Genetic Analysis of Human Esophageal Tumors from Two High Incidence Geographic Areas: Frequent p53 Base Substitutions and Absence of ras Mutations

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Abstract

Esophageal squamous cell carcinoma (ESC) samples from patients residing in Uruguay and in Normandy, France, where alcoholic beverages and tobacco smoke are major risk factors, were analyzed for point mutations in the p53 tumor suppressor gene. Among 34 tumors (15 from Normandy and 19 from Uruguay) 15 point mutations in the p53 gene that result in amino acid substitutions or chain termination were identified by polymerase chain reaction amplification of exons 5-8 and direct DNA sequencing. Base substitutions in ESC from these high-incidence areas are dispersed over the midregion of the p53 gene. There are differences between ESC and other types of gastrointestinal cancer in the nature of frequent base substitutions. CpG toTpG transitions were far less prevalent in these ESC than in colorectal tumors, whereas G to T transversions, rarely found in colon cancers, were found in one-fourth of the ESC samples. Base substitutions at A:T pairs constitute an important fraction of ESC p53 mutations, in contrast to mutation patterns in most other types of solid tumors. In contrast to the frequent mutation of the p53 gene in these samples, no mutations in the H-, K-, or N-ras genes were found in 16 tumors from Uruguay by direct sequencing of exons in which transforming mutations are known to occur. A previous study on ras mutations in ESC from France was also negative (M. C. Hollstein et al., Cancer Res., 48: 5119-5123, 1988). The role of distinct etiological factors in generating these differences and the potential for linking patient exposure histories with patterns of p53 mutations in high risk populations are considered.

Introduction

Cancer is the result of an accumulation of genetic alterations that disrupt control of cell growth and terminal differentiation. The most common specific gene changes known in human tumors that contribute to the disease process are point mutations in the p53 tumor suppressor gene and in the ras protoonogenes (1). More than one-half of the world cancer burden is composed of malignancies in which a mutation at the p53 locus or in a ras gene, or both, have occurred. Whereas activating mutations in the H-, K-, and N-ras genes are confined to a few critical sites, principally codons 12, 13, and 61 (1), mutations in the p53 gene thought to interfere with control of cell proliferation are dispersed over several hundred base pairs in the midregion of the gene, mostly in exons 5-8 (2). The p53 gene thus represents a broad target for mutation events in which mutational specificities of exogenous chemical agents and endogenous cellular mutagenic processes can be examined. Studies using prokaryotic and eukaryotic organisms, as well as in vitro mutagenesis assays with mammalian cells, have shown that carcinogens produce characteristic mutational spectra with respect to the type and location of point mutations they induce in a defined sequence (4-8).

Research efforts were initially directed toward identifying the prevalence of ras and p53 mutations in different types of malignancies. Of current interest is a molecular epidemiology approach in which different cancer populations exposed to defined risk factors are studied for the pattern of these tumor mutations (9). Information on base substitution mutations in tumors of diverse types may offer new insights on the origins of genetic changes in human cancers.

Esophageal cancer is an interesting model for the examination of mutational spectra in human tumors because of the dramatic geographical variations in the incidence in populations of similar ethnic origin (10) and because dietary factors and cultural habits associated with elevated risk have been the subject of intensive epidemiological study (11, 12). In addition, cancer of the esophagus is among the ten most frequent cancers in the world and the prognosis is very poor (13).

Esophageal tumor samples were collected from patients residing in Uruguay and in northwestern France, two areas where esophageal cancer risk is elevated and is attributed to the consumption of alcoholic beverages and to tobacco smoking (14, 15). In South America, consumption of hot maté tea has also been shown to increase esophageal cancer risk, and a dose-response relationship has been observed (14).

p53 mutations have been found in several types of human cancer, including cancers of the colon (16, 17), lung (17, 18), and liver (19, 20). Allelic deletion analysis of ESC from Japan has demonstrated allelic losses on chromosome 17p in about 45% of the patients (21). Recently we reported on the presence of p53 mutations in 5 of 14 ESCs from patients in Lyon, France, where incidence of this cancer is not unusual when compared to many areas of Western Europe (22). The nature and location of ESC p53 mutations found among 35 primary tumor specimens from two high incidence areas, Uruguay and Normandy, are reported here and the mutation pattern in ESC is compared against other cancers with different etiologies.

Analysis for ras mutations has been conducted in ESC from France (Normandy and Lyon) (3), a high incidence region in the Peoples Republic of China (23) and the Transkei, South Africa (24). In these studies, no ras mutations at codons 12, 13, or 61 were detected in the tumors. In the present work the search for ras mutations in ESC has been extended to tumor samples from Uruguay because the patients are from a cancer group with a unique risk factor, hot maté tea. In addition, the

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direct sequence analysis of the H-, K-, and N-ras genes was performed on approximately 100 base pairs surrounding each region where transforming mutations are known to occur in the event that DNA-damaging agents involved in ESC demonstrate sequence preferences that target ras codons less frequently tested for transforming mutations, such as codons 59, 63, and 146.

Materials and Methods

Tissues

Normandy. Primary tumor tissues were from esophageal cancer patients at University Hospital and Centre F. Baclesse, Caen, France. Upon surgical removal, samples were frozen in liquid nitrogen and stored at —70°C. Information on tobacco and alcohol consumption was retrieved from hospital archives.

Uruguay. Tumor biopsies and biopsies from tissue adjacent to the lesions were obtained from patients at the Center of Digestive Diseases, Montevideo. Information from patients on consumption of alcoholic beverages, tobacco smoking, and hot maté tea was recorded. All tumors reported in this study were squamous cell carcinomas of the esophagus.

There was no preselection of ESC on the basis of either patient exposure history or other demographic parameter. Samples were obtained chronologically from patients in the order of admission for medical care.

For five of the patients with a p53 mutation in their ESC, a DNA sample from uninvolved mucosa was also available for analysis.

Preparation of DNA

Tissues were placed in lysis buffer (Applied Biosystems) and incubated at 55°C for 1 h. After phenol and chloroform extractions, DNA was precipitated in ethanol and resuspended in sterile water. DNA stocks were kept physically separate from areas where PCR reaction products were handled. Separate pipetting devices and laboratory materials were set aside to be used exclusively for working with DNA stocks.

PCR (25) and Primers

Amplifications were performed in 100-µl volumes with 25–500 ng of genomic DNA and 1 µM primers essentially as described previously (22). PCR primer synthesis and sequences for the p53 gene have been described (22). PCR primers for direct sequencing of exons 1 and 2 of the H-, N-ras genes and exons, 1, 2, and 3 of the K-ras gene were described (22).

PCR (25) and Primers

The sequences are:

**H-ras:**

- #52 CGATGTCGAGCGGAGCGGACCTGGAGAGGA
- #53 GTATCCCTGCACTGACCCGGGACCGCCCGCTAG
- #57 CGATGTCGAGCGGAGCGGACCTGGAGAGGA
- #58 GTATCCCTGCACTGACCCGGGACCGCCCGCTAG

**K-ras:**

- #03 CGCATTCTAGGACATGTTCTAATATAGTCA
- #04 CGCATTCTAGGACATGTTCTAATATAGTCA
- #05 CGCATTCTAGGACATGTTCTAATATAGTCA
- #06 CGCATTCTAGGACATGTTCTAATATAGTCA

**N-ras:**

- #61 TACTAATGACTGTGCTATAA
- #62 TACTAATGACTGTGCTATAA
- #63 TACTAATGACTGTGCTATAA
- #64 TACTAATGACTGTGCTATAA

To control for DNA contamination of PCR reactions, all experiments included one or more reaction tubes in which no DNA was added. PCR materials were kept strictly separated from any PCR products. All mutations were confirmed by a complete repeat of the experimental procedure: amplification of stock genomic DNA; fragment purification; and sequencing of the DNA strand complementary to that sequenced in the initial experiment. PCR reagents were handled in a tissue culture exhaust hood.

Dideoxy Sequencing of Amplified DNA

PCR-amplified DNA was purified as described in Ref. 22. Recovered material was sequenced directly without any intermediate cloning steps. Sequencing was by the dideoxy chain termination procedure (26) and reagents were purchased from United States Biochemical. Radioactive label incorporation was achieved by a 2-min 42°C preincubation of the sequencing reaction containing the α-35S-labeled deoxyxynucleotide (New England Nuclear) corresponding to the first base of the nascent chain as the only deoxynucleotide. Reactions were electrophoresed on 8% polyacrylamide-urea gels for 1–3 h. Dried gels were exposed to Kodak X-AR 5 film at room temperature for 1–3 days.

Results

Analysis of Tumor Samples for p53 Mutations. In our evaluation of 48 esophageal tumors, including 14 from earlier work, we identified 20 mutations in exons 5–8 of the p53 gene, 19 of which are single base substitution mutations that result in an amino acid substitution or a chain-terminating codon (Table 1). Missense mutations are all at amino acids conserved in 4 mammalian species (human, monkey, rat, and mouse) (27), and more than one-half occur within highly conserved domains previously identified as preferred sites of human tumor mutations (17). Identical mutations (nature of change and position) in two different tumor samples were found at both codons 194 and 305 (Table 1). There are also mutations clustered at sequences comprising codons 192–194 and 244–249.

Analysis of ESC from Uruguay for ras Mutations. Mutations in ras protooncogenes shown to confer transforming activity to the protein occur in exon 1 (codons 12 and 13) and exon 2 (primarily 61; also 59 and 63) of the H-, K-, and N-ras genes and codon 146 (exon 3) of the K-ras gene (28). Negative ras mutation analyses of ESC reported previously were performed with techniques involving oligonucleotide hybridization or other methods.
primer-directed restriction fragment length polymorphism designed to identify mutations specifically at codons 12, 13, and 61, leaving the possibility that the mutational specificity of factors contributing to ESC could target additional codons of ras genes that confer transforming activity when mutated, e.g., codons 59, 61, 63 (H-, K-, N-ras) (1), 146 of the K-ras gene (28), or the activator domain of ras proteins in exon 1 (29). We sequenced all of these regions in 16 tumor samples; however, mutations resulting in an amino acid substitution were not found in codons 12, 13, or 61, or elsewhere in flanking sequences of H-, K-, and N-ras, or in the codon 146 region of the K-ras gene.

Discussion

p53 Mutations in ESC. Mutated p53 alleles are found frequently in human ESC. Forty% (20 of 48) of tumors tested contained one or more mutations in the midregion of the gene. The number of tumors with base substitutions was particularly high (9 of 15) in samples from Normandy which could reflect the fact that most patients in this group were tobacco smokers (Table 2) and tobacco smoke is mutagenic (30). It is also conceivable that the resected tumors from Normandy were at a more advanced stage than lesions biopsied in the diagnostic screening program in Uruguay in which the frequency of mutations detected was lower (5 tumors positive of 19 tested).

This set of 20 ESC mutations describes a p53 base substitution pattern that is different from that seen in another cancer of the digestive tract: in comparison to colorectal tumor mutations, the frequency of ESC CpG toTpG transitions is low (15% in ESC and 70% in colorectal tumors) (16), at P < 0.01; G → T transversions comprise one-fourth of the mutations in these ESC yet no G → T transversions were detected among 30 colorectal tumors (16), at P < 0.01. A high frequency of G to T transversions in non-small cell lung cancer, another tobacco-related cancer, was reported by Chiba et al. (18), whereas the frequent G-T transversions in hepatocellular carcinomas could be largely attributed to aflatoxin B1 exposure (19, 20). Many patients in the present study were consumers of both tobacco and alcoholic beverages (Table 2), the two most widespread risk factors for ESC.

p53 analysis of colon tumors has shown that in this tumor type mutations typically arise relatively late in the progression of the disease (16). In the development of esophageal squamous cell carcinoma there may be greater variability in the timing of these events. In some instances at least, the mutation may be present in early neoplastic lesions of the esophagus since immunohistochemical staining of carcinoma in situ with a polyclonal antibody raised against the human p53 protein has been observed (56).4

A major fraction of ESC cases in Normandy and Uruguay is attributable to tobacco and alcoholic beverage consumption, which suggests that some of the mutations observed may be directly or indirectly the result of exposure to these carcinogenic risk factors. Comparisons of p53 mutations in ESC with mutation patterns of other types of cancers would be expected to reflect similarities or differences in environmental etiologies of the cancer considered. Possible explanations for the mutation trends observed in tumors will be worth exploring as more samples are examined. G → T transversions, frequent in tobacco-related cancers of the esophagus and lung, may be attributable to mutagenic components of cigarette smoke that induce this transversion, such as the polycyclic aromatic hydrocarbon benzo(a)pyrene (4, 31). Carcinogenic N-nitrosamines are also present in tobacco (32) and induce transitions primarily at G:C pairs, although the number of substitutions at A:T pairs increases with substituent size of the alkylating moiety generated by metabolic activation of the N-nitrosamine (33). We have previously shown that human esophageal and bronchial exfoliants can metabolically activate benzo(a)pyrene and N-nitroso- dimethylamine to form promutagenic DNA adducts (34–38).

DNA depurination from irritants to the mucosa, including ethanol or the scalding temperatures at which maté tea is consumed in some regions of South America may be important, particularly among nonsmoking, heavy drinkers of alcoholic beverages, who have a considerably increased risk of esophageal cancer (14, 39). Alcoholic beverages may also contain numerous carcinogenic contaminants, e.g., urethan, but in trace quantities (40). Carcinogenicity may be attributable to ethanol per se, or to the principal metabolite acetaldehyde. Indirect effects of carcinogen exposure on DNA repair activity would also influence the tumor mutation spectrum; Aldehydes have been shown to inhibit repair by alkyltransferase of the promutagenic DNA lesion O6-methyldeoxyguanosine (41).

Current efforts are to examine larger numbers of ESC patients with documented exposure histories so that individuals consuming alcoholic beverages can be distinguished from smokers and from patients that consume both alcoholic beverages and tobacco. Direct exposure measurements in patients, such as levels of O6-guanine in DNA (42), or albumin-carcinogen

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* Numbers in parentheses, number of cigarettes smoked per day.

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4 Bennett and C. C. Harris, unpublished observations.
adducts (43) and correlations with molecular changes in tumor DNA of the same individuals (44) should improve the power of mutational spectral analysis.

Absence of ras Mutations in Tumors from Patient Groups with Frequent p53 Mutations. Our results suggest that in the high incidence region of Uruguay, where tobacco smoking, alcoholic beverages, and mate tea are risk factors, ras mutations do not play an important role in the pathogenesis of the disease. This appears to be true also in ESC from high incidence areas of the Peoples Republic of China and Africa where N-nitrosamine and/or fungal toxin exposure could be important (45, 46) and in ESC from Normandy and Lyon (1). Although ras mutations might be expected to occur in the squamous epithelium of the esophagus, human ESC does not typically proceed by this molecular pathway. This inference has important ramifications regarding tissue specificity in molecular mechanisms of cancer. While some changes in specific genes, e.g., p53 appear to be implicated in most cancer types, others are associated primarily with a specific cancer or with some groups of cancers, e.g., N-myc amplification in neuroblastoma (47) or frequent Ki-ras mutations in pancreatic (48) and colon cancer (49) but not breast tumors and ESC. One early speculation for the differences in ras gene involvement was that mutagenic activity is inherently higher in some tissues than others due to metabolic activation capacities, promutagenic cellular processes, etc. The fact that p53 mutations but not ras mutations occur in human ESC in the same patient groups argues rather that, in this cancer type, p53 mutations contribute to malignancy whereas transforming ras mutations do not. A second possibility is that the mutational specificity of environmental factors contributing to esophageal carcinogenesis in the populations studied does not target potentially transforming ras sequences.

It is noteworthy that ras mutations are activated experimentally in N-nitrosamine-induced rat esophageal tumors (50). This difference between experimental animal models and human cancers is not without precedent: in human breast cancer ras mutations are extremely rare (3) and p53 mutations are frequent (51, 52); while carcinogen-induced ras mutation in rat mammary tumors has been a paradigm of animal carcinogenesis models (53). It will be of interest to study other populations where ESC is high and suggested risk factors are distinct, e.g., in the Caspian littoral of Iran, where opium tar resins (54) are possible factors, and in Kashmir, where N-nitroso compounds are considered important elements in elevating ESC risk (55).

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