Alterations in *c-myc*, *Ha-ras*, and *Ki-ras* Protooncogenes in Experimental Rat Mesothelioma

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Analysis of the polymorphism of restriction fragments or sequencing of *Ha-* and *Ki-ras* gene segments containing codon 12 or 61, amplified in the polymerase chain reaction, failed to detect point mutations in any of seven rat mesothelioma cell lines or in 20 mesotheliomas induced in rats by erionite or chrysotile asbestos. These data indicate the absence of *ras* activation caused by point mutations in the "critical codons" in rat mesothelioma. A twofold increase in the content of *c-myc* mRNA in 5 out of 8 examined tumors appears to reflect a more intensive proliferation of mesothelioma cells in comparison with normal mesothelium, but not their amplification. This is confirmed by the results of Southern blot-hybridization.

Key Words: *ras and myc protooncogenes; point mutations; expression; rat mesothelioma*

The development of a mesothelioma, a tumor originating from mesothelial cells, most probably indicates a history of exposure to asbestos or fibrous zeolites. Cytogenetic analysis of human and rodent mesotheliomas has revealed a variety of chromosome abnormalities [13], but no specific changes which might serve as mesothelioma markers. Protooncogene status in this tumor has virtually never been studied. The *ras* protooncogenes containing point mutations (PM) in codon 12 or 61 can cause abnormal growth and differentiation of cells in culture [5]. The presence of these mutant genes in the tumor is believed to play an important role in its origin. Transfection of *EJ-ras* in human mesothelial cells (HMC) rendered them independent of growth factors and transformed the immortalized HMC into malignant ones [12]. Injection of immortalized HMC expressing *EJ-ras* to nude mice led to the development of tumors in almost of 100% of cases [10]. Although no point mutations were found in *Ki-ras* in 20 human mesothelioma cell lines [9], the question of point mutations in mesothelioma *ras* is still disputed.

Increased expression of the *c-myc* protooncogene has been recorded in almost all tumors examined, which may be due to structural changes (translocation or amplification) or may reflect active proliferation of tumor cells. Experiments with transfection of mutant *ras* and of expressed *c-myc* showed that these protooncogenes may interact during the transformation of certain cell types [8].

In order to verify the hypothesis about the possible participation of activated *ras* and *c-myc* in the genesis of rat mesotheliomas, we compared the level of *c-myc* expression in primary mesotheliomas and in a normal mesothelial culture and examined the primary structure of codons 12 and 61 of *Ha* and *Ki-ras* protooncogenes in mesotheliomas and mesothelioma cell lines.

MATERIALS AND METHODS

Tumor induction and morphology. Mesotheliomas were induced by the acicular zeolite erionite and chrysotile asbestos [1,2], which were injected intraperitoneally to groups consisting of 50 2-month-old

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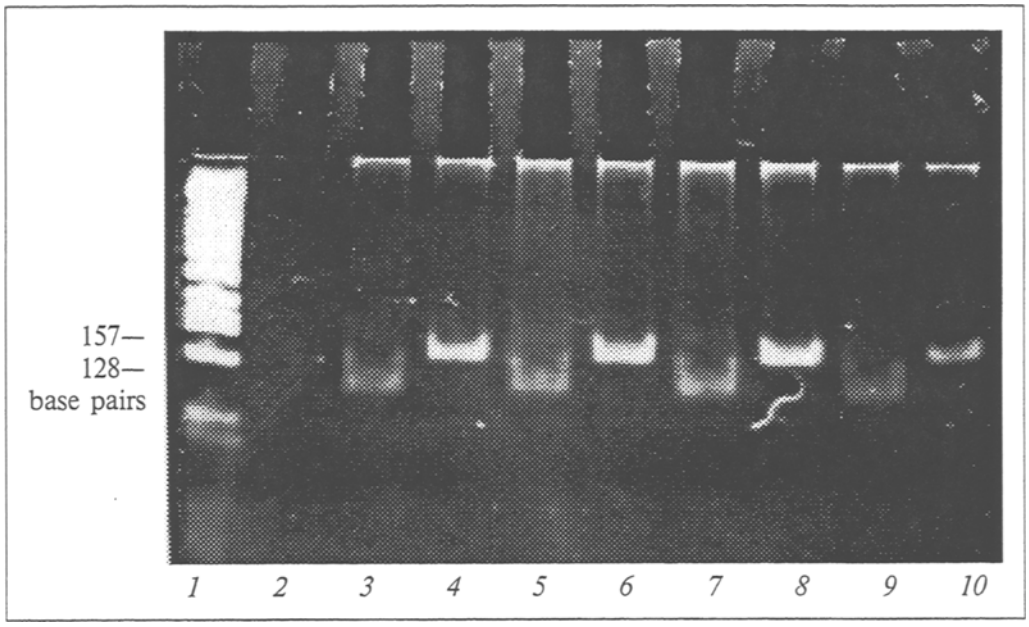

Fig. 1. Product of PCR amplification of DNA fragments containing *c–Ki–ras* codon 12. *1*) phage λ DNA, Hind III; *2*) reamplification of a DNA fragment of erionite–induced mesothelioma (MC 15). PCR product I was purified from high–molecular DNA, treated with restriction enzyme, and then amplified again; *3*) restriction of *Mnl I* amplified DNA fragment MC 15; *4*) product of DNA fragment MC 15 PCR; *5*) restriction of *Mnl I* amplified DNA fragment of rat mesothelioma cell line (T 10); *6*) product of DNA fragment T 10 PCR; *7*) restriction of *Mnl I* amplified DNA fragment of chrysotile asbestos–induced mesothelioma (Masb 3); *8*) product of DNA fragment Masb 3 PCR; *9*) restriction of reamplified DNA fragment Masb 3. High–molecular DNA was not removed; *10*) product of secondary PCR of DNA fragment Masb 3. High–molecular DNA was not removed.

Wistar females weighing 180 to 200 g. Erionite was injected three times at 1-month intervals, the single dose per animal being 20 mg and the total dose 60 mg. Chrysotile asbestos was injected in a single dose of 60 mg. Of the resultant tumors 15 mesotheliomas were induced by erionite and 12 with chrysotile asbestos. The histological types of the tumors varied: carcinoma-like, sarcoma-like, and mixed. In addition, 7 peritoneal mesothelioma cell lines [6] and a normal rat mesothelial cell line [4] were studied.

Polymerase chain reaction (PCR). DNA and RNA were extracted from tumors and cell cultures routinely [3]. The primary structure of codons 12 and 61 of *Ha-* and *Ki-ras* was tested in genomic or complementary DNA (cDNA). The synthesis of cDNA was carried out at 37°C for 1 hour; 20 ml of reaction mixture contained: 1 μg RNA, 5 U reverse transcriptase, 20 U RNAzine, 50 μM random primer, 1 μM of each deoxyribonucleotide, 5 μM $MgCl_2$, and 2 μl of RT-PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$) (Gene AMP RNA PCR Kit, Perkin Elmer Cetus).

Primers for amplification of *ras* fragments containing codon 12 or 61 are listed in Table 1. PCR was carried out in 30 cycles: 1-min denaturation at 95°C, 2-min calcification of primers at 51°C, and 3-min synthesis at 72°C. Fifty microliters of reaction mixture contained 1 mg DNA or the whole volume of synthesized cDNA, 15 μM $MgCl_2$, 5 μl RT-PCR buffer, 50 pM of each primer, 5 U *Tag* polymerase (Gene AMP RNA PCR Kit, Perkin Elmer Cetus, or *Tag* polymerase kit, Promega).

Sequencing. PCR-amplified *ras* fragments were purified from primers and high-molecular DNA us-

TABLE 1. Primers

Ha–ras,	codon 12	ATG ACA GAA TAC AAG CTT GT CTC TAT AGT GGG ATC ATA CT
	codon 61	GAC TCC TAC CGG AAA CAG GT C TGT ACT GAT GGA TGT CTC
Ki–ras,	codon 12	TTA TTG TAA GGC CTG CTG AAA ATG ACT GAG T TAC CTC TAT CGT AGG GTC GTA CTC ATC CA
	codon 61	TAC AGG AAA CAA GTA GTA ATT GAT GGA GAA ATA ATG GTG AAT ATC TTC AAA TGA TTT AGT
Ki–ras,	codon 12, for restriction analysis	ACT GAG TAT AAA CTT GTG GTA GTT GGA CCT

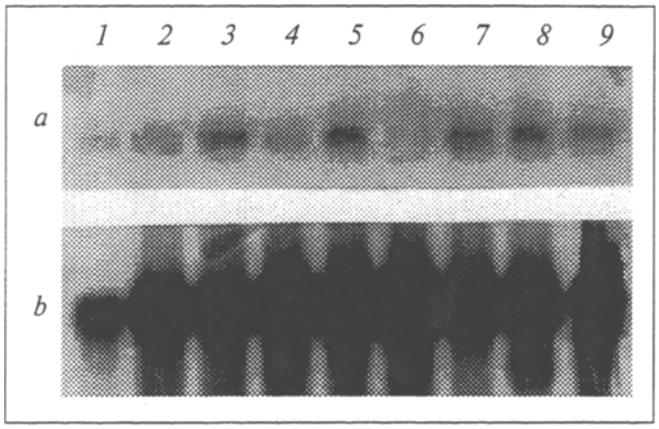

Fig. 2. Northern blot–hybridization with *c–myc* (*a*) and actin (*b*) samples of rat mesothelioma. *1*) normal rat mesothelial cell culture, passage 15; *2*) liver of intact rat; *3*) regenerating liver of intact rat; *4–9*) erionite–induced mesotheliomas (MC 15, MC 18, MC 21, MC 24, MC 31, MC 33, respectively).

ing a Wizard PCR Preps DNA Purification System (Promega). Cyclic sequencing with terminator deoxynucleotides was carried out as described previously [11] and in accordance with the manufacturer's instructions for the commercial kit DS DNA Cycle Sequencing System (Gibco BRL, Life Technologies). One of the primers for PCR served as the sequence primer. The primer was labeled for 30 min with radioisotope at 37°C. Five microliters of reaction mixture contained 10 mM primer, 5 U T_4-kinase (Pharmacia LKB), 1 μl T_4-buffer (300 mM Tris-HCl, pH 7.8, 50 mM $MgCl_2$, 1 M KCl), and 10 μCi γ-^{32}P-deoxyadenosine (Amersham). The synthesis of fragments with dideoxynucleotides was carried out in 25 cycles: 30-sec denaturation at 95°C, 1-min primer calcification at 50°C, and 1-min synthesis at 70°C. PCR products were separated in sequencing gel which was then dried and exposed for 1-2 days with an x-ray film with an amplifying screen at -70°C. Nucleotide sequences were assessed visually.

Restriction analysis. The recognition site of restriction enzyme *Mnl I* coincides with the normal sequence of codons 12 and 13 of rat *c-Ha-ras*. For restriction analysis of *Ki-ras* codon 12 some scientists [7] synthesized a specific primer overlapping this codon and containing a G → C substitution, creating a restriction site for *Mva I* restriction enzyme (the substituted nucleotide is underlined in Table 1). Amplified *ras* fragments were subjected to restriction, and then the whole of the reaction mixture was used for secondary PCR. Half of the secondary PCR volume was again incubated with the respective restriction enzyme, electrophoresis was carried out, and the size of amplified fragments before and after restriction was assessed [7]. Restriction enzymes *Mnl I* (Biolab) and *Mva I* (Promega) were used.

Hybridization. The content of *c-myc* in mesothelioma and normal mesothelial DNA was assessed by Southern blot-hybridization using *EcoRI* restriction enzyme (Fermentas); RNA expression was studied by Northern blot-hybridization [3]. For probes, ^{32}P-labeled *c-myc* and human actin samples (Oncor) were used. Autoradiograms were processed by a GS 408 scanning densitometer (Hoefer).

RESULTS

Point mutations in codons 12 and 61 of *Ha* and *Ki-ras* genes were sought in 20 induced mesotheliomas and 7 rat mesothelioma cell lines by sequencing and restriction analysis of PCR-amplified DNA fragments (Table 2). The sequencing method showed only the normal nucleotide sequence of all tested codons in all the samples examined. Figure 1 shows a photograph of gel with amplified fragments containing codon 12 of *c-Ha-ras* before and after restriction. Fragments that failed to be cleaved were absent after both primary and secondary PCR, this indicating that the primary structure of *c-Ha-ras* codon 12 was intact. Identical results were obtained for fragments containing *Ki-ras* codon 12.

Hence, despite the use of two different highly sensitive methods, we failed to find point mutations in codons 12 or 61 of *Ha* and *Kiras* protooncogenes in primary mesotheliomas and rat mesothelioma cell lines. These data correlate well with results on human mesothelioma cell lines [9].

TABLE 2. Incidence of Point Mutations (PM) in Different Samples of Rat Mesothelioma

Sequence	Number of cases with PM/total number of samples	Mesothelioma samples	
		cell lines	primary tumors
Ha–ras			
codon 12	0/23	0/3 (1)	0/20 (20)
codon 61	0/16	0/5	0/11
Ki–ras			
codon 12	0/25	0/7 (1)	0/18 (13)
codon 61	0/15	0/5	0/10

Note. The number of cases studied by restriction analysis is shown in parentheses.

The level of *c-myc* RNA expression was studied in normal mesothelium and in 8 erionite-induced rat mesotheliomas. Expression of *c-myc* mRNA was recorded for all the samples examined. In 5 mesotheliomas its level was increased 1.8 times in comparison with normal mesothelium (Fig. 2). The size of the RNA transcript, comprising 2.3 thousand base pairs and typical of normal tissues, was preserved in all tumor samples. Southern blot-analysis did not reveal any differences in the size or number of *c-myc* copies in mesotheliomas in comparison with normal mesothelium.

Our results seem to indicate that no activation of *Ha* and *Ki-ras* protooncogenes occurs in rat mesothelioma due to point mutations in the "critical codons." The absence of point mutations in *ras* and their relatively low incidence in suppressor gene p53 in mesotheliomas of humans [9] and rodents support the hypothesis that carcinogenic mineral fibers, in contrast to many chemical carcinogens, do not induce point mutations. At the same time, it is possible that the activity of regulatory genes other than *ras* or p53 is disrupted by point mutations in fibrous carcinogenesis.

The result of Southern blot-hybridization and the recorded expression of *c-myc* mRNA reflect either more intensive proliferation of mesothelioma cells in comparison with normal mesothelium or repair synthesis, but not amplification.

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