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OECD化学物質対策の動向（第11報）
第19回OECD高生産量化学物質初期評価会議（2004年ベルリン）

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Progress on OECD Chemicals Programme (11) — SIAM 19 in Berlin, 2004

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The 19th Screening Information Data Set (SIDS) Initial Assessment Meeting (SIAM 19) was held in Berlin, Germany, hosted by the Germen Federal Agency for the Environment. The initial assessment documents of four substances (CAS numbers: 92-70-6, 126-33-0,131-17-9, 7580-85-0) and one category (High Molecular Weight Phthalate Esters) at SIAM 19 were submitted by the Japanese Government with or without the International Council of Chemical Associations (ICCA) and all of them were agreed at the meeting. In this report, the documents of these substances are introduced.

Keywords: OECD, HPV programme, SIDS Initial Assessment Meeting

1. はじめに

経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）加盟各国における高生産量化学物質（High Production Volume Chemical：HPV）について，1992年に始まったOECD高生産量化学物質点検プログラム（HPV programme）により安全性の評価が行われている[1]．日本政府は初回より評価文書を提出しており，第18回までの初期評価会議（Screening Information Data Set (SIDS) Initial Assessment Meeting: SIAM）において日本政府が担当し結論及び勧告が合意された化学物質の評価文書のヒトの健康影響または環境影響・曝露情報については既に紹介してきた[2-8]．また，SIAM 19[9]，SIAM 20[10]及びSIAM 21[11]の会議内容，SIAM 1からSIAM 18までの会議の結果の概要[12]についても紹介してきた．

国際化学工業協会協議会（International Council of Chemical Associations：ICCA）による評価文書の原案作成に伴い，日本においても2001年から日本政府に加え日本化学工業協会加盟企業も評価文書の原案を作成している．

評価文書は，物性，曝露情報，健康影響及び環境影響に関する記述から構成されている．本稿では第19回SIAM（SIAM 19）で合意に至った化学物質名及び日本担当物質の初期評価文書の概要を紹介する．

2. SIAM 19で合意された化学物質名と日本担当物質の初期評価内容

2004年10月にベルリン（ドイツ）で開催されたSIAM 19において，25物質及び5カテゴリー（構造や毒性の類似した物質をまとめ，カテゴリーとした．それぞれ4，5，6，7及び9物質を含む），計56化学物質の初期評価文書が審議され，表1に示す物質の初期評価結果及び勧告が合意された．SIAMにおける合意はFWまたはLPとして示されている．FWは「今後も追加の調査研究作業が必要である（The chemical is a candidate for further work.）」，LPは「現状の使用状況においては追加作業の必要はない（The chemical is currently of low priority for further work）」ことを示す．

(1) 3-Hydroxy-2-naphthoic acid (92-70-6)（日本及びドイツ政府）

1) 曝露状況

本物質は主に染料や顔料の中間体として，さらに，殺

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Table.1. Chemical substances discussed at SIAM 19 and their outcomes

CAS No.	Name of Substance	Sponsor Country	Outcome
67-48-1	Choline chloride	UK/ICCA	LP
67-56-1	Methanol	US/ICCA	ENV: LP HH: FW
64-17-5	Ethanol	CZ+SK/ICCA	LP
78-83-1	Isobutanol	US/ICCA	LP
92-70-6	3-Hydroxy-2-naphthoic acid	JP+DE	ENV: LP HH: FW
95-53-4	o-Toluidine	DE/ICCA	LP
101-54-2	4-Aminodiphenylamine	DE/ICCA	FW
102-09-0	Diphenyl carbonate	DE/ICCA	LP
108-95-2	Phenol	DE:eu	ENV: LP HH: FW
111-48-8	Thiodiglycol	DE/ICCA	LP
119-64-2	1,2,3,4-Tetrahydronaphthalene	DE/ICCA	FW
126-33-0	Tetrahydrothiophene-1,1-dioxide	JP/ICCA	ENV: LP HH: FW
131-17-9	Diallyl phthalate	JP/ICCA	ENV: LP HH: FW
502-44-3	epsilon-Caprolactone	BE/ICCA	LP
513-35-9	2-Methyl-2-butene	US/ICCA	LP
2530-83-8	Trimethoxy [3-(oxiranylmethoxy)propyl] silane	US/ICCA	LP
6104-30-9	N,N"-(Isobutylidene)diurea	DE/ICCA	LP
6422-86-2	Di(2-ethylhexyl)terephthalate	US/ICCA	LP
7580-85-0	2-tert-Butoxyethanol	JP	ENV: LP HH: FW
7719-12-2	Phosphorus trichloride	DE/ICCA	LP
7758-94-3	Iron dichloride	KO	ENV: FW HH: LP
7775-14-6	Sodium dithionite	DE/ICCA	ENV: LP HH: FW
7783-20-2	Ammonium sulfate	DE/ICCA	LP
10025-87-3	Phosphoryl trichloride	DE/ICCA	LP
85535-85-9	C14-17 chloroalkanes	UK:eu	FW
Name of Category (CAS No.)		**Sponsor Country**	**Outcome**
Amorphous silica silicates (1344-00-9, 1344-95-2, 7631-86-9, 112926-00-8, 112945-52-5)		UK/ICCA	LP
Butenes (106-98-9, 107-01-7, 115-11-7, 590-18-1, 624-64-6, 25167-67-3)		NL/ICCA+FR/ICCA	LP
High Molecular Weight Phthalate Esters (119-06-2, 3648-20-2, 53306-54-0, 68515-41-3, 68515-43-5, 68515-47-9, 85507-79-5)		JP/ICCA+FR/ICCA	LP
Higher olefins (112-88-9, 629-73-2, 25264-93-1, 25339-53-1, 25339-56-4, 25377-83-7, 25378-22-7, 27215-95-8, 85535-87-1)		US/ICCA	LP
Monoethylene glycol ethers (111-76-2, 112-07-2, 112-25-4, 2807-30-9)		US/ICCA+AUS	LP

虫剤や医薬品の中間体としても使用されている．職業曝露の主要経路は経皮と考えられる．

2）環境影響

本物質は環境中で完全に解離しており，解離物質は揮発も吸着もしないことから，主に水圏に分布すると考えられる．本物質は容易に生分解しない（OECD TG 301C）が，水生生物における生物濃縮性は低い（生物濃縮係数BCF：0.5-4，OECD TG 302B）．水生生物に対する急性毒性では，魚類の半数致死濃度（LC_{50}）は68 mg/L（96時間，OECD TG 203），ミジンコの半数影響濃度（EC_{50}）は32.9 mg/L（48時間，遊泳阻害：OECD TG 202），藻類の50%生長阻害濃度（EC_{50}）は65.3 mg/L（72時間，生長速度法：OECD TG 201）であった．慢性毒性では，ミジンコの無影響濃度（NOEC）は10.4 mg/L（21日間，繁殖阻害：OECD TG 211），藻類のNOECは6.8 mg/L（72時間，生長速度法：OECD TG 201）であった．

3）健康影響

ラットの単回経口投与毒性試験における半数致死量（LD_{50}）は823～1,040 mg/kgであり，毒性症状として活動低下，呼吸亢進，閉眼，下痢が認められている．胃腸への刺激と黒色／斑状肝臓が死亡動物にみられた．モルモットの皮膚に24時間密閉塗布した結果，皮膚の壊死や皮下出血がみられ，致死量は約2,000 mg/kgであった．

ウサギの皮膚に対して弱い刺激性，眼に対しては強い刺激性が認められた．モルモットにおいて皮膚感作性が認められた．

ラットに0，12，60及び300 mg/kg/dayを強制経口投与した28日間反復経口投与毒性試験（OECD TG 407）では，雌では60 mg/kg/day以上で副腎の壊死，300 mg/kg/dayで肝重量の増加がみられ，雄では300

mg/kg/dayで血中リン酸塩値の低下，血中・尿中ビリルビン値の上昇が認められた．無毒性量（NOAEL）は雄で60 mg/kg/day，雌で12 mg/kg/dayとされた．二次資料ではあるが，10日間ラットを100 mg/m^{3}に曝露した反復吸入毒性試験では腎臓の壊死が認められた．

ラットの雄に交配前10週間及び交配期間を含め計98日間，雌に交配前2週間及び交配期間を含め分娩後哺育20日まで，0，12.5，50及び200 mg/kg/dayを強制経口投与した一世代生殖毒性試験（OECD TG 415）では，200 mg/kg/dayで親世代の雌雄に流涎，体重増加抑制，前胃粘膜肥厚が認められ，また，前胃扁平上皮過形成が50 mg/kg/day以上の雄，200 mg/kg/dayの雌でみられた．雌雄の生殖能力への影響は認められなかった．児では200 mg/kg/dayで体重低値，発育遅延，短尾及び曲尾が認められた．この試験の結果から，生殖毒性の無影響量（NOEL）は200 mg/kg/day，発生毒性のNOELは50 mg/kg/day，一般毒性のNOELは雄で12.5 mg/kg/day，雌で50 mg/kg/dayとされた．

細菌を用いる復帰突然変異試験は陰性であった．チャイニーズ・ハムスター培養細胞を用いる染色体異常試験では，S9 mix非存在下で染色体異常の誘発作用が認められた．*In vivo*でのチャイニーズ・ハムスター骨髄細胞の染色体異常試験では陰性であったが，有糸分裂中期細胞の観察数が少なく，また，標的組織における化学物質曝露証明が不明であったことから，この試験は評価には不十分とされた．

4）結論と勧告

本物質の健康影響はFWと勧告され，標準的な遺伝毒性試験（OECD TG 474または475）が推奨された．環境影響はLPと勧告された．

(2) Tetrahydrothiophene-1,1-dioxide（126-33-0）
（ICCA日本企業）

1）曝露状況

本物質は主に石油や酸性ガス精製時の芳香族炭化水素の抽出溶媒として使用される．溶媒として使用されるので消費者曝露は起こりにくいが，精製工場付近では飲料水や農作物経由での間接曝露の可能性がある．閉鎖系で使用されるので職業曝露の可能性は低いが，ドラム詰めの際に曝露の可能性がある．

2）環境影響

本物質が水圏に放出された場合，ほぼ全て水圏にとどまる．大気または土壌に放出された場合，または，大気・水圏・土壌に同時に放出された場合，土壌と水圏に等しく分布する．本物質は容易に生分解しない（OECD TG 301C）が，水生生物における生物濃縮性は低い（BCF：＜13）．水生生物に対する急性毒性では，魚類のLC_{50}は＞100 mg/L（96時間，OECD TG 203），ミジンコのEC_{50}は852 mg/L（48時間，遊泳阻害：OECD TG 202），藻類のEC_{50}は＞1,000 mg/L（72時間，生長速度法：OECD TG 201）であった．慢性毒性では，ミジンコのNOECは25 mg/L（21日間，繁殖阻害：OECD TG 211），藻類のNOECは556 mg/L（72時間，生長速度法：OECD TG 201）であった．

3）健康影響

本物質はラットにおいて代謝が飽和する可能性がある．ウサギ，イヌ，リスザルでは本物質は全身に速やかに分布され，半減期3.5～5時間で血漿から除去される．ウサギにおける代謝産物は3-hydroxysulfolaneである．

ラットの単回経口投与毒性試験（OECD TG 401）でのLD_{50}は雄では2,006 mg/kg，雌では2,130 mg/kg，ラットの単回経皮投与毒性試験でのLD_{50}は2,000 mg/kg以上，ラットの単回吸入毒性試験でのLC_{50}は12,000 mg/m^{3}以上と報告されている．

モルモットとウサギの皮膚，ウサギの眼に対して刺激性は認められなかった．モルモットにおいて皮膚感作性はみられなかった．

ラットに0，60，200及び700 mg/kg/dayを強制経口投与した28日間反復経口投与毒性試験では，700 mg/kg/dayにおいて，雌で一過性の自発運動低下が投与初期にみられ，また，雌雄の体重増加の抑制及び摂餌量の減少，血液生化学的検査では雄でコリンエステラーゼ活性及び総ビリルビン値の増加，塩素の減少，雌でGPT活性増加，グルコース値の減少が認められた．さらに，雄では200 mg/kg/day以上で腎臓の近位尿細管上皮における硝子滴及び好酸性小体の増加がみられ，700 mg/kg/dayで腎臓重量が増加した．雌では700 mg/kg/dayで脾臓重量の減少が認められた．これらの結果から，NOAELは雄で60 mg/kg/day，雌で200 mg/kg/dayとされた．

雌雄ラットに交配前2週間から交配期間を含み，雄では計49日間，雌では分娩後哺育3日まで，0，60，200及び700 mg/kg/dayを強制経口投与した簡易生殖毒性試験（OECD TG 421）では，700 mg/kg/dayの雌雄において，1例ずつ死亡し，交配前に体重の増加抑制，摂餌量の減少が認められた．生殖発生毒性については，700 mg/kg/dayにおいて発情回数の低値がみられ，また，新生児が哺育期に全例死亡した母動物が4例認められ，さらに，生児分娩率（生児数／着床痕数×100），分娩時生存率（生児数／総産児数×100），哺育4日の生児数，生存率，哺育0及び4日の雌雄別体重の低値，死産児数の高値がみられた．200 mg/kg/dayでは生児分娩率の低値がみられた．これらの結果から，母体毒性のNOAELは200 mg/kg/day，生殖発生毒性のNOAELは60 mg/kg/dayとされた．

細菌を用いる復帰突然変異試験及びチャイニーズ・ハ

ムスター培養細胞を用いる染色体異常試験では陰性であった．

4）結論と勧告

本物質の健康影響はFWと勧告され，産業的使用者の曝露評価や飲料水からの間接曝露評価を行うことが推奨された．環境影響はLPと勧告された．

(3) Diallyl phthalate（131-17-9）(ICCA日本企業)

1）曝露状況

本化学物質は多種多様の用途を持ち，主にdiallyl phthalateプレポリマーのモノマーや他のポリマー製造中における架橋剤として使用されている．職業曝露の主要経路は吸入及び経皮と考えられる．また，本物質を含む製品から，吸入及び経皮経路による消費者曝露の可能性がある．

2）環境影響

本物質が水圏に放出された場合，主に水圏にとどまる．大気または土壌に放出された場合，主に土壌に分布する．本物質は容易に生分解する（OECD TG 301C）．水生生物における生物濃縮性は低い（BCF：61.3）．水生生物に対する急性毒性では，魚類のLC_{50}は0.23 mg/L（96時間，OECD TG 203），ミジンコのEC_{50}は5.5 mg/L（48時間，遊泳阻害：OECD TG 202），藻類のEC_{50}は5.5 mg/L（72時間，生長速度法，DIN 38412 L9 Part 9）であった．慢性毒性では，ミジンコのNOECは1.16 mg/L（21日間，繁殖阻害：OECD TG 211），藻類のNOECは2.4 mg/L（72時間，生長速度法：OECD TG 201）であった．

3）健康影響

強制経口投与後24時間以内に，ラットでは25-30％が揮発性代謝物（CO_2）として，また，50-70％が尿中に排泄され，マウスでは6-12％が揮発性代謝物（CO_2）として，また，80-90％が尿中に排泄された．ラットとマウスに静注した場合，血中から速やかに除去され（半減期：2分間），30分後には血液，肝臓，腎臓，筋肉，皮膚，小腸で検出されなかった．また，静注後に両動物の尿でmonoallyl phthalate（MAP），allyl alcohol（AA），3-hydroxypropylmercapturic acid（HPMA），極性代謝物（AAの代謝産物）が検出された．

本物質はマウスよりラットへの肝毒性作用が強く，同様の種差はAAでもみられた．AAは門脈域への肝毒性の可能性がある．マウスは第二相代謝の副生成物として，ラットより多くのHPMAを生成するので，本物質の肝毒性の種差はAAまたはacrolein（AAの活性代謝産物）のグルタチオン抱合が関与していると考えられた．単回経口投与毒性試験でのLD_{50}は，ラットの雄では891 mg/kg，雌では656 mg/kg，マウスの雄では1,070 mg/kg，雌では1,690 mg/kg，イヌの雌雄ではおよそ800 mg/kgであった．ウサギの単回経皮投与毒性試験でのLD_{50}は3,300 mg/kg，ラットの単回吸入毒性試験でのLC_{50}は，雄では10,310 mg/m^3，雌では5,200 mg/m^3，雌雄合算した場合は8,300 mg/m^{3}であった．

ウサギの皮膚及び眼に対して刺激性は認められなかった．マウスにおいて皮膚感作性はみられなかった．

ラットに0，25，50，100，200及び400 mg/kg/dayを週5日13週間強制経口投与した反復投与毒性試験では，400 mg/kg/dayで雄の8/10例が死亡または瀕死状態であり，体重の増加抑制もみられた．200 mg/kg/day以上で雌雄に下痢，頭部の被毛の乱れや脱毛，円背，削痩がみられた．400 mg/kg/dayで死亡した雄全8例で肝臓に肉眼的異常（腫大，斑紋，褪色）がみられ，そのうち3例にはさらに多発性腎皮質尿細管壊死が認められた．肝臓の肉眼的異常は400 mg/kg/dayで生存していた雄と400 mg/kg/dayの雌及び200 mg/kg/dayの雄でも認められた．重篤度は用量に依存し，また，雌より雄で重症であった．雌雄の肝臓において，200 mg/kg/dayで門脈周囲の肝細胞変性，壊死，線維化，胆管増殖及び肝細胞過形成が，400 mg/kg/dayでこれらの病変の観察を妨げる肝硬変が認められた．門脈周囲の肝細胞変性は雄の50 mg/kg/dayと雌雄の100 mg/kg/dayでも観察されたが，その発生頻度と重篤さは減少した．NOAELは雌で50 mg/kg/dayとされた．雄のNOAEL及びLOAELは，25 mg/kg/dayでの病理組織学的検査が行われていないので決定されなかった．

雌雄ラットに交配前2週間から交配期間を含み，雄ではおよそ50日間，雌では分娩後哺育4日まで，0，16.7，50及び150 mg/kg/dayを強制経口投与した簡易生殖毒性試験（OECD TG 421）では，150 mg/kg/dayにおいて難産によると考えられる雌の死亡例が3例認められ，また，雌雄の肝臓に門脈周囲肝細胞の壊死，腫脹及び好塩基球の浸潤，胆管増殖及び門脈周囲の線維化の増加が認められた．発生毒性に関する影響は認められなかった．これらの結果から，一般毒性及び生殖毒性のNOAELは50 mg/kg/dayとされた．

細菌を用いる複数の復帰突然変異試験ではS9 mix存在下及び非存在下において陰性または弱い陽性であった．*In vitro*のマウスリンパ腫細胞を用いる突然変異試験ではS9 mix存在下及び非存在下において陽性であった．チャイニーズ・ハムスター培養細胞を用いる染色体異常試験，姉妹染色分体交換試験及び小核試験ではS9 mix存在下において陽性であった．*In vivo*でのマウス小核試験では陰性であったが，マウスを用いた染色体異常試験では陽性であった．これらの結果から，*in vitro*では遺伝毒性があるが，*in vivo*では明白ではないとされた．

雌雄マウスに0，50及び100 mg/kg/dayを週5日で103週間強制経口投与した発がん性試験では，300

mg/kg/dayで雄にリンパ腫発症率の高値が認められたが，統計学的には有意ではないため，疑わしい結果とされた．雌雄ラットに0，150及び300 mg/kg/dayを週5日で103週間強制経口投与した発がん性試験では，100 mg/kg/dayで雌に単核細胞白血病発症率の高値が認められたが，統計学的には有意ではないため，疑わしい結果とされた．これらの結果から，発がん性については曖昧な証拠があるとされた．

4) 結論と勧告

本物質の健康影響はFWと勧告され，職業曝露量の調査が推奨された．環境影響はLPと勧告された．

(4) 2-tert-Butoxyethanol (7580-85-0)(日本政府)

1) 曝露状況

本化学物質は主に塗料用溶剤として使われている．職業曝露の主要経路は吸入及び経皮と考えられる．また，本物質を含む製品から，吸入及び経皮経路による消費者曝露の可能性がある．

2) 環境影響

本物質は，ほぼ全てが水圏及び土壌に等しく分布する．本物質は容易に生分解しない (OECD TG 301C) が，水生生物における生物濃縮性は低い (BCF：3.16)．水生生物に対する急性毒性では，魚類のLC_{50}は＞100 mg/L (96時間，OECD TG 203)，ミジンコのEC_{50}は＞891 mg/L (48時間，遊泳阻害：OECD TG 202)，藻類のEC_{50}は＞866 mg/L (72時間，生長速度法，OECD TG 201) であった．慢性毒性では，ミジンコのNOECは94.2 mg/L (21日間，繁殖阻害：OECD TG 211)，藻類のNOECは291 mg/L (72時間，生長速度法：OECD TG 201) であった．

3) 健康影響

ラットの単回経口投与毒性試験 (OECD TG 401) でのLD_{50}は雌雄で2,000 mg/kg以上，雄マウスに単回経口投与した試験でのLD_{50}は1,328 mg/kgと報告されている．

ラットに交配前2週間及び交配期間を含め，雄では計37日間，雌では分娩後哺育4日まで，0，4，20及び100 mg/kg/dayを強制経口投与した反復投与毒性・生殖発生毒性併合試験 (OECD TG 422) では，100 mg/kg/dayにおいて雌雄に着色尿が認められ，雌雄の赤血球数，ヘモグロビン濃度及び赤血球血色素濃度の低値，赤血球容積，赤血球血色素量及び網状赤血球数の高値がみられた．20 mg/kg/dayにおける雌でも赤血球血色素量の高値を除く血液学的検査値に同様の変化が認められた．その他，100 mg/kg/dayにおいて雄のヘマトクリット値及び白血球数の低値，雌雄の脾臓重量の高値，雌雄の大腿骨骨髄における赤血球系造血細胞の増加，肝臓におけるクッパー細胞のヘモジデリン沈着，腎臓における尿細管上皮細胞のヘモジデリンの沈着，雄の脾臓におけるヘモジデリン沈着，雌の肝臓における髄外造血が認められた．また，雄の100 mg/kg/day，雌の20 mg/kg/day以上で脾臓における赤血球系髄外造血が認められた．生殖発生に関する影響は認められなかった．これらの結果から，反復投与毒性のNOAELは雄で20 mg/kg/day，雌で4 mg/kg/day，生殖発生毒性のNOAELは100 mg/kg/dayとされた．

細菌を用いる復帰突然変異試験及びチャイニーズ・ハムスター培養細胞を用いる染色体異常試験では陰性であった．

4) 結論と勧告

本物質の健康影響はFWと勧告され，職業曝露量及び消費者曝露量の調査が推奨された．環境影響はLPと勧告された．

(5) カテゴリー：High Molecular Weight Phthalate Esters (7 chemicals: 119-06-2, 3648-20-2, 53306-54-0, 68515-41-3, 68515-43-5, 68515-47-9, 85507-79-5) (原案作成：ICCA日本及びICCAフランス企業)

本カテゴリーは，炭素数7以上でアルキル炭素骨格を持つ，7種類の高分子量フタル酸エステル (HMWPE)，つまり，1,2-benzenedicarboxylic acid, di-2-propylheptyl ester(Di-phC10 PE; 53306-54-0)，1,2-benzenedicarboxylic acid, di-C7-9-branched and linear alkyl esters(Di-C7-9 PE; 68515-41-3)，1,2-benzenedicarboxylic acid, di-C11-branched and linear alkyl esters(Di-C11 PE; 85507-79-5)，1,2-benzenedicarboxylic acid, di-C9-11-branched and linear alkyl esters(Di-C9-11 PE; 68515-43-5)，1,2-benzenedicarboxylic acid, di-C11-alkyl ester(Di-C11 PE; 3648-20-2)，1,2-benzenedicarboxylic acid, di-C11-14-branched alkyl esters, C13 rich(Di-C13 PE; 68515-47-9)，1,2-benzenedicarboxylic acid, di-C13-alkyl ester(Di-C13 PE; 119-06-2) から成る．本カテゴリー物質は，2個の分岐または直鎖アルキルアルコールで1個のbenzenedicarboxylic acidをエステル化することにより生産される．

フタル酸エステル類 (PEs) の特記すべき毒性は生殖発生毒性であり，その毒性は構造に依存し，炭素数4～6の骨格を持つ部分構造と関連している．一方，炭素数7以上の骨格を持つPEsにおいて生殖毒性や発生毒性は認められない．さらに，炭素数5以上の骨格を持つPEsには環境影響はみられない．Di-isononyl phthalate ester (DINP; 68515-48-0及び28553-12-0) とdi-isodecyl phthalate ester(DIDP; 68515-49-1及び26761-40-0) は，本カテゴリーの定義に合致するので，データの利用は可能であるが，既にOECD HPV programmeで評価されているので，本カテゴリーには含まれない．

1) 曝露状況

本カテゴリー物質はポリマー産業で添加物として使用

され，ポリ塩化ビニル樹脂に柔軟性を与える．また，潤滑油の添加剤としても使用される．職業曝露の主要経路は経皮及び吸入と考えられる．また，本物質を含む製品から，吸入及び経皮経路による消費者曝露の可能性がある．

2）環境影響

本カテゴリー物質は約98％が土壌に，約2％が底質に分布する．di-phC10 PE，di-C11 PE(3648-20-2)，di-C13 PEs(68515-47-9及び119-06-2）の生分解率は13～75％（28日間）であった．分子量の比較的大きいdi-C13 PEsの生分解率は低いが，試験期間を56日に延長した場合，di-C13 PE(68515-47-9）では13％から63％に上昇した．また，本カテゴリー物質の水生生物における生物濃縮性は低いとされた．

本カテゴリー物質の水生生物に対する急性・慢性毒性は低く，魚類及びミジンコへの毒性は低い．また，藻類でも，本カテゴリー物質の水溶解度（0.017 mg/L以下）を超える濃度設定区（di-phC10 PE：NOEC＝25 mg/L, di-C11 PE（3648-20-2）：同2.1 mg/L, di-C13 PE (68515-47-9)：同0.6 mg/L）においてのみ影響がみられた．

3）健康影響

げっ歯類に経口投与されたDINPは，消化管で速やかに代謝されてモノエステルとなり，吸収され，尿中に排泄される．投与直後，主に肝臓と腎臓に分布するが，他の臓器には分布しない．皮膚からの吸収はほとんどないが，一旦吸収されると経口投与と同様の過程をたどる．一方，ヒトや霊長類への経口投与では，低用量での吸収は少なく，高用量でさえ吸収量は限られている．実際，霊長類はフタル酸エステル類をモノエステルに代謝する効率が低いように思われ，高用量では霊長類によるモノエステルの吸収は飽和している．げっ歯類と霊長類の結果の差異はフタル酸エステル類の加水分解速度の差によるものと考えられる．従って，ヒトにおけるHMWPEの吸収はげっ歯類より少ないと考えられる．

あらゆる曝露経路においてHMWPEの急性毒性は低い．本カテゴリー物質は皮膚及び眼に対して刺激性は認められず（di-C13 PE（68515-47-9）のみ結膜への弱い刺激性があった），皮膚感作性もみられない．

ラットへの反復投与試験では，主に肝臓と腎臓に，そして，より程度は低いが甲状腺に毒性影響が認められた．肝臓への影響はパルミトイル補酵素（PCoA）や肝重量の増加や肝肥大を含むペルオキシソーム増殖を示し，ヒトでは発現しない（これらの影響に介在するペルオキシソーム増殖因子活性化受容体α（PPARα）のレベルはげっ歯類で非常に高く，ヒトでは低い）．腎臓への影響は，用量依存的なα-2u-グロブリン腎症の結果であり，雄ラットに特異的なので，ヒトでは発現しない．雌ラットで散発的にみられた腎臓重量増加との関連性は明らかではない．甲状腺への影響は肝臓のペルオキシソーム増殖に関連した代償作用と思われる．実験結果は全カテゴリー物質において一貫し，NOAELは肝臓や腎臓への影響から導かれ，その範囲は10～282 mg/kg/dayであった．試験毎に用量の設定が異なるため各物質のNOAELが大きく異なっている．最低値の10 mg/kg/dayはdi-C13 PE（119-06-2）の反復投与毒性・生殖発生毒性併合試験（OECD TG 422）から得られた．この試験では，ラットに交配前2週間及び交配期間を含め，雄では計42日間，雌では分娩後哺育3日まで，0，10，50及び250 mg/kg/dayが強制経口投与され，50 mg/kg/day以上の雌，250 mg/kg/dayの雄にペルオキシソーム増殖に関連すると思われる肝臓重量の増加，50 mg/kg/day以上の雌雄に小葉中心性肝細胞肥大が認められた．

本カテゴリーにおいて分子量のより小さい物質（di-C7-9 PE），中程度の物質（di-C9-11 PE），より大きい物質（di-C13 PE; 119-06-2）の生殖毒性に関する試験が行われ，500 mg/kg/day（di-C7-9 PE及びdi-C9-11 PE）あるいは250 mg/kg/day（di-C13 PE）まで生殖毒性は認められなかった．一過性の体重減少や卵巣，精巣上体重量のわずかな増加がみられたが，これらの影響は軽微であり，生殖毒性には間接的にのみ関与する可能性がある．さらに，より新しい試験においてdi-C7-9 PE及びdi-C9-11 PEは生殖能に影響しないことが示された．DINPとDIDPでも同様に生殖影響はみられない．

ラットを用いてdi-phC10 PE，di-C7-9 PE，di-C9-11 PE及びdi-C13 PE（119-06-2）の発生毒性試験が行われ，最高用量は1,000 mg/kg/day（di-phC10 PE, di-C7-9 PE, di-C9-11 PE）または250 mg/kg/day（di-C13 PE）であった．di-phC10 PEにおいて最高用量で軽微な母体毒性（摂餌量及び体重の減少）がみられ，また，吸収胚数の増加及び生存胎児数の減少がみられた．di-C7-9 PEとdi-C9-11 PEでは母体毒性は最高用量までみられず，また，中用量（500 mg/kg/day）以上で胎児にしばしば観察される腎盂拡張や腰肋がみられた．上述のdi-C13 PEにおける併合試験では250 mg/kg/dayで産児の生存数が減少し，発生毒性のNOAELは50 mg/kg/dayとされたが，これは母動物の哺育不良に起因していた．また，DIDPにおける二世代生殖毒性試験ではF2にのみ生存児数の減少が認められた．これらの児への影響は生物学的に有意とはみなされず，本カテゴリー物質はげっ歯類において生物学的に有意な発生生殖毒性を示さないと結論された．

本カテゴリー物質における*in vitro*の遺伝毒性試験の結果及びDINPとDIDPにおける*in vivo*の小核試験の結果から，本カテゴリー物質は遺伝毒性を示さないと結論された．

本カテゴリー物質の発がん性試験は行われていないが，フタル酸エステル類では高用量でげっ歯類にペルオキシソーム増殖に関連すると思われる肝臓の変化が認められ，DINPの試験でも，主に肝臓や腎臓で変化がみられ，ペルオキシソーム増殖に関連する肝腫瘍（雌雄のラット及び雌雄のマウス）とα-2u-グロブリン腎症（雄ラット）が認められたが，これらの影響はヒトでは発現しない．

4) 結論と勧告

本物質の健康影響及び環境影響はLPと勧告された

3. おわりに

本稿では，SIAM 19で合意された化学物質名及び日本担当物質の初期評価文書について紹介した．SIAMで合意された物質の初期評価文書は出版され，また，インターネットのOECD webサイト（http://cs3-hq.oecd.org/scripts/hpv/）でも入手が可能である．

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Lack of potential of low dose *N*-nitrosodimethylamine to induce preneoplastic lesions, glutathione *S*-transferase placental form-positive foci, in rat liver

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Abstract

Induction of liver lesions in male F344 rats by the genotoxic and carcinogenic *N*-nitrosodimethylamine (NDMA) was studied at a wide range of dose levels, i.e. from 0.001 to 10 ppm, in drinking water for 16 weeks. Dose related and statistically significant increase of glutathione *S*-transferase placental form-positive foci, endpoint markers for hepatocarcinogenesis in rats, at 1 and 10 ppm dose groups was obtained, but no increment in foci could be detected with the lower doses (0.001, 0.01, and 0.1 ppm). This finding of a no-observed effect level supports our hypothesis that a threshold, at least in practical terms, exists in carcinogenesis proposed on the basis of extensive wide range dose-dependence studies of other genotoxic carcinogens.

Keywords: *N*-nitrosodimethylamine; Risk assessment; Carcinogenicity dose threshold

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1. Introduction

Chemical carcinogens are generally classified into two categories, genotoxic and non-genotoxic. Concerning cancer risk assessment, it is considered that genotoxic carcinogens exert carcinogenic potential regardless of the animal species, so that chemicals which are carcinogenic to rodents are carcinogenic to humans as well. Because genotoxic carcinogens are mutagenic and seem to act through interaction with DNA to produce irreversible genetic changes in target organ cells, it has been generally concluded that they have no dose threshold in their carcinogenic potential [1,2]. Therefore, there is widespread acceptance of a linear curve extending to zero at very low doses for chemicals found to be carcinogenic with in vivo carcinogenicity tests. However, while there are data supporting the non-threshold theory [3–5], and we recently provided evidence of thresholds for the hepatocarcinogenicity of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and *N*-nitrosodiethylamine (NDEA) in rats [6,7]. Williams et al. [8,9] also earlier reported the existence of similar thresholds for NDEA and 2-acetylaminofluorene hepatocarcinogenicity.

N-nitrosodimethylamine (NDMA), an *N*-nitroso compound, is well established as a hepatocarcinogen in rodents. Humans are exposed to NDMA from occupational and environmental sources and through in vivo formation of ingested precursor amines and nitrosating agents [10]. In particular its endogenous formation from ingested precursors has been indicated to be a major source of exposure. Previously Peto et al. [5] reported non-threshold of NDMN hepatocarcinogenicity based on statistical analysis of results from a long-term carcinogenicity test at low doses in 4080 rats.

Recently in vivo medium-term bioassays for carcinogens have become accepted as alternatives to long-term carcinogenicity tests. Particularly, the liver medium-term bioassay has been developed as useful for detecting hepatocarcinogenicity of chemicals [11]. Recently we found a 21-day-old rat, a medium-term model to be very useful for assessment of low dose carcinogenicity of hepatocarcinogens such as MeIQx and NDEA because of high sensitivity [6]. In this medium-term bioassay, the animal treatment duration was 16 weeks and glutathione *S*-transferase placental form (GST-P)-positive foci, established preneoplastic lesions in the livers of rats [11,12], were taken as end-point markers of carcinogenicity.

In the present study, we examined low dose carcinogenicity of NDMA in the rat liver from viewpoint of 'weight of evidence' for clarification of human risk assessment of genotoxic carcinogens. For this purpose we employed the same experimental protocol with which MeIQx and NDEA were earlier examined for low dose carcinogenicity [6].

2. Materials and methods

2.1. Animals and chemicals

A total of 540 male 20-day-old F344 rats were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan) and housed in rooms maintained on a 12 h light/dark cycle, at constant temperature and humidity, and observed daily. Numbers of the rats employed in the present study were decided on the basis results of previous, low dose carcinogenicity studies [6,7]. NDMA (purity >99%) was purchased from Sakai Research Laboratory (Fukui, Japan).

2.2. Experimental procedures

The experiment was started when the animals were aged 21 days. They received NDMA at doses of 0 (group 1, a control, 90 rats), 0.001 (group 2, 89 rats), 0.01 (group 3, 89 rats), 0.1 (group 4, 90 rats), 1 (group 5, 91 rats), or 10 ppm (group 6, 91 rats), in drinking water for 16 weeks. The lowest level, 0.001 ppm, was established with reference to daily exposure of humans to this carcinogen [10]. The animals had free access to Oriental MF diet (Oriental Yeast Co., Tokyo, Japan) throughout the experiment and were killed at the end of week 16 under ether anesthesia for examination of lesion development.

Ten percent phosphate-buffered formalin-fixed liver tissues (a total of 9 slices per animal, 3 each from the left lateral, medial, and right lateral lobes) were embedded in paraffin wax for immunohistochemical examination of GST-P-positive foci in the liver, as described previously [6]. Hepatocellular foci comprising of two and more cells were counted under a light microscope. They were categorized as

Table 1
Final average body, absolute and relative liver weights, and average total NDMA intakes

Groups	NDMA doses (ppm)	No. of rats	Final body weights (g)	Liver		Total NDMA intake (mg/rat)
				Absolute (g)	Relative (%)	
1	0	90	327±15[a]	9.6±0.9	3.0±0.2	0
2	0.001	89	325±17	9.6±0.7	2.9±0.2	0.00151
3	0.01	89	325±16	9.5±0.7	2.9±0.2	0.0145
4	0.1	90	327±18	9.5±0.8	2.9±0.1	0.1505
5	1	91	326±19	9.9±1.0	3.0±0.2	1.5117
6	10	91	315±19	9.1±0.9	2.9±0.2	15.0680

[a] Values are mean±SD.

having a total of 11 and more cells. Total areas of livers were measured using a color image processor (IPAP, Sumica Technos, Osaka, Japan) and the numbers of foci per cm^2 of liver tissue were calculated.

2.3. Statistical analysis

Statistical analysis of the data was performed using the StatView-J 5.0 program (Abacus Concepts, Inc., Berkeley, CA). Differences from control values were evaluated for significance with the Dunnett two-tailed post hoc test.

3. Results

3.1. General findings

All the rats survived in good condition until the scheduled sacrifice. No macroscopic lesions were apparent in any organ including the liver. No adverse effects on average body weight gain were observed in rats treated with NDMA at any of the doses (Table 1). Absolute liver weights were not increased in the groups given NDMA and relative liver weights did not differ among the groups. Average total NDMA intake in each group was dose-dependent.

3.2. Induction of GST-P-positive foci in the liver

In livers of rats treated with NDMA, total numbers of GST-P-positive foci per unit area in the groups receiving 0.001 to 0.1 ppm of the carcinogen did not differ from the control value (non-treatment group, Table 2 and Fig. 1), in contrast to the significant increase observed in rats treated with the 1 and 10 ppm doses. In fact, total values in groups treated with NDMA at a dose of 0.01 ppm showed a slight decrease as compared to the control value. Moreover, numbers of GST-P-positive foci comprising ≥11 cells in the groups given 0.001–0.1 ppm NDMA were not different from the control values, while these values in rats treated with 1 ppm NDMA, and particularly 10 ppm NDMA, were significantly increased.

Table 2
The development of GST-P-positive foci in the livers of rats treated with NDMA at various doses

Groups	NDMA doses (ppm)	No. of rats	Size distribution of GST-P-positive foci (no./cm^2)	
			Total	≥11 cells
1	0	90	0.375±0.545[a]	0.012±0.066
2	0.001	89	0.366±0.586	0.018±0.077
3	0.01	89	0.276±0.582	0.011±0.056
4	0.1	90	0.377±0.519	0.025±0.074
5	1	91	1.905±2.399*	0.117±0.200*
6	10	91	24.875±13.267*	11.063±6.986*

*$P<0.01$ (vs. group 1).
[a] Values are mean±SD.

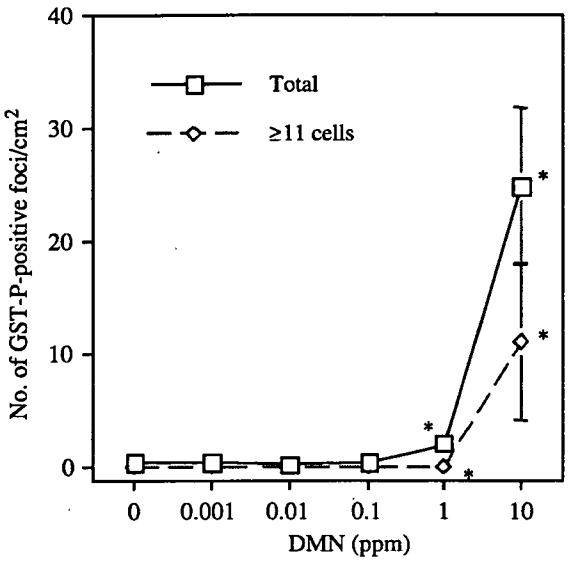

Fig. 1. Induction of GST-P-positive foci in the liver of rats treated with NDMA. *$P < 0.01$ (vs. group 1). Numbers of rats are shown in Table 1. Bars, SD.

4. Discussion

Previously Peto et al. [5] examined the carcinogenicity of NDMA or NDEA at low doses (the so-called ED01 study) and found no indication of any threshold for liver tumor induction of rats. They speculated that NDMA or NDEA at 0.1 ppm in drinking water cause about 2.5% of animals to develop liver tumors and therefore a dose of 0.01 ppm would yield a 0.25% incidence. However, the issue of true and practical thresholds for carcinogenicity has attracted increasing interest [13] and recently Waddell [14] reanalyzed data of the rat carcinogenicity study using NDEA at low doses. His speculation pointed to the existence of a threshold for NDEA carcinogenicity in the liver and esophagus. Recently Williams et al. [9] also suggested the existence of threshold for NDEA hepatocarcinogenicity in rats. In the present study, a dose related and statistically significant increase of GST-P-positive foci in the liver, established endpoint markers for hepatocarcinogenesis in rats [11,12], was obtained with the 1 and 10 ppm doses of NDMN, but the lower doses (0.001, 0.01, and 0.1 ppm) did not cause significant increment in the foci, in line with thresholds found for MeIQx and NDEA previously [6].

Recently we documented that MeIQx and NDEA do not induce GST-P-positive foci in rat liver at very low doses [6]. Moreover, formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), the most abundant species of adduct associated with oxidative stress, also demonstrated a no-observed effect level. We also reported that the curve for induction of aberrant crypt foci, preneoplastic markers in the colon of rats by 2-amino-1-methyl-6-phenolimidazo[4,5-*b*]pyridine (PhIP) is not linear down to zero [15]. Similarly, no-response levels were evident for both PhIP–DNA adducts and 8-OHdG formation. The present study also clearly indicates that the curve for induction of GST-P-positive foci in the liver is not linear down to zero. Taking all the evidence together, we conclude that genotoxic carcinogens have a threshold, at least in practical terms, for their carcinogenicity.

The question of whether there is a threshold for chemical carcinogenesis, particularly with genotoxic agents is clearly controversial in risk assessment and the non-threshold theory continues to hold sway in the regulatory area for carcinogenic toxicology. However, the findings for a threshold in the genotoxicity of MeIQx [16,17] and the evidence of practical thresholds for genotoxic carcinogenicity from recent in vivo studies including the present result [6–9,15] indicates that this area requires more attention and careful consideration.

In conclusion, the present finding of no-observed effect level on induction of GST-P-positive foci supports our hypothesis that a threshold, at least in practical terms, exists with regard carcinogenesis due to genotoxic agents, from our extensive wide range dose-dependence studies of a variety of carcinogens.

Acknowledgements

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School, Nagoya, Japan) and Dr T. Kitagawa (Director, Cancer Institute, Tokyo, Japan).

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Review Article

High susceptibility of human c-Ha-ras proto-oncogene transgenic rats to carcinogenesis: A cancer-prone animal model

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Transgenic animals carrying human c-Ha-ras proto-oncogene, v-Ha-ras transgenic mice, pim-1 transgenic mice and several knockout mice deficient of tumor suppressor genes, such as p53, have been shown to exhibit increased carcinogen susceptibility. As a result, studies into practical application and medium-term screening of environmental carcinogens are under way. Given the advantages of rat models characterized by larger organ size, abundant information regarding preneoplasias and virus-free constitution, we have concentrated on the generation of transgenic rats bearing copies of the human c-Ha-ras proto-oncogene and shown the Hras128 strain to be extremely sensitive to the induction of mammary carcinomas, and to a lesser extent, lesions in the urinary bladder, esophagus and skin. In most, if not all, the mammary cancers mutations of the transgene but not the endogenous H-ras gene are present, appearing to occur early in the process of tumorigenesis, which involves proliferation of cells in TEB and intraductal hyperplasia before carcinomas arise. Preliminary findings suggest that this is independent of endogenous ovarian hormones, although inhibited by soy isoflavones and promoted by atrazine and nonylphenols. Although further studies of the mechanisms are clearly necessary, the model appears to have great potential for screening purposes, not only for modifiers active in the breast, but also other organs where tumors characterized by ras gene mutations develop. (*Cancer Sci* 2005; 96: 309–316)

Transgenic mice carrying the human c-Ha-ras proto-oncogene,[1–3] v-Ha-ras transgenic mice,[4,5] pim-1 transgenic mice[6,7] and several knockout strains of mice deficient in tumor suppressor genes such as p53 have been shown to exhibit increased carcinogen susceptibility. Therefore, there is a great deal of interest in their practical application for medium-term screening of environmental carcinogens, for example with rasH2 and Tg.AC mice.

For studies of chemical carcinogenesis, however, rats rather than mice are generally more frequently used for various reasons. For example, in addition to the benefits accruing with size, abundant information is available regarding biological characteristics of preneoplastic lesions that can be used as endpoint lesions appropriate as surrogate markers for cancer development.[8–11] Furthermore, tumors of the mammary glands and other organs can be induced without the complication of a possible viral etiology, which is not the case with mice. However, only limited types of transgenic rats have so far been developed for studying carcinogenesis. In the majority of established models, the transgene is under the control of an SV40 T antigen gene such as the probasin/SV40 T antigen gene for the prostate,[12,13] the albumin-SV40 T antigen gene for the liver,[8,14,15] and the phosphoenolpyruvate carboxykinase (PEPCK)-SV40 T antigen gene for pancreas islet cells.[16] This is clearly not optimal. In oncogene transgenic rats, the c-erbB-2 and TGFα-MMTV[17] and Neu proto-oncogene[18] have been applied for the study of mammary carcinogenesis. An example of a transgene related to thymus carcinogenesis is the pX gene encoding a major product of human T lymphocyte virus type I (HTLV-I). Others include the glutathione S-transferase (GST-P) gene[19] for the liver, and the Tsc2 gene[20,21] for the kidney but these are not directly relevant to enhancement of carcinogenesis, unlike the case with tumor oncogenes (Table 1).

We have concentrated attention on the generation of transgenic rats with the same human c-Ha-ras proto-oncogene used for establishment of transgenic mice.[3] As this transgene is under the control of its own promoter region, it is expressed in the whole body, allowing the study of carcinogenesis in different organs. Two rat lines have been found to exhibit very high susceptibility to chemically induced mammary carcinogenesis, with development of multiple carcinomas within an extremely short period.[23,30] Less remarkably, one has also been found to demonstrate increased sensitivity to skin, bladder and esophagus carcinogens.[24–26] Here we report our experience regarding susceptibility of our transgenic rats to chemically induced carcinogenesis, analysis of the mechanisms, and possible application as an animal model for short-term evaluation of carcinogenicity of chemical compounds.

Generation of H-ras transgenic rats

The DNA construct used for transgenic rats has been described in a previous study.[31] For the purpose of genarating the transgenic rata, a 6.8 kb *Bam*HI fragment of the human c-Ha-ras proto-oncogene with its own promoter region eluted from agarose gel was purified and then injected into the pronuclei of rat

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Abbreviations: TEB, terminal end buds; Hras128, human c-Ha-ras proto-oncogene transgenic rat; MNU, *N*-methyl-*N*-nitrosourea; DMBA, 7,12-dimethylbenz[a]anthracene; PhIP, 2-amino-1-methl-6-phenylimidazo[4,5-b]pyridine; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; PCNA, proliferating cell nuclear antigen; NLRR-3, neuronal leucine-rich repeat protein-3; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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Table 1. Transgenic rats generated for carcinogenesis studies

Carcinogenesis	Transgene	Promoter	Strain	Tumor site	Reference
Enhancement/ induction†	SV40	Albumin	SD	Liver‡	Hully *et al.* (1994)[8]
		Probasin	SD	Prostate	Asamoto *et al.* (2001)[12]
		PEPCK	SD	Pancreas islet	Haas *et al.* (1999)[22]
	Human c-Ha-ras¶	Human Hras	SD	Mammary gland‡	Asamoto *et al.* (2000)[23]
				Bladder‡	Ota *et al.* (2000)[24]
				Esophagus‡	Asamoto *et al.* (2002)[25]
				Skin‡	Park *et al.* (2004)[26]
	Neu	MMTV	SD	Mammary gland‡	Watson *et al.* (2002)[18]
	c-erbB2	MMTV	SD	Mammary gland‡	Davies *et al.* (1999)[17]
	TGFα	MMTV	SD	Mammary gland‡	Davies *et al.* (1999)[17]
	px(HTLV-1)	p53lck	F344	Thymus	Kikuchi K (2002)[27]
	HLA-B27	HLA-B27	F344	Colon	Hammer RE (1995)[28]
Inhibition†	Tsc2	Tsc2	Eker	Kidney	Kobayashi *et al.* (1997)[20]
	Rat H-, K-ras§	Rat H-ras	SD	Mammary gland‡	Thompson *et al.* (2002)[29]
	GST-P	GST-P	Wistar	Liver‡	Nakae *et al.* (1998)[19]

†As compared to wild-type rats; ‡chemically induced tumor; §domionant negative: ¶protooncogene. SD, Sprague-Dawley; PEPCK, phosphoenolpyruvate carbokinase; MMTV, mouse mammary tumor virus; GST-P, glutathione S-transferase.

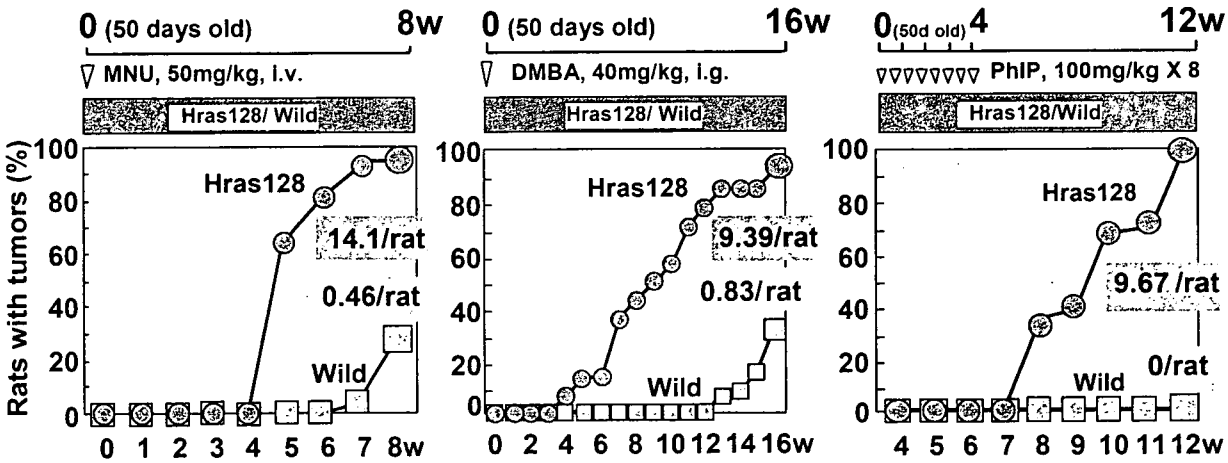

Fig. 1. Rapid development of mammary carcinomas with three different carcinogens, MNU, DMBA and PhIP. Hras128 and its wild-type counterparts were given a single dose of MNU (50 mg/kg, i.v.) or DMBA (40 mg/kg, i.g.) on day 50 after birth, then killed on week 8 or 12, respectively. PhIP (100 mg/kg) was given i.g. eight times over a 4 week-period followed by death on week 12. Multiple mammary carcinomas developed in almost all the transgenic rats, whereas the incidence and multiplicities were far lower in the wild-type rats.

embryos from Sprague-Dawley female rats. Two male progeny were shown to carry the transgene, one having three copies (Hras128) and the other one copy (Hras104).[23] Subsequent matings have been carried out between transgenic males and non-transgenic Sprague-Dawley female rats to maintain the heterozygote transgenic Hras128 strain. Expression of the transgene has been repeatedly detected in all organs by northern blot analysis. The strain is now being maintained and bred by Clea Japan (Tokyo, Japan).

Mammary carcinogenesis

To examine the susceptibility of the transgenic rat to mammary carcinogens, females at 50 days of age were treated with a single dose of N-methyl-N-nitrosourea (MNU) (50 mg/kg, i.v.), 7,12-dimethylbenz[a]anthracene (DMBA) (40 mg/kg, i.g.) or multiple doses (100 mg/kg, i.g., eight times in 4 week-period) of the food contaminant carcinogen, 2-amino-1-methl-6-phenylimidazo[4,5-b]pyridine (PhIP).[23,30,32] Almost all the transgenic rats rapidly developed multiple mammary carcinomas within 8–12 weeks (see Fig. 1). The histological appearance of the tumors was solid, tubular, papillary and, less frequently, undifferentiated and sarcomatous, as previously reported to be typical in rats.[33,34] As it has been established that the mode of DNA modification differs among the three compounds, O^6-methylguanine formation resulting with MNU,[29,35] depurinating adduct formation with DMBA[36] and guanine deletion in the 5′-GGGA-3′ sequence with PhIP,[37] our results indicate that Hras128 rats are highly susceptible to chemically induced mammary carcinogenesis, irrespective of the carcinogen applied.

Mutation of the transduced c-Ha-ras proto-oncogene and demonstration of activated ras in induced mammary tumors

PCR-SSCP and PCR-RFLP analysis and direct sequencing of the transgene indicated the large majority of carcinomas induced with MNU, DMBA and PhIP to contain cells with mutations,

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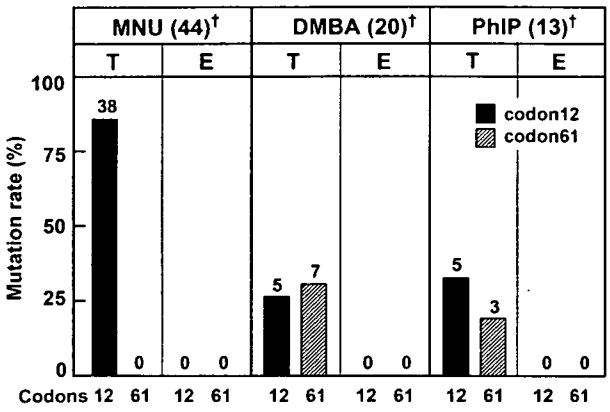

Fig. 2. Data for mutations that occurred in the transgene but not the endogenous Hras gene in all treatment groups. T, transgene; E, endogenous Hras gene; †numbers indicate the number of tumors examined.

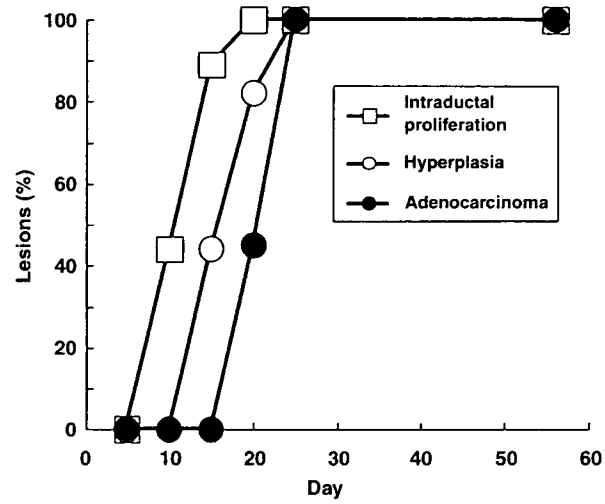

Fig. 3. Results of periodical observation of preneoplastic and carcinoma lesion development in abdominal-inguinal mammary glands after injection of N-methyl-N-nitrosourea (MNU) in Hras128.

many featuring GG → GA at codon 12[23,30] (Fig. 2). Furthermore, activated form ras protein could be detected in carcinomas induced by PhIP.[32] In contrast, no mutations whatsoever were found in the endogenous c-Ha-ras gene in the mammary tumors arising in the Hras128 strain.[23] Therefore, the results indicate that preferential mutation and activation of the transduced human c-Ha-ras proto-oncogene play dominant roles over the endogenous c-Ha-ras gene. It is of interest in this context that treatment with *d*-limonene, an inhibitor of ras protein isoprenylation, clearly inhibited tumor induction.[38]

Our results are in contrast to the observation that transgenic rats with copies of rat H- and K-ras gene showed less carcinoma development than their non-transgenic littermates following MNU exposure, with less transgene mutation than in the endogenous ras gene.[29] The findings indicate that particular mutations of the transgene are important for its functions as a modifier gene in mammary carcinogenesis.

Elevated cell proliferation and mutation of the transgene in TEB cells as early events

The observed transgene mutations as early events in carcinogenesis, periodic observation of mutations and whether proliferative focal lesions were performed in TEB were studied.[39] As TEB cells are precursors for mammary exocrine glands, they are the most likely targets of chemical carcinogens applied before sexual maturation, at 50 days old.[39] We focused on analysis of numbers, proliferative status and presence of any mutations in the transgene.

Number count and proliferative potential of TEB. Counts of TEB in the abdominal mammary glands of 49–91-day-old female Hras128 were compared with those in wild rats. The numbers were significantly greater in Hras128 rats until 81 days after birth. Confocal microscopy further revealed that the level of active protein kinase is clearly elevated in TEB cells. Thus, an increase in number with elevated proliferation activity would appear to play an important role in observed rapid tumor development.[40] As TEB is a precursor tissue for mammary glands, duct and acini, the induced tumors comprised epithelial, stromal and transitional cells. Indeed, a variety of histological patterns, from epithelial to stromal and epithelial-mesenchymal type, was found,[32] in line with observations in wild rats.[41,42]

Alteration of sensitivity to DMBA carcinogenesis during sexual maturation. With a time sequence observation of sensitivity to DMBA administration, made by shifting the application time from 7 to 25 weeks of age, the tumor yield was clearly decreased in line with the evolutionary decrease in the number of TEB.[40]

Early transgene mutations in TEB cells induced by MNU. Transgene mutations in codon 12 could be demonstrated in the laser captured normal-looking TEB from female Hras128 rats as early as 5 days after application of MNU, providing compelling evidence that the TEB is indeed a target of the carcinogen and that transgene mutation is an early event in carcinogenesis.[40]

Rapid development of tumors in abdominal-inguinal mammary glands. Sequential histological observation of the abdominal-inguinal mammary glands, taken as a representative of all six pairs of mammary glands, at different time points after a single injection of MNU showed rapid development of intraductal epithelial proliferation on day 5, atypical hyperplasia on day 15 and adenocarcinoma on day 20, as shown in Fig. 3. The data are clearly of great significance for application as a rapid carcinogen assay model for detection of environmental carcinogens. The postulated sequence of events is depicted in Fig. 4.

Spontaneous tumor development. To determine carcinoma development without exposure to carcinogens, we conducted a study to observe spontaneous lesions in virgin Hras128 rats. The tumor yield was 52.8% at week 40, slightly increased as compared to the value for parent Sprague-Dawley females. As preneoplastic lesions, incidences of intraductal epithelial and acinar cell hyperplasia, with increased PCNA labeling, were two-fold greater than in wild rats at week 10. Similar data were obtained for subsequent atypical hyperplasias. The mRNA and protein levels of cyclins D1 and D2 started to increase from week 17. The results confirmed proliferative features of TEB at early stages followed by duct epithelial and alveolar cell hyperplasia with elevated expression of the c-Ha-ras protooncogene are background lesions for carcinoma development.[40,43] Thus, the transduced human c-Ha-ras proto-oncogene in the TEB is a target of carcinogens and mutations may occur before obvious proliferative changes become evident. Studies of spontaneous carcinogenesis indicated that carcinomas directly arise from the duct and acinar cells, corresponding to human 'ductular' and 'lobular' carcinomas, respectively.

Cloning of a new gene involved in the ras-MAPK pathway

In a search for genes involved in ras gene activation and MAPK transduction, the rat neuronal leucine-rich repeat protein-3

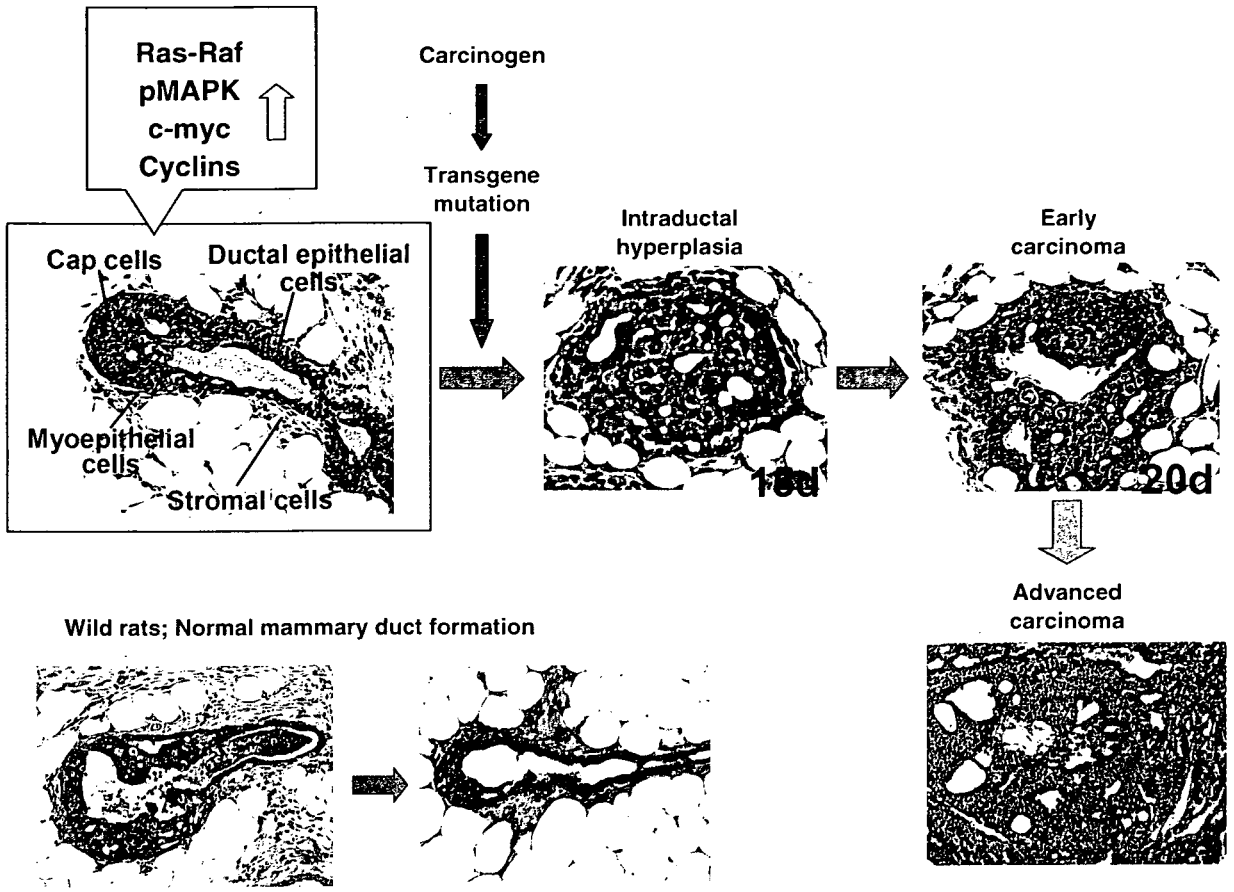

Fig. 4. Schematic presentation of carcinogenesis. Terminal endbuds, targets of carcinogens, have proliferative potential as a result of expression of the mutated transgene. Hyperplastic change occurs rapidly after application of carcinogens. The TEB shown at the lower left indicates normal mammary duct development in wild-type rats.

(NLRR-3) gene was cloned in a sarcoma spontaneously developing in the mammary region of an Hras128 rat. Stable expression of constitutively active forms of Ras (H-RasV12 or v-H-Ras) and treatment of epidermal growth factor (EGF) led to increase in NLRR-3 mRNA expression in normal fibroblasts. The carboxyl-terminal 30-aa region of NLRR-3 is responsible for the amplification of MAPK phosphorylation through association with endocytotic vesicles and NLRR-3 may amplify MAPK activity resulting in growth stimulation/Ras activation in malignant tumors.[44,45]

Modification of mammary carcinogenesis in the Hras128 rat

Effects of ovarian hormones. Effects of ovarian hormones on induction of mammary carcinogenesis were examined by performing ovariectomy before treatment with MNU. Although ovariectomy completely inhibited development of mammary carcinomas in wild counterparts, it did not affect either the incidence or the multiplicity of mammary carcinomas in the Hras128 rats,[38] indicating the high susceptibility to MNU carcinogenesis to be independent of ovarian hormones, including estrogens.

Environmental compounds: Possible application as a medium-term detection model for carcinogenesis modifying agents. After administration of MNU, suppressive effects of soy isoflavones could be clearly shown within 20 days in the Hras128 rat. Numbers of lesions, atypical hyperplasias and adenocarcinomas, in the isoflavone fed group in the post-initiation stage were clearly decreased as compared to the basal diet group (40 vs 100% incidence). The results indicate that this model may be useful for short-term screening for chemopreventive agents for mammary carcinogenesis.[43] To assess this possibility, effects of environmental compounds with estrogenic action, 4-n-octylphenols, atrazine and nonylphenols, were investigated. Female transgenic rats were given a single oral dose of DMBA and thereafter received diets containing one of these compounds. Although 4-*n*-octynophenols proved inactive,[46] atrazine at a dose of 50 p.p.m. and nonylphenol at 10 p.p.m. increased the incidence and multiplicity of adenocarcinomas ($P < 0.05$, by trend analysis).[47] These results suggest that endocrine disruptors may enhance mammary carcinogenesis, although the doses applied were extremely high as compared with feasible environmental human exposure levels, and that our transgenic rat can be used for medium-term assessment of the modification potential of environmental compounds.

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Summary of tumor induction and transgene mutation

Organs	Tumors: Chemically-induced	Tumors: Spont.†	Mutation: Hras128 Trans-gene	Mutation: Hras128 Endo-genous	Mutation: Wild Endo-genous
Mammary gland	⬆	⬀	+	—	—/+
Soft tissue	⬀	⬀	+	—	—
Esophagus	⬆	—	+	+	+
Bladder	⬀	—	+	+	—
Skin Back	⬆	—	+	—	—
Skin Scrotum	⬆	⬀	+	—	—
Liver (Foci)	—	—	*	*	*

⬆, Increase; ⬀, slight increase; — no change relative to wild rats
+, positive; —, negative; *, not examined; †spontaneous tumors

Fig. 5. Summary of data for the organ specificity for susceptibility to tumor induction, transgene mutations and endogenous ras gene mutations.

Other organ carcinogenesis

Skin. We have been able to establish a novel rat skin carcinogenesis model using males of our transgenic strain. Male and female transgenic rats were topically treated with DMBA on the back skin at 50 days after birth, and thereafter were similarly exposed to 2-o-tetradecanoylphorbol 13-acetate (TPA), three times a week for 31 weeks. Squamous cell papillomas and carcinomas, were preferentially induced at the painting sites with DMBA followed by TPA, 100%; DMBA, 75%; TPA, 16.7%. Unexpectedly, a high incidence of skin tumors was also noted on the remote scrotal skin. Furthermore, in females, mammary carcinomas, distant from the painting sites, were primarily induced with a few skin lesions. The results indicate that in addition to mammary cells, epidermal cells are also susceptible to initiation by DMBA.[26]

Urinary bladder. To examine susceptibility to N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)-induction of urinary bladder carcinogenesis, male transgenic and wild-type littermates were treated with 0.05% BBN in their drinking water for 10 weeks and killed at week 20. The number of bladder tumors, transitional cell papillomas and carcinomas, were greater in the transgenic rats than in their wild-type counterparts. Two cases of transitional cell carcinomas exhibiting invasion of the bladder muscle layer, were also observed to be limited to Hras128 rats. These results indicate that the Hras128 rat is highly susceptible to BBN carcinogenesis and may be used as a rat model for analysis of bladder tumor development.[24]

Esophagus. The transgenic rats were also found to be highly susceptible to N-nitrosomethylbenzylamine induction of esophageal carcinogenesis. Multiple esophageal lesions, squamous cell papillomas and carcinomas, rapidly developed within 10-weeks after application of the carcinogen, at 7-fold the number of tumors found in wild-type rats. Codon 12 GGC to GAC mutations of the transgene were detected at high incidence (76%), along with less frequent mutations of endogenous rat c-Ha-ras gene (8%).[25]

Discussion

Although the human c-Ha-ras proto-oncogene is transduced with its own promoter region, and therefore the gene mRNA is expressed, in all organs, the Hras128 transgenic rat does not exhibit enhanced carcinogenesis independent of the tissue but rather shows organotropic effects (see Fig. 5 for a summary of the findings so far). For example, intestine and prostate tumor induction in male rats was not different from that in wild type littermates after treatment with PhIP.[32] Similarly, no differences were observed in the incidences of liver, lung and thyroid tumors with dihydroxy-*di-n*-propyl nitrosamine. It is interesting that all of the organs that exhibit enhanced susceptibility to carcinogens are known as organs in which endogenous c-Ha-ras gene mutations have been found in chemically induced tumors in wild rats. For example, this is the case in the mammary gland,[48–52] esophagus,[53,54] bladder[55,56] and skin.[57] This is one conceivable reason for the organ-specific predisposition of organotropic oncogenicity with the c-Ha-ras gene (Table 2).

In the mammary gland, the transgene itself appears to increase the number of TEB and enhance proliferation, as evidenced by an increase in activated MAPK and expression of CyclinD2 and D3. Such proliferation-prone conditions are associated with mutations of the transgene in normal-looking endbuds.[40] From our results and the known pathways involving ras (Fig. 6), we speculate that mutated H-ras causes chronic upregulation of signaling through Raf and, therefore, transcription of genes responsible for cell growth. This may correspond to human cases with diseased conditions featuring chronically elevated cell turnover, which may facilitate induction of mutations in cancer related genes, including the ras gene,[58–61] by environmental agents. Actually, enhanced cell proliferation of human mammary tissue cells was shown to be relevant to carcinogenesis.[39,62] Our results may support the hypothesis proposed by Kumar *et al.* that normal physiological proliferative processes can lead to development of carcinomas if the targeted cells harbor latent ras oncogenes under conditions with some stimulus for cell proliferation.[63]

Table 2. **Correlation of susceptibility of Hras128 rats to carcinogens and H- or K-ras mutations in tumors in the rat, mouse and human**

Tumor site	Enhanced tumor induction in Hras128 rats[†]	H- or K-ras mutation in tumor of		
		Rat	Mouse	Human
Mammary gland	Yes	Hras	Hras	–
Skin	Yes	Hras	Hras	–
Colon	No	Kras	Kras	Kras
Bladder	Yes	–	–	Hras
Pancreas	No	–	–	Kras
Lung	No	Kras	Kras	Kras
Esophagus	Yes	Hras	–	–

[†]As compared to wild-type rats; –, not reported or no mutations found.

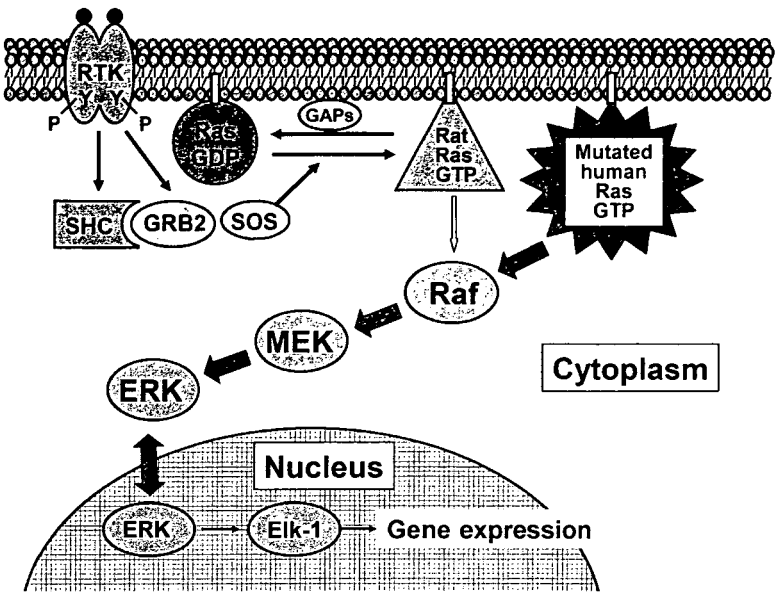

Fig. 6. Schematic presentation of relevant signal pathways in Hras128 rats. The mutated c-Ha-ras oncogene plays a dominant role in activation of the MAPK pathway by binding to Raf.

In addition to mammary carcinogenesis, the Hras128 transgenic rat was found to be susceptible to esophageal and urinary bladder and provides the first rat model featuring rapid generation of skin tumors. The latter has particular advantages in terms of wide application (painting) area and therefore can be used for the testing of compounds for carcinogenicity. For dominant activity in inducing tumors in some limited organs, studies on the possible involvement of some competitive inhibition mechanisms in activation of the ras gene are obviously required.

In conclusion, irrespective of the mechanisms involved in its high susceptibility to chemical carcinogenesis in particular organs, the transgenic rat offers a good model of human mammary carcinogenesis and promising short-term *in vivo* assay model for environmental carcinogens and modifying agents.

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