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Distinct Pattern of Allelic Loss and Inactivation of *Cadherin 1* and *5* Genes in Mammary Carcinomas Arising in *p53*$^{+/-}$ Mice

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Mammary carcinoma/Loss of heterozygosity/*p53*$^{+/-}$ mice/*Cadherin* gene/Tumor suppressor gene.

p53 is one of the most frequently mutated genes in mammary carcinomas (MCs). To detect tumor suppressor genes cooperating with a hetero-deficient *p53* gene in mammary carcinogenesis, we first examined allelotypes in MCs from (BALB/cHeA × MSM/Ms) F_1- *p53*$^{+/-}$ and (BALB/cHeA × 129/SvEv) F_1- *p53*$^{+/-}$ female mice, and then surveyed down-regulated genes in the allelic loss regions. Genome-wide screening at 42 loci identified frequent (more than 30%) loss of heterozygosity (LOH) on chromosomes 5, 8, 11, 12, 14 and 18 in the MCs from either of the F_1 mice. The MCs in the *p53*$^{+/-}$ mice indicated highly frequent LOH, especially on chromosomes 8, 11 and 12, distinct from other mouse tumors. More than 60% of the 38 MCs from (BALB/cHeA × MSM/Ms) F_1- *p53*$^{+/-}$ mice showed LOH in a region ranging from *D8Mit85* (105.0 Mb from centromere) to *D8Mit113* (111.8 Mb) on chromosome 8, a region syntenic to human chromosome 16q22.1, on which LOH has been found in breast cancers. RT-PCR analyses revealed that the LOH of chromosome 8 was associated with the reduced and/or complete loss of expression of *Cdh1* and *Cdh5* genes in 15 (58%) and 8 (31%) of 26 MCs derived from the F_1 mice, respectively. Thus, inactivation of *Cdh1* and *Cdh5* is likely to cooperate with the loss of *p53,* suggesting a possible tumor suppressive function of these genes in mammary carcinogenesis.

INTRODUCTION

A number of studies have been performed to clarify the molecular mechanism underlying the development of breast cancer. The *p53* gene is mutated in approximately 50% of human cancers[1,2] and sporadic breast cancers show a high frequency of *p53* mutations.[3] In fact, the frequency of *p53* mutations varies from 20 to 40% in breast cancers, depending on the tumor size and the stage of the disease.[4] Li-Fraumeni syndrome is due to germline mutations of *p53*, and patients are predisposed to a high incidence of breast cancer, sarcomas and other neoplasms.[5,6] Loss of function of *p53*, by both genetic and epigenetic changes, is thus crucial in mammary carcinogenesis. In addition, some other genetic events are required for the development of mammary carcinomas (MCs) since the carcinogenesis is multi-step. In fact, analyses of knockout (KO) mice revealed that *Brca1* and *Brca2* tumor suppressor genes cooperate with *p53*.[7,8] Studies of transgenic mice also indicated that *neu, Wnt1, ras* and *IGF-1* oncogenes also cooperate with *p53* to accelerate tumor formation.[9] Furthermore, *p53* mutations are highly frequent in breast cancers of those patients carrying germline mutations of *BRCA1* or *BRCA2*.[10,11] In *p53*-heterozygous deficient BALB/c mice (BALB/c-*p53*$^{+/-}$), almost all MCs exhibit a loss of the remaining wild-type *p53* allele.[12] As the function of *p53* is completely lost in these MCs, additional genetic events related to other tumor suppressor genes can be detected by analyses of allelotypes and alterations of gene expression in the tumors.

Various genetic events result in a loss of heterozygosity (LOH) in cancers,[13] and the LOH regions often contain tumor suppressor genes in human and in experimental animals.[14,15] Therefore, the genome-wide search for the tumor suppressor genes can be made by allelotype analysis in F_1 hybrid mice between two different subspecies,[16,17] supple-

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mented with the gene expression profile analyses by microarray techniques.

In this study, we first examined allelotypes to identify the region of LOH in the MCs from (BALB/cHeA × MSM/Ms) F_1-*p53*$^{+/-}$ and (BALB/cHeA × 129/SvEv) F_1-*p53*$^{+/-}$ female mice, and those from doubly heterozygous deficient (BALB/cHeA × MSM/Ms) F_1-*p53*$^{+/-}$*Atm*$^{+/-}$ mice. Then, the regions with LOH were analyzed for the down-regulated genes for possible genes cooperating with the loss of *p53* for the development of MCs. We found that highly frequent LOH appeared on chromosomes 8, 11 and 12 in the MCs. The LOH of a region of chromosome 8 was found to be associated with the reduced and/or complete loss of expression of the *Cdh1* and *Cdh5* genes.

MATERIALS AND METHODS

Mice

The *p53* targeted allele originally generated by Donehower *et al.*[18] was introduced into the BALB/cHeA mouse at The Netherlands Cancer Institute (Amsterdam). The *p53*-heterozygous deficient mice (*p53*$^{+/-}$) were repeatedly backcrossed to BALB/cHeA mice, and maintained at the animal facility of Osaka Prefecture University. The MSM/Ms mice used in the production of F_1 mice in this study were *Atm*-heterozygous deficient mice (MSM/Ms-*Atm*$^{+/-}$). The *Atm* targeted mouse (129/SvEv-*Atm*$^{tm1Awb/+}$ mouse) was originally generated at the Jackson Laboratory.[19] The *Atm*-heterozygous deficient mice (*Atm*$^{+/-}$) were repeatedly backcrossed more than ten times to MSM/Ms mice. The BALB/cHeA-*p53*$^{+/-}$ mice were crossed with MSM/Ms-*Atm*$^{+/-}$ or 129SvEv-*Atm*$^{+/-}$ mice, and females of the F_1 progeny [(BALB/cHeA × MSM/Ms)F_1 and (BALB/cHeA × 129SvEv)F_1] were used in the experiments. The conditions for breeding were described previously.[20] All animal experiments were carried out in accordance with the *Standards Relating to the Care and Management of Experimental Animals* (Japan) and the *Guidelines for Animal Care and Use of Osaka Prefecture University.*

Induction of MC and histological examination

Mice with genotypes of *p53*$^{+/-}$*Atm*$^{+/+}$ or *p53*$^{+/-}$*Atm*$^{+/-}$ were exposed to 5Gy X-rays at five weeks of age. MCs arising in the irradiated mice were used in the present study. Additional 11 MCs were those from non-irradiated F_1 mice with the same genotypes. Developed MCs and normal tissues were removed, and immediately frozen and kept at –80°C until isolation of DNA and RNA. Histological examination was described previously.[21] Briefly, the tissues were fixed in 10% neutralized formalin solution and embedded in paraffin. Thin sections were prepared from the paraffin blocks. They were stained with hematoxylin and eosin for microscopic examination.

DNA isolation, LOH analysis and genotyping

Isolation of DNA, PCR amplification, electrophoresis of PCR products and the assessment of allelic losses were performed according to the procedures described previously.[20] The chromosomal locations (cM) of the microsatellite markers were based on the 2000 Chromosome Committee Reports in the Mouse Genome Database (Mouse Genome Informatics; Jackson Laboratory, Bar Harbor, ME). The chromosomal locations (Mb) of the markers and genes were based on Ensemble and UCSC databases. Genotypes for the wild-type and targeted alleles of *p53* and *Atm* genes were determined by analyzing the PCR products for these alleles. Amplification of the *p53* alleles was described elsewhere.[22] Similarly, amplification of the wild-type and the targeted allele of the *Atm* gene were performed by using primers IMR0640 (5'-GCTGCCATACTTGATCCATG-3') and IMR-0641 (5'-TCCGAATTTGCAGGAGTTG-3'), and primers IMR0641 and AtmNeo410 (5'-CGGTGGATGTGGAATGTGTG-3'), respectively.

Expression analysis of the Cadherin family by RT-PCR

The tissues were stored in RNA*later* RNA Stabilization Reagent (QIAGEN). Total RNA was isolated from the tissues using RNeasy Protect Mini Column Kit (QIAGEN) as recommended by the manufacturer. cDNA was synthesized using SUPERSCRIPT II Amplification Kit (Invitrogen). The reaction volumes were 50 µl and the reaction tubes contained SUPERSCRIPT™ reverse transcriptase/Platinum *taq* DNA polymerase (1 µl), dNTPs (0.2 mM of each), magnesium sulfate (1.2mM) and gene specific sense/anti-sense primers (0.2 µM) at concentrations that were recommended by the manufacturer. The primers for RT-PCR of cadherin family members are shown as below.

Cadherin 1	Forward:	GCCAAGGGCTTGGATTTTGAGGCCAA
	Reverse:	GGTCCAGTTGACGCTGGCCCC
Cadherin 5	Forward:	CAGCTTCACTGTCAAGGTGC
	Reverse:	GGTACAAGACAGTGGCGTGGC
Cadherin 3	Forward:	CAGAGCTGAGTGCTGTGTGGC
	Reverse:	CGTGGCACGCTGCATCTGACT
Cadherin 8	Forward:	TGGAAACAGTGCAAAGTTGG
	Reverse:	TTGTGGTTCCAGACAGACCA
Cadherin 11	Forward:	GAGCCCAGTACACACTGATGGC
	Reverse:	GGGATCATCTGCATCAGAGGC
Cadherin 16	Forward:	CTCAGGCAGTGTAACACTGGG
	Reverse:	GTGGCCATGAGTGTTGCCAC
Gapdh (control)	Forward:	CATCACCATCTTCCAGGAGCGAGA
	Reverse:	GTCTTCTGGGTGGCAGTGATGG

The RT-PCR conditions consisted of 2 min at 94°C, followed by 35 cycles of a denaturation step of 30 sec at 94°C, an annealing step of 1min at adequate temperatures for each primer set, an extension step of 1 min at 72°C, and a final extension step of 10 min at 72°C.

Exon analysis of Cdh5 gene

Tumor and normal tissue DNA were amplified using the primer sets for each exon as described below. Sequences of the primers were designed using a software Primer 3 based on DNA sequences shown on NCBI Sequence Viewer.

Exon 1	Forward:	AAGGTGCAGAGGCTCACAG
	Reverse:	GGGTTCTCTTCATCGATGTGT
Exon 2	Forward:	TCAGCTCATGTTGCCTGTTC
	Reverse:	TGAGGTGGCTGATGTGAGAG
Exon 3	Forward:	CATGAGCGGACATACCAGAA
	Reverse:	CCATCCCTCCAAATGGGTA
Exon 4	Forward:	TGGGACACTCTTCTTCTCAGG
	Reverse:	TGTCCACTTAAAGGCCTATGG
Exon 5	Forward:	TGCGGTATCCTATGCACATT
	Reverse:	GCTCGGTTCTGCAGGTCTAC
Exon 6	Forward:	GGAGGACTGGGCCTAAGTGT
	Reverse:	GTCCTCCCTTGAGCCTTGAT
Exon 7	Forward:	GTCAAGGAGAGCCATGAAGC
	Reverse:	AGGTAGCCTGGAAGGTGGAT
Exon 8	Forward:	CAGGTAACCCTGTAGGGAAA-GA
	Reverse:	AAACACACGACTCCCTAGTCC
Exon 9	Forward:	CACATTCCAGCTGGTAGTACA-GA
	Reverse:	TTCTCAGAGCCAACCGTCTT
Exon 10	Forward:	GGGAACAAAGAAGGCAGTGA
	Reverse:	TTCTGTGAAGCCCTCTCGAT
Exon 11F	Forward:	CTGGTCCCATGAACCTGTCT
	Reverse:	TTTCTTCACGTCGATCATGG
Exon 11R	Forward:	AGTGTACACGCAGGTGCAGA
	Reverse:	CCTAGATGATGAGTTCCTCCTG

The PCR conditions consisted of 3 min at 94°C, followed by 35 cycles of a denaturation step of 1 min at 94°C, an annealing step of 1min at 58°C, an extension step of 75 sec at 72°C, and a final extension step of 3 min at 72°C.

RESULTS

Genome-wide search for LOH in MCs from (BALB/cHeA × MSM/Ms)F_1 and (BALB/cHeA × 129SvEv)F_1 mice

To detect tumor suppressor genes cooperating with the *p53* gene in the mammary carcinogenesis, LOH analysis was first carried out for 23 MCs from 5 Gy-irradiated (BALB/cHeA × MSM/Ms) F_1-$p53^{+/-}$ mice at 42 polymorphic microsatellite loci. Fig. 1A depicts the highest frequency of LOH found in the analyses of 2 to 4 loci on each chromosome. In (BALB/cHeA × MSM/Ms) F_1-$p53^{+/-}$, highly frequent allelic losses were found at *D8Mit270* (56 cM from centromere) on chromosome 8, *D11Mit12* (75.4 cM) on chromosome 11 and *D12Nds1* (27 cM) on chromosome 12, and the frequencies of LOH at these loci were 52%, 57% and 65%, respectively. LOH of lower frequencies was observed at several loci; 26% at *D3Mit6* (23.3 cM), 22% at *D4Mit13* (71 cM), 30% at *D5Mit81* (28 cM), 20% at *D9Mit20* (61 cM), 28% at *D10Nds2* (58 cM), 35% at *D14Mit7* (44.7 cM), 26% at *D16Mit7* (57.7 cM) and 22% at *D18Mit8* (47 cM) (Fig. 1A).

LOH analysis was then performed for 13 MCs from 5 Gy-irradiated (BALB/cHeA × 129SvEv)F_1-$p53^{+/-}$ mice at 42 polymorphic microsatellite loci. Fig. 1B demonstrates the highest frequency of LOH at 1 to 4 loci on each chromosome. Highly frequent LOH was again found on chromosome 8 at *D8Mit87* (56 cM), on chromosome 11 at *D11Mit51* (15 cM) near *p53* and on chromosome 12 at *D12Mit132* (52 cM), and the frequencies of LOH at these loci were 92%, 92% and 77%, respectively. LOH with a lower frequencies was observed in several loci; 23% at *D4Mit31* (51 cM), 23% at *D6Mit55* (50 cM), 23% at *D10Nds1* (6 cM), and 31% at *D18Mit8* (47 cM) (Fig. 1B).

Thus, more than 30% LOH was observed in MCs on chro-

Table 1. LOH frequency of microsatellite markers on chromosome 8 in 38 mammary carcinomas from X-irradiated (BALB/cHeA × MSM/Ms)F_1 mice

Locus	Position (Mb)	Position (cM)	$p53^{+/-}Atm^{+/-}$ mice		$p53^{+/-}Atm^{+/+}$ mice		$(p53^{+/-}Atm^{+/-}) + (p53^{+/-}Atm^{+/+})$mice	
			No. (n=25)	Frequency (%)	No. (n=13)	Frequency (%)	No. (n=38)	Frequency (%)
D8Mit4	33.5	14	10	40	4	31	14	37
D8Mit6	50.9	30	11	44	2	15	13	34
D8Mit11	99.2	46	10	40	6	46	16	42
D8Mit85	105	47	18	72	7	54	25	66
D8Mit212	107	51	18	72	8	62	26	68
D8Mit12	109.6	53.3	15	60	8	62	23	61
D8Mit113	111.8	52	17	68	9	69	26	68
D8Mit319	117.4	58	13	52	7	54	20	53
D8Mit120	121.2	61	8	32	5	38	13	34

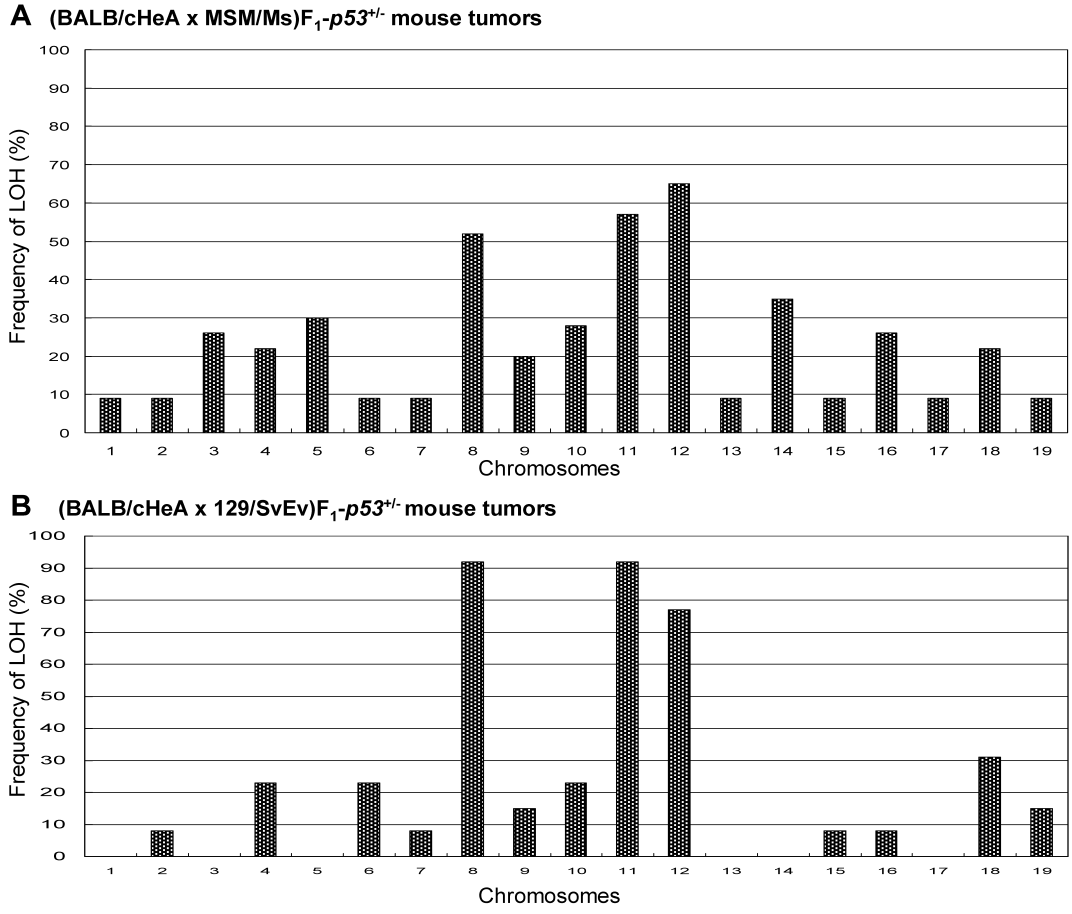

Fig. 1. Genome-wide search for LOH conducted on 23 mammary carcinomas (MCs) from irradiated (BALB/cHeA × MSM/Ms)F_1-*p53*$^{+/-}$ (A) and on 13 MCs from irradiated (BALB/cHeA × 129SvEv)F_1-*p53*$^{+/-}$ (B) mice. The maximum frequency obtained on each chromosome is shown. (A) Loci examined were as follows: *D1Mit9* (52 centimorgans from the centromere: 52 cM), *D2Mit15* (69 cM), *D3Mit6* (23.3 cM), *D4Mit13* (71 cM), *D5Mit81* (28 cM), *D6Mit16* (30 cM), *D7Mit9* (59 cM), *D8Mit270* (56 cM), *D9Mit20* (61 cM), *D10Nds2* (58 cM), *D11Mit12* (75.4 cM), *D12Nds1* (27 cM), *D13Mit263* (71 cM), *D14Mit7* (44.7 cM), *D15Mit15* (64.8 cM), *D16Mit7* (57.7 cM), *D17Mit221* (56.7 cM), *D18Mit8* (47 cM), *D19Mit32* (0 cM). (B) Loci examined were as follows: *D1Mit18* (30 cM), *D2Mit14* (49 cM), *D3Mit14* (64 cM), *D4Mit31* (51 cM), *D5Mit13* (20 cM), *D6Mit55* (50 cM), *D7Nds4* (72.4 cM), *D8Mit87* (56 cM), *D9Mit18* (77 cM), *D10Nds1* (6 cM), *D11Mit51* (15 cM), *D12Mit132* (52 cM), *D13Mit13* (21.9 cM), *D14Mit34* (40 cM), *D15Mit33* (49 cM), *D16Mit73* (10 cM), *D17Mit16* (18 cM), *D18Mit8* (47 cM), *D19Mit56* (2.2 cM).

mosomes 5, 8, 11, 12, 14 and 18 in the MCs from either of the F_1 mice, and it was especially high on chromosomes 8, 11 and 12 in both F_1 mice. This pattern was distinct from other mouse tumors.[16,17,23,24] Meanwhile, the LOH patterns in the *Atm*$^{+/-}$ and *Atm*$^{+/+}$ mice of both of the two F_1 did not differ markedly (data not shown). There was no correlation between the chromosome regions of LOH and the pathological characteristics such as the latency of tumors and histopathological features.

Allelotype analysis of microsatellite loci on chromosome 8

Frequent LOH found at some loci on chromosome 8 was further studied for the precise allelotype of this chromosome on 38 radiation-induced MCs from (BALB/cHeA × MSM/Ms)F_1-*p53*$^{+/-}$ mice (Table 1 and Fig. 2). Frequent LOH spanned wide regions from *D8Mit11* (99.2 Mb, 46 cM,) to *D8Mit319* (117.4 Mb, 58 cM) in both 25 tumors from *p53*$^{+/-}$ *Atm*$^{+/-}$ and 13 tumors from *p53*$^{+/-}$*Atm*$^{+/+}$ mice (Table 1). No significant difference of LOH pattern was observed in the mice between heterozygous deficient and wild-type for *Atm* gene. More than 60% of 38 MCs from *p53*$^{+/-}$*Atm*$^{+/-}$ and *p53*$^{+/-}$*Atm*$^{+/+}$ mice showed LOH in a region ranging from *D8Mit85* (105.0 Mb, 47 cM) to *D8Mit113* (111.8 Mb, 52 cM) on chromosome 8, a region syntenic to human chromosome 16q22.1 where frequent LOH has been reported for human breast cancer cases (Table 1 and Fig. 2).

Among the loci tested, the highest frequency of LOH was found at two loci, and the frequency was 68% (26 of 38 MC) (Fig. 2). One was *D8Mit212* (107.0 Mb, 51 cM), which is a

MC No.	*p53* genotype	*Atm* genotype	*D8Mit4* (14 cM) 33.5Mb	*D8Mit6* (30 cM) 50.9Mb	*D8Mit11* (46 cM) 99.2Mb	*D8Mit85* (47 cM) 105.0Mb	*D8Mit212* (51 cM) 107.0Mb	*D8Mit12* (53.3 cM) 109.6Mb	*D8Mit113* (52 cM) 111.8Mb	*D8Mit319* (58 cM) 117.4Mb	*D8Mit120* (61 cM) 121.2Mb
1238	KO/Wild	KO/Wild	R	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻
1249	KO/Wild	KO/Wild	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R	R
1301	KO/Wild	KO/Wild	C⁻	R	R	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻
1082	KO/Wild	KO/Wild	C⁻	C⁻	C⁻	C⁻	R	C⁻	C⁻	C⁻	C⁻
1083	KO/Wild	KO/Wild	C⁻	M⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻
1103	KO/Wild	KO/Wild	R	R	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R
1104	KO/Wild	KO/Wild	C⁻	M⁻	R	C⁻	C⁻	R	C⁻	C⁻	R
1106	KO/Wild	KO/Wild	R	M⁻	R	C⁻	C⁻	R	C⁻	R	R
1173	KO/Wild	KO/Wild	R	R	R	R	C⁻	R	C⁻	R	R
1245	KO/Wild	KO/Wild	C⁻	R	R	C⁻	R	C⁻	C⁻	C⁻	R
1107	KO/Wild	KO/Wild	C⁻	M⁻	M⁻	R	C⁻	R	C⁻	R	R
1264	KO/Wild	KO/Wild	M⁻	R	M⁻	M⁻	M⁻	M⁻	C⁻	M⁻	M⁻
1216	KO/Wild	KO/Wild	C⁻	M⁻	R	R	R	R	R	R	R
1109	KO/Wild	KO/Wild	R	R	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻
1172	KO/Wild	KO/Wild	R	R	R	M⁻	M⁻	M⁻	M⁻	R	R
1251	KO/Wild	KO/Wild	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	R	R	R
1304	KO/Wild	KO/Wild	R	R	R	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻
1209	KO/Wild	KO/Wild	R	M⁻	R	R	M⁻	M⁻	M⁻	M⁻	R
1252	KO/Wild	KO/Wild	R	R	M⁻	M⁻	M⁻	M⁻	R	M⁻	R
1180	KO/Wild	KO/Wild	R	R	R	M⁻	M⁻	R	R	R	R
1242	KO/Wild	KO/Wild	R	R	R	R	M⁻	R	R	R	R
1250	KO/Wild	KO/Wild	R	M⁻	R	M⁻	R	R	M⁻	M⁻	M⁻
1108	KO/Wild	KO/Wild	R	R	R	M⁻	R	M⁻	R	R	R
1202	KO/Wild	KO/Wild	R	R	R	R	R	R	R	R	R
1218	KO/Wild	KO/Wild	R	R	R	R	R	R	R	R	R
1241	KO/Wild	Wild	R	R	C⁻	R	C⁻	C⁻	R	R	R
1300	KO/Wild	Wild	R	R	C⁻	R	R	R	C⁻	R	C⁻
1244	KO/Wild	Wild	R	R	R	R	R	C⁻	C⁻	M⁻	R
1296	KO/Wild	Wild	R	M⁻	R	R	C⁻	R	C⁻	R	R
1081	KO/Wild	Wild	M⁻	C⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻
1295	KO/Wild	Wild	M⁻	R	M⁻	M⁻	R	M⁻	M⁻	M⁻	M⁻
1203	KO/Wild	Wild	M⁻	R	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻
1208	KO/Wild	Wild	M⁻	R	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	R
1110	KO/Wild	Wild	R	R	R	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻
1201	KO/Wild	Wild	R	R	R	M⁻	M⁻	M⁻	M⁻	R	R
1294	KO/Wild	Wild	R	R	R	M⁻	M⁻	R	R	M⁻	R
1197	KO/Wild	Wild	R	R	R	R	R	R	R	R	R
1112	KO/Wild	Wild	R	R	R	R	R	R	R	R	R
LOH (%)			37	34	42	66	68	61	68	53	34

Fig. 2. Schematic representation of the allelotype in the frequent LOH regions on chromosome 8 in 38 mammary carcinomas (MC) from (BALB/cHeA × MSM/Ms) F_1-*p53*$^{+/-}$ mice. The names of microsatellite markers and their positions in centiMorgan (cM) and megabase (Mb) from the centromere are indicated at the top. Numbers at the left of the fig. represent tumor numbers. Black (C^-) and stippled (M^-) boxes represent loss of alleles derived from BALB/cHeA and MSM/Ms, respectively, and white boxes represent the retention of both alleles. Genotypes of *p53* and *Atm* of mice bearing tumors are shown at the left of the fig.. The frequency of LOH at each marker is written below.

marker within the *Cdh5* gene (107.0 Mb), and is also near the *Cdh16* gene (107.5 Mb). The other locus was *D8Mit113* (111.8 Mb, 52 cM) near the *Cdh1* gene (109.4 Mb) and *Cdh3* genes (109.1 Mb). The above mentioned LOH locus, *D8Mit85* (105.0 Mb, 47 cM), is located near the *Cdh8* (101.9 Mb) and *Cdh11* genes (105.5 Mb). Spontaneously developed 11 MCs also showed similar LOH patterns (data not shown). The frequencies were 73% (8 of 11 MC) at both *D8Mit85* and *D8Mit113*, and 55% (6 of 11 MC) at the loci *D8Mit212* and *D8Mit12*.

Inactivation of cadherin (Cdh) family genes

D8Mit212, one of the two loci exhibiting the most frequent LOH, is a marker within the *Cdh5* gene. Other members of the cadherin family, such as *Cdh1*, *Cdh3*, *Cdh8*, *Cdh11* and *Cdh16*, are also located in the frequent LOH region. The consequence of LOH was tested by analyzing the expression of these cadherin genes, *Cdh1*, *Cdh3*, *Cdh5, Cdh8, Cdh11* and *Cdh16*, which exist in the chromosomal region syntenic to human chromosome 16q22.1, on 26 MCs from (BALB/cHeA × MSM/Ms) F_1-*p53*$^{+/-}$ mice. Representative examples of RT-PCR of cadherin mRNA are shown in Fig. 3. Expression of the *Cdh1* and *Cdh5* genes was not detected in 15 (58%) and 8 (31%) of the 26 tumors, respectively (Fig. 4). The expression of *Cdh3* and *Cdh8* mRNA was also absent in 4 (15%) and 7 (27%) of the 26 tumors, respectively (Fig. 4). No inactivation of *Cdh11* and *Cdh16* genes was observed.

The integrity of all 11 exons of the *Cdh5* gene was examined by PCR of DNA segments with primers designed within each exon in the 8 tumors in which down-regulation of *Cdh5* was observed by RT-PCR. PCR products of exon 7, 8 or 10 were not detected in 4 of 8 tumors (data not shown). Thus, abnormality of some of the exons of the *Cdh5* gene was found to be associated with the loss of expression in MCs arising in *p53*$^{+/-}$ mice.

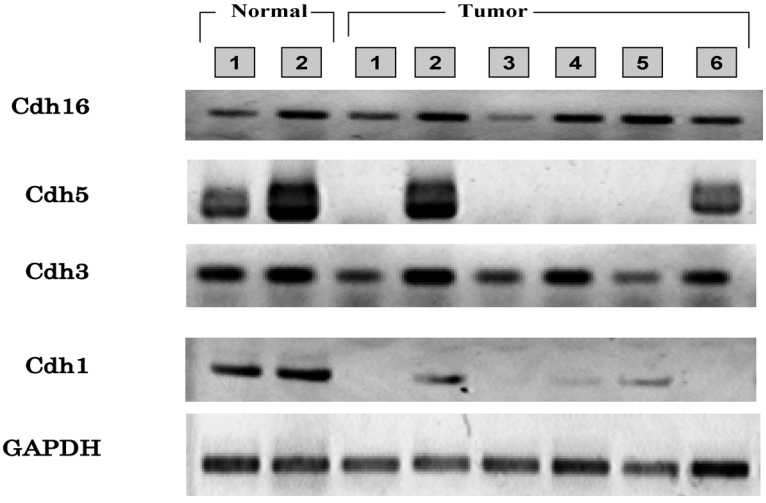

Fig. 3 Representative examples of mRNA detection of *Cdh 1, 3, 5* and *16* genes by RT-PCR in 2 normal mammary glands and 6 mammary carcinomas. Control: GAPDH

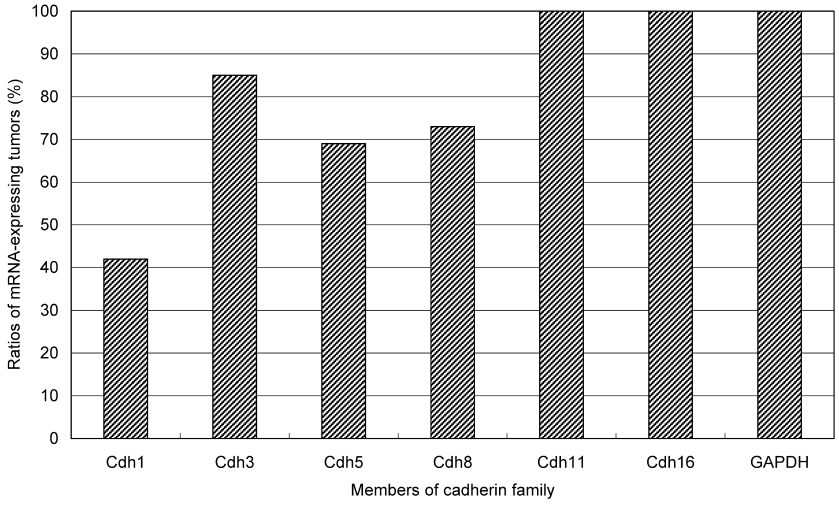

Fig. 4 Detection of mRNA expression of the *Cdh1*, *Cdh3*, *Cdh5, Cdh8, Cdh11* and *Cdh 16* genes by RT-PCR in 26 mammary carcinomas from (BALB/cHeA × MSM/Ms) F_1-*p53*$^{+/-}$ mice. Ratios of mRNA-expressing mammary carcinomas are indicated in the several members of cadherin. Control: GAPDH.

MC No.	*p53* genotype	*Atm* genotype	*Mit37* (1)	*Mit9* (2)	*Mit283* (11)	*Mit2* (19)	*Mit64* (22)	*Mit4* (34)	*Mit3* (32)	*Mit178* (43)	*Mit117* (44)	*Mit6* (45)	*Mit278* (50)	*Mit30* (46)	*Mit166* (50)	*Mit233* (52)	*Mit50* (52)	*Mit28* (52)	*Mit132* (52)	*Mit279* (53)	*Nds2* (60)
1082	KO/Wild	KO/Wild	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
1083	KO/Wild	KO/Wild	R	R	R	R	R	M⁻	R	R	R	R	R	R	R	R	R	R	R	R	R
1103	KO/Wild	KO/Wild	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻
1104	KO/Wild	KO/Wild	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻
1106	KO/Wild	KO/Wild	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R	C⁻	C⁻	C⁻	C⁻	C⁻
1107	KO/Wild	KO/Wild	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻
1108	KO/Wild	KO/Wild	R	C⁻	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
1109	KO/Wild	KO/Wild	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻
1172	KO/Wild	KO/Wild	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
1173	KO/Wild	KO/Wild	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
1180	KO/Wild	KO/Wild	C⁻	R	C⁻	C⁻	C⁻	C⁻	R	R	R	R	C⁻	C⁻	R	R	R	R	C⁻	C⁻	R
1201	KO/Wild	KO/Wild	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R	R	C⁻	C⁻	C⁻	C⁻	C⁻
1202	KO/Wild	KO/Wild	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R	C⁻	C⁻	C⁻	C⁻	R	R	R	C⁻	C⁻	C⁻	C⁻
1203	KO/Wild	KO/Wild	R	R	R	R	R	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R	R	R	C⁻	C⁻	C⁻	C⁻
1209	KO/Wild	KO/Wild	C⁻	C⁻	C⁻	C⁻	C⁻	R	C⁻	C⁻	C⁻	C⁻	R	R	R	R	R	R	R	R	R
1216	KO/Wild	KO/Wild	M⁻	M⁻	M⁻	M⁻	R	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	R	M⁻	R	M⁻	M⁻	M⁻
1224	KO/Wild	KO/Wild	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
1243	KO/Wild	KO/Wild	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R	C⁻	C⁻	C⁻	C⁻	C⁻
1304	KO/Wild	KO/Wild	R	C⁻	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
1081	KO/Wild	Wild	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻
1110	KO/Wild	Wild	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
1112	KO/Wild	Wild	R	C⁻	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
1197	KO/Wild	Wild	C⁻	R	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R	C⁻	C⁻	C⁻	C⁻	C⁻
1208	KO/Wild	Wild	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R	C⁻	C⁻	C⁻	C⁻	C⁻	R	C⁻	C⁻	C⁻	C⁻	C⁻	R
1218	KO/Wild	Wild	C⁻	C⁻	C⁻	R	R	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	C⁻	R	C⁻	C⁻	C⁻	C⁻	C⁻
1294	KO/Wild	Wild	R	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	R	M⁻	M⁻	M⁻	M⁻
1295	KO/Wild	Wild	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	R	M⁻	R	M⁻	R
1296	KO/Wild	Wild	R	R	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	M⁻	R	R	M⁻	R	R	R	R	R
LOH (%)			57	64	64	61	57	68	61	61	64	64	64	61	43	29	43	54	57	61	46

Fig. 5. Schematic representation of the allelotype in the frequent LOH regions on chromosome 12 in 28 mammary carcinomas (MC) from (BALB/cHeA × MSM/Ms) F1-*p53*$^{+/-}$ mice. The names of microsatellite markers are presented in abbreviated forms such as *Mit37* and *Nds2* at the top. Their genetic distances (cM) from the centromere are also indicated in parentheses at the top. Numbers at the left of the fig. represent tumor numbers. Black (C^-) and stippled (M^-) boxes represent loss of alleles derived from BALB/cHeA and MSM/Ms, respectively, and white (R) boxes represent the retention of both alleles. Genotypes of *p53* and *Atm* of mice bearing tumors are shown at the left of the fig.. The frequency of LOH at each marker is written below.

Frequent LOH on chromosomes 12

Since frequent LOH extended to some loci on chromosome 12 in genome-wide screening described earlier, precise allelotype analysis on chromosome 12 was also examined for 28 radiation-induced MCs from (BALB/cHeA × MSM/Ms) F1-*p53*$^{+/-}$ mice (Fig. 5). Highly frequent LOH was found at many loci throughout chromosome 12. Two regions are of particular interest; a wide region from *D12Mit37* (1 cM) to *D12Mit30* (46 cM) and a near telomeric region centered at *D12Mit279* (53 cM). No strain preference for the allelic loss was observed on this chromosome.

DISCUSSION

p53 is one of the most frequently mutated genes in MC. However, the MC development in *p53*$^{+/-}$ mice is greatly modified by genetic background. Mammary tumors develop at less than 1% of all tumors in *p53*$^{+/-}$ mice of the 129/Sv and C57BL/6 × 129/Sv backgrounds.[25] In contrast, the *p53*-heterozygotes of the BALB/c genetic background spontaneously develop mammary tumors at a high incidence.[12, 26, 27] In addition, the mammary epithelia of BALB/c-*p53*$^{-/-}$ mice develop into mammary tumors at high incidence when transplanted into mammary fat pads of *p53*$^{+/+}$ BALB/c hosts.[28] Thus, genetic components cooperating with the p53 deficiency must be present in BALB/c to promote mammary tumor formation. In the analysis of N2 backcross mice [(C57BL × BALB/c) × BALB/c], BALB/c alleles for *Prkdc* and *Cdkn2a* do not bring about a difference in mammary tumor susceptibility.[29] Thus, the BALB/c genetic components remain to be elucidated. Therefore, the search for allelic loss in MC from F_1-*p53*$^{+/-}$ mice heterozygous with the BALB/c were carried out to find some tumor suppressor genes cooperating with both *p53*$^{+/-}$ and the BALB/c genetic components in mammary carcinogenesis.

By genome-wide LOH screening, we found highly frequent LOH on chromosomes 8, 11 and 12 (Fig. 1). The wild-type allele of *p53* on chromosome 11 was lost in almost all the MCs from *p53*$^{+/-}$ mice (data not shown), and so LOH at the *p53* locus was nearly 100%. A considerable portion of the LOH found on chromosome 11 is thought to accompany the loss of the *p53* wild allele. Therefore, important tumor suppressor genes cooperating with homozygously deficient *p53* gene and the BALB/c genetic components in the mammary carcinogenesis are expected to reside in the regions of LOH of chromosomes 8 and 12. In these allelic loss regions, one allele of the recessive tumor suppressor genes can be inactivated by methylation, base substitutions or microdeletions. The other allele can be subsequently lost by the mechanisms operating at the chromosome level such as large rearrangements due to mitotic recombination.[30] Although the effect of X-irradiation on tumorigenesis is partially changed by genetic background,[31] highly frequent LOH on chromosomes 8 and 12 were found in MCs from two different F_1-*p53*$^{+/-}$ mice.

More than 60% of the MCs showed LOH in a region rang-

ing from *D8Mit85* (105.0 Mb, 47 cM) to *D8Mit113* (111.8 Mb, 52 cM) on chromosome 8, a region syntenic to human chromosome 16q22.1 (Table 1 and Fig. 2). A number of studies on breast cancer showed frequent LOH on human chromosomes 16q22.1 and 16q24.3.[32–35] Many genes of the *CDH* family, *CDH1, 3, 5, 8, 11* and *16*, exist in human chromosome 16q22.1. Among the mouse loci tested on chromosome 8, the highest frequency of LOH was found at two loci; one was *D8Mit212* (107.0 Mb, 51 cM), a marker within the *Cdh5* gene (107.0 Mb), and the other was *D8Mit113* (112 Mb, 52 cM) with the nearby *Cdh1* gene (109.4 Mb) and *Cdh3* gene(109.1 Mb).

Expression of the *Cdh1* (E-cadherin) gene was not detected by RT-PCR in 15 (58%) of the 26 tumors showing LOH on chromosome 8 (Fig. 4). Inactivation of *CDH1*, a tumor suppressor gene, has been reported in many human breast cancers.[36,37] On the other hand, the expression pattern of *Cdh5* (VE-cadherin) was more complex. *Cdh5* expression is up-regulated in invasive breast carcinomas which contributes for neovascularization of the tumors.[38] VE-cadherin was exclusively expressed by highly aggressive melanoma cells and yet it was undetectable in the poorly aggressive tumor cells, suggesting the difference in ability to induce tumor angiogenesis.[39] In contrast, the endothelial specific VE-cadherin is low or absent in angiosarcomas, suggesting an inhibitory role for this protein in tumor progression.[40] In MCs from (BALB/c × MSM)F_1-*p53*$^{+/-}$ mice, the expression of *Cdh5* gene was not detected in 8 (31%) of the 26 tumors showing LOH on chromosome 8 (Fig. 4). Furthermore, exons 7, 8 or 10 of *Cdh5* failed to be amplified in 4 of the 8 MCs, indicating some impairment of the gene.

The overexpression of *CDH3* (P-cadherin) in breast cancer is strongly associated with tumor aggressiveness, and the aberrant expression might be regulated by hypomethylation of gene prompoter region.[41] In the present analyses, expression of *Cdh3* mRNA was absent in only a few MCs from the F_1 mice with BALB/c-*p53*$^{+/-}$ mice (Fig. 4). Screening of 16 cell lines from renal cell carcinomas revealed a complex pattern of cadherin expression, and cadherin 8 may be involved in tumorigenesis in some types of renal cell carcinomas.[42] The expression of *Cdh8* mRNA was absent in 7 (27%) of the 26 tumors in our study (Fig. 4). These discrepancies between the previous reports and our study remain to be clarified in further studies. *Cdh11* mRNA and protein are expressed in the most invasive cell lines but not in any of the noninvasive cell lines, suggesting that the *Cdh11* expression may well correlate with the invasive phenotype in cancer cells.[43] Inactivation of *Cdh11* and *Cdh16* genes was not observed in our study.

Another highly frequent LOH on chromosome 12 was observed in at least two regions; a wide region from *D12Mit37* (1 cM) to *D12Mit30* (46 cM) and at *D12Mit279* (53 cM) which is close to the telomeric region. The former region is very large, and may contain more than one tumor suppressor gene. However, no major tumor suppressor gene has been reported yet in this region of MC. The latter region contains *Rit1* (*Bcl11b*), a major tumor suppressor gene found in mouse thymic lymphomas.[44,45] Future studies on the role of this gene in mammary carcinogenesis are likely to yield important insight of the mechanism of radiation-induced mammary carcinogenesis.

Our genome-wide screening at 42 loci identified frequent LOH (more than 30%) in chromosomes 5 (*D5Mit81,* 28 cM), 11 (*D11Mit12*, 75.4 cM), 14 (*D14Mit7,* 44.7 cM) and 18 (*D18Mit8,* 47 cM) in addition to chromosomes 8 and 12. The use of KO mice indicated that *Brca1* (60.5 cM on chromosome 11) and *Brca2* (88 cM on chromosome 5) cooperate with *p53*.[7,8] Another tumor suppressor gene, *Rb1*, is located at 41 cM on chromosome 14. However, the contribution of the *Brca1, Brca2 and Rb1* genes to tumor development in the BALB/c- *p53*$^{+/-}$ mice remains to be determined.

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