

# 培養細胞を用いた発がん性予測試験法

## Bhas 42形質転換試験法について

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2012年11月28日

# 変異発がん物質と非変異発がん物質の検出

## 変異発がん物質

### 遺伝毒性試験法

初期 DNA 傷害

コメット・アッセイ

UDS 試験



遺伝子突然変異

エームス試験

マウスリンフォーマ試験



染色体切断作用

染色体異常試験

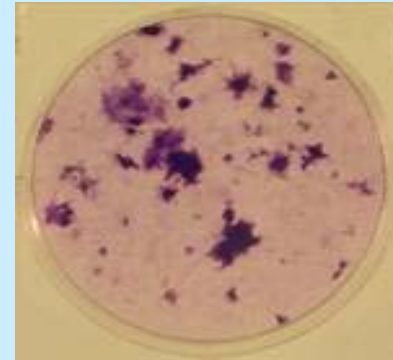
小核試験



## 非変異発がん物質

### 形質転換試験 (CTA)

Bhas 42 cells



(3 weeks)

Human non-genotoxic carcinogens were present in 12%(45/371) of IARC's Groups 1, 2A and 2B carcinogens (Hernández LG *et al.*, Mutat Res 682, 94-109, 2009)

# 形質転換試験とは？

細胞の形態や性質の悪性転換を指標とする試験法



- 1) BALB/c 3T3 や Bhas 42 細胞では、**フォーカスの形成** (増殖の接触阻止の喪失)を指標
- 2) SHE 細胞では、**コロニーの形態変化**を指標
- 3) JB6 細胞では、**軟寒天内増殖** (足場依存性の喪失)を指標



これらの形質転換細胞は、**造腫瘍性**を示す

# 発がん細胞の形質転換の関連を示すいくつかのエビデンス

1. 形質転換したBhas 42 細胞はヌードマウスへの移植で腫瘍を形成する。このことより、化学物質による細胞の形質転換が発がんの直接的なエビデンスになり得る。



$10^6$  cells/site were implanted to nude mice.

2. NIH 3T3 を用いた細胞の形質転換試験によりoncogenes (*ras*、*myc* 等)や oncovirus が特定され、またBALB/c 3T3を用いて発がん物質の検出に応用されてきた歴史がある。
3. 正常細胞から悪性細胞へ至る形質転換は、動物における正常細胞から腫瘍にいたるinitiationや promotionなどの多段階的発がん過程をsimulateしていると考えられている。

# OECDへ提案された3種の形質転換試験の特性比較

| CTA               | 提案国             | 細胞種          | 培養期間             | コスト | 計測難易度                       |
|-------------------|-----------------|--------------|------------------|-----|-----------------------------|
| SHE               | France &<br>USA | Primary cell | 1 週間<br>* 短期培養   | Low | 難<br>* 形態観察                 |
| BALB/c 3T3<br>厚労省 |                 | Cell line    | 5-6 週間<br>* 長期培養 | Mid | 易<br>* フォーカス数カウント           |
| Bhas 42           | 経産省             | Cell line    | 3 週間             | Low | 易<br>* フォーカス数カウント<br>* 高感受性 |

# Bhas 42 細胞の開発

BALB/c 3T3 cells

Transfection

v-Ha-ras

Isolation of clones containing *ras*

Selection of non-transformed clones

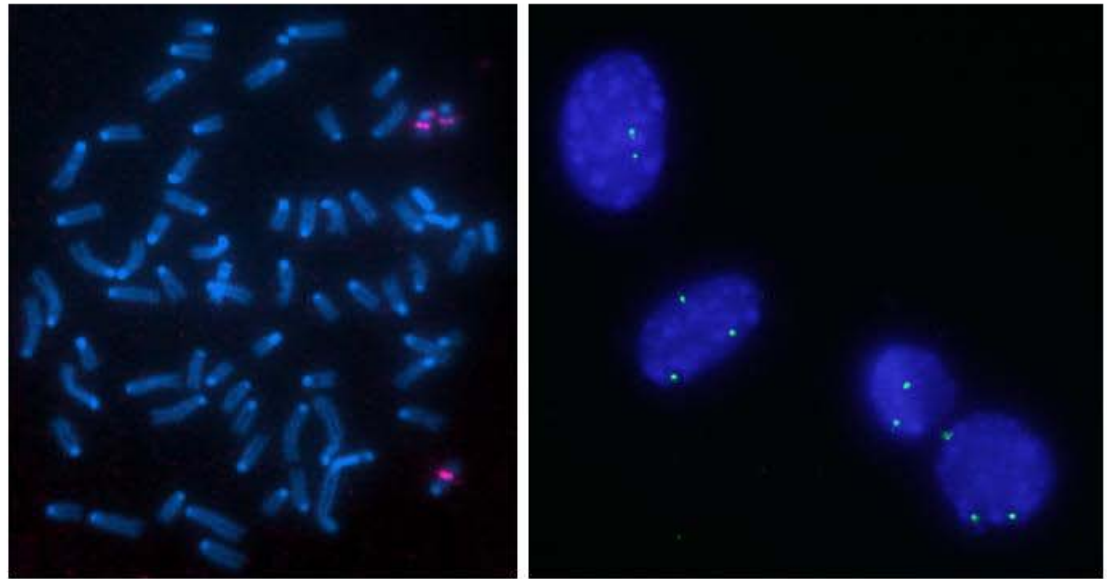
Selection of TPA-sensitive clones

**Bhas 42 cells** : Initiated cells

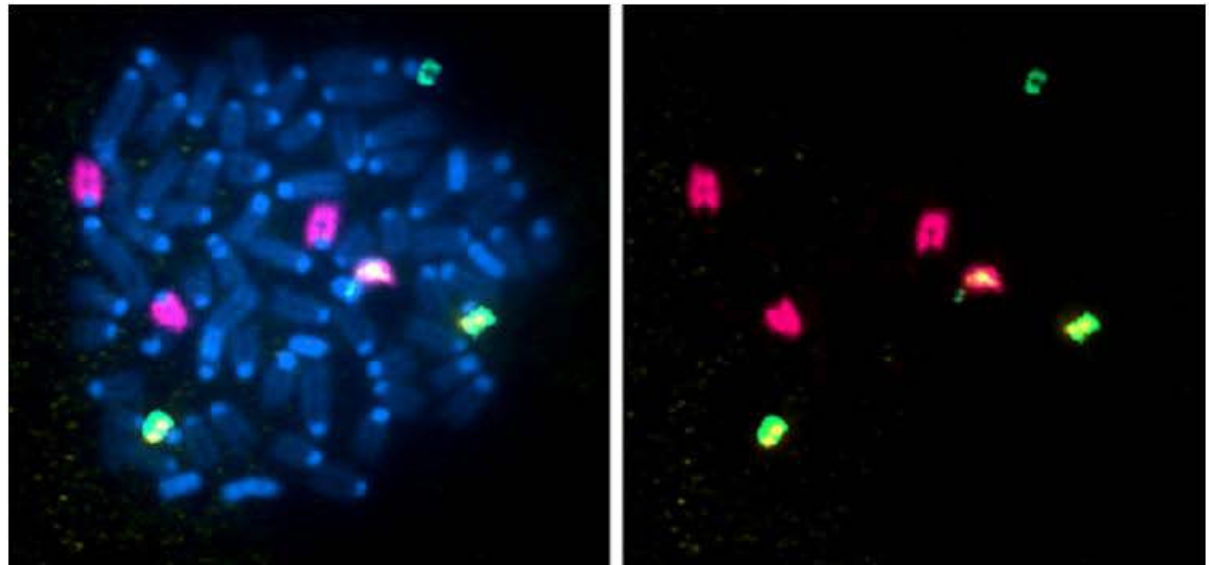
Sasaki *et al.*, *Jpn. J. Cancer Res.*, 79 , 921-930 (1988).

## Bhas 42 細胞 における v-Ha-ras の保持と局在 (Fish染色)

全ての細胞にv-Ha-ras gene  
がみられる。  
(平均 = 2.4 コピー/核)



# 17 染色体 (red) と  
# 19 染色体 (green) 上に  
V-Ha-ras gene (yellow) が  
局在。





## Bhas 42 CTA の開発経緯

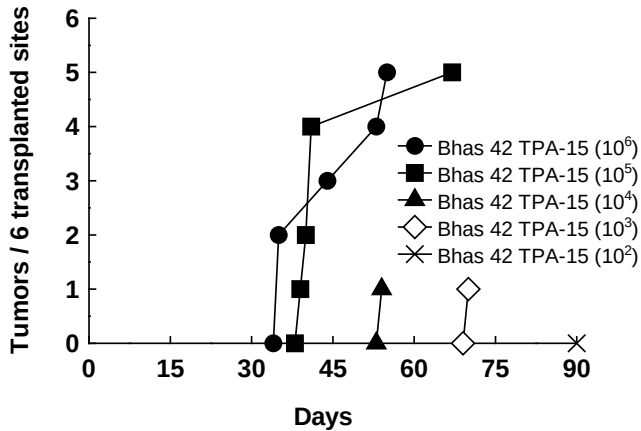
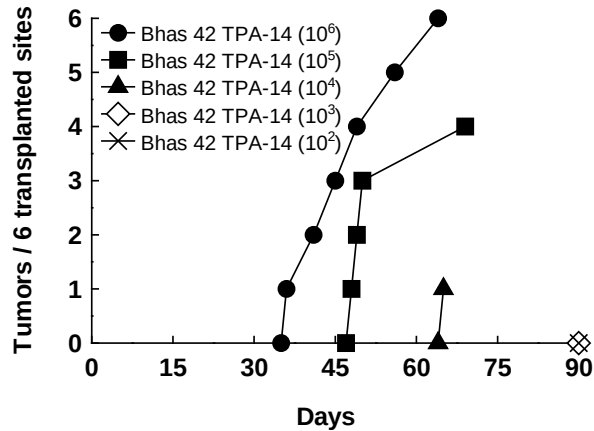
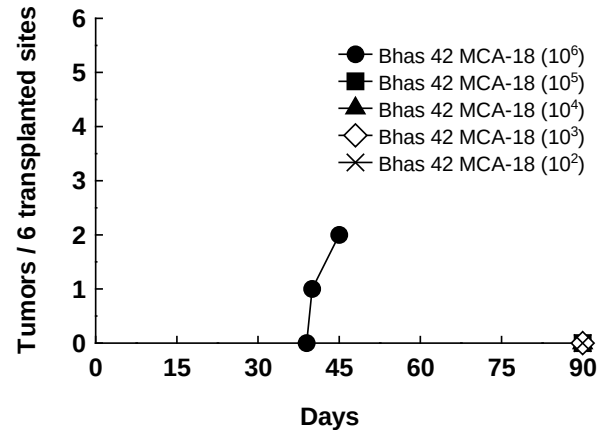
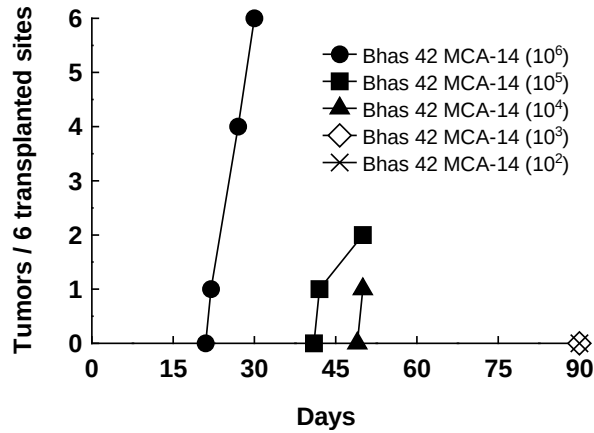
1. Bhas 42 CTAは、tumor-promotersを検出  
*Ohmori et al., Mutat. Res., 557, 191-202 (2004)*
2. Bhas 42 CTAは、tumor-promotersに加えtumor-initiators も検出  
*Asada et al., Mutat. Res., 588, 7-21 (2005)*

## Bhas 42 CTA の利点

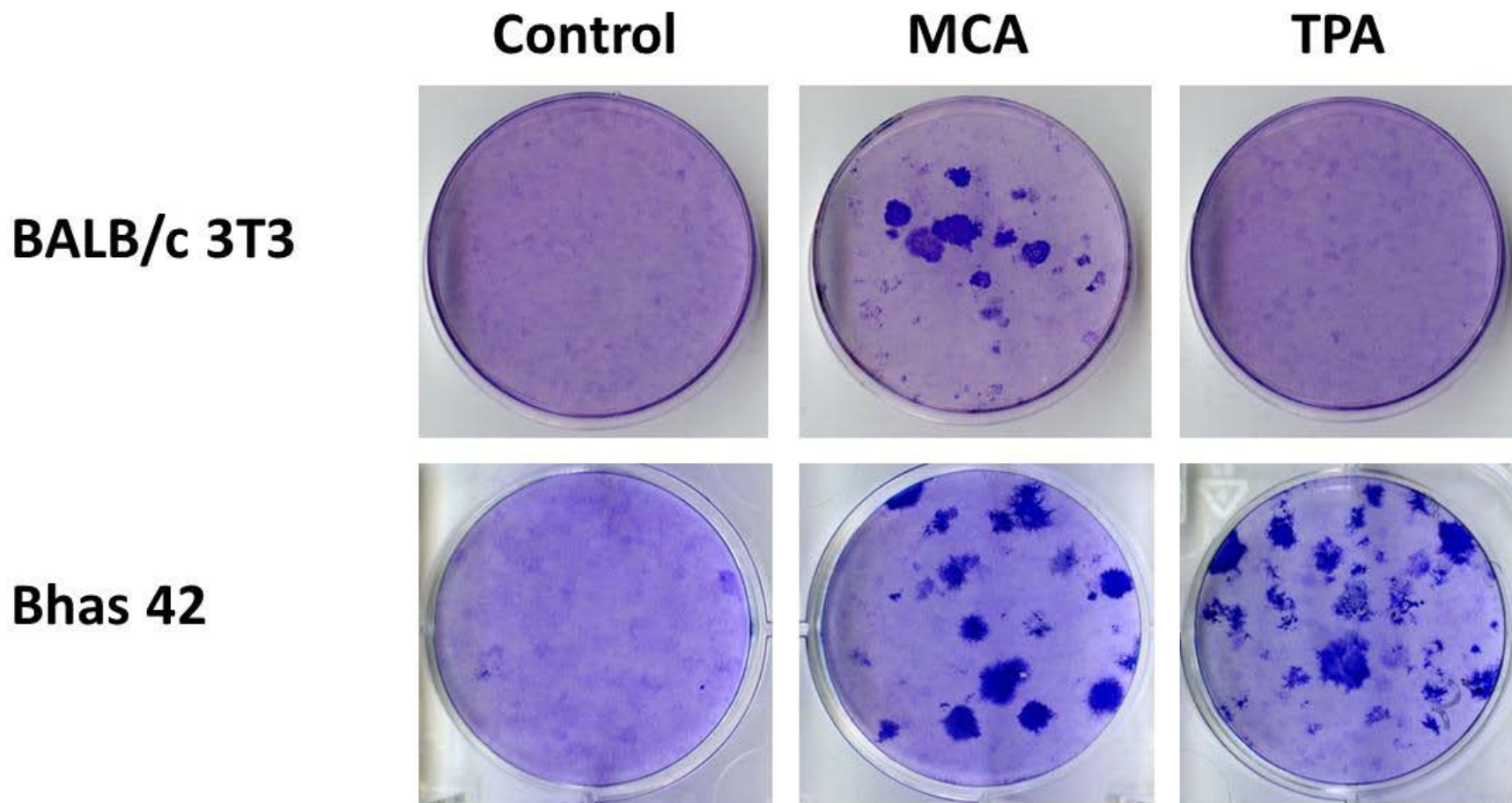
1. 培養期間が3 週間と従来の BALB/c 3T3 法の6 週間に比べ短い.
2. 作業時間が短く、器材や培地量が少なく経済的.
3. Bhas 42 CTA は、プロトコールを少し変えることで、発がん物質の Initiation活性 と Promotion 活性をそれぞれ区別して検出できる.



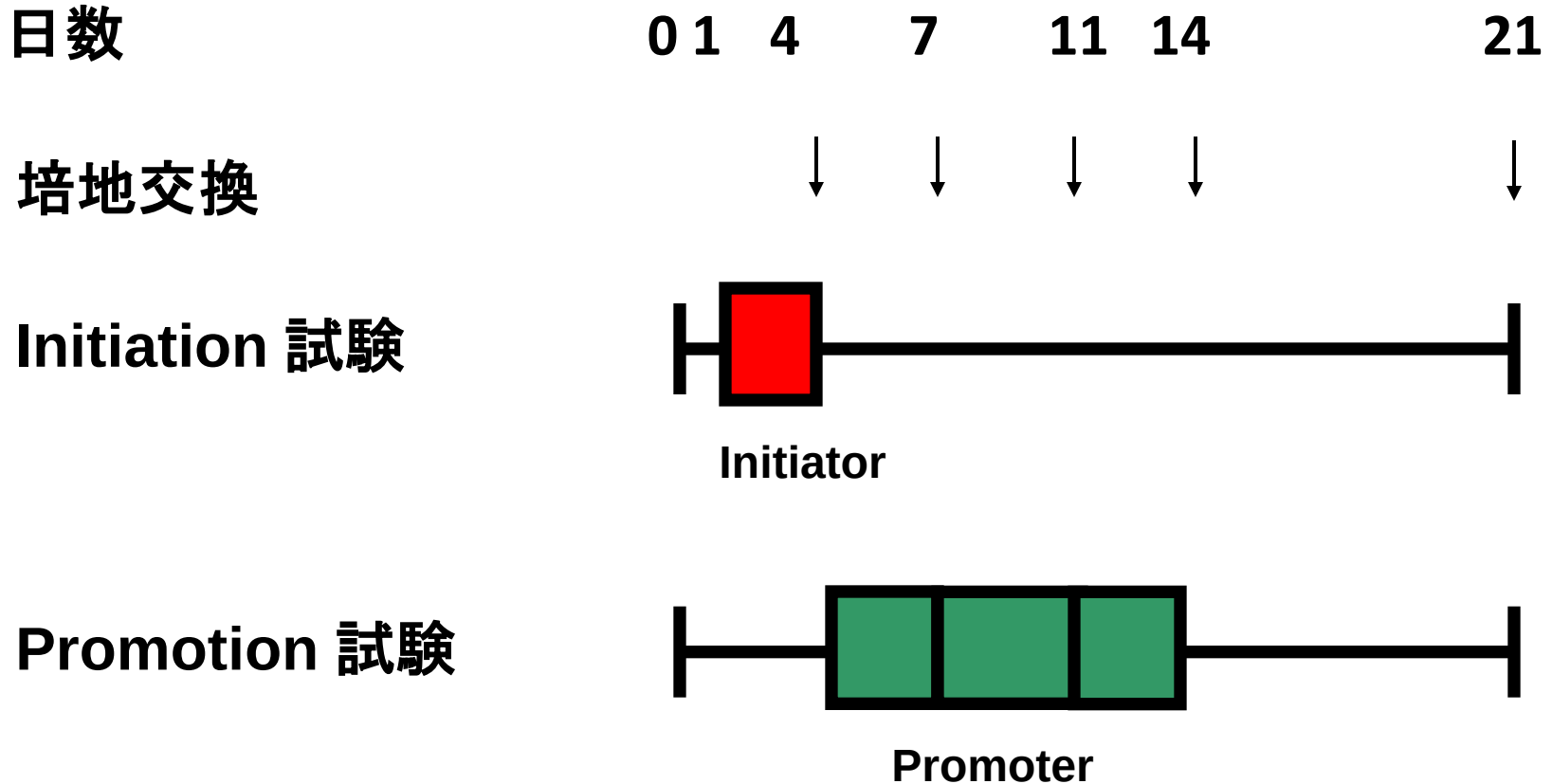
# 形質転換したBhas 42 細胞 はヌードマウスへの移植で造腫瘍性を示すが、正常 Bhas 42細胞やBALB/c 3T3 細胞は造腫瘍性なし



# BALB/c 3T3細胞 と Bhas 42 細胞における形質転換誘発



# Bhas 42 CTAにおける処理と試験期間

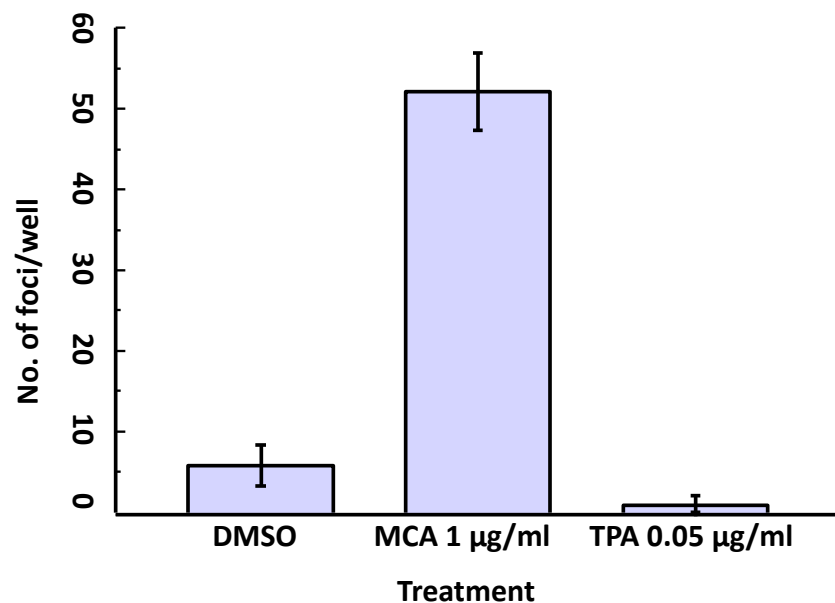


6-well法 または 96-well 法に適用

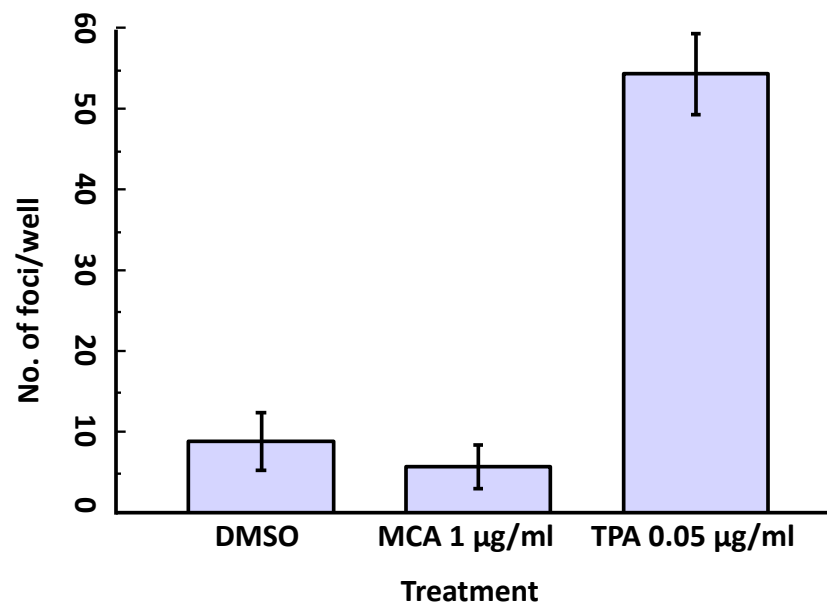
細胞増殖試験用の細胞は7日目に固定

## Bhas 42 CTAにおけるinitiator と promoter の分別

### Initiation 試験



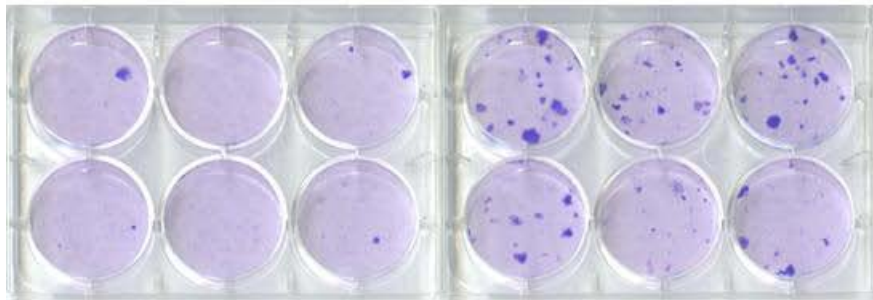
### Promotion 試験



## 6-well 法と 96-well 法による形質転換頻度の表現

Control

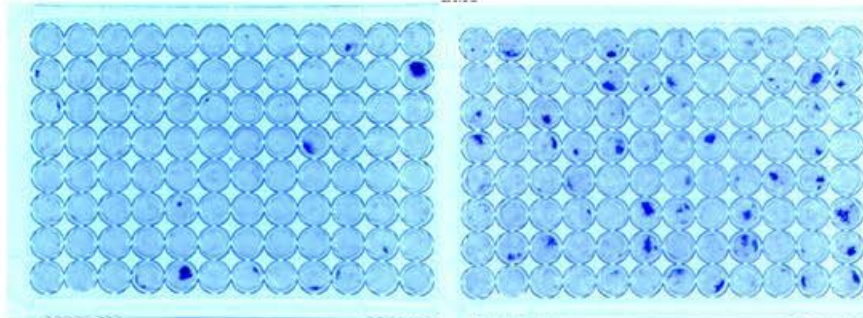
MCA 1  $\mu\text{g}/\text{ml}$



### 6-well 法

フォーカス 数/ **Well**

例) 14 個/ **Well**



### 96-well 法 (自動化への応用が可能)

フォーカスを有するwell数/ **96 wells**

例) 52 wells/ **96 wells**

# Bhas 42 CTAの発がん物質検出への利用法

- \* OECD/TG化し、発がん物質スクリーニングへ利用
- \* 安衛法でのスクリーニング試験としての利用
- \* 産業界でのスクリーニングへの利用



従来の  
変異原性試験と併用

Initiators & Promoters の検出

発がんPromotersの 検出

# Bhas 42 CTA の特性とパフォーマンス 98化学物質についての結果から

*Sakai et al., Mutat. Res., 702, 100-122 (2010)*



# Bhas 42 CTA における試験結果と遺伝毒性試験データとの比較

## 発がん物質 (52 物質)

| Chemical                                          | Bhas 42 CTA |           | Ames            | ML        | Chrom. Ab. |         | MN         |
|---------------------------------------------------|-------------|-----------|-----------------|-----------|------------|---------|------------|
|                                                   | Initiation  | Promotion |                 |           | in vitro   | in vivo |            |
| 2-Acetylaminofluorene                             | +           | +         | +               | +,+,+     | +          | +       | +          |
| Benz[a]anthracene                                 | +           | +         | +,+,+           | +,+,+,+/- | +/-        | +/-     | +          |
| 2-Amino-3-methylimidazo-<br>[4,5-f]quinoline (IQ) | +           | -         | +               |           |            |         | -          |
| 5-Azacytidine                                     | +           | -         | +,+/-           | +,+,+,+   | +,+/-      |         | +          |
| Barium chromate                                   | +           | -         | -               |           |            |         |            |
| Benzo[a]pyrene                                    | +           | -         | +,+             | +,+,+     | +          | +       | +          |
| Cyclophosphamide                                  | +           | -         | +,+/-, +/-      | +,+,+,+   | +,+        | +,+/-   | +,+,+      |
| Cyclosporin A                                     | +           | -         | -               |           |            |         | -          |
| 2,4-Diaminotoluene                                | +           | -         | +,+             | +         | +          |         | -          |
| Dibenz[a,h]anthracene                             | +           | -         | +,+/-, +        | +,+       | +          |         |            |
| Melphalan                                         | +           | -         | +,+,+,+/-       | +         | +,+        | +       | +          |
| 3-Methylcholanthrene (MCA)                        | +           | -         | +,+             | +         | -, -       |         |            |
| Mitomycin C                                       | +           | -         | +               | +,+       | +,+        | +,+,+   | +,+,+      |
| MNNG                                              | +           | -         | +,+             | +,+       | +/-        |         | +          |
| Sterigmatocystin                                  | +           | -         | +               |           |            |         | +          |
| Thio-TEPA                                         | +           | -         | +               |           | +          | +       | +          |
| Cadmium chloride                                  | -           | +         | -, -            | +         | +, -       |         | -          |
| Chenodeoxycholic acid (TP)                        | -           | +         | -/+             |           |            |         |            |
| 4-Chloro-o-toluidine HCl                          | -           | +         | -               | +         | +W         |         |            |
| Cholic acid (TP)                                  | -           | +         | +/-             |           |            |         |            |
| Deoxycholic acid (TP)                             | -           | +         | -/+             |           |            |         |            |
| Dichlorvos                                        | -           | +W        | +,+             | +,+,+     | +          | -, -    | -          |
| Epichlorohydrin                                   | -           | +         | +,+,+/-         | +,+       | +,+/-      | +,+/-   | -          |
| D-Limonene                                        | -           | +         | -, -            | -, +      | -          |         |            |
| Lithocholic acid (TP)                             | -           | +         | -, -            | +/-, +/-  | -          |         |            |
| Methapyrilene HCl                                 | -           | +         | -               | -, +, +/- | +          |         |            |
| Methylarsonic acid (TP)                           | -           | +         | -               | +         | +          |         | +          |
| Mezerein (TP)                                     | -           | +         | -, -            |           |            |         |            |
| 2-Naphthylamine                                   | ±           | +         | +,+,+/-, +, +/- | +,+,+     | +,+        |         | -, +, -, + |
| Phorbol 12,13-didecanoate (TP)                    | +           | +         |                 |           |            |         |            |
| Quercetin                                         | -           | +         | +,+,+/-, +/-    | +         | +,+        | +       | +,+        |
| Sodium arsenate                                   | -           | +         | -               | +         |            |         | -          |
| Sodium arsenite                                   | ±           | +         | -               | +,+       |            |         | +          |

Red chemical: Carcinogens that were positive in the Bhas 42 CTA but are negative or discordant in the Ames assay.

TP: Tumor promoter

# (続き)

## 発がん物質 (52 物質—続き)

| Chemical                    | Bhas 42 CTA |           | Ames          | ML               | Chrom. Ab. |         | MN        |
|-----------------------------|-------------|-----------|---------------|------------------|------------|---------|-----------|
|                             | Initiation  | Promotion |               |                  | in vitro   | in vivo |           |
| <b>Sodium saccharin</b>     | -           | +         | -,-           |                  |            |         |           |
| Styrene oxide               | -           | +         | +,+,+/-,+/-   | +,+,+            | +          | +/-     | -         |
| <b>TPA (TP)</b>             | ±           | +         | -             |                  |            |         |           |
| <b>o-Toluidine</b>          | -           | +         | +, -          | +, -, +/ -, +/ - | +, +/ -    |         | +/-, -    |
| Zinc chloride               | -           | +         | +             |                  | -          |         |           |
| o-Anisidine                 | -           | -         | +             | +,+,+            | +          |         |           |
| Benzene                     | -           | -         | -             | -,+,+/-,+/-      | -,+,+      | +,+     | +,+       |
| Cobalt sulfate heptahydrate | -           | -         | +W            |                  |            |         |           |
| Diethylstilbestrol (DES)    | -           | -         | -, -, -       | +,+,+/-,+/-      | +, +/ -    | +       | +/-, +, - |
| Dimethylarsinic acid        | -           | -         | -             | +                | +          | +       | +/-       |
| 1,4-Dioxane                 | -           | -         | -, -          | -, -             | -          |         | -         |
| Ethyl carbamate (Urethane)  | -           | -         | +/-, -, -     | -                | -, +       |         | +, +      |
| Formaldehyde                | -           | -         | +, +/ -, +/ - | +                | +, +       | +/-     | +/-       |
| Furylfuramide (AF-2)        | -           | -         | +,+,+         | +,+              | +          |         |           |
| Methyl carbamate            | -           | -         | -, -, -       | -, -             | -          |         | -         |
| Nickel (II) chloride        | -           | -         | -             | +                | +          | +       | -         |
| Nickel monoxide             | -           | -         | -             |                  |            |         | -         |
| Phenobarbital sodium        | -           | -         | -, +/ -       |                  |            |         |           |
| p-Toluidine                 | -           | -         | -             |                  |            |         |           |

**Red chemicals:** Carcinogens that were positive in the Bhas 42 CTA but are negative or discordant in the Ames assay.

**Blue chemicals:** Carcinogens that were negative in Bhas 42 CTA and are negative or discordant in the Ames assay.

# (続き)

## 非発がん物質 (37 物質)

| Chemical                           | Bhas 42 CTA |           | Ames       | ML             | Chrom. Ab. |         | MN   |
|------------------------------------|-------------|-----------|------------|----------------|------------|---------|------|
|                                    | Initiation  | Promotion |            |                | in vitro   | in vivo |      |
| 4-Acetylaminofluorene              | -           | -         | +          | +/-,+,+/-      | -          | -       |      |
| Acid red 14                        | -           | -         | -, -       | -, -           | -          |         | -    |
| Ampicillin sodium salt             | -           | -         | -, -       | -, -           | -          |         |      |
| Anthracene                         | -           | -         | +W, -, +/- | +,+,+,+/-      | -          |         |      |
| L-Ascorbic acid                    | -           | -         | +W/-, -    |                | -          | +       | +    |
| Aspartame                          | -           | -         | -          |                |            |         | -    |
| Benzoin                            | -           | -         | +W/-, +/-  | +, -, +/-      | -          | -       | -    |
| Caffeine                           | -           | -         | -, -, -    |                | +          |         | +    |
| Caprolactam                        | -           | -         | -, -       | -, -, -, -     | -          | -       | -, - |
| 2-Chloroethanol                    | -           | -         | +,+, +/-   | +, +           | +          | -       | -    |
| Chromium (III) Chloride, anhydrous | -           | -         | -          |                | +          | -       |      |
| 2,6-Diaminotoluene                 | -           | -         | +          |                |            |         |      |
| Diazepam                           | -           | -         | -, -       |                | -          |         |      |
| N,N-Dimethylformamide              | -           | -         | -, -       | +/-, -, -, +/- | -          |         |      |
| Eugenol                            | -           | -         | -          | +,+, +         | +          |         | -, + |
| HC Blue 2                          | -           | -         | +          | +, +           | -          | -       |      |
| Hydrocortisone                     | -           | -         |            |                | +          |         |      |
| D-Mannitol                         | -           | -         | -, -       | -, -           | -          | -       | -    |
| Methotrexate                       | -           | -         | -          | +, +           | +          | +       | +    |
| 1-Naphthylamine                    | -           | -         | +,+, +/-   |                | +,+/-      |         | +, + |
| Phenanthrene                       | -           | -         | +W, -, +/- |                | -          |         |      |
| Phenol                             | -           | -         | -          | +,+, +         | +, +       | +       | +    |
| p-Phenylenediamine 2HCl            | -           | -         | +          | +,+/-, +       | +          |         |      |
| Phthalic anhydride                 | -           | -         | -, -       | +              | -          |         |      |
| Rotenone                           | -           | -         | -          | +              | +          |         | +    |
| Sodium chloride                    | -           | -         | -          | +/-            |            |         |      |
| Sodium nitrite                     | -           | -         | +, +       | +              | +          | +       | -, + |
| Sunset yellow FCF                  | -           | -         | -          | +, -           | -          | -       | -    |
| Thiabendazole                      | -           | -         | +          |                |            |         | +    |
| m-Toluidine                        | -           | -         | -          |                |            |         |      |
| Triphenyltin hydroxide             | -           | -         | -, -       | +              | -          |         |      |
| Barium chloride dihydrate          | -           | +         | -, -       | +              | -          |         |      |
| tert-Butylhydroquinone             | -           | +         | -, -       |                | +          | -       | -    |
| 8-Hydroxyquinoline                 | -           | +         | +, +       | +, +           | +W         | -       | -    |
| Propyl gallate                     | +           | +         | -, -       | +, +           | +          | +       | +/-  |
| Sodium fluoride                    | -           | +         | -, -       | +,+, +         | +/-        | -       | -, + |
| Tetracycline HCl                   | -           | +         | -, -       | -, +/-         | -          |         |      |

# 発がん性予測法としてのBhas 42 CTAのパフォーマンス

|             |          | <i>In vivo</i> carcinogenicity |                |
|-------------|----------|--------------------------------|----------------|
|             |          | Carcinogen                     | Non-carcinogen |
| Bhas 42 CTA | Positive | 38                             | 6              |
|             | Negative | 14                             | 31             |

89 chemicals in total  
(Out of 98 chemicals)

|                       |      |
|-----------------------|------|
| Concordance           | 78 % |
| Sensitivity           | 73   |
| Specificity           | 84   |
| Positive predictivity | 86   |
| Negative predictivity | 69   |
| False negative        | 27   |
| False positive        | 16   |

## Performance of CTAs for the prediction of carcinogenicity

| CTAs            | Bhas 42 | SHE    |         | BALB/3T3* | C3H10T1/2* |
|-----------------|---------|--------|---------|-----------|------------|
|                 |         | pH6.7* | pH>7.0* |           |            |
| Total chemicals | 89      | 88     | 204     | 149       | 96         |
| Concordance     | 78 (%)  | 74     | 85      | 68        | 73         |
| Sensitivity     | 73      | 66     | 92      | 75        | 72         |
| Specificity     | 84      | 85     | 66      | 53        | 80         |
| +Predictivity   | 86      | 88     | 88      | 77        | 95         |
| - Predictivity  | 69      | 62     | 75      | 50        | 34         |
| False +         | 16      | 15     | 34      | 47        | 20         |
| False -         | 27      | 33     | 8       | 25        | 28         |

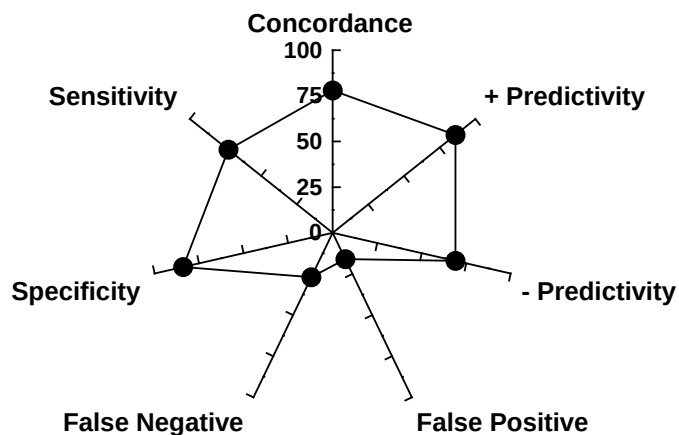
\* Data were obtained from OECD DRP 31 (2007)

## Comparison of performance of Bhas 42 CTA with that of *in vitro* genotoxicity assays

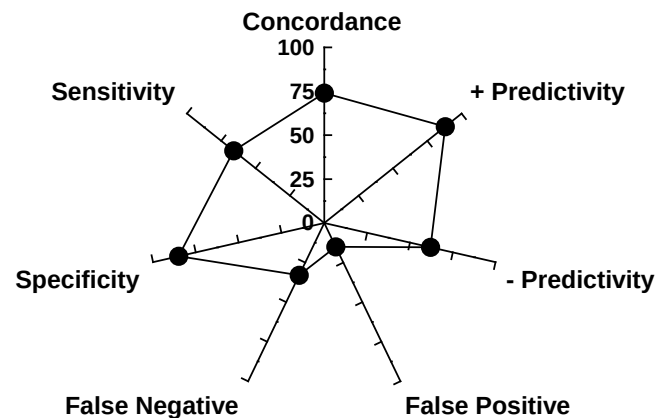
| In vitro assay  | Bhas 42 CTA   | Ames* | Mouse lymphoma* | HPRT* | Chromosomal aberration* |
|-----------------|---------------|-------|-----------------|-------|-------------------------|
| Total chemicals | <b>89</b>     | 315   | 215             | 135   | 238                     |
| Concordance     | <b>78 (%)</b> | 52    | 72              | 78    | 62                      |
| Sensitivity     | <b>73</b>     | 39.5  | 86              | 80    | 65                      |
| Specificity     | <b>84</b>     | 79    | 26              | 70    | 59                      |
| +Predictivity   | <b>86</b>     | 81    | 79              | 91    | 76.5                    |
| - Predictivity  | <b>69</b>     | 37    | 36              | 48.5  | 45                      |
| False +         | <b>16</b>     | 21    | 74              | 30    | 41                      |
| False -         | <b>27</b>     | 60.5  | 14              | 20    | 35                      |

\* Data were obtained from OECD DRP 31 (2007)

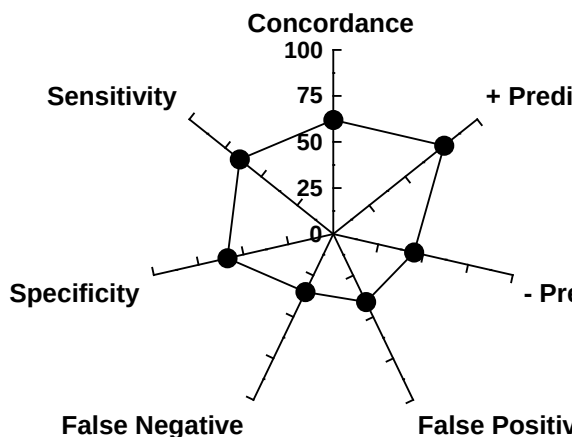
# 他の試験法とのパフォーマンスの比較



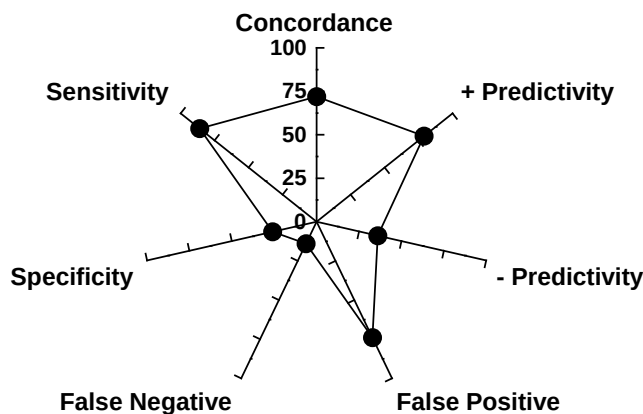
Bhas 42 (98 Chemicals)



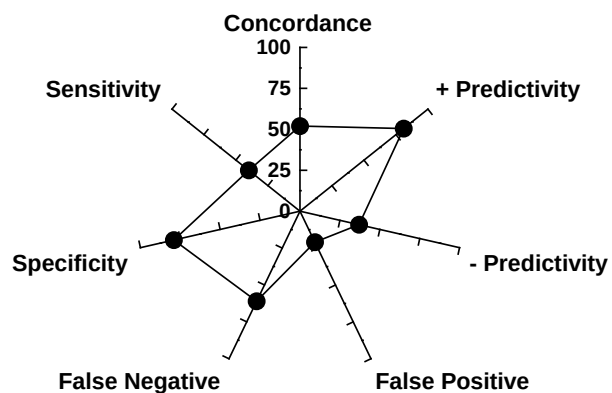
SHE pH 6.7 (88 Chemicals)



CA (238 Chemicals)



ML (215 Chemicals)



Ames (315 Chemicals)

## **Bhas 42 CTAについてのValidation studies**

- **Pre-validation study of the 6-well method**
- **Validation study of the 6-well method**
- **Validation study of the 96-well method**
  - Pre-validation phase**
  - Validation phase I**
  - Validation phase II**



## **Bhas 42 CTA Validation Study ReportのESAC Peer Reviewにおける 評価の要点**

**Module 1: Test definition (定義)**

**Module 2: Within-laboratory reproducibility (施設内再現性)**

**Module 3: Transferability (技術移転性)**

**Module 4: Between-laboratory reproducibility (施設間再現性)**

**Module 5: Predictive capacity (予測能)**

**Option: Similarity of the 6- and 96-well protocols (6-wellと96-well  
法の同等性)**

### **ESAC OPINION**

**2013年3月ごろ公表される予定**