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Advances in Brief

Cell-type-specific *ras* Mutations But No Microsatellite Instability in Chemically Induced Mouse Skin Tumors and Transformed 3T3 Cells[1]

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Abstract

In mouse skin, both papillomas/carcinomas or fibrosarcomas can be induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) depending on the mode of administration. Thus, upon DMBA painting (or transplacental exposure by i.p. injection to pregnant mothers) followed by 12-*O*-tetradecanoylphorbol-13-acetate applications to the skin of CD1 mice, papillomas and carcinomas appeared, whereas fibrosarcomas were induced when DMBA was s.c. injected. Molecular analysis of these tumors revealed that the majority of papillomas (17/20) and carcinomas (9/10) showed DMBA-specific mutations (A to T transversion at the 61st codon) in the Ha-*ras* gene. On the other hand, many fibrosarcomas (5/9) showed the same mutation only in the Ki-*ras* gene. When microsatellites were studied in these tumors at nine loci containing CA repeats, none of them showed an instability. In addition, when we analyzed 14 BALB/c 3T3 cell lines transformed by various carcinogens (including 3 clones induced by DMBA which have the A to T mutation in the Ki-*ras* gene), no changes in CA repeats were observed. These results suggest that DMBA-induced mouse tumors/transformed cells show cell-type-specific *ras* gene mutations, and these occur independently in the absence of microsatellite instability. While murine cells are considered to be relatively susceptible to cancer induction partially due to genomic instability, our results indicate that microsatellite instability is not induced in these cells by chemical carcinogens.

Introduction

Molecular genetic analysis of tumor samples suggests that the accumulation of multiple genetic changes is essential for a normal cell to progressively acquire malignant phenotypes (1). Thus, mutations in various oncogenes and tumor suppressor genes have been identified in various types of cancers (1, 2). Another type of genetic change recently found in tumors is microsatellite instability, in which simple di- and trinucleotide repeats are altered in the number of their repeats. Such microsatellite instability was first discovered in hereditary and sporadic forms of human colon tumors (3, 4) and then in other types of sporadic cancers (5–7). Microsatellite instability is often found to occur at multiple repeat loci in a given tumor, and thus can be considered as a marker of genomic instability. The origin of the microsatellite instability is believed to be from functional damage of genes involved in mismatch DNA repair, *e.g.*, *hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2* (8–10).

Experimental animal models are useful for studying molecular genetic changes associated with exposure to specific carcinogens. Mouse skin is among the first models in which carcinogen-specific oncogene mutation patterns were demonstrated (2, 11). Thus, it has been shown that skin papillomas and carcinomas induced by DMBA[4] often contain A to T transversion mutations at the 61st codon of the Ha-*ras* gene, whereas G to A transition at the 12th codon of the same gene was highly prevalent when *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was used as a carcinogen (2, 11). Interestingly, however, there is no evidence that DMBA-induced fibrosarcomas contain such specific Ha-*ras* gene mutations (12).

Recent studies also indicated that microsatellite instability is present in chemically induced animal tumors (13, 14). We have shown that MBNA-induced esophageal papillomas in the rat show CA repeat changes (13), and Canzian *et al.* (14) reported that rat colon tumors induced by the α-heterocyclic amine 2-amino-1-methyl-6-phenylimidazol-[4,5-b]pyridine, but not those induced by another heterocyclic amine, 2-amino-3-methylimidazol-[4,5-f]quinolinine, contain microsatellite instability. These results suggest that animal experimental models are useful for the study of carcinogen-induced microsatellite instability. Whether genomic instability revealed by microsatellite changes is involved in the generation of other types of mutations, *e.g.*, point mutations in *ras* or *p53* genes, is not known.

In the present study, we used *in vivo* and *in vitro* experimental mouse carcinogenesis models to study whether carcinogen-induced tumors contain specific mutations and to study their relationship to microsatellite instability. We report here that carcinogen-induced mouse tumors or transformed cells show cell-type-specific *ras* gene mutations, but no microsatellite instability.

Materials and Methods

Chemical Initiation and Tumor Production in Mouse Skin. For the production of skin papillomas and carcinomas, dorsal skin of CD1 female mice (4–7 weeks old) was treated with a topical application of DMBA (200 μg/0.2 ml acetone; Sigma Chemical Co., St. Louis, MO) followed by painting of TPA (10 μg/0.2 ml acetone twice a week; CCR, Inc., Eden Prairie, MN). Fibrosarcomas were also obtained in CD1 mice by s.c. injection of DMBA (150 μg/0.1 ml olive oil) in the dorsal neck region.

Additional samples of skin papillomas and carcinomas used for microsatellite DNA analysis were from our previous studies in which tumors were induced by transplacental DMBA initiation and postnatal TPA promotion (15, 16).

DNA Extraction. Genomic DNA was isolated from tumor cells as described previously (5) with reagents supplied by Applied Bio-Systems, Inc. (Foster City, CA).

Detection of A to T Mutations in H- and Ki-*ras* Genes. The presence of a typical DMBA-induced *ras* gene mutation, A to T transversion at the 61 st codon, was analyzed by PCR-RFLP described previously (17) with some modifications. In brief, we amply the H- or Ki-*ras* 61st codon flanking sequences and digest with *Xba*I, which cleaves only if the 61st codon contains A to T transversion mutation. The amplimers (Operon Technologies, Inc., Alameda, CA) used for the H-*ras* gene amplification were 5'-GAC TCC TAC CGG AAA CAG GT-3' and 5'-AGG AAG CCC TCC CCT GTG CG-3', and

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[4] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

those used for the K-*ras* gene were 5′-GAC TCC TAC AGG AAA CAA GT-3′ and 5′-CTA TAA TCG TGA ATA TCC TC-3′.

Analysis of Microsatellite Instability. Microsatellite instability in mouse tumors and transformed cells was examined at nine separate CA repeat loci (18, 19): Igh (chromosome 12), Orm 1 (chromosome 4), MUSANTP91A (chromosome 11), D4Nds3 (chromosome 4), hr (chromosome 14), Il-2 (chromosome 3), Ii (chromosome 18), Int1 (chromosome 15), and Thy-1 (chromosome 9).

PCR was carried out in a 25-μl reaction mixture containing 20 ng DNA, 100 ng of each oligonucleotide primer, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 2.5 μM dATP, 1 μCi [α-^{35}S]dATP, 50 mM KCl, 10 mMTris (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin, and 0.75 units *Taq* polymerase (Boehringer). PCR was carried out for 30 cycles comprising 1 min at 93°C, 2 min at 55°C, and 2 min at 72°C. Aliquots of amplified DNA were electrophoresed on standard denaturing 8% polyacrylamide DNA sequencing gels.

Results

A total of 9 fibrosarcomas induced by s.c. injection of DMBA and 11 papillomas/carcinomas induced by skin painting of DMBA and TPA were analyzed for the presence of *ras* gene mutations. Since a vast amount of literature indicates that DMBA-induced tumors predominantly contain A to T transversion at the 61st codon of *ras* genes (2, 11, 15), we concentrated our effort to examine this specific mutation using PCR-RFLP method (17). Our previous experiments have shown that this method is specific and sensitive to detect the mutation (17). As shown in Fig. 1 and Table 1, there was a clear cell-type-specific activitation of *ras* genes. While many fibrosarcomas contained Ki-*ras* and no Ha-*ras* mutations, most of the papillomas contained Ha-*ras* mutations. There was no fibrosarcoma with the Ha-*ras* mutation, nor any papillomas or carcinomas with the Ki-*ras* mutation.

To examine whether microsatellite instability was induced in these mouse skin tumors, we compared nine loci containing CA repeats in tumors and normal cells. As shown in Fig. 2 and summarized in Table 1, none of the samples had any change in CA repeat sequences. Similarly, the analysis of 11 additional papillomas and 8 carcinomas induced by transplacental initiation/postnatal promotion (DMBA/TPA) and other 9 papillomas and 2 carcinomas revealed no change in any of the 9 loci examined; most of them also had A to T mutations at the 61st codon of the Ha-*ras* gene (Refs. 15 and 16; Table 1).

Since microsatellite instability is considered to represent one form of genomic instability and since some murine cells are believed to be readily transformed due to their genomic instability, we studied possible CA repeat changes in BALB/c 3T3 cells (clone A31–1–1) before and after transformation. Again, none of the 14 BALB/c 3T3 transformed cell lines showed changes in their CA-repeat sequences at any of the nine loci studied (Table 2). The cell lines studied include those transformed by various agents, *i.e.*, DMBA, 3-methylcholanthrene, MNNG, nitrosomethyl urea, and UV-B (17). In addition, we also analyzed BALB/c 3T3 clone A31–1–13 cells, which are extremely susceptible to chemical and UV induction of transformation (20), and

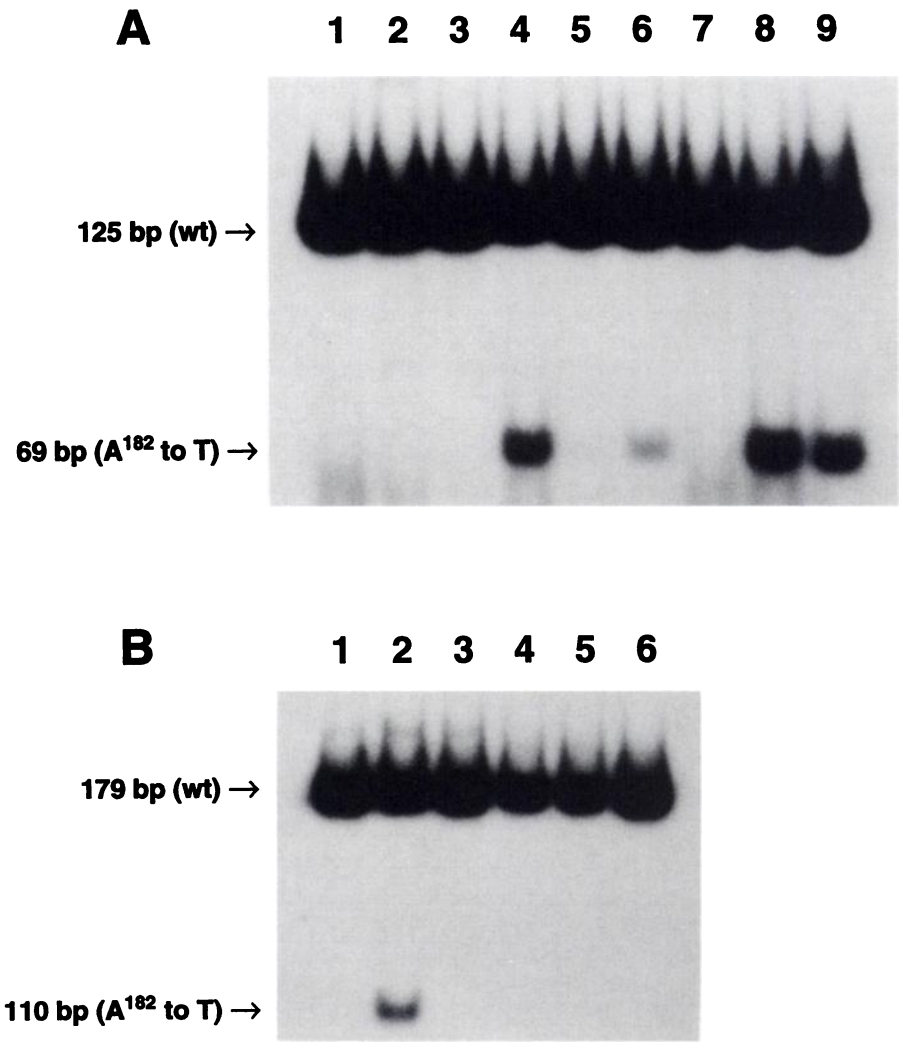

Fig. 1. Cell-type-specific induction of *ras* gene mutations (A^{182} to T) in DMBA-induced mouse skin papillomas, carcinomas, and fibrosarcomas. Ha- and Ki-*ras* genes encompassing the 61st codon were amplified with 5′-end-labeled primers ("Materials and Methods"), and the PCR products were digested with *Xba*I enzyme to separate wild- and mutant-type alleles before PAGE (6%). *A*, analysis of Ha-*ras* gene mutations. Wild-type (125-bp) and mutant (69-bp) bands are indicated. *Lane 1*, normal liver (control); *Lane 2*, fibrosarcoma; *Lanes 3–6*, papilloma; *Lanes 7–9*, carcinoma. Tumors were induced by s.c. (*Lane 2*), painting (*Lanes 3*, *4*, *7*, and *8*), and transplacental (*Lanes 5*, *6*, and *9*) applications of DMBA. *B*, analysis of Ki-*ras* mutations. Wild-type (179-bp) and mutant (110-bp) bands are indicated. *Lane 1*, normal liver (control); *Lane 2*, fibrosarcoma; *Lanes 3* and *4*, papilloma; *Lanes 5* and *6*, carcinoma. Tumors were induced by s.c. (*Lane 2*), painting (*Lanes 3* and *5*), and transplacental (*Lanes 4* and *6*) applications of DMBA. *wt*, wild type.

Table 1 *ras X gene mutations and microsatellite instability in chemically induced tumors of CD1 mice*

Tumors	Treatment[a]	No. of tumors	A to T mutation at 61st codon of		Microsatellite changes								
			Ki-*ras*	Ha-*ras*	Chromosome Locus	3 *Il-2*	4 *Orm-1*	4 *D4Nds3*	9 *Thy-1*	11 *MUS*[a]	12 *Igh*	14 *hr*	15 *Intl*	18 *Ii*
Fibrosarcoma	DMBA (s.c.)	9	5/9	0/9		0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Papilloma	DMBA + TPA (pt)	9	0/9	7/9		0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
	DMBA (tp) + TPA	11	0/11[b]	10/11[b]		0/11	0/11	0/11	0/11	0/11	0/11	0/11	0/11	0/11
Carcinoma	DMBA + TPA (pt)	2	0/2	1/2		0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	DMBA (tp) + TPA	8	0/8[b]	8/8[b]		0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

[a] MUS, MUSANTP91A; pt, painting; tp, transplacental.
[b] Data from Ref. 15 and 16.

found that their CA-repeat sequences are the same as those in clone A31–1.

Discussion

We have shown that a single carcinogen, namely, DMBA, induces different tumors which contained different types of *ras* gene mutations, depending on the route of administration. Tissue-specific mutation of *ras* genes has been shown from our own and other laboratories using transplacental exposure (16, 21). For example, transplacental exposure of mice to DMBA results in induction of various tumors; liver and skin tumors contained A to T transversion at the 61st codon of the Ha-*ras* genes, but tumors in the lung contained instead Ki-*ras* gene mutations (16). Our studies have also shown that such a cell-type-specific *ras* gene mutation is due to biological differences in the process of carcinogenesis. When BALB/c 3T3 cells were exposed to DMBA, both Ha- and Ki-*ras* genes were mutated and detected as early as 3 days after exposure. However, only Ki-*ras* gene mutated cells were fully transformed; our results suggested that such a specific recruitment of Ki-*ras*, but not Ha-*ras*, gene mutations into cell transformation was due to a much higher expression of Ki-*ras* gene in these cells (17). These results clearly indicated that DMBA induces both Ha- and Ki-*ras* gene mutations but only Ki-*ras* gene mutation contributed to the BALB/c 3T3 cell transformation process. This seems to mirror *in vivo* fibroblast transformation since in the present study DMBA-induced fibrocarcomas contained Ki-*ras* but not Ha-*ras* mutation.

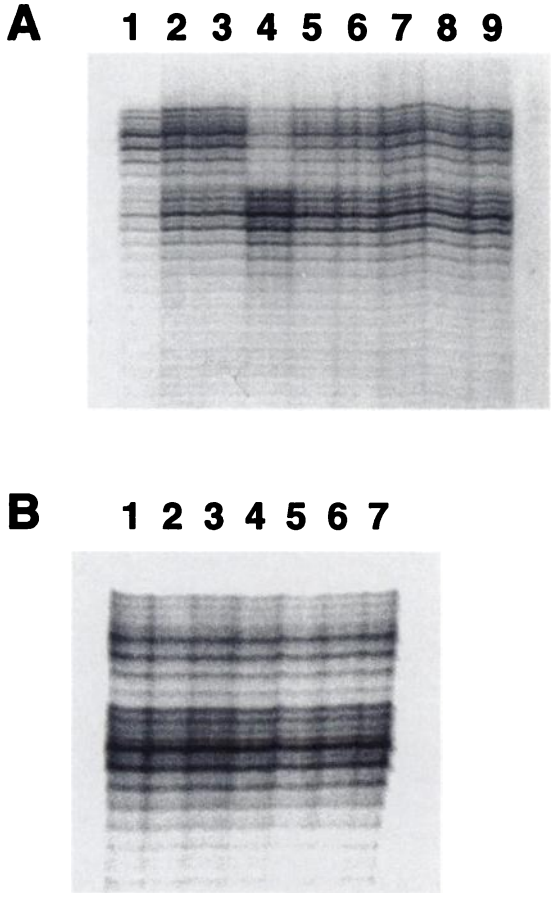

Fig. 2. Lack of microsatellite instability in chemically induced mouse skin tumors and transformed BALB/c 3T3 cells at the *Orm-1* locus. Labeled PCR products were electrophoresed, followed by autoradiography. *A*, CD1 mouse skin tumors. *Lanes 1*, *4*, and *7*, normal liver (control); *Lanes 2*, *5*, and *8*, fibrosarcoma; *Lanes 3*, *6*, and *9*, papilloma. *B*, transformed BALB/c 3T3 cells. *Lanes 1* and *2* are nontransformed A31–1–1 and A31–1–13 cells, respectively. Transformed BALB/c 3T3 cells were produced by treatment with DMBA (*Lane 3*), 3-methylcholanthrene (*Lane 4*), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (*Lane 5*), Nitrosomethyl urea (*Lane 6*), or UV (*Lane 7*).

Mice and murine cells *in vitro* are often considered to be sensitive targets for chemical carcinogenesis, and one of the reasons for their sensitivity is believed to be the relative instability of their genome. However, we found no CA-repeat changes in any mouse skin tumors or transformed BALB/c 3T3 cells. These results suggest that if genomic instability is responsible for the high susceptibility of murine cells to transformation, then genomic instability traits other than those represented by CA-repeat changes must be considered.

Previous studies in rats showed that microsatellite instability is present in some chemically induced tumors (13, 14). In these cases, it is likely that chemical carcinogens induced mutations in one of the mismatch repair genes responsible for the maintenance of microsatellite stability. Since DMBA-induced mouse skin tumors contained DMBA-specific base substitution mutation in *ras* genes, it is possible that mismatch repair genes were also mutated by DMBA in the skin cells. However, no microsatellite instability was observed in the present study. One possible reason for this apparent discrepancy may be due to organ specificity; in the rats, microsatellite instability has been seen in esophageal and colon tumors. The way in which microsatellite stability is controlled and altered during carcinogenesis may differ from other organs; in fact, there is thus far no microsatellite changes reported in any skin cancers.

The microsatellite instability in various tumors does not necessarily correlate with other types of genetic changes. Thus, in human colon tumors, the prevalence of *p53*, *APC*, or Ki-*ras* gene mutations was not higher in those tumors with microsatellite instability when compared with that of tumors without such instability (3). Similarly, our previous results suggested no correlation between the presence of *p53*, *APC*, or *Cx 32* gene mutations and microsatellite instability in human gastric tumor samples (5). Our results presented here also clearly showed that *ras* gene mutations are not associated with microsatellite instability. Thus, the results thus far suggest that microsatellite and oncogene/tumor suppressor gene mutations occur independently from each other. On the other hand, several *in vitro* studies indicate that those cells defective in their mismatch repair systems show not only microsatellite instability, but also other types of mutations such as base transitions and transversions (22). Further use of multistage

Table 2 *ras gene mutations and microsatellite instability in BALB/c 3T3 transformed cells by various carcinogens*

Treatment	No. of transformed clones	A to T mutation[a] at 61st codon of		Microsatellite changes									
		Ki-*ras*	Ha-*ras*	Chromosome Locus	3 *Il-2*	4 *Orm-1*	4 *D4Nds3*	9 *Thy-1*	11 *MUS*[b]	12 *Igh*	14 *hr*	15 *Intl*	18 *Ii*
DMBA (10 nM)	3	3/3	0/3		0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
3-Methylcholanthrene (3 nM)	3	0/3	0/3		0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
N-methyl-*N'*-nitro-*N*-nitrosoguanidine (10 nM)	3	0/3	0/3		0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Nitrosomethyl urea (10 nM)	2	0/2	0/2		0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
UV (20 mJ)	3	0/3	0/3		0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

[a] Data from Nakazawa *et al.* (17).
[b] MUS, MUSANTP91A.

models of experimental animal carcinogenesis for the analysis of microsatellite and cancer gene mutations should shed light on their temporal relationships.

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