GENETICS

Mutations in Codon 61 of H-*ras* Gene in Mouse Liver Tumors Induced by Potent and Weak Carcinogens

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PCR with subsequent sequencing showed that codon 61 of H-*ras* gene is presented by mutant variants in 7 of 13 liver tumors induced by neonatal injection of potent hepatocarcinogens o-aminoazotoluene or nitrosoethylurea in CBA mice, while in ICR mice tumors induced by these carcinogens carried only wild-type allele. Only mutant alleles of the studied codon were detected in 8 tumors induced with a weakly carcinogenous mutagen ethylmethansulfonate in ICR mice. In 7 of 15 cases these alleles were presented by two variants: AAA and CTA simultaneously (normal codon CAA) in CBA mice injected with o-aminoazotoluene and by CGA and CTA in ICR mice receiving ethylmethansulfonate.

Key Words: *mouse liver tumors; H-ras gene; codon 61 mutations*

Cancer is now believed to be a genetic disease, and tumor cells develop as a result of accumulation of mutations in critical protooncogenes and tumor suppressor genes [11]. In mice such genes are the *ras* genes: H-*ras* in the liver and K-*ras* in the lungs; in humans it is antioncogene p53 [11]. Mutations in certain positions of these genes are detected in more than half of the respective tumors [8]. Many tumors contain no such mutations, and this probably means that mutations in these genes are not responsible for tumors which are negative or positive with respect to such mutations. However, high incidence of mutations of these genes in tumors indicates their positive relationship with neoplastic status of the cell, associated with some advantages for cell multiplication and growth. Mutations in these genes can be selected in the course of cell selection during tumor progress. The above-reported results were obtained at late stages of tumor growth (in mice aged about 2 years). On the other hand, in 38-week-old mice activated H-*ras* gene is present in only 10% liver tumors, while in 65-week-old animals it is present in 28% tumors [10]; in the skin, mutant alleles of this gene are detected as early as 7 days after exposure to carcinogen [9]. R. Maronpot *et al.* [8] summed up the data on the frequency of H-*ras* gene mutations in liver tumors induced in mice of more than 10 inbred strains and hybrids by almost twenty various carcinogenic compounds. The effect depended on animal genotype, type of carcinogen, dose, and duration of exposure, and other factors, but the role of this oncogene in hepatocarcinogenesis remained unclear. We analyzed the incidence of mutations in codon 61 of H-*ras* gene in 37 liver tumors induced in mice of two strains never used in such experiments by three compounds, whose mutagenic activity towards codon 61 of H-*ras* gene has not been studied before.

MATERIALS AND METHODS

CBA/LacSto (CBA) and ICR mice bred at the Institute of Cytology and Genetics were used. On day 12-14 after birth, male mice were intraperitoneally (through hip muscles) injected with one of the carcinogens:

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o-aminoazotoluene (OAT, Shostka Chemical Plant), 225 μg/g; nitrosoethylurea (NEM, 50 μg/g); or ethylmethansulfonate (EMS, 100 or 200 μg/g).

At the age of 1 month the mice were separated from parents and kept 6-10 per cage with free access to water and food. At the age of 40-45 weeks the animals were sacrificed by cervical dislocation, opened, and large tumor nodules (>1 mm) in the liver was counted under magnifying glass, after which the liver were fixed in 10% formalin. For the analysis tumors were isolated from fixed liver and DNA was extracted by a standard method [4]. Mutant H-*ras* alleles were detected by highly sensitive PCR [9]: primer with 3'-terminal fragment complementary to specific gene mutation enables elongation of only mutant DNA during amplification. First, we amplified genome DNA of tumors with primers flanking codon 61 of H-*ras* gene: direct (393) AAACAGGTGGTCAT TGATGGG and reverse (394) GCAAATACACAGA GGAAGCC. Amplification was performed under standard conditions [10] as follows: 95°C for 3 min, 58°C for 0.5 min, 72°C for 1 min, and then 34 cycles: 94°C for 1 min, 58°C for 0.5 min, and 72°C for 1 min. Amplification products were analyzed in 7% polyacrylamide gel. A fragment of appropriate size was restricted, eluted from the gel, and reamplified with direct primers 408, 409, and 410 detecting changes in codon 61 (normal sequence C*A**A and substitutions C*—A, A**—G, and A**—T). Primer sequences: 408 — GACACAGCAGGT**A**, 409 — GACACAGCAGGT **C**G, and 410 — GACACAGCAGGTC**T**. Amplification was carried out under the following conditions: 95°C for 3 min, 62°C for 0.5 min, and 72°C for 1 min, and then 28 cycles: 94°C for 1 min, 62°C for 0.5 min, 72°C for 1 min. Nucleotide sequence of DNA fragments containing mutations in the region of codon 61 was confirmed by direct DNA sequencing with Taq polymerase [7].

RESULTS

The known carcinogens OAT and NEM caused liver tumors in 100% mice of both strains. The multiplicity of tumors induced by NEM was the same in animals of both strains, while that of tumors induced by OAT in CBA mice was almost 3-fold higher than in ICR mice (Table 1). EMS, a potent mutagen characterized by carcinogenic activity towards the lungs, also induced liver tumors in 30-56% animals in a dose-dependent manner, with multiplicity of 1-4 nodules per tumor carrier (0.3-1.4 per mouse). The latter parameter seems to be the maximum, because EMS in a single dose of 200 mg/kg is poorly tolerated and the dose cannot be increased. OAT and NEM in the studied doses were nontoxic.

It was interesting to compare the carcinogenic effect of test compounds with the mutagenic, which, as many scientists believe, is the only primary effect of carcinogens. To this end we studied the structure of codon 61 of H-*ras* gene in 10 tumors induced by NEM, 19 induced by OAT, and 8 induced by EMS (a total of 37 tumors). Mutant alleles of this gene were detected in 2 (20%), 5 (27.8%), and 8 (100%) tumors, respectively, and their frequency largely depended on the mouse strain. OAT is a potent hepatocarcinogen for CBA mice and relatively weak for ICR strain. Half OAT-induced tumors in CBA mice contained mutations in the studied codon, while OAT-induced tumors in ICR mice contained no mutations. However NEM, equally carcinogenic for ICR and CBA mice also induced tumors containing (in CBA mice) and not containing (in ICR mice) mutations in this codon. H-*ras* gene in all tumors in ICR mice injected with the weakest carcinogen EMS was presented only by alleles mutant in codon 61. Therefore, our findings do not allow us to deduce a relationship between mutations induced by carcinogens in this codon and liver tumor induction.

According to published reports, the most frequent changes in H-*ras* gene codon 61 (normal structure CAA) are C to A substitution in the first position and A to G and A to T substitutions in the second position; in spontaneous tumors these substitutions occur in a ratio of 5:2:1, respectively [5]. In induced tumors the CGA and CTA variants predominate [8]. In OAT-induced tumors in CBA mice we detected 3 cases with AAA mutation, which never occurred in NEM-induced tumors in these animals and in ICR mice exposed to EMS. S. Manam *et al.* [7] previously detected these mutations in the liver of CD-1 mice after neonatal injection of aminoazobenzene. The fact that such mutations predominate in spontaneous tumors confirms our previous hypothesis that neonatal exposure to aminoazo stains induces liver tumors in the same way as such tumors develop spontaneously, or that they just stimulate the development of spontaneous tumors [2]. This explains relatively weak carcinogenicity of OAT for ICR mice, in which the incidence of spontaneous liver tumors was two times lower than in CBA mice

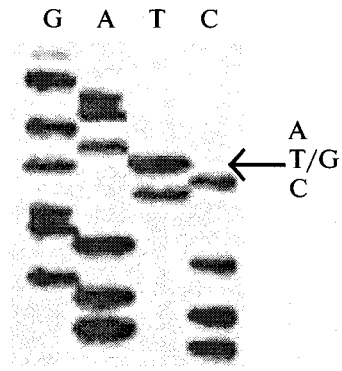

Fig. 1. H-*ras* gene sequence containing 2 substitutions in the second position of codon 61 CAA.

TABLE 1. Mutations in H-*ras* Gene Codon 61 in Liver Tumors Induced in Mice of Different Strains by Potent and Weak Carcinogens

Mouse strain	Carcinogen, dose, mg/kg	Number of mice		Number of tumors per *mouse*	Tumors studied			Number of mutations		
		total	with tumors, %		total	with mutations, %	containing	CAA-AAA	CAA-CGA	CAA-CTA
							2 mutations / 1 mutation			
CBA	OAT, 225	11	11 (100)	14.3±2.2	10	5 (50)	3 / 2	3	1	4
ICR	OAT, 225	22	22 (100)	5.3±1.5	9	0	— / —	—	—	—
CBA	NEM, 50	10	10 (100)	21.9±5.3	3	2 (66.7)	0 / 2	0	0	2
ICR	NEM, 50	11	11 (100)	24.8±6.1	7	0	— / —	—	—	—
ICR	EMS, 100	23	7 (30.4)	0.35±0.14	4	4 (100)	2 / 2	0	4	2
ICR	EMS, 200	22	14 (56.0)	1.4±0.3	4	4 (100)	2 / 2	0	3	3
Total:					37	15 (41.7)	7 / 8	3	8	11

(27.4 and 64.5%, respectively) [1]. Judging from other mutations (CGA and CTA), tumors developed after NEM and EMS are truly induced, but not spontaneous tumors. It is confirmed by similar carcinogenicity of NEM for CBA and ICR mice and by extremely high content of CGA and CTA mutations in tumors developed after injection of EMS: 4 of 8 tumors contained both mutations (Fig. 1). Three tumors in CBA mice injected with OAT contained two mutations each (AAA and CTA in two cases and CGA and CTA in one). Hence, double mutations were recorded in 20% tumors and in almost 50% tumors with mutant allele of H-*ras* gene. We found only one report about simultaneous presence of CGA and CTA variants of H-*ras* gene codon 61 in the tumor [8]; the authors hypothesized that two tumors with independent mutations developed in parallel. This hypothesis does not explain our findings, particularly for EMS, which even in high doses induced the development of no more than 4 tumor nodules per liver.

High incidence of H-*ras* gene mutations in induced tumors can be a result of chemical effect of the carcinogen, because mutations in this gene reflect the formation of the known or expected DNA adducts. Double mutations develop independently and, apparently, in different cells, and their presence in mature tumors indicates their polyclonal origin. It is however possible that mutations in the studied gene are not induced directly by carcinogens, but results from genome stabilization [3] and their role in hepatocarcinogenesis is not crucial. Probably further studies will help to define it.

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