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Differential selection for *B-raf* and *Ha-ras* mutated liver tumors in mice with high and low susceptibility to hepatocarcinogenesis

Albrecht Buchmann *, Züleyha Karcier, Benjamin Schmid, Julia Strathmann, Michael Schwarz

Institute of Pharmacology and Toxicology, Department of Toxicology, University of Tübingen, Wilhelmstr. 56, 72074 Tübingen, Germany

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Abstract

Activation of the Ras/Raf/MEK/ERK pathway is frequently observed in animal and human tumors. In our study, we analyzed *B-raf* codon 637 (formerly 624) and *Ha-ras* codon 61 mutations in liver tumors from C3H, B6C3F1 and C56BL mice which differ considerably with regard to their susceptibility to hepatocarcinogenesis. In total, 73% (102/140) of tumors induced by a single application of *N*-nitrosodiethylamine or 7,12-dimethylbenz[a]anthracene contained either *B-raf* or *Ha-ras* mutations and only <3% (4/140) were mutated in both genes. In addition, *B-raf* mutations were present in 76% (19/25) of early precancerous liver lesions. The prevalence of *Ha-ras* mutated tumors was significantly higher in the susceptible C3H and B6C3F1 mouse strains (39–50%) than in the comparatively resistant C57BL mouse (7%). *B-raf* mutated tumors, by contrast, were more frequent in C57BL mice (68%) than in the other two strains (17–45%). Taken together, our findings indicate that alterations affecting the Ras/Raf/MEK/ERK signalling pathway are a hallmark of carcinogen-induced liver tumors in mice. Moreover, our results show that mutational activation of *B-raf* in liver tumors of different mouse strains is, by contrast to *Ha-ras*, inversely related to their susceptibility to hepatocarcinogenesis. Although activated Ras and Raf proteins are assumed to have similar biological effects because they feed into the same signalling pathway, there seem to be subtle strain-specific differences in selection processes favouring the preferential outgrowth of either *B-raf* or *Ha-ras* mutated tumor populations in mouse liver.

Keywords: Mouse hepatocarcinogenesis; Ha-ras; B-raf; Gene mutation; Strain-specific selection

1. Introduction

Rodent carcinogenesis models are useful tools to analyze molecular alterations during tumor formation and it is now generally accepted that tumors develop in a stepwise process involving genetic alterations and selection processes which drive the outgrowth of defined tumor cell populations [1,2]. Spontaneous and chemically induced liver tumors from certain strains of mice frequently harbour mutations in the *Ha-ras* proto-oncogene (for a review see [3]), and recent studies have shown a strong correlation between the animals' susceptibility to hepatocarcinogenesis and the occurrence of *Ha-ras* mutations in hepatocellular tumors [4,5].

* Corresponding author. Tel.: +49 7071 29 74946; fax: +49 7071 29 2273.
E-mail address: albrecht.buchmann@uni-tuebingen.de (A. Buchmann).

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Table 1
PCR primers used for amplification of *Ha-ras* and *B-raf*

Gene	PCR primer sequence (5′ → 3′)	Length of PCR product
Ha-ras forward[a,b]	GAGACATGTCTACTGGACATCTT	166 bp
Ha-ras reverse[a,b]	GCTAGCCATAGGTGGCTCACCTG	
B-raf forward1[a]	TCAAAATGCTTTCTCTAATAGGA	426 bp
B-raf reverse1[a]	TGTTCTGGAACTATATAGACAG	
B-raf forward2[b]	TTCCTTTACTTACTGCACCTCAGA	202 bp
B-raf reverse2[b]	AGCAGTCACTAGTTTAGGACTG	

[a] Primers were used for PCR with isolated tumor DNA.
[b] Primers were used for PCR with punched tissue samples and also in some analyses with isolated tumor DNA.

Ha-Ras is a member of the Ras protein family which comprises a group of small GTPases that act as molecular switches and cycle between a GDP-bound, inactive, and a GTP-bound, active, conformation. Activated Ras proteins transmit their signals via several effector pathways, including the Raf/MEK/ERK cascade, which regulate fundamental cellular processes such as proliferation, survival and differentiation (reviewed in [6–8]). Activating mutations in *ras* genes result in expression of aberrant forms of Ras proteins which are locked in their GTP-bound state and thus cause a constitutive activation of the down-stream effector pathways in tumor cells. Among the various Ras effectors, Raf kinases are the most extensively studied mediators of Ras signalling, and B-Raf, one of the three mammalian Raf family proteins, has gained particular attention during the last years since activating mutations in the *B-raf* gene have been detected in various human tumor types (reviewed in [8–11]). The vast majority of these mutations occur at a hotspot site in codon 600 (formerly 599; [10]) and cause a single amino acid change (V600E) that results in elevated kinase activity and transformation of rodents cells [12–14]. Notably, most of the *B-raf* V600E mutated tumors did not contain concomitant mutations in *ras* genes, suggesting that mutated Ras and B-Raf proteins exert similar biological effects [12,15–17].

In a previous study, we observed that mouse liver tumors lacking detectable *ras* mutations often contained mutations in the *B-raf* gene which correspond to the human V600E B-Raf mutant [18]. In that study, we used C3H mice which are highly susceptible to spontaneous and chemically induced hepatocarcinogenesis and ∼50% of liver tumors from C3H mice induced by a single application of *N*-nitrosodiethylamine (DEN) contain *Ha-ras* codon 61 mutations (e.g. see [3,4]). This is in contrast to other mouse strains, such as C56BL mice, which are comparatively resistant to hepatocarcinogenesis and rarely carry *Ha-ras* mutated liver tumors [3,4]. The mechanisms responsible for the differences in liver tumor susceptibility between different strains of mice are only partly understood. Genetic analyses have demonstrated the existence of both susceptibility and resistance loci which determine the incidence of liver tumors in mice [19–23] and may also modulate the selective pressure for hepatocytes carrying *Ha-ras* mutations [4,20]. Since Ras and Raf proteins are both members of the MAP kinase signalling pathway, which is constitutively activated in carcinogen-induced mouse hepatomas [24], we reasoned that the observed strain-specific differences in liver tumor susceptibility and *ras* mutation frequency would be paralleled by similar differences in the mutation frequency of *B-raf*. To test this hypothesis we comparatively analyzed tumor samples from C3H and C57BL mice and their F1 cross (B6C3F1) for the presence of *B-raf* and *Ha-ras* mutations. The results of our study show, however, that the frequency of *B-raf* mutated liver tumors is significantly higher in the resistant C57BL strain than in the susceptible C3H and B6C3F1 mice, suggesting that mutational activation of *B-raf* in liver tumors of different mouse strains is inversely related to their susceptibility to hepatocarcinogenesis.

2. Materials and methods

2.1. Liver tumor material

Frozen liver tumors that had been induced by a single application of two different carcinogens, either *N*-nitrosodiethylamine (DEN; CAS Number 55-18-5) or 7,12-dimethylbenz[a]anthracene (DMBA; CAS Number 57-97-6), were available to us from previous studies of our laboratory [4,24]. In brief, male C3H/He, C57BL/6J and B6C3F1 mice were injected i.p. with a single dose of DEN (20 μg/g body weight) or DMBA (20 nmol/g body weight) at 2 weeks of age. After weanling, mice were housed individually in macrolon cages and kept on a standard diet without further treatment. Mice were killed at various times after carcinogen application and larger liver tumors were excised and immediately frozen in liquid nitrogen. Liver tissue containing small tumors and