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Preferential Loss of Heterozygosity of Chromosome 7 Loci in Simian Virus 40 t/T Antigen-Induced Mouse Hepatocellular Carcinomas Does Not Involve H-*ras* Muatations

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Genetic complementation experiments have indicated that both a maternal and a paternal copy of the distal region of mouse chromosome 7 are essential for normal development [1]. This suggested the presence of genes whose expression is dependent on the gamete of origin in this chromosomal region. Two such imprinted genes, namely insulin-like growth factor II (*Igf2*) and *H19*, have been identified so far [2, 3]. The first encodes a peptide with mitotic activity towards several cell types, that contributes significantly to prenatal growth of mammals, whereas the second has, as yet, no defined role and seems not to encode any protein, but works as RNA. (*Igf2*) and *H19* are located 90 kb apart, have similar expression patterns during development and are reciprocally imprinted, since the maternal *Igf2* and the paternal *H19* alleles are inactive in most fetal tissues [4, 5].

The human chromosome 11p15.5, homologous to distal mouse chromosome 7, undergoes frequent loss of heterozygosity (LOH) of polymorphic markers with preferential retention of maternal alleles in several embryonal neoplasms [6]. This has led to the hypothesis that the natural or aberrant imprinting of a tumour suppressor gene might contribute to the progression of some human cancers. Later, a relaxation of the imprinting state of the IGF2 gene with activation of the maternal allele has been observed in embryonal and adult tumours, raising the hypothesis that loss of imprinting (LOI) is the functional equivalent of maternal LOH with paternal duplication and that an increase in the number of active IGF2 genes is a favorable step in the progression of the neoplasms [7-10]. More intriguingly, two groups have recently reported that H19 expression is severely down-regulated in the cases of Wilms' tumour showing LOI of the IGF2 gene or LOH of 11p15.5 markers [11, 12]. This result together with the observation that H19 is capable of inhibiting the transformed phenotype when transfected in a tumour cell line [13] have suggested the possibility that H19 might possess a growth-suppressing activity

and that its expression may be coordinately regulated with IGF2 by an enhancer competition mechanism which exclusively activates one of the two genes on each chromosome [4].

We have developed an experimental carcinogenesis system in which the allele-specific expression of *Igf2* and *H19* could be followed by means of polymorphisms present in the transcribed region of the genes. For this purpose, a line of transgenic mice carrying the simian virus 40 oncoprotein large T antigen under an inducible and liver-specific promoter and developing hepatocellular carcinomas (HCC) [14] was crossed to an inbred laboratory strain and first-generation progeny were used for the imprinting study. Although the expression *Igf2* and *H19* genes was completely silent in the livers of adult mice, we observed a time-dependent activation and the conservation of parental imprinting of both genes in the course of the hepatocarcinogenesis. Allelic imbalances of and *H19* genes were found in more than one third (13/36) of the HCC sanalysed. The analysis of several other markers suggested that the imbalances very likely extended to the entire chromosome 7 and measurement of *Igf2* gene dosage indicated that loss of the maternal allele with or without duplication of the paternal chromosome occurred in HCC. A strong bias on the parental origin of the allele retained in the neoplasms was observed, since underrepresentation or complete loss of maternal chromosome 7 was recognised in 12/13 imbalances. The expression of the *H19* gene was severely attenuated in the carcinomas lacking the maternal chromosome 7, whereas *Igf2* mRNA levels were high in most liver tumours, including those with the allelic imbalances.

Chromosome 7 imbalances have previously been observed in mouse skin tumours [15, 16]. In these cases, chromosome non-disjunctions and mitotic recombinations were recognised as the molecular mechanisms responsible for the duplication of the mutated H-*ras* oncogene and/or the loss of the normal H-*ras* allele in a high percentage of these neoplasms. Interestingly, no bias in the parental origin of the allele preferentially retained or duplicated was observed in the skin tumours.

We investigated the incidence of H-*ras* mutations in our hepatocarcinogenesis system and compared the HCCs showing allelic imbalances with those having anormal maternal/paternal chromosome 7 ratio. Mutations of the *ras* proto-oncogene have been previously observed in both chemically induced and spontaneous mouse liver tumours [17]. The most frequent mutations described alter codon 61 of H-*ras* from CAA to AAA, CGA or CTA. To evaluate the presence of such mutations in HCC, we amplified by polymerase chain reaction (PCR) a 13-bp fragment encompassing codon 61 o the H-*ras* gene from genomic DNA of the 36 liver tumours already analysed for chromosome 7 imbalances. The presence of the wild-type sequence CAA and of the mutated AAA in the PCR products was investigated by allele-specific oligonucleotide hybridisation, whereas the CGA and CTA mutations were analysed for the restriction fragment length polymorphisms they create, after digestion of the amplified fragments with *Taq*I and *Xba*I, respectively [18]. The results, summarised in Table 1, indicate that the wild-type CAA sequence, and none of the most common mutations of H-*ras* codon 61, is present in the 36 HCCs analysed. No difference was therefore observed between the carcinomas with and those without chromosome 7 imbalances.

These findings ruled out a role for H-*ras* in the progression of these SV40 Tantigen-induced HCCs and indicate that gene activities other than H-*ras* must be affected by the chromosome 7 imbalances observed in the liver tumours. The non-equivalent behaviour

Table 1 - Chromosome 7 imbalances and the H-*ras* codon 61 of SV40 T antigen-induced HCCs

		H-ras codon 61[a]			
Tumor[b]	Genotype[c]	CAA	AAA	CGA	CTA
SP	ND	2/2	0/2	0/2	2/2
♂ CRP-T antigen 60-3 × ♀ B/c cross					
HCC	*b/t*	21/21	0/21	0/21	0/21
HCC	*-/t*	6/6	0/6	0/6	0/6
HCC	*$_{l}$/t*	3/3	0/3	0/3	0/3
HCC	*b/$_{t}$*	1/1	0/1	0/1	0/1
♂ B/c × ♀ CRP-T antigen 60-3 cross					
HCC	*b/t*	*2/2*	*0/2*	*0/2*	*0/2*
HCC	*b/-*	1/1	0/1	0/1	0/1
HCC	*b/$_{t}$*	2/2	0/2	0/2	0/2

[a]Number of positive samples per group of tumours.

[b]Two skin papilloma cell lines (SP), kindly provided by Dr. A. Balmain were used as positive controls. The HCCs were derived from animals obtained by reciprocally crossing the CRP-Tantigen 60.3 line and the Balbc strain. The genotype was determined by typing eight polymorphic markers located between 21.3 and 72.0 cM from the top of chromosome 7, including *Igf2* and *H19*. All chromosome 7 markers gave similar results. Alleles reduced to <15% of the intensity of the other allele were not reported and are substituted by a dash. Alleles present at 30-40% the amount of the other allele indicated by a smaller character. *b*=B/c alleles; t=CRP-T antigen 60-3 alleles; ND= not determined.

of paternal and maternal chromosome 7 alleles suggests that an imprinted gene(s), the expression of which is relevant to tumour progression, is involved in the allelic imbalances. It has been proposed that the activation of growth factor receptors, such as the insulin-like growth factor receptor, is necessary for the transformed cells to avoid apoptosis [19]. *Igf2* is progressively activated in the course of liver carcinogenesis and maintains high mRNA levels in the majority of HCCs, Since the maternal *Igf2* allele is kept inactive because of parental imprinting, it would be highly disadvantageous for the neoplastic cell to lose the paternal chromosome 7. So, if the chromosome 7 loss causes the inactivation of a tumour suppressor allele in the HCCs, linkage of the growth inhibitory gene to *Igdf2* might limit the favourable allelic losses to the maternal chromosome. Alternatively, loss of maternal chromosome 7 could remove a negative regulator of the *Igf2* gene and more firmly establish at elevated levels the expression of this growth factor.

More difficult to understand is the role of *H19* in the hepatocarcinogenesis. The observed activation in the preneoplastic steps and the reported up-regulation detected in regenerating liver [20] suggest that H19 RNA plays a functional role during proliferation of liver cells. On the other hand, the possibility of a selective advantage conferred by loss of *H19* expression to the neoplastic cell in an advanced step of liver carcinogenesis cannot be excluded.

The presence of a growth suppressor linked to the human IGF2 gene has been suggested by experiments of subchromosomal fragment transfers into rhabdomyosarcoma cells [21]. Since synteny of several loci is conserved in human chromosome 11p15 and distal mouse chromosome 7 [22], it is possible that the genetic events occurring in the experimental tumours we described and the preferential 11p15.5 LOH observed in the human embryonal neoplasms reflect the involvement of homologous genes.

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