Short Communication

p53 Mutations in Esophageal Tumors from a High Incidence Area of China in Relation to Patient Diet and Smoking History

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Abstract

Esophageal tumors from 29 patients residing in Guangzhou, China, were examined for mutations in exons 5–8 of the p53 tumor suppressor gene and for p53 protein accumulation in tumor cell nuclei. Anamnestic data for each patient, which included information on family history of cancer, tobacco smoking, drinking of alcoholic beverages, and dietary habits such as consumption of pickled vegetables, were recorded. Screening of DNA from tumor cells microdissected from biopsies was performed by PCR amplification of p53 gene exons 5–8, denaturing gradient gel electrophoresis analysis, and DNA sequencing. Mutations were identified in 20 of 29 tumors (69%). All tumors harboring a missense mutation in the p53 gene also showed nuclear accumulation of the tumor suppressor protein by immunohistochemistry. The most common p53 mutations in these tumors were guanine to adenine (G→A) transitions (10 of 20 tumors; 50%). We did not find multiple mutations at codon 176, in contrast to Lung et al. in their recent study of esophageal cancer patients from Guangzhou (M. L., Lung et al., Cancer Epidemiol. Biomark. Prev., 5: 277–284, 1996).

The mutation prevalence was high both in smokers (13 mutations in 20 smokers; 65%) and in nonsmokers (7 of 9 tumors with mutations; 78%), an observation that differs from that of studies in European and North American patients, which demonstrate a much higher prevalence of p53 mutations in smokers than in nonsmokers (reviewed in R. Montesano et al., Int. J. Cancer Predict. Oncol., 69: 225–235, 1996.).

Our findings in this pilot study of tumor suppressor gene mutations in patients from Guangzhou support a large body of epidemiological observations pointing to dietary mutagenic carcinogens peculiar to populations in China at high risk of esophageal cancer.

Introduction

Over 300,000 new cases of esophageal cancer arise each year, with a predominance of cases in developing countries, and a striking regional variation in incidence (1, 2). Tobacco and alcoholic beverages are the major known risk factors in Europe and North America, where the age-adjusted incidence rates for SCC of the esophagus are in the range of 5–25 cases/100,000. In contrast, in areas of the world with high esophageal cancer incidence figures, such as Linxian and Guangzhou, China (>100/100,000), dietary items, for example pickled cabbage and other vegetables preserved by this method, are also implicated in the etiology and may be among the dominant risk factors among nonsmoking patients, particularly women (Refs. 3 and 4 and references therein). Among male patients from these provinces who are smokers, both smoking/drinking habits and dietary preferences may be critical. The number of men in China who are regular smokers has been rising considerably in recent years, such that tobacco consumption may become a predominant risk factor in men and may drive the cancer incidence figures still higher in the future, as tobacco consumption increases.

p53 point mutations are the most common specific gene lesions found in human cancers and are directly linked to pathogenesis (5, 6). The point mutations found in tumors, which occur at any of more than 250 bp and encompass a full variety of sequence changes (all types of base substitutions, for example), cause disruption of tumor suppressor functions (7, 8). The prevalence of mutations or the pattern (composed from assembling the type and location of mutations) can be quite different in two groups of patients with the same type of malignancy but different cancer risk factor exposure histories (9). These pattern differences are being used to explore clues to etiology gained by classical epidemiology studies (10).

In Europe and North America, the prevalence of p53 mutations in malignancies associated with consumption of tobacco products (e.g., lung, esophageal, and bladder cancer) is clearly higher in patients who are smokers (4, 11). In lung tumors of smokers, this is paralleled by an increase in G to T transition mutations in the p53 gene with increasing cigarette consumption (12). Metabolites of carcinogenic mutagens from cigarette smoke found in the lung, such as benzo(a)pyrene-7,8-diol 9,10-epoxide, may be responsible for inducing these mutations (13), whereas in other organs, for example, the urinary bladder, different carcinogens and their mutagenic metabolites seem to be critical (9).

We undertook a pilot study in a well-defined patient group with esophageal cancer from Guangzhou, China, for which we were able to obtain anamnestic data on habits associated with cancer risk and on family history of cancer. Our objectives were: (a) to determine the tumor p53 mutation frequency and...
pattern; (b) to correlate this with exposure information; (c) to compare these data with mutation patterns in patients reported for Europe and North America; and (d) to look for evidence of a putative p53 hot spot mutation at codon 176 recently described by a Lung et al. (14) in their survey of esophageal tumors from China.

Materials and Methods

Tissue Collection. Specimen donors were provided with cancer research information and given the opportunity to refuse participation in the project, which was approved by local boards reviewing human research risks. All 29 patients (20 males and 9 females; age range, 34–69 years) were from Guangzhou, People’s Republic of China (Table 1). Twenty-four patients were diagnosed with SCC of the esophagus, and five were diagnosed with ADC. Case-by-case dietary information as well as other anamnestic data for these patients was obtained, including consumption of pickled vegetables, consumption of hot foods or beverages, long-term dysphagia or other symptoms of esophagitis, smoking history, and consumption of alcoholic beverages (mostly beer and wine). Three patients reported having a first-degree family member with esophageal cancer: (a) patient HE91-07, esophageal cancer (mother) and liver cancer and nasopharyngeal cancer (two siblings); (b) patient HE91-26, esophageal cancer (grandfather) and nasopharyngeal cancer (father and uncle); and (c) patient HE91-27, esophageal cancer (sister).

Ethanol-fixed tumor tissues from each patient were collected for DNA extraction and for paraffin embedding in preparation for immunohistochemistry. Tissue blocks were sectioned serially at 5 or 10 μm and mounted with minimal heating onto poly-L-lysine-coated slides. DNA was extracted from ethanol-fixed microdissected tumor cells according to standard procedures.

Immunohistochemistry. Immunohistochemical analysis was performed essentially as described previously (15). Briefly, after dewaxing, inactivating endogenous peroxidase activity, and blocking with normal goat serum, the sections were incubated overnight at 4°C with CM-i rabbit polyclonal antibody against p53 (a gift of D. Lane, University of Dundee, Dundee, Scotland). Localization of the primary antibody was achieved using a horseradish peroxidase-antiperoxidase kit; Vector Laboratories, Burlingame, CA), and diaminobenzidine together with nickel chloride. H&E-stained sections were reviewed to confirm the presence of malignant tissue. Staining

### Table 1 Patient histories and p53 analysis

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<td>Ins +1 NA f</td>
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aAge at diagnosis.
bLocation (designated as upper, middle, or lower), histology, and stage of tumor.
cCigarettes/day.
dPounds/month.
eMissense; s, stop; f, frameshift; sp, splice; NA, not applicable.
fg, G to A transition.
hND, not done.
iint, intron.
was recorded numerically according to: (a) intensity, 0–3 (for none, equivocal, moderate, and intense); (b) distribution, 0–4 (for the percentage of cells stained; none, <10%, 10–50%, 50–90%, and >90%); and (c) the sum of intensity and distribution, reported on a 4-point scale as negative (0) or positive (1+ , sum = 1–3; 2+, sum = 4–7; or 3+, sum = 8–10).

**PCR and Mutational Analysis.** DNA fragments were amplified in a DNA thermocycler (Perkin-Elmer Corp.) in 50-μl reaction mixtures containing up to 200 ng of genomic DNA, 20 pmol of each primer, 160 μM each deoxynucleotide triphosphate, and 1 unit of Taq polymerase (Boehringer Mannheim) in buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 2.5 mM magnesium chloride, 2.5% formamide, and 0.02% BSA. The DNA fragments were screened for mutations by DGGE as described by Hamelin et al. (16) and Renault et al. (17). For DGGE analysis, the sequences of the primers used for exons 5, 7, and 8 and their cycling conditions were in accordance with the method of Hamelin et al. (16), which allows for the detection of mutations in the entire coding segment of each exon. For exon 6, primers and conditions were as described by Renault et al. (17). All primers were purchased at Biometra (Goettingen, Germany). Samples were loaded onto 6 or 6.5% polyacrylamide gels containing the appropriate denaturant gradient. The gels were run in a C.B.S. DGGE system (C.B.S. Scientific Co., Del Mar, CA) at 60°C and 150 or 160 V submerged in 1× TAE buffer [40 mM Tris acetate, 20 mM sodium acetate, and 1 mM EDTA (pH 7.4)] for 3–16 h. After electrophoresis, the gels were stained with ethidium bromide and photographed using an UV transilluminator. A second PCR with genomic DNA was performed for sequencing those exons showing an abnormal migration pattern on the DGGE gels. Primers and sequencing conditions were as described previously (18), except that dideoxysequencing was performed on biotinylated PCR product affixed to streptavidin magnetic bead support (Dynal, Oslo, Norway). DNA stocks and PCR reagents were kept physically separated from areas where PCR products were handled, and setup was performed in an Oncor Template Tamer hood (Oncor, Inc., Gaithersburg, MD) equipped with UV light.

**Results**

In this group of 29 patients, composed of consecutive incoming hospital cases of diagnosed esophageal cancer (24 SCCs and 5 ADCs), the majority of individuals were male cigarette smokers (20 of 29 patients). Three-fourths of the smokers also reported consumption of alcoholic beverages, mostly wine or beer. There were 21 men and 8 women, and none of the women were smokers or reported drinking beverages containing alcohol. Most patients (23 of 29; 80%) frequently ate pickled cabbage and/or ingested food or beverages at scalding temperatures, dietary factors that are thought to play an important role in the etiology of esophageal cancer in China. Three patients had first-degree relatives who had been diagnosed with esophageal cancer (see “Materials and Methods”).

A total of 69% (20 of 29) of the tumors harbored a mutation in coding sequences of the p53 gene (19 patients) or at a splice site (Table 1; Fig. 1). Most of the mutations (16 of 20) were base substitutions at highly conserved codons, leading to missense mutations and accumulation of the p53 protein in tumor nuclei. The most common mutation (10 of 20; 50%) was guanine to adenine transition, which occurred frequently (6 of 10 G-to-A mutations) at CpG dinucleotides, sites in the p53 gene where cytosine is methylated (19). The CpG site at codon 175 was the location at which multiple (more than two) mutation occurrences were observed in this patient group (four tumors with this mutation). Codon 175 is one of the locations in the p53 gene where mutations in human cancers occur most frequently; transitions at this site account for approximately 5% of the >6000 human p53 tumor mutations identified thus far (20) and can arise through the spontaneous deamination of 5-methylcytosine (19, 21). Three of the four tumors with base transition at this site were from nonsmokers and nondrinkers. Seven of the nine nonsmoking patients (78%) had a mutated p53 gene in their tumors. Unexpectedly, this frequency was comparable to that found among smokers (13 tumor mutations from 20 smokers; 65%; Table 1).

**Discussion**

In a group of North American patients with head and neck cancer, Sidransky and colleagues (11) effectively demonstrated that tobacco smokers were much more likely to have p53 mutations in their tumors than nonsmokers, and the p53 mutation frequency increased with dose (number of pack-years smoked) at time of diagnosis. A review of publications describing p53 mutations in patients with SCC of the esophagus reveals a similar association between tumor mutation frequency and cigarette consumption (4). Fifty-five percent of esophageal cancer patients who had smoked up to 20 cigarettes/day had p53 tumor mutations, and 4 of every 5 individuals smoking more than this amount per day had a tumor mutation. In contrast, only one of every five nonsmoking esophageal cancer patients had a p53 tumor mutation. Similarly, head and neck
cancers of patients without exposure to this known risk factor are also unlikely to harbor p53 mutations (<18%; Ref. 11). These data argue that in the absence of exposure to potent mutagenic carcinogens, such as those derived from tobacco smoke (12, 13), point mutations in the p53 gene rarely arise in spontaneous tumors of the upper aerodigestive tract.

Although no conclusions can be drawn from a patient group of this size with respect to the nutritional components that may have contributed to the tumor mutations, the relatively high overall mutation frequency (20 of 29; 69%) we observed, particularly among the nonsmokers (7 of 9; 78%), is in keeping with epidemiological findings that dietary carcinogens capable of damaging genetic material and inducing point mutations in DNA may indeed be important esophageal cancer risk factors in Guangzhou. An expanded investigation in this geographical area with an epidemiological study design and including analysis of genetic changes in tumor material would be of value.

The performance of additional studies of p53 mutations in esophageal cancer patients from Guangzhou is also important to clarify whether there is indeed a mutation hot spot at codon 176 in cancers from Guangzhou. We found only 1 such mutation among the 20 point mutations we identified, a result that clearly differs from the report that 12 of 39 esophageal tumor specimens from Guangzhou harbored an identical mutation (14). Of all of the many cancer populations around the world and the cancer types studied that have yielded the >6000 p53 human tumor mutations in the European Bioinformatics Institute database (20), only 2 instances of a mutation hot spot of this magnitude have been described: (a) a codon 249 Arg→Ser mutation in up to 50% of hepatocellular carcinomas from individuals exposed through their diet to hepatocarcinogenic aflatoxins (22, 23); and (b) a codon 249 Arg→Met mutation in over 25% of lung tumors of uranium miners putatively associated with radon gas exposure (24). Whereas the radon-linked mutation has thus far proved elusive in investigations seeking to substantiate the finding (25–27), the hot spot associated with hepatocellular carcinoma and aflatoxin exposure has been amply corroborated by investigators in many laboratories.

References
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