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Protection against Malignant Progression of Spontaneously Developing Liver Tumors in Transgenic Mice Expressing O^6-Methylguanine-DNA Methyltransferase

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To study the effect of O^6-methylguanine-DNA methyltransferase (MGMT) on carcinogenesis, we have previously generated MGMT transgenic mice overexpressing the bacterial MGMT gene, *ada*, and demonstrated that high MGMT levels in the liver suppress induction of liver tumors after treatment with an alkylating hepatocarcinogen. To examine the effects of life-long elevation of MGMT activity on mouse spontaneous liver tumor development, ada-transgenic and control non-transgenic mice were compared. We also examined mutations at codon 61 of the *H-ras* oncogene, reported as a hot spot in mouse liver tumors, using a direct DNA sequencing method. The results revealed no significant difference in tumor incidence or mutation spectrum, but interestingly, ada-transgenic mice were found to have fewer malignant tumors and survived longer, indicating a possible protective role of MGMT against malignant conversion.

Key words: O^6-methylguanine-DNA methyltransferase — Transgenic mice — Liver tumors — Malignant conversion

Chemical modification of cellular DNA is one of the most significant events in carcinogenesis, and alkylating carcinogens present in the environment produce various kinds of alkylated purine and pyrimidine bases.[1,2] Among the alkylated bases formed in DNA, O^6-methylguanine (O^6-meG) is regarded as being of particular importance for the induction of mutations and cancers in organisms. O^6-meG preferentially pairs with thymine instead of cytosine during DNA replication, resulting in G:C to A:T transition mutations. Mutations may lead to activation of proto-oncogenes and cause malignant transformation. For example, such mutations have been typically detected at the second guanine of codon 12 of the *K-ras* or *H-ras* oncogenes in rodent tumors induced by alkylating carcinogens.[3–5]

O^6-meG itself is specifically repaired by a DNA repair enzyme,[6] termed O^6-methylguanine-DNA methyltransferase (MGMT), which transfers methyl groups from O^6-meG moieties of double-stranded DNA to its cysteine residue at codon 145.[7] As each MGMT protein molecule can transfer only one methyl group from O^6-meG, MGMT activity depends on the number of molecules in the cell. Generally MGMT activity is several times higher in humans than in rodents and is regulated at appreciable levels throughout life.[8–10] Carcinogenic N-alkyl-N-nitrosoureas are known to induce tumors preferentially in tissues with low MGMT activity.[11,12] After alkylating carcinogen exposure, rodent cell variants with low MGMT activity also undergo malignant conversion with much higher frequency than their high MGMT activity counterparts, indicating that repair of O^6-alkylguanine protects the cell.[13] Moreover, it has been documented that MGMT activity is decreased in non-cancerous tissues derived from patients with cancer.[14] Transgenic mice expressing the bacterial MGMT gene, *ada*, have a higher level of MGMT activity in liver and are resistant to liver carcinogenesis induced by low-dose exposure to the alkylating carcinogens, dimethylnitrosamine (DMNA) or diethylnitrosamine (DENA). Transgenic mice expressing the human MGMT gene in the thymus, lung, colon and skin also develop fewer tumors than normal non-transgenic mice after an application of a single dose of alkylating carcinogens. Thus, increased MGMT activity in target organs may actually decrease the susceptibility of animals to neoplasia induced by alkylating carcinogens.[15–20] However, the role of MGMT, particularly in cases of spontaneous carcinogenesis, remains to be elucidated. Long-term liver tumor incidence is high in the founder mice of the C3H/HeN strain, and mutation at

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codon 61 of the *H-ras* oncogene is seen in nearly 40% of tumors.[21] Therefore we compared the *H-ras* gene mutation spectrum between ada-transgenic and non-transgenic control mice.

Male and female reproductive C3H/HeN mice were purchased from Japan SLC (Hamamatsu) as non-transgenic control animals because ada-transgenic mice had been established by microinjection of C3H/HeN mouse eggs from the same breeder. Characterization of the transgenic mice has been reported in detail elsewhere.[22] Briefly, germ-line transmission of the transgenic mice was confirmed, with the chimeric gene copy number in the lineage used in this study estimated to be about 100. Southern blot analysis indicated that the injected DNA was tandemly rejoined in the same orientation at the site of integration. The animals were bred and maintained in our laboratory as a homozygous colony with respect to the integrated *ada* gene. Liver extracts from transgenic homozygotes showed about 3 times the control MGMT activity (Fig. 1), determined as previously described.[22] Parent transgenic mice were checked for gene integration before mating. All the animals including both transgenic and non-transgenic mice were housed in a controlled environment at 23±1°C and fed on CE-2 standard laboratory diet (CLEA Japan, Tokyo) and autoclaved tap water *ad libitum*. The prevalence of spontaneous liver tumors in C3H/HeN strain is much higher in male mice and this sex was therefore chosen for investigation here. In total, 130 ada-transgenic and 48 non-transgenic mice were examined daily throughout the experimental period. Except for the immediate necropsy of the dead or moribund animals all the mice were killed under ether anesthesia at the end of the 2-year observation period. The survival curves are shown in Fig. 2. More non-transgenic mice died or were killed after becoming moribund between 14 to 24 months of age, compared to the transgenic mice ($P<0.025$, χ^2 test). Liver tumors were detected in all animals that died or were killed between 14 to 24 months.

At necropsy, livers were removed, weighed and carefully examined for grossly visible lesions. Parts of the large tumors (>5 mm diameter) were frozen in liquid nitrogen and stored at −70°C for direct sequencing of the *H-ras* oncogene. Remaining liver and all other major organs were fixed in 10% neutralized formaldehyde solution, each liver lobe being completely cut into 2 mm thick slices, and routinely processed for light microscopy. Sections were cut at 3 μm, stained with HE and examined by three pathologists, using a blind method. All liver tumors were confirmed by histopathologic examination and classified into adenoma or hepatocellular carcinoma (HCC), which has an unequivocally malignant appearance. Histopathological examination occasionally revealed tiny adenomas (>1.0 mm in diameter), which were added to the tumor scores. Incidences of tumor-bearing male mice were

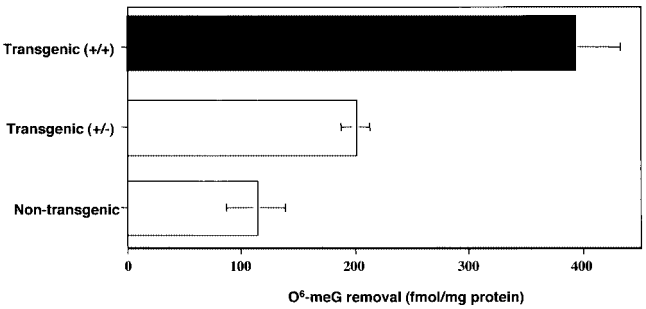

Fig. 1. Comparative MGMT activity levels in liver extracts from non-transgenic control, heterozygous ada-transgenic and homozygous transgenic mice. Data are average values for 4 animals±SD.

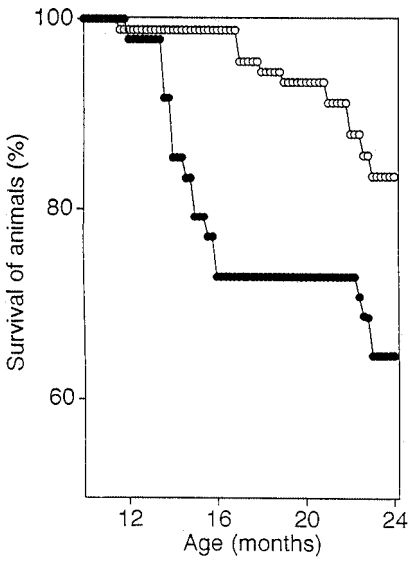

Fig. 2. Survival curve of ada-transgenic and non-transgenic mice. ● non-transgenic mice, ○ ada-transgenic mice.

similar in both groups (48.5%, transgenic; 47.9%, non-transgenic mice). This is consistent with our previous observation for the C3H mouse strain. Spontaneous liver tumors developed in 10% of mice aged 11 months and in about 40 to 50% of the mice aged 16 months or more. However, significant differences between the two groups in the proportions of hepatocellular adenomas and HCC were demonstrated. The malignant lesions were significantly fewer in the ada-transgenic mouse group (28.6%) than in the non-transgenic mouse group (60.9%) (Table I, $P<0.05$, χ^2 test).

A 129-bp fragment of mouse *H-ras* gene was amplified by polymerase chain reaction (PCR) with the primers 5′-

Table I. Incidence and Histologic Classification of Spontaneous Liver Tumors in Ada-transgenic and Non-transgenic Mice

Animals	No. of mice	No. of tumor-bearing mice (%)[a)]	Classification of the tumors (%)[b)]		
			Adenomas	HCC[c)]	Hyperplastic foci
Ada-transgenic	130	63 (48.5)	40 (63.5)	18 (28.6)	5 (7.9)
Non-transgenic	48	23 (47.9)	9 (39.1)	14 (60.9)	0

a) There is no significant difference between ada-transgenic mice and non-transgenic mice ($P>0.1$).
b) The proportions were significantly different between ada-transgenic and non-transgenic mice ($P<0.025$, χ^2 test).
c) HCC, hepatocellular carcinoma ($P<0.05$, χ^2 test).

Table II. H-ras Activation at Codon 61 in Hepatocellular Adenomas and Carcinomas

Animals	No. of tumors with *H-ras* activation at codon 61			Codon 61 (CAA) mutation		
	No. of tumors examined			AAA	CGA	CTA
Ada-transgenic	10/22 (45.5%)	Adenomas	5/15 (33.3%)	4	0	1
		Carcinomas	5/7 (71.4%)	1	3	1
			Total	5	3	2
Non-transgenic	8/19 (42.1%)	Adenomas	2/6 (33.3%)	2	0	0
		Carcinomas	6/13 (46.2%)	3	3	0
			Total	5	3	0

GCAGGACTCCTACCGG-3′ and 5′-AGGAAGCCCTCC-CCTGTGCG-3′. PCR was performed for 40 cycles of 92°C for 1 min, 52°C for 1 min and 72°C for 2 min, and products were analyzed by agarose gel electrophoresis. The amplified DNA was directly sequenced by the dideoxy method using a Sequenase sequencing kit (Amersham-Pharmacia Biotech).

Twenty-two out of 41 hepatomas from ada-transgenic and 19 out of 41 from non-transgenic mice showed mutations of the *H-ras* oncogene in codon 61, as summarized in Table II. The incidences of activation of *H-ras* oncogene in transgenic and non-transgenic mouse liver tumors were similar (45.5% in transgenic mice and 42.1% in non-transgenic mice). Activation was found in both adenomas and carcinomas, with only a slightly higher frequency in the latter. With regard to the types of mutations, CAA to AAA, CAA to CTA and CAA to CGA were the main patterns, being consistent with those reported for spontaneous liver tumors in mice.[23–25)]

Low levels of alkylating agents are present in food, water, air, tobacco smoke, and industrial and consumer products. They are also formed endogenously in reactions mediated by gastric floral bacteria and macrophages. They may thus contribute to human cancer development. As regards hepatocarcinogenesis in C3H/HeN mice, the Hcs (hepatocarcinogen sensitivity) loci appear to be responsible for the approximately 50-fold higher susceptibility of male C3H/HeN mice to spontaneous, DENA-induced or ethylnitrosourea-induced hepatocarcinogenesis than male C57Bl/6 mice.[26)] In the present study, the survival profile demonstrated ada-transgenic mice to be more viable than non-transgenic mice under the same living conditions, and histopathologic classification of the tumors demonstrated the occurrence of less malignant liver tumors. The incidences of activation of *H-ras* oncogene in transgenic and non-transgenic mouse liver tumors were similar. Overexpression of human MGMT transgene in homozygous PMS2 knockout mice was proved to protect the mice from induction of thymic lymphomas by N-methylnitrosourea (MNU) treatment, compared to their non-transgenic PMS2 $-/-$ counterparts, although the incidences of background spontaneous lesions were similar.[27)] The incidences of activation of *K-ras* oncogene were also similar. These data are consistent with our observations. MGMT-deficient mice, generated by Tsuzuki *et al.*, develop normally, but show hypersensitivity to MNU.[28)] Thus, strong myelosuppression was observed after treatment with MNU at 50 mg/kg (body weight), indicating that MGMT normally protects the reproductive capacity of hematopoietic stem cells.[27)] The present results are therefore in line with the literature, suggesting that MGMT may play an important role in protecting animals from low-level exposure to naturally occurring alkylating agents.

Many investigators have documented that mouse liver tumors often feature *H-ras* mutations, especially of the first C and the second A of codon 61 (CAA). The mutation frequencies for overall tumors in ada-transgenic and non-transgenic mice were 45.5% and 42.1%, respectively,

and the mutation spectrum was consistent with the previous reports. There was no difference in mutation frequency between HCC and hepatocellular adenomas. These results provide support for the concept that *H-ras* mutation events are important for early stages of carcinogenesis, rather than for the later conversion from adenomas to carcinomas. Based on the present results we speculate that MGMT could hinder the malignant conversion of adenomas by repair of O^6-meG endogenously produced in the tumor cells, which may cause mutations of oncogenes other than *H-ras*, or suppressor genes or other DNA repair genes involved in carcinogenesis.

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