



Targeted deletion of the H-*ras* gene decreases tumor formation in mouse skin carcinogenesis

Kazuhiro Ise^{4,7}, Kenji Nakamura¹, Kazuki Nakao¹, Seiichiro Shimizu³, Hosami Harada¹, Taeko Ichise¹, Jun Miyoshi⁵, Yoichi Gondo⁶, Takatoshi Ishikawa³, Atsu Aiba¹ and Motoya Katsuki^{*1,2}

¹Division of DNA Biology and Embryo Engineering, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108-8639 Japan; ²Core Research for Evolutional Science and Technology (CREST), Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108-8639 Japan; ³Department of Pathology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan; ⁴Department of Cell Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan; ⁵Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka 537-8511, Japan; ⁶RIKEN Genomic Sciences Center, Kanagawa 244-0804, Japan

To clarify the role of the H-Ras *in vivo*, we generated H-*ras* null mutant mice by gene targeting. In spite of the importance of the Ras in cell proliferation and differentiation, H-*ras* null mutant mice grew normally and were fertile. The oldest H-*ras* mutant mice grew to be more than 30 months old. We used the H-*ras* deficient mice to study the importance of the H-*ras* and other *ras* genes in the development of skin tumors induced by initiation with 7,12-dimethylbenz(a)anthracene (DMBA) followed by promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). We showed that H-*ras* null mutant mice develop approximately six times less papillomas compared with wild-type littermates after 20 weeks of TPA treatment. While all papillomas examined (17 out of 17) in wild-type mice have mutations of H-*ras* at codon 61, 13 (62%) out of 21 papillomas in H-*ras* null mutant mice have mutations of K-*ras* gene at codon 12, 13, or 61 and another eight (38%) papillomas have no mutations in these codons of K-*ras* or N-*ras* genes. This suggests that the activation of H-*ras* gene is critical in the wild-type mice, but the activation of K-*ras* gene can replace the H-*ras* activation in the initiation step of skin tumor development in the H-*ras* deficient mice. *Oncogene* (2000) 19, 2951–2956.

Keywords: H-*ras* mutant; skin papilloma; chemical carcinogenesis

Introduction

The *ras* family genes are the oncogenes that have been detected most frequently both in human and in animal tumors (Barbacid, 1987; Guerrero and Pellicer, 1987; Balmain and Brown, 1988). There are three members among *ras* family genes in mammals. H-*ras* and K-*ras* were firstly identified as the transforming genes of the Harvey and Kirsten rat sarcoma viruses, respectively (Harvey, 1964; Kirsten and Mayer, 1967), and N-*ras* was

initially isolated from a human neuroblastoma (Shimizu *et al.*, 1983). These *ras* genes encode the 21 kDa proteins and these proteins are members of the superfamily of small GTP-binding proteins. Mutations of the *ras* genes frequently found in mammalian tumors produce the oncogenic Ras proteins which are constitutively in the active GTP-bound conformation and give uncontrolled output signals (Barbacid, 1987; Lowy and Willumsen, 1993; Balmain and Brown, 1988; Sekiya *et al.*, 1984). Three *ras* genes are thought to be functionally redundant by our genetic analysis, but tumorigenic mutations of each gene are related to the specific tissue types (Barbacid, 1987; Balmain and Brown, 1988).

The mouse model system for the skin tumorigenesis (Boutwell, 1974; Hecker *et al.*, 1982) has been invaluable in formulating the principles of initiation, promotion, and progression of tumors (Yuspa and Poirier, 1988; Yuspa, 1994). Molecular analyses of the stages of chemical carcinogenesis in the skin have indicated that the H-*ras* gene is a major target for a mutational event that takes place at the time of initiation (Balmain and Brown, 1988). Mutant H-*ras* alleles are found in a high proportion of premalignant tumors, indicating that the H-*ras* mutation is an early event (Balmain *et al.*, 1984). Furthermore, the specific mutations observed depend on the nature of the carcinogen as an initiator (Zarbl *et al.*, 1985; Quintanilla *et al.*, 1986; Bizub *et al.*, 1986; Brown *et al.*, 1990), suggesting a direct interaction between the carcinogen and the H-*ras* gene.

Availability of several mutant mice generated by gene targeting has enabled us to investigate the functional role of the oncogene, tumor suppressor gene, or cancer modifier in the initiation, the promotion and the progression of carcinogenesis *in vivo*, using a mouse skin tumorigenesis as a model system. Analysis of p53 tumor suppressor deficient mice treated with DMBA and TPA showed that absence of p53 does not augment the frequency of initiation or the rate of promotion but enhances malignant progression (Kemp *et al.*, 1993). Upon treatment with TPA, *c-fos* mutant mice carrying a v-H-*ras* transgene were able to develop benign skin tumors, but *c-fos* deficient tumors failed to undergo malignant conversion, suggesting that *c-fos* is required for malignant progression of skin tumors (Saez *et al.*, 1995). TNF- α mutant mice were resistant to development of benign and malignant skin tumors induced by DMBA and TPA, suggesting a proinflammatory cytokine, TNF- α

*Correspondence: M Katsuki, Division of DNA Biology and Embryo Engineering, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8638, Japan

⁷Current address: Center of Medical Information Science, Kochi Medical School, Kochi 783-8505, Japan

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is important to the early stages of tumor promotion (Moore *et al.*, 1999). Thus, genetic approach is valuable in elucidating the role of the specific genes in the specific stages during tumorigenesis.

We generated H-ras null mutant mice by gene targeting to examine the function of H-ras in carcinogenesis and development. To test directly whether H-Ras levels can influence skin tumor in an intact animal model, we have examined the incidence of epidermal papillomas formed in H-ras null mutant mice after a carcinogenesis protocol with DMBA and TPA. We also determined *ras* mutations in the tumor DNAs by sequencing to examine if activation of H-ras predominantly found in wild-type mice could be replaced by the activated mutation of K-ras or N-ras genes in the H-ras deficient tumors. We demonstrated that H-ras mutant mice display a decreased predisposition to tumor formation after the treatment. Interestingly, more than half of tumors formed in H-ras null mutant mice carried mutations in K-ras gene. These results are consistent with the idea that the H-ras gene is the initiator gene in skin carcinogenesis, although activation of H-ras genes can be replaced by K-ras gene mutations.

Results

Generation of the H-ras mutant mice

We generated a null H-ras allele by deletion of the exon ϕ to 5' part of exon IV, which contain initiation codon and the first 160 amino acid residues of H-Ras protein (Figure 1). The genetic background of the mice used in these studies was a mixture of 129/Sv, C57BL/6 and DBA/2 strains. To confirm that the allele has a null mutation, we carried out Western blot analysis with anti-H-Ras antibody. No H-Ras protein was found in the lysate from the H-ras homozygous mutant [H-ras (-/-)] skin (Figure 2). On the other hand, amounts of K-Ras and N-Ras protein were significantly increased in H-ras(-/-) skin, although the total amount of Ras proteins detected by anti-pan-Ras antibody was not significantly different between wild-

type and H-ras(-/-) mice (Figure 2). H-ras(-/-) mice grew normally and were fertile. The oldest H-ras(-/-) mice grew to be more than 30 months old and appeared indistinguishable from their wild-type littermates. They do not exhibit any predisposition to spontaneous tumor formation.

Skin tumorigenesis

To induce the skin tumors in mice, we used DMBA/TPA tumor induction protocol. Since H-ras gene is expected to play as an initiator gene through the point mutation in the specific codon, a decrease in gene dosage, as in the H-ras(+/-) and H-ras(-/-) mice, might lead to a decrease in the incidence of chemically induced papillomas. Groups of wild-type, H-ras(+/-)

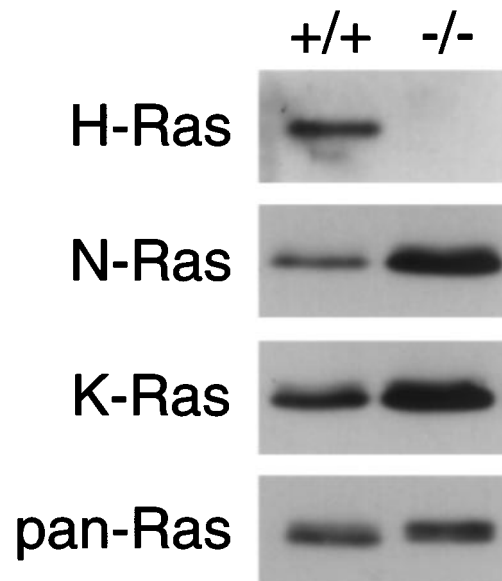


Figure 2 Expression of Ras proteins in the wild-type and H-ras(-/-) skin. 10 μ g (H-Ras and pan-Ras) or 40 μ g (K-Ras and N-Ras) of lysates from the wild-type and H-ras(-/-) skin were analysed by Western blotting with anti-H-Ras, anti-N-Ras, anti-K-Ras and anti-pan-Ras antibodies. Abbreviations: +/+, wild-type; -/-, H-ras(-/-) skin extract

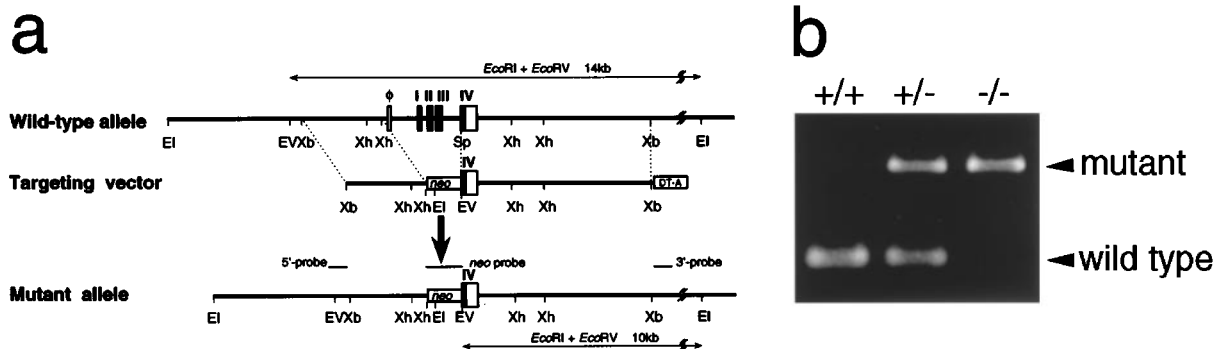


Figure 1 Production of H-ras null mutant mice. (a) A targeting construct of the H-ras. Schematic drawings of a region of the H-ras gene that contains exon ϕ to exon IV are shown. The targeting vector of the H-ras gene has a neomycin resistance (*neo*) gene to disrupt the exon ϕ to 5' part of exon IV, which contain initiation codon and the first 160 amino acid residues of H-Ras protein. The H-ras probes used for screening of ES cell clones as well as mice are indicated as bars, together with the expected sizes of hybridizing restriction fragments from wild-type and mutant alleles, respectively. Abbreviation for the restriction enzyme sites: EI, *EcoRI*; EV, *EcoRV*; Xb, *XbaI*; Xh, *XhoI*; Sp, *SpeI*. (b) PCR analyses of representative tail biopsies. Genomic DNA was isolated from mice derived from intercrosses of H-ras(+/-) mice. Abbreviations: +/+, wild-type; +/-, H-ras(+/-); -/-, H-ras(-/-) tail DNA

mouse and H-ras(-/-) mouse were treated with DMBA and then with the tumor promoter TPA twice weekly for 20 weeks. The animals treated with DMBA and TPA were monitored twice a week for the appearance of papillomas. The time to appearance of the first tumor was indistinguishable among three genotypes, following 5–6 weeks of promotion. No obvious differences in tumor size could be observed among mice of different genotypes. However, at all the time points, H-ras(-/-) mice had fewer papillomas than wild-type mice did (Figure 3). The numbers of papillomas per mouse were decreased according to the loss of H-ras wild-type allele. At the end of 20 weeks of promotion, average papilloma yield was approximately sixfold less in H-ras(-/-) mice compared with wild-type control mice, although H-ras(+/-) mice demonstrated an intermediate response: wild-type mice (16.7 tumors per mouse), H-ras(+/-) mice (6.8 tumors per mouse) and H-ras(-/-) mice (2.6 tumors per mouse) (Figure 3). Histological analyses showed that types of papillomas were indistinguishable among three genotypes (Figure 4).

Two conclusions can be drawn from these data regarding the role of H-ras genes in the early stages of mouse skin carcinogenesis. Firstly, the absence of H-ras greatly reduces the number of tumors, suggesting that H-ras gene is important for tumor formation presumably as an initiator gene. Secondly, H-ras gene is not essential for tumor formation, since H-ras(-/-) mice could develop tumors.

By the end of the study (week 30), malignant progression had been observed in two of 10 (20%) surviving wild-type mice, in two of 12 (17%) H-ras(+/-) mice, and in one of 11 (9%) H-ras(-/-) mice. Thus, H-ras gene is not essential for malignant conversion.

ras mutations in the tumor DNA

We evaluated the tumors for the presence of activating mutations at codons 12, 13 or 61 of the H-ras, N-ras and K-ras genes by PCR amplification

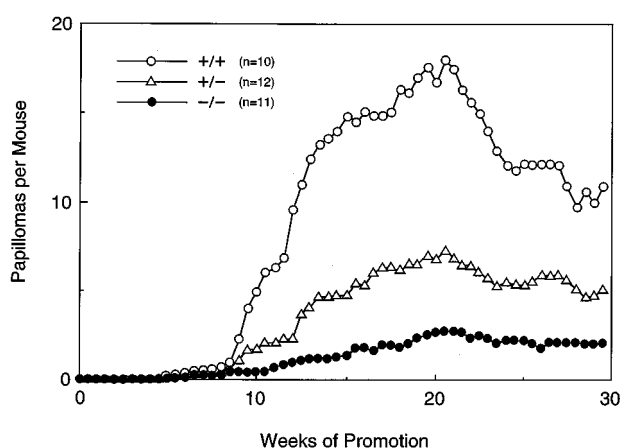


Figure 3 Rate of appearance of papillomas. The average number of papillomas per mouse is plotted versus the number of weeks of promotion. Mice were treated with twice weekly application of TPA for 20 weeks and caged individually to prevent wound-induced tumors. Animals were examined twice weekly for tumor formation. ○, wild-type(+/+); Δ, H-ras(+/-); ●, H-ras(-/-) mice

and direct sequencing of PCR products (Table 1). We found all tumors in wild-type mice examined have missense mutations at codon 61 of H-ras genes in accordance with previous results using DMBA on wild-type mice (Quintanilla *et al.*, 1986; Bizub *et al.*, 1986). Seventeen of 18 tumors contain A→T transversion and one tumor contains A→G transition in the middle base of codon 61. On the other hand, two papillomas out of 23 and one carcinoma in the H-ras(+/-) mice carry the missense mutations at codon 13 or 61 of K-ras genes. This is the first demonstration that chemically induced skin papillomas and carcinoma with activated mutation in the K-ras gene. Furthermore, 14 out of 22 tumors in the H-ras(-/-) mice have mutations at codons 12, 13 or 61 of K-ras genes. While activated H-ras mutations in the wild-type tumor are predominantly A→T transversion, 10 of 14 tumors with K-ras mutation contain G→C transversion in the first or second base of codon 12 or 13. For the other eight papillomas, we could not find any mutations at codon 12, 13 or 61 of K-ras or N-ras genes. We determined the sequence for whole coding region of K-ras genes, but no mutations were detected in the DNA from these tumors at all. Histological analyses showed that no obvious differences among the tumors with activation of H-ras, K-ras and no mutation (Figure 4), suggesting that activation of K-ras or other genes could replace the function of activated H-ras gene as the initiator gene in skin tumorigenesis.

Discussion

In this study, we showed that the absence of H-ras greatly reduces the number of papillomas, but H-ras(-/-) mice did develop tumors with activated K-ras mutations in skin carcinogenesis induced with DMBA and TPA. These results clearly showed that activation of H-ras gene is strongly responsible for initiation of tumor cells in the wild-type mice, but H-ras gene activation is not an absolute prerequisite for DMBA/TPA induced tumorigenesis.

We first demonstrated that skin papillomas and carcinomas could be developed with activated K-ras mutation instead of H-ras mutation in H-ras deficient mice. Genetic analysis could reveal the tumor-inducing properties of the specific genes among the oncogene families by knocking out the predominant cause of the oncogenes which belong to the same gene family. The appearance of K-ras mutations in H-ras deficient tumors could raise two possibilities since ras genes can acquire transforming properties by qualitative and quantitative mechanisms. While missense mutations within certain domains yield highly efficient transforming ras genes, the increased expression of ras oncogenes was observed in the tumors. Therefore, one possibility is that mutation rate in the K-ras genes are not different between wild-type and H-ras(-/-) cells, but total activity of mutated K-Ras in terms of the proliferation is increased compared with those in the wild-type mice, since expression level of K-Ras was increased in the H-ras(-/-) skin. In the wild-type cells, even though K-ras could be activated by mutation, the activity of mutant K-Ras proteins could not reach the threshold

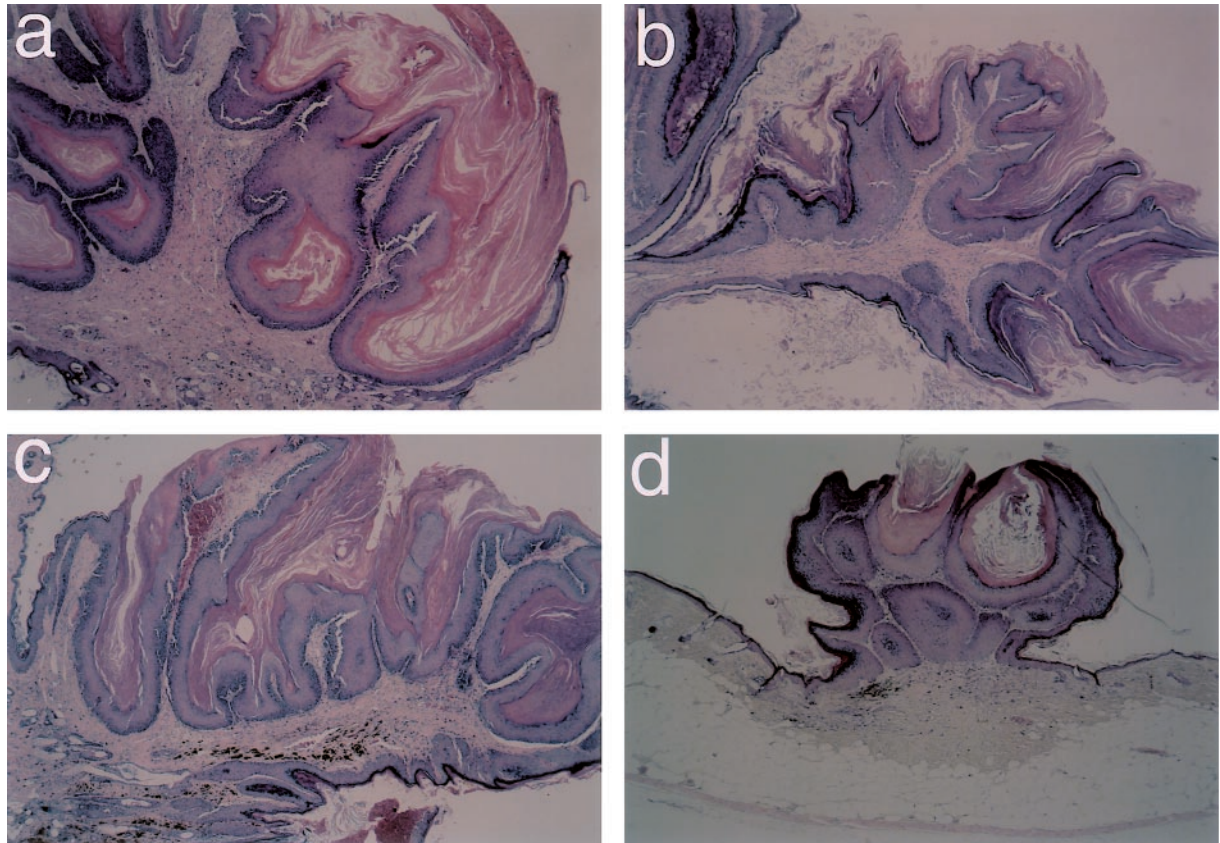


Figure 4 Histology of papillomas. Hematoxylin and eosin-stained paraffin sections were used for morphological evaluation of material. (a) A papilloma carrying mutation at codon 61 of H-ras on a wild-type mouse. (b) A papilloma carrying mutation at codon 13 of K-ras on an H-ras(+/-) mouse. (c) A papilloma carrying mutation at codon 13 of K-ras on an H-ras(-/-) mouse. (d) A papilloma carrying no mutations in ras genes on an H-ras(-/-) mouse

Table 1 ras gene mutations in skin tumors

Genotype	Histology	Mutant gene and codon	Mutation ^a	Amino acid substitution	Total number tested
Wild-type	Papilloma	H-ras 61	CAA→CTA	Gln→Leu	16
	Papilloma	H-ras 61	CAA→CGA	Gln→Arg	1
	Carcinoma	H-ras 61	CAA→CTA	Gln→Leu	1
	Total				18
H-ras(+/-)	Papilloma	H-ras 61	CAA→CTA	Gln→Leu	18
	Papilloma	H-ras 61	CAA→TTA	Gln→Leu	1
	Papilloma	H-ras 61	CAA→CAT	Gln→His	1
	Papilloma	H-ras 13	GGC→CGC	Gly→Arg	1
	Papilloma	K-ras 61	CAA→CAT	Gln→His	1
	Papilloma	K-ras 13	GGC→GCG	Gly→Ala	1
	Carcinoma	K-ras 61	CAA→CAT	Gln→His	1
	Total				24
H-ras(-/-)	Papilloma	no mutation ^b			8
	Papilloma	K-ras 13	GGC→CGC	Gly→Arg	7
	Papilloma	K-ras 61	CAA→CTA	Gln→Leu	2
	Papilloma	K-ras 12	GGT→GTT	Gly→Val	2
	Papilloma	K-ras 13	GGC→GCG	Gly→Ala	1
	Papilloma	K-ras 12	GGT→TGT	Gly→Cys	1
	Carcinoma	K-ras 13	GGC→CGC	Gly→Arg	1
	Total				22

^aThe indicated sequences correspond to the codons. Bold letters are nucleotides involved in the mutation. ^bNo mutations were detected in codon 12, 13 or 61 of K-ras or N-ras genes

for cell proliferation. Another possibility is that the mutation rate of K-ras gene was increased in H-ras(-/-) cells. A mutation rate or a site preference of the genes could be changed in the H-ras(-/-) cell with unknown reason.

DMBA is thought to have a strong preference for chemical interaction with adenine, and predominant mutation detected in mouse skin induced by DMBA is an A→T transversion in the second base of H-ras codon 61 (Quintanilla *et al.*, 1986; Brown *et al.*, 1990).

However, we found that the majority of DMBA-induced tumors in H-ras(-/-) mouse skin had G→C transversion in the K-ras codon 12 or 13, which have not been reported previously in the skin tumors induced by DMBA. Mutations at guanine residues by DMBA are not unprecedented, since *ras* mutations detected in primary bladder epithelial cells treated with DMBA *in vitro* were invariably G→A transitions in K-ras codon 12 (Brookes *et al.*, 1988) and the majority of DMBA-induced liver tumors had a G→C transversion in K-ras codon 13 (Manam *et al.*, 1992). These results show that DMBA causes guanosine adducts as well as adenosine adducts.

We found two carcinomas with activated K-ras mutations, which have not been reported previously. Brown *et al.* (1990) showed that different spectra of activated H-ras mutations were induced by different carcinogens and these initiating events can affect the probability of progression to malignancy. For example, 3-methylcholanthrene (MCA) initiation produced both codon 13, G→T, and codon 61, A→T transversion, of H-ras in papillomas; only the G→T mutation, however, was found in carcinomas, suggesting that tumors with codon 13, G→T, have higher probability of progressing into carcinomas than those with codon 61, A→T. Identification of H-ras-deficient tumors containing a single mutation at codon 12, 13 or 61, as described in this report, should permit more detailed analyses of the critical changes in *ras* family genes that lead to malignancy.

Materials and methods

Generation of ras mutant mice

H-ras genomic clones were isolated from a 129/Sv mouse genomic library (Stratagene) using v-Ha-ras as a probe. The targeting vector was constructed using the following DNA fragments: a 2.7 kb *XbaI*-*XhoI* fragment located 5' of exon ϕ , a 5.8 kb *SpeI*-*XbaI* fragment located 3' region of exon IV, a 1.1 kb fragment containing a *neo* gene derived from pMC1neo Poly A (Stratagene), a 1.0 kb fragment containing *DT-A* gene for negative selection (a gift of Dr S Aizawa; Yagi *et al.*, 1990) and the plasmid pBluescript (Stratagene). CCE ES cells (a gift of Dr E Robertson; Kuehn *et al.*, 1987), obtained from an inbred mouse line (129/SvJ), were cultured essentially as described previously (Koera *et al.*, 1997). Fifty μ g of the linearized targeting vector DNA was transfected into 4.7×10^7 ES cells by electroporation at 270 V/1.8 mm and 500 μ F capacitance (ECM600; BTX Inc.). Forty-eight hours after electroporation, G418 (250 μ g/ml; Gibco-BRL) was added to the medium. DNA from G418 resistant ES clones was digested, and hybridized with a 5'-probe and 3'-probe as illustrated in Figure 1. A targeted clone was injected into C57BL/6J blastocysts and chimaeric mice were thus generated (Bradley, 1987). Chimaeric males were mated with (C57BL/6 \times DBA/2)F1 females, and the tail DNA of the agouti offspring was analysed to confirm the germline transmission of the targeted allele by Southern blotting. Heterozygous mutant mice were interbred to generate homozygous mutant mice lacking the H-Ras protein.

PCR for genotyping

The allele specific primers are H1; 5'-GCTCCCTATTTGTGTTGGTTTTGC-3', H2; 5'-CCGCAATTTATGCTGCCGATCTC-3' and neo; 5'-TCCTGCCGAGAAAGTATCCA-3'.

The wild-type band is generated with the primers; H1 and H2, and the targeted band is generated with the primers; H2 and neo. Amplification conditions included pre-melting at 94°C for 2 min, 30 cycles of melting at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min and a final extension at 72°C for 7 min in the last cycle.

Western blotting

The skin of 6 week-old male mice was homogenized in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 20 μ g/ml aprotinin, 20 μ g/ml leupeptin). Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred to an Immobilon membrane (Millipore) and immunoblots were probed with monoclonal anti-pan Ras (Calbiochem), polyclonal anti-H-Ras (Santa Cruz), monoclonal anti-N-Ras (Santa Cruz) and monoclonal anti-K-Ras (Santa Cruz), antibodies and were visualized by enhanced chemiluminescence (ECL, Amersham).

Production of skin tumor

At 8 weeks of age, male mice were shaved, and their dorsal epidermis was treated with a single dose of 25 μ g of DMBA dissolved in 100 μ l of acetone followed by twice weekly application of TPA (0.2 mM dissolved in 100 μ l of acetone) for 20 weeks. Mice were caged individually to prevent wound-induced tumors. There were 10 wild-type, 12 H-ras(+/-) and 11 H-ras(-/-) mice at the outset of the experiments. The number and size of papillomas on each mouse were recorded twice weekly.

Tissue samples

Papillomas and carcinomas in the skin and tumor tissue were taken for routine histological examination and for *ras* gene mutation analysis (frozen in liquid nitrogen and stored at -70°C). Hematoxylin and eosin-stained paraffin sections were used for morphological evaluation of material. Histological classification of skin lesions is as follows. Papilloma is a cauliflower-like structure with a base consisting of a series of folds united to the underlying skin by one or a few common stalks. Each of these folds consists of a central connective tissue core covered by stratified squamous epithelium. Squamous cell carcinoma is an endophytic growth of atypical epithelial cells which invades the dermis and subcutaneous tissue. Carcinoma is distinguished from papilloma by its nuclear atypia, pleomorphism and nuclear hyperchromasia.

Sequencing analysis of ras gene mutation

Genomic DNA isolated from papillomas and carcinomas were used as a template for polymerase chain reaction (PCR). Exon sequences (exon I) containing codons 12 and 13, and exon sequences (exon II) containing codon 61 of H-ras, N-ras and K-ras gene were amplified and nucleotide sequences of both strands of these fragment DNA were determined by dye terminator cycle sequencing (Applied Biosystem). The primers for amplifying the exon sequences are as follows: H-ras exon I: 5'-GGCCTTGGCTAAGTGTGCTTCTCAT-3', 5'-TGGTCATTTACCCATGACCACTGCC-3'; H-ras exon II: 5'-CCCCACTAAGCCGTTGTTTTGC-3', 5'-TCAGTGTGCACACGGAACCTTCCT-3'; N-ras exon I: 5'-TAT-TGTAGGTTTGGTTTGCC-3', 5'-CTCTATGGTGGGATC-ATATT-3'; N-ras exon II: 5'-TCCTCACTCTTTCATAT-TCC-3'; AATATCCCCAGTACCTGTAG-3'; K-ras exon I: 5'-GAGTCTTACACACAAGGTG-3', 5'-GCAGCGTT-ACCTCTATCGTA-3', K-ras exon II: 5'-CCAGACTGT-GTTTCTCCCTT-3', 5'-CCCACCTATAATGGTGAATA-3'.

Amplification conditions included premelting at 94°C for 2 min, 30 cycles of melting at 94°C for 1 min, annealing at 55°C (N-ras and K-ras) or 60°C (H-ras) for 1 min and extension at 72°C for 1 min, and extension at 72°C for 7 min in the last cycle. The primers for sequencing are as follows: H-ras exon I: 5'-AAGGGCCTTGGCTAAGTGTG-3', 5'-ACCTCTGGCAGGTAGGCAG-3'; H-ras exon II: 5'-AG-GACTCCTACCGGAAACAG-3', 5'-TCACGGGCTAGCC-ATAGGTG-3'; N-ras exon I: 5'-CGTAATTGCTGCTTTTC-TAC-3', 5'-CATCCACAAAGTGGTTCTGG-3', N-ras exon II: 5'-TTCTTACCGAAAGCAAGTGG-3', 5'-TGATGGCA-AATACACAGAGG-3'; K-ras exon I: 5'-GTAAGGCCTGC-TGAAAATGA-3', 5'-GGGTGCTACTCATCCACAAA-3',

K-ras exon II: 5'-GGACTCCTACAGGAAACAAG-3', 5'-GGCAAATACACAAAGAAAGC-3'.

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References

- Balmain A, Ramsden M, Bowden GT and Smith J. (1984). *Nature*, **307**, 658–660.
- Balmain A and Brown K. (1988). *Adv. Cancer Res.*, **51**, 147–182.
- Barbacid M. (1987). *Annu. Rev. Biochem.*, **56**, 779–827.
- Bizub D, Wood AW and Skalka AM. (1986). *Proc. Natl. Acad. Sci. USA*, **83**, 6048–6052.
- Boutwell RK. (1974). *CRC Crit. Rev. Toxicol.*, **2**, 419–443.
- Bradley A. (1987). *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. Robertson EJ. (ed.). IRL Press: London, pp. 113–151.
- Brookes P, Cooper CS, Ellis MV, Warren W, Gardner E and Summerhayes IC. (1988). *Mol. Carcinog.*, **1**, 82–88.
- Brown K, Buchamann A and Balmain A. (1990). *Proc. Natl. Acad. Sci. USA.*, **87**, 538–542.
- Guerrero I and Pellicer A. (1987). *Mutat. Res.*, **185**, 293–308.
- Harvey JJ. (1964). *Nature*, **204**, 1104–1105.
- Hecker E, Fusenig NE, Kunz W, Marks F and Thielmann HW (eds). (1982). *Carcinogenesis: A Comprehensive Survey*. Raven Press: New York.
- Kemp CJ, Donehower LA, Bradley A and Balmain A. (1993). *Cell*, **74**, 813–822.
- Kirsten WH and Mayer LA. (1967). *J. Natl. Cancer Inst.*, **39**, 311–335.
- Koera K, Nakamura K, Nakao K, Miyoshi J, Toyoshima K, Hatta T, Otani H, Aiba A and Katsuki M. (1997). *Oncogene*, **15**, 1151–1159.
- Kuehn MR, Bradley A, Robertson EJ and Evans MJ. (1987). *Nature*, **326**, 295–298.
- Lowy DR and Willumsen BM. (1993). *Annu. Rev. Biochem.*, **62**, 851–891.
- Manam S, Storer RD, Prahallada S, Leander KR, Kravnak AR, Ledwith BJ, van Zwieten MJ, Bradley MO and Nichols WW. (1992). *Cancer Res.*, **52**, 3347–3352.
- Moore RJ, Owens DM, Stamp G, Arnott C, Burke F, East N, Holdsworth H, Turner L, Rollins B, Pasparakis M, Kollias G and Balkwill F. (1999). *Nat. Med.*, **5**, 828–831.
- Quintanilla M, Brown K, Ramsden M and Balmain A. (1986). *Nature*, **322**, 78–80.
- Saez E, Rutberg SE, Mueller E, Oppenheim H, Smoluk J, Yuspa SH and Spiegelman BM. (1995). *Cell*, **82**, 721–732.
- Sekiya T, Fushimi M, Hori H, Hirohashi S, Nishimura S and Sugimura T. (1984). *Proc. Natl. Acad. Sci. USA*, **81**, 4771–4775.
- Shimizu K, Goldfarb M, Suard Y, Perucho M, Li Y, Kamata T, Feramisco J, Stanvnezer E, Fogh J and Wigler MH. (1983). *Proc. Natl. Acad. Sci. USA*, **80**, 2112–2116.
- Yagi T, Ikawa Y, Yoshida K, Shigetani Y, Takeda N, Mabuchi I, Yamamoto T and Aizawa S. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 9918–9922.
- Yuspa SH and Poirier MC. (1988). *Adv. Cancer Res.*, **50**, 25–70.
- Yuspa SH. (1994). *Cancer Res.*, **54**, 1178–1189.
- Zarbl H, Sukumar S, Arthur AV, Martin-Zanca D and Barbacid M. (1985). *Nature*, **315**, 382–385.