against the WT1 protein would have little risk of escape from immune surveillance following loss of the WT1 Ag.

It has been well documented that tolerance to self-peptides is induced by deletion of self-reactive T cells in the thymus (48) as well as by deletion or exhaustion of such cells in the periphery (49), and that self-reactive T cells which have escaped deletion are functionally anergized or silenced by down-regulation of coreceptor molecules (50, 51). Since WT1 is a self-protein, it is considered to become tolerant in classical immunology. However, increasing evidence promoted us to accept that a large quantity of antigenic determinants of the self have not induced self-tolerance and thus that a substantial number of self-reactive clones must exist in healthy individuals and have the potential to elicit immune responses directed against tumors. These potentially self-reactive T cell clones are either anatomically secluded (52) or can be simply ignorant of their targets (53–55). It is probably possible to break tolerance especially if the self-proteins are not expressed at sufficient levels at the time and place of tolerance induction. The WT1 peptides are likely to be subdominant self-peptides so that the epitopes are probably ignored by the immune system under physiological conditions, although CTL precursors responsible for the WT1 peptides are present.

WT1 peptides that were predicted on the basis of the peptide library-based scoring system of MHC class I-binding peptides (25, 26, 29) actually showed comparatively higher binding affinity for $H\text{-}2D^b$ molecules, confirming that this scoring system is useful for finding candidates for MHC class I-binding peptides. Dyall et al. (56) designed a few artificial variants of MHC class I-binding self-peptides . Since these variant peptides are obviously foreign to the host immune system, a strong CTL response can be induced. Unlike weak T cell responses to self-MHC complexes, CTL responses to variant peptides can be sustained for a longer period without causing annihilation of the clones due to insufficient signals for cell division or survival (57, 58). Since a substantial fraction of such CTLs cross-reacts against nonmutated self-peptides expressed in tumor cells in much smaller amounts, immunization with variant peptides may be a more efficient method to induce CTLs against tumors. The scoring system for MHC class I-binding peptides should provide a convenient design of cross-reactive self-mimicking peptides for immunization.

We have recently reported that in vitro stimulation of HLA-A2.1-positive PBMC with WT1 peptides, Db126, RMFPNAPYL, or WH187, SLGEQQYSV, both of which contain anchor motifs needed for binding to HLA-A2.1 molecules and actually bind to HLA-A2.1 molecules, elicits CTLs against each WT1 peptide (59). The CTLs specifically killed the WT1 peptide-pulsed target cells and endogenous WT1-expressing leukemic cells in an HLA-A2.1-restricted fashion. Thus, the WT1 peptide Db126 that was a shared

sequence between murine and human WT1 protein was immunogenic for the induction of CTLs in both mice and humans. These accumulated data obtained from both human and murine settings suggested a successful clinical application of WT1 protein-directed immunotherapy for patients with leukemia and solid tumors. We are now planning clinical trials of this immunotherapy for patients with leukemia or lung cancer.

Acknowledgments

We thank Tsuyomi Yajima for preparation of this manuscript and Machiko Mishima for her skillful technical assistance. We also thank Dr. O. Chisaka for instructing histological procedures. We gratefully acknowledge Drs. B. Chesebo, M. Miyazawa, K. Kärre, H.-G. Rammensee, D. Sachs, and H. Stimpfling for sending cell lines and Drs. D. Houseman and G. Nolan for giving cDNA and vectors.

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文献 8

インターフェロンβによる WT1 ペプチドワクチンの腫瘍免疫増強

腫瘍関連抗原特異的 CTL の誘導や活性化のためには、強力な CTL エピトープの選択とともに、適切な免疫増強剤の併用も重要である。今回我々はインターフェロンβによる腫瘍免疫の誘導について検討した。試験治療群では、C57BL/6 マウスに WT1 ペプチドワクチンを 2 回事前に投与し、WT1 が発現した C1498 細胞を移植し、1 週間間隔で 4 回 WT1 ペプチドワクチンを投与する治療を行った。ワクチンの投与期間中、インターフェロンβを 1 週間に 3 回投与した。一方コントロール群のマウスは WT1 単独、インターフェロンβ単独、またはリン酸緩衝食塩水（PBS）単独で治療した。試験治療群のマウスは腫瘍を拒絶し、コントロール群のマウスより有意に長期生存した。Day 75 の生存率は、試験治療群では 40%であったのに対して、WT1 単独、インターフェロンβ単独、PBS 単独ではそれぞれ 7、7、0%であった。WT1 特異的 CTL の誘導や NK 活性の増強が試験治療群のマウスの脾臓細胞において認められた。さらにインターフェロンβの投与は移植腫瘍細胞における MHC クラス I 分子の発現を増強した。WT1 ペプチドワクチンとインターフェロンβの併用投与は腫瘍免疫を増強しており、この増強効果は主として WT1 特異的 CTL の増強、NK 活性の誘導並びに腫瘍細胞における MHC クラス I 分子の発現の促進によると考えられた。以上より WT1 ペプチドワクチン治療におけるインターフェロンβの併用は WT1 免疫療法の臨床的な効果を増強することが期待される。

Vaccine xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Enhanced tumor immunity of WT1 peptide vaccination by interferon-β administration☆

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ARTICLE INFO

Article history:
Received 18 August 2011
Received in revised form
18 November 2011
Accepted 19 November 2011
Available online xxx

Key words:
WT1
IFN-β
Immunotherapy
Cancer vaccine
Adjuvant

ABSTRACT

To induce and activate tumor-associated antigen-specific cytotoxic T lymphocytes (CTLs) for cancer immunity, it is important not only to select potent CTL epitopes but also to combine them with appropriate immunopotentiating agents. Here we investigated whether tumor immunity induced by WT1 peptide vaccination could be enhanced by IFN-β. For the experimental group, C57BL/6 mice were twice pre-treated with WT1 peptide vaccine, implanted with *WT1*-expressing C1498 cells, and treated four times with WT1 peptide vaccine at one-week intervals. During the vaccination period, IFN-β was injected three times a week. Mice in control groups were treated with WT1 peptide alone, IFN-β alone, or PBS alone. The mice in the experimental group rejected tumor cells and survived significantly longer than mice in the control groups. The overall survival on day 75 was 40% for the mice treated with WT1 peptide + IFN-β, while it was 7, 7, and 0% for those treated with WT1 peptide alone, IFN-β alone or PBS alone, respectively. Induction of WT1-specific CTLs and enhancement of NK activity were detected in splenocytes from mice in the experimental group. Furthermore, administration of IFN-β enhanced expression of MHC class I molecules on the implanted tumor cells. In conclusion, our results showed that co-administration of WT1 peptide + IFN-β enhanced tumor immunity mainly through the induction of WT1-specific CTLs, enhancement of NK activity, and promotion of MHC class I expression on the tumor cells. WT1 peptide vaccination combined with IFN-β administration can thus be expected to enhance the clinical efficacy of WT1 immunotherapy.

1. Introduction

Induction and activation of tumor-associated antigen (TAA)-specific cytotoxic T lymphocytes (CTLs) is essential for cancer immunotherapy. For this purpose, it is important to co-administer appropriate immunopotentiating agents, including adjuvants or cytokines, together with a TAA-derived peptide that serves as a CTL epitope, because injection of a CTL epitope alone cannot sufficiently induce and activate the TAA-specific CTLs. Furthermore, if the co-administered agents not only help induction/activation of the CTLs but also activate other effector cells such as NK cells, this may further enhance anti-tumor responses.

The Wilms' tumor gene *WT1* was originally isolated as a gene responsible for Wilms' tumor, a pediatric renal cancer [1,2]. This gene encodes a zinc finger transcription factor involved in organ development, cell proliferation and differentiation, as well as apoptosis. The *WT1* gene product regulates the expression of various genes either positively or negatively, depending upon how it combines with other regulatory proteins in different types of cells. Although *WT1* was categorized at first as a tumor suppressor gene [3], we have proposed that the wild-type *WT1* gene plays an oncogenic rather than a tumor-suppressor gene function in leukemogenesis/tumorigenesis on the basis of the following

☆ This study was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor, and Welfare, Japan.
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0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.11.074

findings: (i) the wild-type *WT1* gene was highly expressed in leukemias and solid cancers [4–17]; (ii) high expression levels of *WT1* mRNA correlated with poor prognosis in leukemia and several kinds of solid cancer [4]; (iii) growth of *WT1*-expressing leukemia and solid cancer cells was inhibited by treatment with *WT1* antisense oligomers *in vitro* [18]; and (iv) in wild-type *WT1* gene-transfected myeloid progenitor cells, differentiation was blocked but proliferation was induced in response to granulocyte colony-stimulating factor [19,20]. These findings indicate that *WT1* over-expression and leukemogenesis/tumorigenesis may be closely related, which suggests that the wild-type *WT1* gene product could be a promising tumor rejection antigen for cancer immunotherapy. In fact, we [14–17,21] and others [22,23] have generated human WT1-specific CTLs *in vitro*, and we were able to show that mice immunized with MHC class I-restricted WT1 peptide or with *WT1* plasmid DNA elicited WT1-specific CTLs and rejected the challenge of *WT1*-expressing cancer cells *in vivo* [14–17,24,25], while the induced CTLs did not damage normal tissue cells that physiologically expressed *WT1*, including kidney podocytes and bone marrow (BM) stem/progenitor cells. Furthermore, we demonstrated that WT1 peptide vaccination combined with *Mycobacterium bovis* bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) [26], which was injected one day previously at the same site as the WT1 peptide was more effective for eradication of *WT1*-expressing tumors than treatment with WT1 peptide alone or BCG-CWS alone [27]. BCG-CWS strongly activated dendritic cells (DCs) of the injection sites, i.e. activated of innate immunity, and also induced/activated of TAA (WT1)-specific CTLs.

Interferon-β (IFN-β) is a Type I interferon, and is known for its various immunopotentiating properties: (i) enhancement of the expression of many surface molecules that are essential for binding and/or activation of CTLs, in particular the major histocompatibility complex (MHC) class I as well as the receptors B7-1 (CD80) and intercellular adhesion molecule-1 (ICAM-1) [28,29], on antigen-presenting cells (APCs) or cancer cells; (ii) activation of NK, B, and T cells [30,31]; (iii) a direct anti-proliferation effect on cancer cells by promoting cell cycle arrest at the G1 phase [32]; (iv) induction of apoptosis of cancer cells [33]; and (v) inhibition of angiogenesis [34]. In fact, it was reported in mouse models that type I interferon was essential in the induction of CTL and eradication of EG-7 tumors expressing ovalbumin in mice by vaccination with CpG-adjuvanted ovalbumin [35], and that type I interferon augmented induction of CTL through DNA-based vaccination [36]. Furthermore, IFN-β has already been in use for cancer immunotherapy in clinical settings [37–40], and the mechanism for the enhancement of immunity against cancer has been thoroughly investigated. The results show that IFN-β should be considered as one of the most promising immunopotentiating agents for use with TAA-directed cancer vaccines.

We examined whether WT1 peptide vaccination combined with IFN-β administration leads to greater enhancement of tumor cell rejection than WT1 peptide vaccination alone in a mouse model and we tried to elucidate the mechanisms of enhancement of WT1 immunity by the co-administration of IFN-β.

2. Materials and methods

2.1. Mice

Male C57BL/6 (H-2D^b) mice were purchased from Clea Japan, Inc. (Tokyo, Japan), maintained in a specific pathogen-free (SPF) containment facility in accordance with the guidelines of Osaka University, and used for experiments at 6–8 weeks of age.

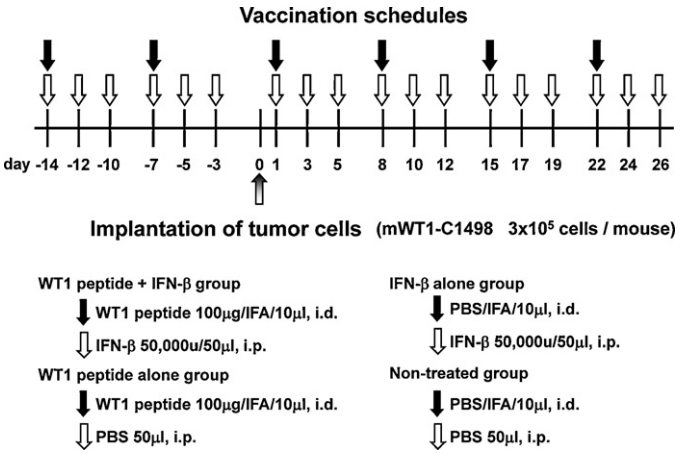

Fig. 1. *In vivo* tumor cell challenge and vaccination schedule. Mice were intradermally (i.d.) and abdominally pre-immunized with 100 μg WT1 peptide emulsified in incomplete Freund's adjuvant (IFA, Montanide ISA51) on day −14 and −7. Concomitantly, 50,000 units of murine IFN-β was intraperitoneally (i.p.) injected three times per week during the two weeks before tumor cell implantation. On day 0, mice were subcutaneously implanted with 3×10^5 mWT1-C1498 cells in 100 μl of PBS. This was followed by abdominal injection of 100 μg WT1 peptide emulsified in IFA on days 1, 8, 15 and 22. In addition 50,000 units of murine IFN-β was also i.p. injected three times per week until day 26 (WT1 peptide + IFN-β group). Mice in the control groups were injected with WT1 peptide emulsified in IFA and PBS (WT1 peptide alone group), PBS emulsified in IFA and IFN-β (IFN-β alone group), or PBS emulsified in IFA and PBS (non-treated group).

2.2. Reagents

An MHC class I (H-2D^b)-binding peptide, Db126 peptide (a.a.126-134 RMFPNAPYL), was synthesized by SIGMA Genosys (Ishikari, Japan) [24]. The peptide was dissolved in PBS and stored at −20 °C until use. Murine IFN-β was kindly donated by Toray Industries (Tokyo, Japan). Montanide ISA 51, an incomplete Freund's adjuvant (IFA), was purchased from Seppic S.A. (Orsay, France). Anti-CD8 and anti-NK1.1 mAbs for cell depletion were produced by 53-6.7.2 and PK136 hybridoma clones, respectively. Both hybridoma were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

2.3. Cells

C1498, a *WT1*-nonexpressing murine leukemia cell line of C57BL/6 origin, was obtained from ATCC (Rockville, MD, USA). *WT1*-expressing murine WT1-C1498 (mWT1-C1498) was generated by transduction of C1498 cells with CMV promoter driven murine WT1 17AA(+)KTS(+) isoform full length cDNA that was inserted into pcDNA3.1(+) mammalian expression vector (Invitrogen, Tokyo, Japan). YAC-1 cells that were used as target cells for NK activity were obtained from ATCC. RMAS, a TAP-deficient subline of RMA (Rauscher leukemia virus-induced lymphoma cell line of C57BL/6 origin), was kindly provided by Dr. K. Kärre (Karolinska Institute, Sweden) through Dr. H.-G. Rammensee (University of Tübingen, Germany) [24].

2.4. In vivo tumor cell challenge and vaccination schedule

The implanted dose of the tumor cells was optimized by preliminary experiments in which more than 90% of the non-treated mice transplanted with the tumor cells died within two months due to tumor development. We therefore adopted an observation period of 75 days after the tumor cell implantation (day 0). Tumor implantation and vaccination schedule are shown in Fig. 1. Mice were intradermally (i.d.) pre-immunized with an abdominal injection of 100 μg WT1 peptide emulsified with IFA on days −14 and

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−7. During the same period, 50,000 units of murine IFN-β was intraperitoneally (i.p.) injected three times per week. On day 0, mice were subcutaneously (s.c.) implanted with 3×10^5 mWT1-C1498 cells in 100 μl of PBS, followed by abdominal i.d. injection of 100 μg WT1 peptide emulsified with IFA on days 1, 8, 15, and 22. In addition, 50,000 units of murine IFN-β was also injected i.p. three times per week until day 26. Mice in control groups were vaccinated with WT1 peptide + IFA + PBS (WT1 peptide alone group); PBS + IFA + IFN-β (IFN-β alone group); and PBS + IFA + PBS (non-treated group). Tumor growth was monitored by measuring the longest diameter of the palpable mass.

For the assessment of immunological effector cells, we performed *in vivo* experiments independently from those for the assessment of survival. Splenocytes and bone marrow cells from mice immunized as shown in Fig. 1 were recovered on day 30 (8 days after the last vaccination) and used for ^{51}Cr release cytotoxicity assay (CTL and NK activities) and colony assay, respectively. Furthermore, the resected tumors were used for analysis of MHC class I expression.

2.5. ^{51}Cr release cytotoxicity assay and mice treatment schedule for the assay

Splenocytes were stimulated with the 5 μg/ml WT1 peptide and cultured in complete medium containing 10% heat-inactivated FCS, 45% RPMI1640 medium, 45% AIM-V medium, 1× non-essential amino acid (Gibco), 25 ng/ml 2-mercaptoethanol, 50 IU/ml penicillin and 50 μg/ml streptomycin. Two and four days later, recombinant interleukin-2 (rIL-2; kindly donated by Shionogi Biomedical Laboratories, Osaka, Japan) was added to the culture at a concentration of 20 IU/ml. After six days of culture, a ^{51}Cr release cytotoxicity assay was performed against WT1 peptide-pulsed or -unpulsed RMAS cells for WT1-specific CTL activity, and against YAC-1 cells for NK cell activity, as described previously [24]. Target cells (1×10^4 cells) labeled with ^{51}Cr were added to wells containing varying numbers of effector cells in 96-well plates. After 4 h of incubation at 37 °C, cell lysates were centrifuged and 100 μl of the supernatant was collected and measured for radioactivity. The percentage of specific lysis (% specific lysis) was calculated as follows: percentage of specific lysis = (cpm of experimental release − cpm of spontaneous release)/(cpm of maximal release − cpm of spontaneous release) × 100. Radioactivity of the supernatant, either of the target cell cultures without effector cells, or of the target cells that were completely lysed by the treatment with 1% Triton X-100 was used for spontaneous and maximal release, respectively.

2.6. Analysis of MHC class I expression

Tumors were resected from the tumor-bearing mice on day 30, and tumor cell suspensions were prepared with the tissues in the center of the tumor mass. The resected tissues contained only tumor mass with the naked eye. The cells were stained with FITC-conjugated anti-mouse H-2D^b monoclonal antibody (KH-95, BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with the FACSort (BD). Live cells were determined by means of FSC and SSC gating.

2.7. Colony assay

For CFU-GM (colony-forming-unit granulocyte-macrophage) assay, bone marrow cells were recovered from mice on day 30, plated at 1×10^4 cells/plate in methylcellulose medium containing 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml SCF, and 3 U/ml erythropoietin (EPO) (Methocult M3434; Stem Cell Technologies, Vancouver, BC, Canada), and cultured at 37 °C in a humidified incubator under 5% CO_2. Colonies with more than 50 cells were counted on days 8 and 12.

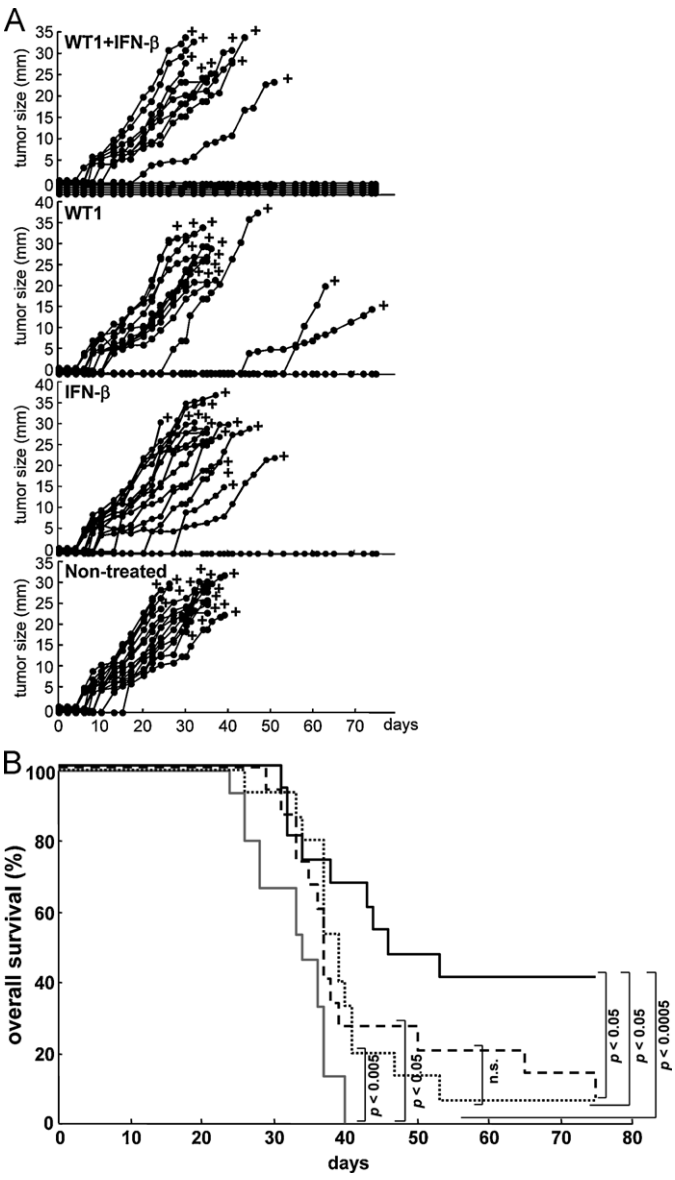

Fig. 2. Effect of WT1 peptide vaccination combined with IFN-β administration on rejection of implanted tumor cells. (A) Time course of size of tumors developed in individual mice of the four groups. Tumor sizes represent the longest diameters. (B) Overall survival curves of the four groups. Solid black, broken, dotted, and solid gray lines represent overall survival curves of mice treated with WT1 peptide vaccine + IFN-β, WT1 peptide vaccine alone, IFN-β alone, and non-treated mice, respectively.

2.8. In vivo CD8$^+$ T and NK cell depletion experiments

Mice were implanted with 3×10^5 mWT1-C1498 cells and treated with WT1 peptide vaccine + IFN-β as shown Fig. 1. The WT1- and IFN-β- treated mice were injected with PBS or 200 μg of anti-CD8 and/or 200 μg of anti-NK mAbs on days −15, −8, −1, 4, 7, 11, 14, 18, 21 and 25 [35,41].

2.9. Statistical analysis

Significant differences in overall survivals among experimental groups were evaluated with the Logrank test. The Student's *t*-test was used to calculate the differences in the expression levels of H-2D^b on tumor cells in mice among experimental groups.

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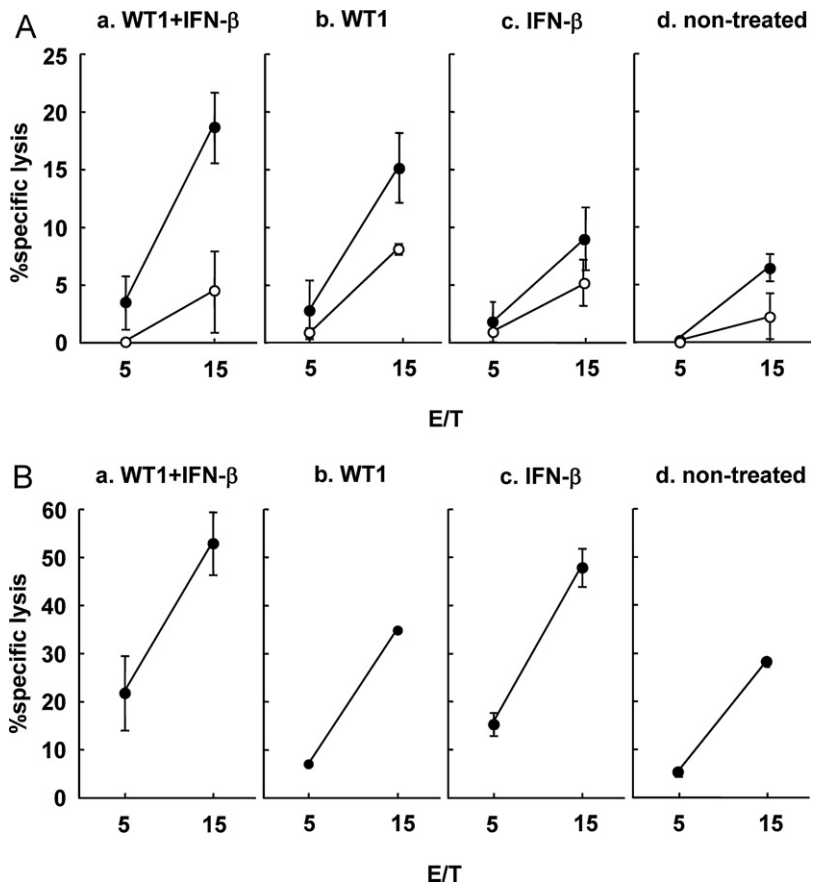

Fig. 3. Induction of WT1-specifc CTLs and enhancement of NK activity by treatment with WT1 peptide vaccine + IFN-β. Eight days after the last vaccination, splenocytes from the mice in each group were stimulated *in vitro* with WT1 peptide-pulsed synergistic splenocytes. WT1-specific CTL and NK cell activities were assayed in triplicate as cytotoxic activities against WT1 peptide-pulsed, -unpulsed RMAS or YAC-1 cells, respectively, at the indicated E/T ratio. (A) WT1-specific CTL activity. Closed and open circles represent cytotoxic activities against WT1 peptide-pulsed or -unpulsed RMAS, respectively. (B) NK activity. NK activity is shown as cytotoxic activities against YAC-1 cells. Bars indicate standard errors.

3. Results

3.1. IFN-β promotes efficacy of WT1 peptide vaccination

To investigate whether IFN-β promoted tumor cell rejection by WT1 peptide vaccination, mice were twice immunized with Montanide ISA51-emulsified WT1 peptide with or without IFN-β administration before transplantation of *WT1*-expressing tumor cells (mWT1-C1498) and then repeatedly WT1-immunized, followed by assessment of the tumor growth and their survival (Fig. 1). Optimization of cell number and determination of the observation period are described in Section 2.

Nine of the 15 mice treated with WT1 peptide vaccine + IFN-β developed tumors and died, while the remaining 6 mice were alive without tumors on day 75 (Fig. 2A). In contrast, 14 of the 15 mice treated with WT1 peptide vaccine alone, 14 of the 15 mice treated with IFN-β alone and all of the 15 non-treated mice had died of tumor growth by day 75. Overall survival rates on day 75 were 40% for mice treated with WT1 peptide vaccine + IFN-β, but 7, 7 and 0% for mice treated with WT1 peptide vaccine alone or IFN-β alone or for non-treated mice, respectively. The overall survival rates of mice treated with WT1 peptide vaccine + IFN-β were significantly higher than those of the other three groups (WT1 peptide vaccine + IFN-β versus WT1 peptide vaccine alone, IFN-β alone or non-treated: $p < 0.05$, $p < 0.05$, and $p < 0.0005$, respectively). The overall survival rates of mice treated with WT1 peptide vaccine alone or IFN-β alone were significantly higher than those of non-treated (WT1 peptide vaccine alone versus non-treated, IFN-β alone versus non-treated: $p < 0.05$ and $p < 0.005$, respectively). There was no significant difference in survival rate between WT1 peptide vaccine alone and IFN-β alone (Fig. 2B).

3.2. WT1 peptide vaccine + IFN-β enhances induction of WT1-specific CTLs and activates NK cell activity

In order to analyze immune responses, tumor-bearing mice treated with WT1 peptide vaccine + IFN-β as shown in Fig. 1 were sacrificed on day 30. The splenocytes of each mouse were stimulated *in vitro* with WT1 peptide and assayed for WT1 peptide-specific CTL activity against WT1 peptide-pulsed and -unpulsed RMAS cells and for NK activity against YAC-1 cells. Representative data are shown in Fig. 3. Splenocytes from mice treated with WT1 peptide vaccine + IFN-β showed the strongest WT1 peptide-specific cytotoxic activity while splenocytes from non-treated mice showed the weakest activity. WT1-specific cytotoxic activity was in the following order: WT1 peptide vaccine + IFN-β > WT1 peptide vaccine alone > IFN-β alone > non-treated. These findings convincingly showed that WT1-specific CTL activity was higher in the two groups with WT1 peptide vaccine than in the two groups without it. It appeared that the WT1-specific CTL activities in splenocytes from IFN-β-treated or non-treated mice were endogenously induced as a result of immunological stimulation by *WT1*-expressing tumor cells implanted.

Next, NK cell activity was examined (Fig. 3B). Mice of all four groups were sacrificed on day 30 and their splenocytes were analyzed for their NK cell activity. NK cell activity was higher in both

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WT1 peptide vaccine + IFN-β and IFN-β alone groups. These results suggested that NK activity was endogenously induced in *WT1*-expressing tumor-bearing mice and that this activity was enhanced by administration of IFN-β, which is a potent enhancer of NK activity.

Taken together, these results indicated that the strongest rejection of implanted tumor cells in the mice treated with WT1 peptide vaccine + IFN-β resulted from the generation of the highest levels of both WT1-specific CTLs and NK cells.

3.3. WT1 specific CTLs and NK cells play crucial roles in the treatment by WT1 peptide vaccine + IFN-β

To confirm that WT1-specific CTLs and NK cells played crucial roles in the tumor rejection, *in vivo* depletion of $CD8^+$ T and/or NK cells was performed. Mice that were implanted with mWT1-C1498 cells and vaccinated with WT1 peptide vaccine + IFN-β as shown in Fig. 1 were treated with both or either of anti-CD8 and anti-NK mAbs.

Tow of five mAb-non-treated mice developed tumors and died, while the remaining three survived without development of tumors. In contrast, all of the mice that were treated with both or either of anti-CD8 and anti-NK mAbs and vaccination-non-treated mice died of tumor development. It should be noted that appearance of tumors in mice treated with both or either anti-CD8 and anti-NK mAbs was earlier than that in mAb-non-treated mice (Fig. 4).

These results strongly indicated that both WT1-specific $CD8^+$ CTLs and NK cells played crucial roles in the rejection of tumor cells.

3.4. Enhancement of MHC class I (H-2D^b) expression on transplanted tumor cells by the administration of IFN-β

Since WT1 (Db126) peptide is produced from WT1 protein through processing in tumor cells and presents on the cell surface in association with MHC class I (H-2D^b) [29,32], H-2D^b expression levels of target cells are thought to exert a major influence on the susceptibility of the cells to attack by vaccination-induced WT1 (Db126)-specific CTLs. For this reason, the H-2D^b expression levels on the transplanted tumor cells (*WT1*-expressing C1498 cells) were examined. Tumor-bearing mice were sacrificed 30 days after tumor cell implantation, the tumors were resected, and the tumor cells were stained with anti-H-2D^b antibody (Fig. 5). The expression levels of H-2D^b on tumor cells was significantly higher in mice treated with WT1 peptide vaccine + IFN-β or IFN-β alone than in those treated with WT1 peptide vaccine alone or non-treated mice ($p < 0.05$) (Fig. 5B). These results indicated that IFN-β administration enhanced the expression of H-2D^b on tumor cells, which should make tumor cells more susceptible to attack by WT1-specific CTLs.

3.5. No inhibition of colony-forming ability of bone marrow cells from mice immunized with WT1 peptide vaccine + IFN-β

WT1 is expressed in some tissues of normal adult mice, including hematopoietic stem/progenitor cells, podocytes of kidney glomeruli, gonads and mesothelial structures. To evaluate the risk of induction of autoimmunity by immunization with WT1 peptide vaccine + IFN-β, the colony-forming ability of bone marrow cells, as shown by the numbers of CFU-GM colonies, were examined. No differences in numbers of CFU-GM colonies were found among the five groups (WT1 peptide vaccine + IFN-β, WT1 peptide vaccine alone, IFN-β alone, tumor-bearing non-treated, and non-tumor-bearing non-treated) (Fig. 6). These results showed that induced

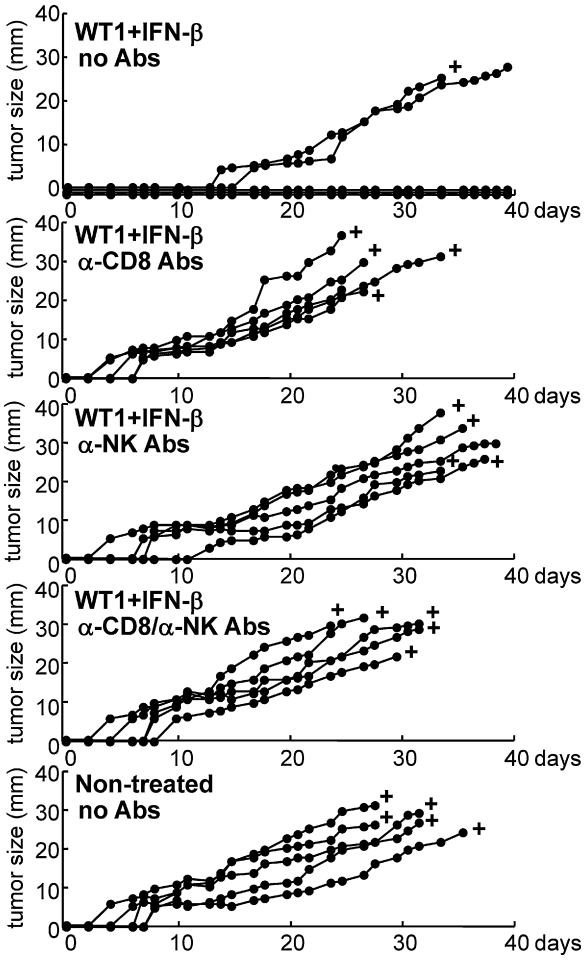

Fig. 4. Cancellation of tumor rejection by WT1 peptide vaccine + IFN-β by the administration of anti-CD8 and/or anti-NK mAbs. Mice were implanted with 3×10^5 mWT1-C1498 cells and treated with WT1 peptide vaccine + IFN-β as shown in Fig. 1. The WT1- and IFN-β- treated mice were injected with PBS or 200 μg of anti-CD8 and/or 200 μg of anti-NK mAbs on days −15, −8, −1, 4, 7, 11, 14, 18, 21 and 25. Time course of size of tumors developed in individual mice from the five groups. Tumor sizes represent the longest diameters.

WT1-specific CTLs did not recognize normal cells that physiologically expressed *WT1*.

4. Discussion

In the study presented here, we demonstrated that co-treatment with WT1 peptide vaccine (Db126; CTL epipope) + IFN-β enhanced rejection of *WT1*-expressing tumor cells in a mouse model. Enhanced induction of WT1-specific CTLs and NK cell activity was considered to be largely responsible for the successful rejection of the implanted tumor cells. The important roles of WT1-specific $CD8^+$ T cells and NK cells in the tumor rejection were confirmed by depletion experiments using anti-CD8 and/or anti-NK mAbs.

The most likely mechanism for the induction of the strongest WT1-specific cytotoxic activity in mice treated with WT1 peptide vaccine + IFN-β is the following: IFN-β activates NK cells [30,42,45], which generate IFN-γ, which in turn activates DCs and T cells [42–44]. Furthermore, IFN-β can also activate T cells directly [30]. These conditions lead to a more efficient induction of WT1-specific CTLs by the WT1 peptide vaccine. The WT1 peptide-specific cytotoxic activity observed in tumor-bearing non-treated mice may be due to the spontaneous induction of WT1-specific CTLs as a result of immune stimulation by implanted *WT1*-expressing tumors. Enhancement of NK cell function induced by *in vivo*

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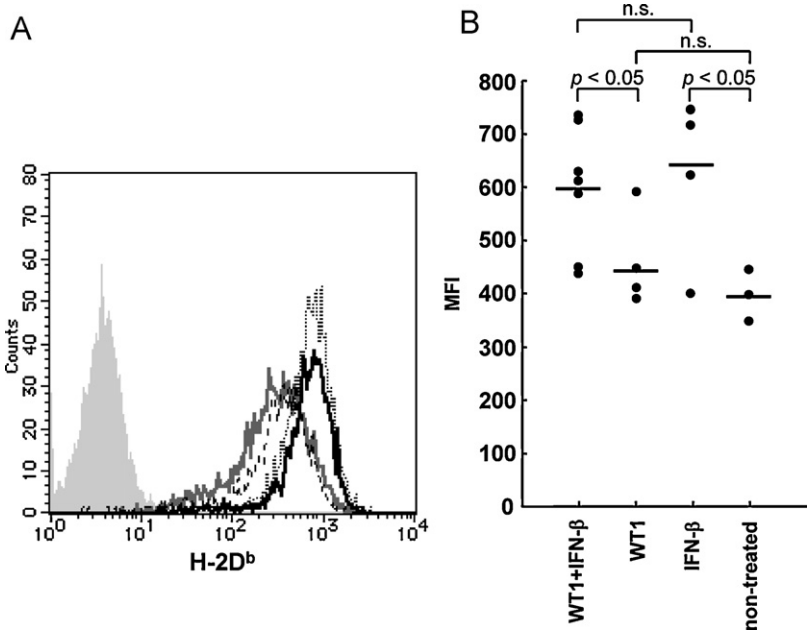

Fig. 5. IFN-β enhanced MHC class I (H-2D^b) expression of tumor cells *in vivo*. (A) H-2D^b expression levels of tumor cells recovered from mice. Solid black, broken, dotted, and solid gray lines represent the expression levels of tumor cells from mice treated with WT1 peptide vaccine + IFN-β, WT1 peptide vaccine alone, or IFN-β alone, and non-treated mice, respectively. (B) The mean fluorescence intensity (MFI) of H-2D^b expression of tumor cells from mice.

administration of IFN-β contributed to a high rejection rate of tumors in the present experiment system. However, the exact mechanism of the enhancement was not addressed in this study, while a series of investigations regarding the effect of IFN-β on NK cells were reported, including that IFN-β upregulated TRAIL on NK cells [45] and enhanced production of IFN-γ from NK cells. Besides NK cells, NKT cells might also have important roles in enhancement of tumor rejection in the present experiment system, considering that it was reported that IFN-β enhanced up-regulation of CD1d on DCs, which leads to NKT cell activation [46]. Further studies are needed to address the mechanism of enhancement of NK and NKT cell function by IFN-β in the context of tumor immunity.

At least two merits of IFN-β administration could be confirmed. One was that, as shown in Fig. 3B, greater enhancement of NK cell activity was observed in mice treated with WT1 peptide vaccine + IFN-β or with IFN-β alone than in the other two groups. This indicates that IFN-β activated NK cells *in vivo*, and that the enhanced NK activity contributed to eradication of MHC class I-negative tumor cells or those with low MHC class I expression. Another merit was that MHC class I expression on the WT1-C1498 leukemia cells was enhanced. WT1 peptides were generated through intracellular processing of the WT1 protein in tumor cells and presented on the surface of these cells in association with MHC class I molecules, followed by the recognition of the WT1 peptide/MHC class I complex by WT1-specific CTLs. Consistent with previously reported findings [28,29], MHC-class I expression on the WT1-C1498 leukemia cells was enhanced in mice treated with WT1 peptide vaccine + IFN-β or IFN-β alone. Higher expression of MHC class I molecules contributes the recognition and attack by CTLs [29]. It is possible that in mice treated with WT1 peptide vaccine + IFN-β MHC class I expression on the WT1-C1498 leukemia cells was enhanced, resulting in a heightened vulnerability to attack by WT1-specific CTLs. Taken together, it seems likely that target cells (mWT1-C1498 cells), of which the MHC class I expression was enhanced by IFN-β, were efficiently killed by WT1-specific CTLs, while the remaining target cells with negative or low MHC class I expression were efficiently killed by NK cells whose activity was enhanced by IFN-β. IFN-α is another type I IFN and has the similar structure and function to IFN-β [31–36,45,47]. Furthermore, both IFN-α and IFN-β were approved for human use [30,37–40,48]. Therefore, it would be interesting to examine, using this experiment system, whether IFN-α, as well as IFN-β, is effective in the context of a combined use with WT1 peptide vaccine for the treatment of malignancies.

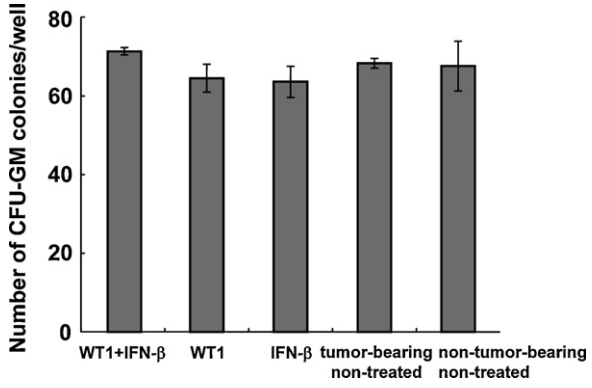

Fig. 6. No inhibition of colony-forming ability of bone marrow cells from mice immunized with WT1 peptide vaccine + IFN-β. Numbers of colonies generated by CFU-GM (colony-forming-unit granulocyte-macrophage) from mouse bone marrow cells on day 30. Values represent the means of the results from four mice in each group. Bars indicate standard errors.

Other functions of IFN-β in tumor rejection enhancement, that is, non-immunological mechanisms such as direct anti-tumor and anti-angiogenesis effect [32–34] may also have contributed to such rejection.

Although WT1 is physiologically expressed in some type of normal cells, including hematopoietic stem/progenitor cells and kidney glomeruli, WT1 vaccination combined with IFN-β treatment did not diminish the GM colony-forming ability of BM cells (Fig. 6), which is in agreement with previous reports [25,27]. These findings indicate that WT1-specific CTLs did not recognize normal cells that physiologically expressed *WT1*. The reason for this lack of recognition appears to be that WT1-specific CTLs can

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discriminate only between *WT1*-expressing tumor cells and physiologically *WT1*-expressing normal cells, resulting in the selective killing of tumor cells with no damage to normal tissues. These results suggested that the mechanisms involved in processing of WT1 protein and/or presentation of WT1 peptide might be different between tumor and normal cells, resulting in no or weak presentation of the WT1 peptide on the cell surface of normal cells. Further studies to address this issue are clearly warranted.

Immunopotentiating agents play a key role in the success of cancer immunotherapy, because injection of CTL epitope peptide alone cannot sufficiently induce and activate the TAA-specific CTLs. Co-administration of CTL epitope peptides and immunopotentiating agents proved to be effective for induction and activation of the CTLs and/or activation of other effector cells such as NK cells. We previously reported that the WT1 peptide vaccine combined with *M. bovis* bacillus Calmette-Guérin cell wall skeleton (BCG-CWS), which activates DCs through TLRs 2 and 4, had a synergistic effect on tumor rejection in mice [27]. In the current study, we could demonstrate the immunopotentiating activities of IFN-β leading to the enhancement of WT1-specific CTLs, NK cells, and MHC class I expression. It is anticipated that WT1 peptide vaccination combined with both IFN-β and BCG-CWS will be more effective for tumor rejection. The combination of CTL epitope vaccine with some immunopotentiating agents with various mechanisms for enhancement of anti-tumor immunity can be expected to become part of effective strategies for the cancer immunotherapy. Clinical trials of WT1 peptide cancer vaccine have already been started, and WT1 peptide vaccination was shown to have good potential for the treatment of cancer [14–17,49–54]. So far, we have performed immunization using WT1 peptide with Montanide ISA 51 adjuvant, and another group used KLH and GM-CSF [55]. Since the safety and toxicity of IFN-β have been confirmed to a considerable extent [37–40], WT1 peptide vaccination combined with IFN-β should be ready for use in the clinical settings in the near future.

Acknowledgement

We would like to thank Ms. Sachie Mamitsuka-Watanabe for preparation of this manuscript.

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第 29 回高度医療評価会議	資料1－3
平成 24 年2月3日	

高度医療審査の照会事項（珠玖技術委員）に対する回答（２）

高度医療技術名：切除不能・再発胆道癌を対象としたゲムシタビン+CDDP+WT1 ペプチドワクチン併用化学免疫療法とゲムシタビン+CDDP 治療の第 I/II 相試験

2012/01/19

国立がん研究センター中央病院　奥坂　拓志

１．2011 年第 70 回日本癌学会学術総会で、北大のグループから胆道癌に於ける WT1 の発現解析についての報告があります。
（70th Annual Meeting of the Japanese Cancer Association P.2314 “WT1 expression in solid cancers of 4 different organs” pp.356）
それによりますと、先回の回答１にありました報告（Modern Pathology(2006) 19,804-814）で用いられている WT1 に対する単クローン抗体（6F-H2）を用いて、胆道癌 95 症例を免疫組織化学的に解析し、WT1 発現症例は、“0”であると報告されています。胆道癌における WT1 の発現について、提示していただいた参考文献以外の文献、報告が存在すれば、提示して下さい。また、これに関しての未発表の解析結果等のデータがありましたら示して下さい。

上記発表がなされたことは、承知しております。
一方で、上記発表内のデータでは、他の研究者が WT1 の発現を確認し、かつ WT1 特異的リンパ球による特異的細胞傷害を確認し、論文化されている肺がん細胞株も陰性とされており、第 3 者の免疫研究者から、そのデータの解釈は、方法論も含め慎重に評価する必要があるとの助言をいただいております。
WT1 の免疫染色法に関しては、固定方法並びに抗原活性化法が標準化されておらず、個々の研究者によってその染色効率が変わるとの指摘がなされております。染色結果の診断法の標準化もなされておりません。前回のご意見に対する回答内に、WT1 の免疫染色法・診断法の国際標準化作業を進めていることを触れさせていただきましたが、我々独自の解析はその上で進めさせていただきたいと思っています。また、国立がん研究センター内に構築中の、がんワクチン開発のための Core Facility でも、客観的評価を行うための独自の系の立ち上げを行っていることを申し添えます。

第 29 回高度医療評価会議	資料1－3
平成 24 年2月3日	

２．前回の質問に対する回答１では、胆道癌に於ける WT1 発現の頻度は 2 種類の抗体（C-19, 6F-H2）で各々80%及び、68%となっています。
　これらの頻度について、今回の臨床試験に於ける臨床効果の統計学的解析への影響につき述べて下さい。

WT1 の免疫組織化学的な発現割合が臨床効果と関連するのかどうか、仮に関連するのであればどの程度であるのか、についてはコンセンサスの確立した結論は得られておらず、今後の研究により明らかにしていく必要のある課題と認識しております。これらの検討は、検査方法の標準化が前提であり、それが確立された時点で、必要に応じて免疫組織化学的解析を行い、臨床結果と照らし合わせて総合的に判断することになります。
なお、今回の臨床試験においては、研究計画書にありますように将来実施可能な規模の第Ⅲ相試験において WT1 ペプチドワクチンの上乗せ効果を統計学的に検出できる差を示すことができるかどうかを探索する（早期探索試験）ことを目的として研究のデザインをしております。

第 29 回高度医療評価会議	資料1－3
平成 24 年2月3日	

高度医療審査の照会事項（山中構成員）に対する回答

高度医療技術名：切除不能・再発胆道癌を対象としたゲムシタビン+CDDP+WT1 ペプチドワクチン併用化学免疫療法とゲムシタビン+CDDP 治療の第 I/II 相試験

日付　2012/01/30

国立がん研究センター中央病院　奥坂　拓志

１．高度医療部分（50 万円×約 50 人分）は研究費負担ということだが、原資は何か。

厚生労働科学研究費補助金（がん臨床研究事業）「切除不能胆道がんに対する治療法の確立に関する研究（H22－がん臨床－一般－013)」です。

２．実施計画書内には「Rubinstein et al（JCO, 2005）のデザインを採用した場合、200 例以上が必要となり、本試験では実現困難な症例数になる」との記載がある。本試験は JCOG 施設とほぼ同様の施設を母体として実施されるもので、質の高い試験を実施できる全国の主要施設を（ある程度は）網羅しているように思われる。今回申請される第 II 相試験をはじめとして開発が進んだ場合、最終的には第 III 相試験による検証が必要になる。その場合、本治療法の effect size の大きさからみて、恐らく進行がん臨床試験における一般的な症例数（300 例、400 例以上）の試験になることが予想される。本邦における開発を念頭におかれているのだと思うが、この規模の試験の適正な期間内での実施可能性について、研究者側の perspective をお聞きしたい。

ご意見をいただき、ありがとうございます。ご指摘のように次相の第Ⅲ相試験は、400 例から 500 例規模になる可能性が高いと予想しております。最近本邦では下記のような 2 つのランダム化第Ⅱ相試験が終了しており、この登録状況を参考に 400 例から 500 例規模の第Ⅲ相試験の実施可能性を勘案いたしますと、参加施設が 20 施設程度であれば 4 年～6 年前後、40 施設以上であれば 2 年～3 年前後で登録が完了することが期待され、本邦での第Ⅲ相試験実施は可能と考えております。

試験名	症例数	施設数	登録期間
進行胆道がんを対象としたゲムシタビン+シスプラチン併用療法とゲムシタビン単独療法のランダム化第Ⅱ相試験（BT22）	83 人	9 施設	2 年 1 月（2006/9～2008/10）
進行胆道がんを対象としたゲムシタビン+S1 併用療法と S-1 単独療法のランダム化第Ⅱ相試験（JCOG0805）	101 人	19 施設	1 年 3 月（2009/2～2010/4）