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Mutation Affecting the 12th Amino Acid of the c-Ha-*ras* Oncogene Product Occurs Infrequently in Human Cancer

Abstract. *A point mutation alters the 12th amino acid of the c-Ha-*ras *oncogene product p21 in a human bladder cancer cell line. This is, at present, the only mutation known to result in a human transforming gene. This mutation may therefore represent a possible target for mutagenesis leading to carcinogenesis in humans. By means of restriction enzyme analysis, 29 human cancers, including 20 primary tumor tissues, derived from organs commonly exposed to environmental carcinogens, were tested for the presence of this mutation. None of ten primary bladder carcinomas exhibited the mutation; nor did nine colon carcinomas or ten carcinomas of the lung. Thus the point mutation affecting the 12th amino acid of the c-Ha-*ras *gene product, while a valuable model for carcinogenesis, does not appear to play a role in the development of most human epithelial cancers of the bladder, colon, or lung.*

It was recently observed that a point mutation in the cellular oncogene c-Ha-*ras* occurs in a cell line derived from a human bladder carcinoma. The c-Ha-*ras* gene product is a protein of 21,000 daltons (p21). The mutation alters this protein by converting its 12th amino acid (normally glycine) to valine. The altered p21 is responsible for the ability of DNA from this bladder tumor cell line to transform NIH 3T3 cells upon DNA transfection (*1*, *2*). Altered p21 proteins of related *ras* genes also seem to be responsible for the transforming activities of lung and colon cancer cell lines, although the genetic change resulting in this alteration has not yet been defined at the molecular level (*3*). It is interesting that all transforming *ras* oncogenes that have been sequenced so far, be they of viral or cellular origin, exhibit a mutation at the 12th amino acid of p21 (*1*, *2*, *4*). On the basis of computer models of the protein, a change in the 12th amino acid is expected to result in a dramatic change in the structure of the protein, this structural change presumably resulting in transforming capacity (*1*).

One of the most important implications of this observed mutation is that the codon for the 12th amino acid of the c-Ha-*ras* gene product may represent a precisely defined target for mutagenesis leading to carcinogenesis. This implication has obvious relevance to the large body of data correlating mutagenicity with carcinogenicity in laboratory animals and man (*5*). It also raises the question of how frequently this mutation occurs in human cancers, especially those that may arise as a result of exposure to environmental carcinogens. To answer this question, we analyzed 29 human cancers for the presence of a mutation of the codon for the 12th amino acid of the c-Ha-*ras* gene product. We

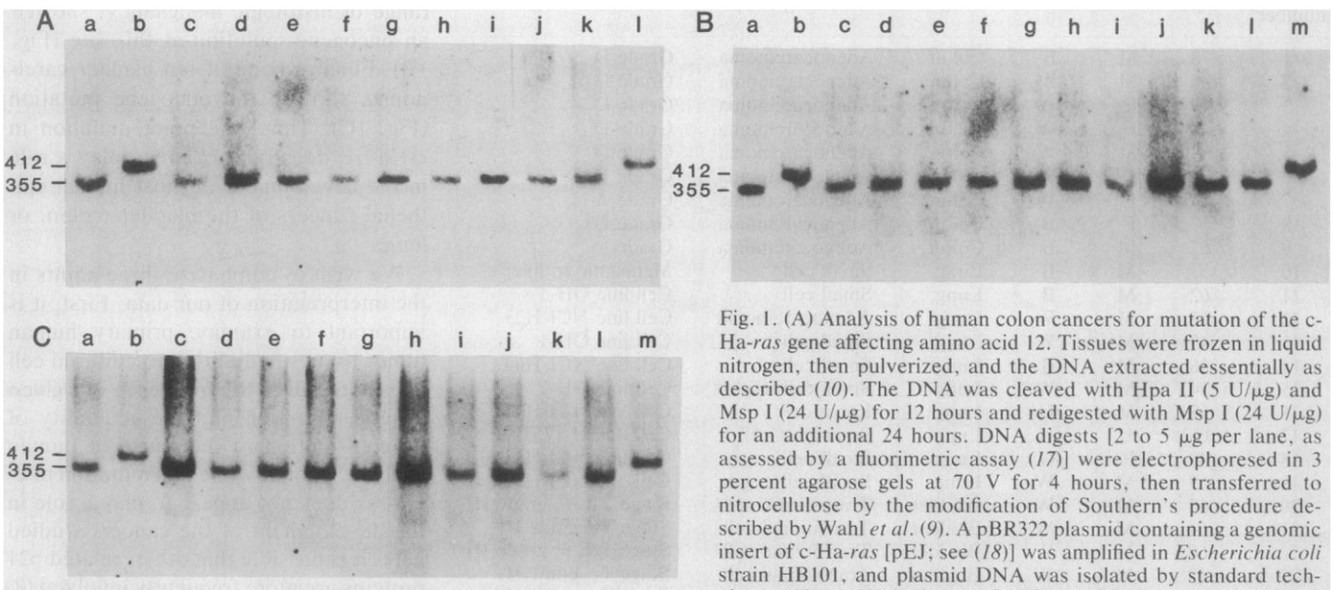

Fig. 1. (A) Analysis of human colon cancers for mutation of the c-Ha-*ras* gene affecting amino acid 12. Tissues were frozen in liquid nitrogen, then pulverized, and the DNA extracted essentially as described (*10*). The DNA was cleaved with Hpa II (5 U/μg) and Msp I (24 U/μg) for 12 hours and redigested with Msp I (24 U/μg) for an additional 24 hours. DNA digests [2 to 5 μg per lane, as assessed by a fluorimetric assay (*17*)] were electrophoresed in 3 percent agarose gels at 70 V for 4 hours, then transferred to nitrocellulose by the modification of Southern's procedure described by Wahl *et al.* (*9*). A pBR322 plasmid containing a genomic insert of c-Ha-*ras* [pEJ; see (*18*)] was amplified in *Escherichia coli* strain HB101, and plasmid DNA was isolated by standard techniques (*19*). A 600-bp insert of pEJ containing the relevant Msp I fragments was obtained by digesting pEJ DNA with Sma I (*20*). The Sma I fragment was purified and labeled to 10^9 dpm/μg with ^{32}P-labeled deoxycytidine triphosphate by a technique described elsewhere (*21*). Hybridization and autoradiography were performed as described (*21–23*). Molecular weight standards consisted of Hinf I–digested pBR322 and Hae III–digested ΦX174 DNA. Lane a, normal placenta; lanes b and l, T24 DNA (the bladder cancer cell line with the c-Ha-*ras* gene mutation); lanes c to k, colon carcinomas from patients 1 to 9, respectively. (B) Analysis of human lung carcinomas for the c-Ha-*ras* mutation. The experiment was performed as described in the legend to (A). Lane a, normal placenta; lanes b and m, T24 DNA; lanes c to l, lung carcinomas from patients 10 to 19, respectively. (C) Analysis of ten human bladder carcinomas for the c-Ha-*ras* mutation. The experiment was performed as described in the legend to (A). Lane a, normal placenta; lanes b and m, T24 DNA; lanes c to l, bladder carcinomas from patients 20 to 29, respectively.

selected three types of cancers—bladder, lung, colon—for this analysis; all three cancers are derived from the epithelia of organs expected to have high levels of exposure to environmental mutagens.

The method we used to detect mutations at this locus is based on the following circumstances (*1, 2, 6, 7*): (i) The normal c-Ha-*ras* gene contains the codon GGC coding for the 12th amino acid glycine. Because of the redundancy of the genetic code, only changes in the first or second base of this codon will result in an amino acid substitution for glycine; (ii) this codon is preceded by the dinucleotide CC, so any mutation resulting in a changed 12th amino acid will result in an alteration of the tetranucleotide CCGG; and (iii) the restriction endonucleases Hpa II and Msp I cleave the sequence CCGG (*8*). Therefore, any mutation altering amino acid 12 will destroy this cleavage site, rendering c-Ha-*ras* resistant to Hpa II and Msp I cleavage at this position. The nearest CCGG tetranucleotides are 57 base pairs 5′ and 355 bp 3′ to this codon. Thus, Msp I will yield a 355-bp fragment and a 57-bp fragment in the absence of a mutation, and a 412-bp fragment in the presence of a mutation for amino acid 12.

An example of this method is shown in Fig. 1A, lanes a and b. Normal human placental DNA and DNA from the bladder tumor cell line T24 [which is the cell line shown to contain the mutation at the 12th amino acid (*1, 2*)] were cleaved with Msp I, electrophoresed through an agarose gel, and transferred to a nitrocellulose filter by the method of Southern (*9*). The DNA on the filter was then hybridized to a ^{32}P-labeled DNA probe which spans the region of interest (see legend to Fig. 1). The autoradiograph of the filter shows that in the normal human placental DNA, a 355-bp fragment is detected (the 57-bp fragment would not be detectable under these gel and hybridization conditions). In the T24 DNA, the relevant Msp I site is lost, resulting in a larger fragment of 412 bp. All alleles of the c-Ha-*ras* gene in the T24 cells are altered at this site, since no normal 355-bp fragment is detected.

Thus, the normal c-Ha-*ras* gene is clearly distinguishable from the mutated form. We examined ten human bladder cancers and nine human colon cancers, processed immediately after surgery, and ten human lung carcinomas (one being a primary tumor and the others cell lines) representing all four major histologic types of lung cancer (Table 1). DNA was extracted from all samples as described (*10*), and then digested with Msp I and Hpa II. We noted during the course of this study that a large excess of restriction endonuclease was required for complete digestion of this gene in the human DNA samples. Several times more Msp I was required for a complete digest of this gene, compared to the amount required to digest other genes in the same DNA samples. However, the excess enzyme did not alter its sequence specificity (as assessed by cleaving pBR322 at varying enzyme concentrations). The reason such a large amount of enzyme is required may be related to the GC-rich sequences that flank this site in the c-Ha-*ras* gene; these flanking sequences may inhibit cleavage if the internal cytosine in the Msp I recognition site is methylated, even though Msp I is normally insensitive to methylation of the internal cytosine residue (*11*). Hence, although an abnormal 412-bp fragment must be interpreted with caution (it could represent mutation or methylation), the normal 355-bp fragment unequivocally rules out a mutation at the codon for the 12th amino acid.

These results show that all of the nine colon cancers yielded a 355-bp fragment on digestion with Msp I and Hpa II (Fig. 1A). Thus, none of these cancers possessed a mutation of the c-Ha-*ras* gene site corresponding to amino acid 12 of p21. In addition, none of the ten carcinomas of the lung, representing a wide range of histologic malignancy, showed an oncogene mutation at this site (Fig. 1B). Finally, none of ten bladder carcinomas showed the oncogene mutation (Fig. 1C). Thus, the point mutation in c-Ha-*ras* does not appear to play a role in the development of most human epithelial cancers of the bladder, colon, or lung.

We wish to emphasize three points in the interpretation of our data. First, it is important to examine primary human tumor tissues in addition to cultured cell lines to establish the frequency of a given genetic alteration and the generality of its contribution to the cause of human cancer. Second, while this mutation of c-Ha-*ras* does not appear to play a role in the development of the cancers studied here, it is possible that other, related p21 proteins are more frequently involved (*3, 12*). Third, mechanisms of carcinogenesis other than mutation should also be considered (*13*). DNA alterations involving mutations, rearrangements (*14*), methylation changes (*15*), and gene amplification (*16*) have all been reported to occur in some examples of human tu-

Table 1. Human cancers analyzed for a mutation altering the 12th amino acid of the c-Ha-*ras* gene.

Case number	Age	Sex	Race	Organ	Histologic type	Notes*
1	78	M	B	Colon	Adenocarcinoma	Grade D
2	45	M	B	Colon	Adenocarcinoma	Grade D
3	49	F	W	Colon	Adenocarcinoma	Grade D
4	44	F	W	Colon	Adenocarcinoma	Grade D
5	69	M	W	Colon	Adenocarcinoma	Grade D
6	75	F	W	Colon	Adenocarcinoma	Grade D
7	79	F	W	Colon	Adenocarcinoma	Grade A
8	67	F	B	Colon	Adenocarcinoma	Grade D
9	67	M	B	Colon	Adenocarcinoma	Grade D
10	53	M	B	Lung	Small cell	Metastatic to liver
11	62	M	B	Lung	Small cell	Cell line OH-3
12	51	M	W	Lung	Adenocarcinoma	Cell line NCI-H23
13	43	M	W	Lung	Small cell	Cell line OH-1
14	56	M	B	Lung	Small cell	Cell line NCI-H64
15	34	M	W	Lung	Small cell origin†	Cell line OH-2
16	59	M	U‡	Lung	Large cell	Cell line NCI-H157
17	U	U	U	Lung	Squamous cell	Cell line U-1752
18	49	F	O	Lung	Small cell	Cell line NCI-H231
19	69	M	W	Lung	Small cell origin	Cell line NCI-H196
20	68	M	W	Bladder	Transitional cell	Stage T_3L+, grade III
21	64	M	W	Bladder	Transitional cell	Stage T_3, grade III
22	60	M	W	Bladder	Transitional cell	Stage T_3L+, grade III
23	42	M	W	Bladder	Transitional cell	Stage T_1, grade II
24	53	M	B	Bladder	Transitional cell	Stage T_1, grade II
25	62	M	W	Bladder	Transitional cell	Stage T_1, grade II
26	82	M	W	Bladder	Transitional cell	Stage T_1, grade II
27	78	F	W	Bladder	Transitional cell	Stage T_1, grade II
28	73	M	W	Bladder	Transitional cell	Stage T_3L+, grade III
29	83	M	W	Bladder	Transitional cell	Stage T_1, grade II

*The histologic classifications of the primary cancer tissues are described in (*24*). The lung cancer cell lines are described in detail in (*25, 26*). †Tumor of small cell origin that acquired a large-cell histology and biochemical phenotype (*26*). ‡Data unavailable.

mors. Whether any of these changes are causally related to the malignancy in which they occur remains a fundamental, unanswered question.

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Hyaluronate in Vasculogenesis

Abstract. *Limb buds of chicken embryos contain within the peripheral mesoderm an avascular zone that is rich in hyaluronic acid. Epithelial tissues that synthesize large amounts of hyaluronic acid relative to other glycosaminoglycans caused avascularity when implanted into normally vascular wing mesoderm. Epithelia that synthesize little hyaluronic acid did not cause avascularity. Elvax implants containing hyaluronic acid caused the formation of avascular zones, whereas similar implants containing other glycosaminoglycans did not give rise to avascular zones. Hyaluronic acid may thus play a role in determining the location of blood vessels in the embryo.*

Vasculogenesis, the development of the vascular system, has been studied by embryologists (*1*) and by those interested in tumor vascularization (*2*). Factors that promote or inhibit blood vessel growth have been described in the tumor system (*2, 3*), but little is known about the way in which tissues interact to form the vascular system in embryos.

Injection of India ink into the vascular system of chicken embryos at successive stages (*4*) of development and clearing (making the embryo transparent with oil of wintergreen) revealed vascular and avascular regions (*1, 5, 6*). The limb bud, as well as the entire trunk region, has a definitive avascular zone within the peripheral mesoderm. This shell of avascular mesoderm lies subjacent to the ectoderm and surrounds a central core of highly vascular mesoderm (Fig. 1, A and B). The only histological difference between these regions is the presence or absence of blood vessels (Fig. 1C). The ectoderm is responsible for the formation and maintenance of this peripheral avascular zone (*7*). When embryonic back-skin ectoderm was implanted into the normally vascularized mesoderm of the wing bud, the tissue surrounding the implants became avascular. The width of this avascular region was similar to that underlying normal wing bud ectoderm.

To determine whether the formation of avascular zones in underlying mesoderm was a universal characteristic of embry-

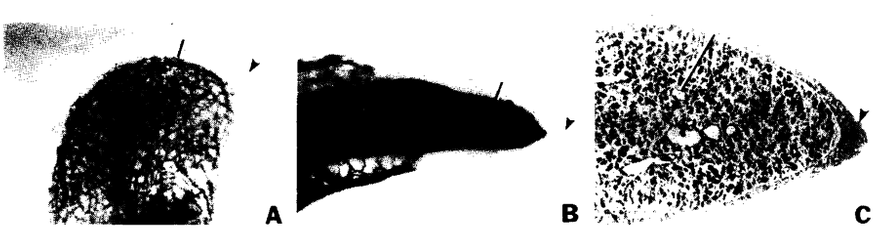

Fig. 1. (A) Dorsal view of the distal portion of a wing bud at stage 25 showing the peripheral mesodermal avascular zone (bar) subjacent to the ectoderm (arrowhead). (B) Side view of a wing bud showing the mesodermal avascular zones subjacent to the dorsal (bar) and ventral surfaces of the limb. These avascular domains merge beneath the apical ectodermal ridge at the distal tip of the limb (arrowhead). (C) A section (1 μm) of a similar subridge region shows the location of the vascularized mesodermal core. The mesenchyme appears similar within the vascular and avascular (bar) parts of the limb. The arrowhead points to the apical ectodermal ridge, which is continuous with the limb ectoderm.