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In vitro Progression-associated c-H-*ras* Activation in Neoplastic Hepatocyte Lines Established from SV40-T Antigen Gene-harboring Transgenic Mice

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In order to investigate the molecular mechanisms of multistep hepatocarcinogenesis in SV40-T antigen gene-harboring transgenic mice, 9 hepatocellular carcinoma (HCC) lines and 10 "preneoplastic" hepatocyte lines were established from the animals and their biological and molecular changes during culture were investigated. Three of the 9 HCC lines showed progression during culture in terms of growth rate and growth capability in soft agar and in nude mice. This progression was associated with the appearance of activated c-H-*ras* oncogene. Including these 3 lines, H-*ras* activation was observed in a total of 7 of the 9 HCC lines (78%), whereas it was found only in 1 of 10 (10%) "preneoplastic" hepatocyte lines. These data thus indicate that H-*ras* activation may be an event occurring at a relatively late stage of hepatocarcinogenesis in this transgenic mouse system and that it may serve towards completion of the carcinogenic process together with the T-antigen.

Key words: H-*ras* activation — Liver cell line — SV40-T transgenic mouse — Progression

It is well documented that simian virus 40 large tumor antigen gene (SV40-T)[4] can easily immortalize mammalian cells but that it requires a long period of time for the immortalized cells to develop true malignant potential.[1-3] Thus the SV40-T antigen by itself may not be sufficient and additional event(s) may be essential for the complete transformation.[4-6] The question arises as to what kind of event(s) are required, occurring at what stage(s) of carcinogenesis.

A transgenic mouse line harboring an albumin promoter-regulated SV40-T was produced by Aizawa's group and sequential morphological observation revealed a distinct multistep progression of hepatocarcinogenesis in remarkable synchrony: normal appearance of the liver tissue in the initial 3 weeks after birth, rapid megalocytic change in almost all the hepatocytes in the 4th to 7th weeks; replacement of the whole liver tissue by regenerative focal growth of normal-sized hepatocytes with a decreased tendency for megalocytic change by 12 weeks; development of neoplastic nodules, some giving rise to macroscopic hepatocellular carcinomas (HCCs) by 24 weeks of age.[7] Thus, this appears to be a promising system for analyzing the mechanisms of multistep progression of carcinogenesis. Similar sequential changes were also reported by other groups with livers of different SV40-T transgenic mice.[8-10] Since one of the advantages of oncogene-introduced transgenic mice is the easiness of culturing "normal" and neoplastic cells,[6] we cultured hepatocytes obtained from the livers of the animals at 3–12 weeks of age as well as HCC cells obtained at around 24 weeks, and established 10 "preneoplastic" and 9 HCC cell lines.

The liver or HCC tissues were minced to small fragments (1 mm^3) and digested with lyophilized crystalline enzyme, Dispase (Godo Shusei Co. Ltd., Tokyo; 1000 U/ml), at 37°C for 2 h. The cells were then harvested by centrifugation at 1500 rpm for 5 min and cultured in Waymouth's medium supplemented with 10% fetal calf serum and ITS (5 μg of insulin, 5 μg of transferrin and 5 ng of selenium/liter). Twenty-four hours later, and twice per week thereafter the medium was changed. For establishing cell lines of homogenous populations, we subcultured cells of individual colonies separately and repeated this procedure 4 to 5 times at the initial phase.

The biological behavior of these cell lines was examined, including growth rate in culture and growth capabilities in soft agar or nude mice. In the negative cases, experiments were repeated several times at regular intervals in order to determine any progression during the long-term culture.

DNAs extracted from each of the cell lines at different passages were tested for transforming activity utilizing Syrian hamster embryo cell (SHOK cells)[11] and for the

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[4]Abbreviations: SV40-T, simian virus 40 large tumor antigen gene; HCC, hepatocellular carcinoma; SHOK, Syrian hamster embryo cell; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Table I. Biological and Molecular Properties of SV40-T Liver Cell Lines in Early and Late Passages

Origin	No. of lines	Early passages				Late passages[a]			
		Soft agar	Nude	Tf.A[c]	*ras*	Soft agar	Nude	Tf.A	*ras*
HCC (24 wk)	9: 4	+	+	+	+	+	+	+	+
	9: 2	+	+	−	−	+	+	+	−
	9: 3	−	−	−	−	+	+	+	+
Hepatocyte (3–12 wk)	10: 9	−	−	−	−	−	−	−	−
	10: 1	−	−	−	−	−	−	−	+[b]

a) Over 20 passages (6 months). *b*) Very weak.
c) Tf.A. Transforming activity in SHOK transfection assay.

Table II. H-*ras* Activation-associated Progression of 3 HCC Cell Lines

Cell line	Passage tested	Culture time (weeks)	Soft agar formation	Tumorigenicity (latent time[b]/days)	Transforming activity	H-*ras* activation	
						Codon	Type[c]
TH-1	10th	28	−	ND[a]	ND	−	−
	13th	32	−	+ (126)	−	−	−
	17th	36	−	+ (72)	−	−	−
	34th	48	+	+ (20)	+	61	CTA
	50th	57	+	+ (21)	ND	61	CTA
T24-2a	6th	12	−	ND	ND	−	−
	13th	27	−	−	−	−	−
	28th	34	+	+ (22)	+	61	CTA
T25-4a	8th	14	−	ND	ND	−	−
	15th	29	−	−	−	−	−
	24th	36	+	+ (25)	+	61	CTA
	35th	42	+	+ (12)	+	61	CTA

a) ND. Not done because of the limited cell number, or for other reason(s).
b) Latent time. Appearance of an obvious tumor.
c) The normal codon of H61 is CAA.

presence of a point mutation of H-*ras* in codon 61 utilizing the polymerase chain reaction (PCR), by the methods described elsewhere.[12–14] The SHOK cells have been described as being as sensitive as NIH3T3 cells in the transformation assay and are specifically useful for detecting incorporated mouse DNA.[11] When the cell numbers were too limited to allow extraction of DNA in some early passage cells, crude cells (2,000–10,000 in number) were directly used for the PCR reaction. The primers used were synthesized by the supplier (Katayama Chem. Co. Ltd., Japan) with sequences of CT AAG CCT GTT GTT TTG CAG GAC from the upstream side and ATG CGC GAC CAG TAC ATG CGC AC from the downstream side. After confirmation of the amplified sequences with 3% Nusieve (FMC, Rockland, MD) gels, the PCR products of each sample were diluted with 1 volume of 20×SSC solution, then 2 μl aliquots of the mixtures were spotted onto Hybond-N^+ filters (Amersham) and fixed by the alkali procedure. Oligonucleotide hybridization was performed as described by Verlaan-de Vries *et al.*,[15] using synthesized DNA probes to detect AAA, CTA and CGA type point mutations at codon 61 of H-*ras*. These point mutations are known to occur by far most frequently in mouse HCCs,[16] either spontaneous or chemically induced, and also in HCCs developed in SV40-T transgenic mice.[14] Efforts to detect other, rather unlikely mutations in H-*ras* codon 61 or 12 or K-*ras* or N-*ras* were not made in the present experiment, because the focus of our interest was in the determination of the phase at which *ras* mutation occurred.

As shown in Table I, among 9 established HCC cell lines, 4 showed growth capabilities in both soft agar and in nude mice from the beginning, as well as H-*ras* activation and transforming activity in the SHOK cell transfection assay. A further 2 HCC cell lines were positive in the

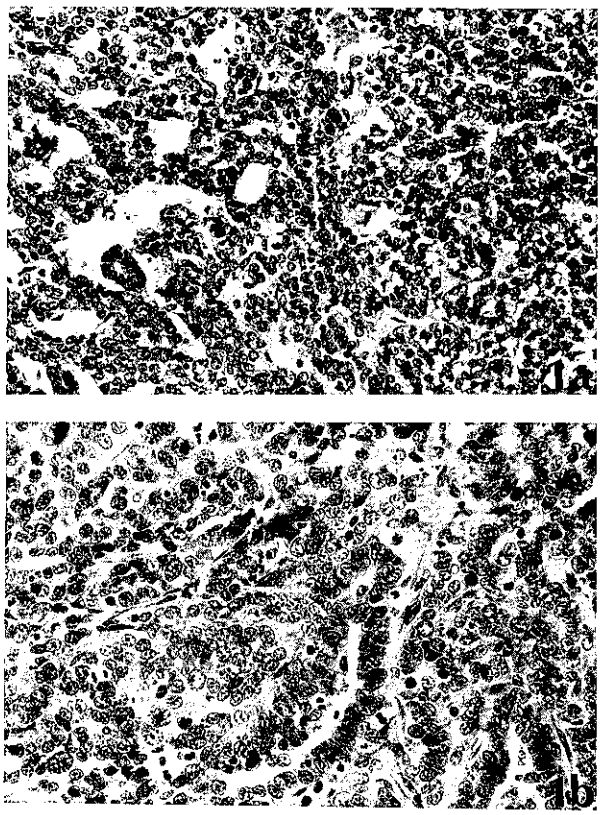

Fig. 1. Histological patterns of the original tumor of 24-2a (a) and the one grown in an athymic nude mouse after inoculating 24-2a cells at late passage (b). Morphological progression is evident in the nuclear atypia. H-E. ×200.

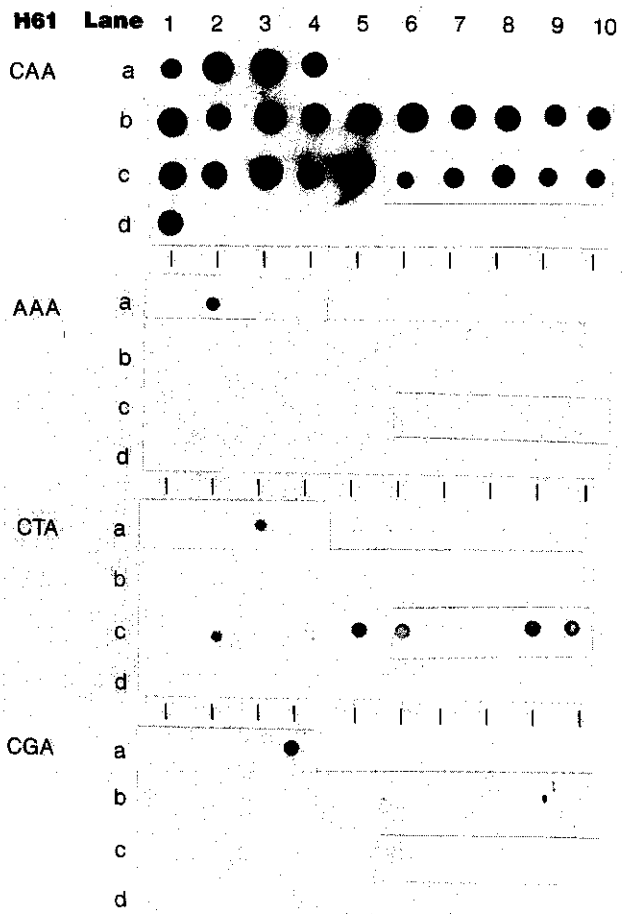

Fig. 2. Oligonucleotide hybridization for detecting *ras* point mutation at H-*ras* codon 61. The samples in each of the filters were applied in the following orders: Lane a: 1. Normal mouse liver tissue (negative control); 2. DEN-induced mouse HCC with AAA-type mutation at H-*ras* codon 61; 3. MDAB-induced mouse skin tumor with CTA-type mutation at H-*ras* codon 61; 4. DEN-induced mouse HCC with CGA-type mutation at H-*ras* codon 61. 5–10: empty. Lane b: 1–10. Samples from "preneoplastic" liver cell lines of SV40-T transgenic mice. Lane c: 1. 24-2a before biological progression; 2. 24-2a after biological progression; 3. 25-4a before biological progression; 4. 25-4a after biological progression; 5. TH-1 after biological progression; 6–10. Other HCC cell lines of SV40-T transgenic mice. Lane d: 1. HCC cell line of SV40-T mice. 2–10: empty.

two biological tests and also in transforming activity from the first, but remained negative for H-*ras* activation all along. In the case of the remaining 3 HCC cell lines, TH-1, 24-2a and 25-4a, which had been negative for all four features at early stages of culture, progression was evident in association with the appearance of ras point mutation in the later passages as shown in detail in Table II. Increment of the growth rate was seen not only in terms of passages but also in a shortening of the latent period required for tumor growth after transplantation. Progression was also evidenced by morphological changes (Fig. 1). In contrast to the findings with the HCC cell lines, the 10 cell lines originating from livers of young animals (3–12 weeks) showed no growth capabilities in soft agar or nude mice throughout the experimental period (over 6 months), confirming their "preneoplastic" status. Only one of them revealed a weakly positive signal for H-*ras* point mutation at the 38th generation when the probe for CTA-type mutation was used (Fig. 2).

The data from this study thus clearly demonstrated different frequencies of H-*ras* activation between HCC (7/9 or 78%) and "preneoplastic" (1/10 or 10%) cell lines from SV40-T transgenic mice and also the simultaneous occurrence of *ras* activation and appearance of transforming activity in 3 cell lines originating from

HCCs. Thus H-*ras* gene activation may be an event occurring in the late stage of hepatocarcinogenesis in the transgenic mouse, although the possibility of the occurrence of point mutation during an earlier phase followed by selection during the culture can not be completely ruled out. This conclusion is in concert with that obtained from our previous studies of carcinogenesis utilizing the "normal" C3H mouse-derived immortal hepatocyte system[17,18] and the HCCs developed in our SV40-T transgenic mice.[14]

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