Prevalence of p53 mutations in patients with squamous cell carcinoma of the esophagus

Squamous cell carcinoma of the esophagus has an uneven geographic distribution with a strong prevalence in the South Carolina Lowcountry. Although many environmental influences and some genetic factors have been implicated in its development, the molecular events required for tumorigenesis have not been defined. Point mutations in the p53 tumor suppressor gene are the most commonly noted genetic defect in human tumors. Our study shows that p53 point mutations occur more frequently in patients with esophageal cancer from this region than in patients from other areas of the world where the disease is prevalent. (J THORAC CARDIOVASC SURG 1994;108:148-52)

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Esophageal cancer is an aggressive tumor of the gastrointestinal tract that claims more than 10,000 American lives each year.¹ Although its overall prevalence worldwide is only 6.4 per 100,000,² in several locations it is much more common. Coastal South Carolina has one of the highest rates in the world. In this geographic region the rate of squamous cell carcinoma of the esophagus in black men, in particular, is more than four times the global average.³

Although environmental exposures to such substances as tobacco, alcohol, opiates, aflatoxin, and caustic agents have been most frequently implicated in the development

of esophageal cancer,⁴ the inherited syndromes of tylosis⁵ and Li-Fraumeni⁶ may support an underlying genetic predisposition for its formation in some cases. Despite the growing epidemiologic data, the precise mechanism by which these influences generate or promote the genetic events required for tumorigenesis have not been delineated.

One area of intense interest has been the p53 tumor suppressor gene. Point mutations in this gene have been found in more tumors across the spectrum of human malignancies than in any other gene.⁷ Several recent studies not only have reported p53 point mutations but, more important, have correlated these genetic lesions to the patients' endogenous and environmental influences.^{8, 9} Our study was designed to determine the frequency of p53 mutations in patients from the Lowcountry of South Carolina who have squamous cell carcinoma of the esophagus and to analyze the characteristics of these mutations to determine if they support a role for p53 in the process of tumorigenesis in these individuals.

Methods

Tumor samples. Tumor biopsy specimens were obtained at esophagoscopy and either grown in tissue culture or cryopre-

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Supported in part by the American Cancer Society Clinical Oncology Career Development Award (P.L.B.).

Read at the Seventy-third Annual Meeting of The American Association for Thoracic Surgery, Chicago, Ill., April 25-28, 1993.

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0022-5223/94 \$3.00 + 0 12/6/55543

Table I. *p53* Point mutations

Patient	Race	Sex	Base change	Codon number	Amino acid change
1	W	M	None	—	—
2	B	M	None	—	—
3	W	M	None	—	—
4	B	M	T to G	270	Phe to Cys*
5	B	F	C to T	241	Ser to Phe*
6	B	M	C to T	282	Arg to Cys*
			A to G	292	Lys to Arg†
7	W	M	G to A	181	Arg to His*
8	W	F	G to A	175	Arg to His*
			T to G	270	Phe to Cys*
9	B	M	None	—	—
10	B	M	G to A	157	Val to Ile†
			G to A	159	Ala to Thr*
11	B	M	G to A	175	Arg to His*
12	B	M	T to A	194	Leu to His*
13	B	M	None	—	—
14	B	M	A to T	193	His to Leu*
15	B	M	A to T	164	Lys to stop

All specimens are squamous cell carcinomas of the esophagus. *B*, Black; *W*, white; *M*, male; *F*, female; *A*, *C*, *G*, and *T*, DNA bases adenine, cytosine, guanine, and thymine; *Phe*, phenylalanine; *Cys*, cysteine; *Ser*, serine; *Arg*, arginine; *Lys*, lysine; *His*, histidine; *Val*, valine; *Ile*, isoleucine; *Ala*, alanine; *Thr*, threonine; *Leu*, leucine.

*Nonconservative amino acid change.

†Conservative amino acid change.

served in liquid nitrogen. Total cellular ribonucleic acid (RNA) was isolated by the acid guanidium phenol/chloroform method of Chomczynski and Sacchi.¹⁰ The RNA was reverse transcribed into complementary deoxyribonucleic acid (DNA) by random primers and the Maloney murine leukemia virus reverse transcriptase.

Polymerase chain reaction. (PCR): PCR¹¹ was performed with oligonucleotide primers that flank a 606 base pair region of *p53* from codon 120 in exon 4 through codon 321 in exon 9. This region has previously been shown to contain the majority (98%) of described point mutations.¹² The PCR products were identified and purified on 2% agarose gels.

Cloning and sequencing. The initial eight cases had the gene-cleaned, blunt-end fragment cloned into the *Sma* I restriction enzyme site of pBluescript II KS+ plasmids. Recombinant plasmids were verified by restriction analysis and sequenced according to the Sanger dideoxy chain termination method with vector-specific primers.¹³ The subsequent seven cases were directly sequenced by a modification of the same method. All data were verified by repeating the PCR and sequencing at least one other time.

Results

Fifteen different squamous cell tumors were sequenced (Table I). Ten specimens had at least one point mutation and three of these specimens had two point mutations each (Fig.). Eight of 11 black patients (72.7%) had point mutations and two of four white patients (50%) had them. Thirteen bases underwent mutation, of which eight were transitions (61.5%) and five (38.5%) were transversions. All but one mutation led to an amino acid change, and this one produced a stop codon. Only two amino acid changes

were strictly conservative and both of these occurred in patients with a nonconservative second mutation. Four mutations were at arginine residues and three had cysteine as the resultant amino acid. Two others had an aliphatic for basic side chain substitution and two resulted in the loss of a phenylalanine residue while one produced it. Specimens RA and HL were from cultured cells and showed no mutations. The other 13 specimens were from cryopreserved tissue.

Discussion

Tumorigenesis occurs when a cell loses its well-regulated growth cycle and clonally expands beyond the control of the host. This is believed to require a critical series of molecular events that cause the cell to divide and escape from normal proliferative control.¹⁴ Squamous cell carcinoma of the esophagus may follow this model. This cancer is of particular interest because epidemiologic data suggest many environmental exposures⁴ that may be associated with an increased risk for its formation. Moreover, its uneven geographic distribution suggests that specific social, dietary, or heritable characteristics of a region may be involved in its development.¹⁵

Although some studies have proposed a role for a *c-ras* gene activation¹⁶ and others have suggested *hst-1* or *int-2* gene amplifications,^{17,18} one area of intense interest is the *p53* tumor suppressor protein. The *p53* protein has been extensively studied because it may play an important role

Fig. 1. A representative point mutation: Sample EH, anti-sense strand, beginning with codon 280 reads: CTC TCT GAC. The accepted sequence is CTC TCT GGC. This G to A transition at codon 282 in the anti-sense strand corresponds to a C to T transition in the sense strand, which results in an arginine to cysteine missense mutation in the p53 protein.

in the negative regulation of cell growth. It appears that wild-type p53 functions by blocking the progression of cells through the G₁ phase of replication. This function may be achieved by oligomers of p53 that bind to specific sequences of DNA and serve as transcription regulators for the expression of particular genes.⁷

Several other investigators have reported p53 mutations in esophageal cancer from other areas around the world. Tamura,¹⁹ Furihata,²⁰ and their associates have reported p53 mutations in 38% and 34% of squamous cell esophageal cancers from Japan. In addition, Huang and colleagues²¹ from Baltimore reported on a loss of heterozygosity in 55% of p53 genes from esophageal tumors against a background loss of heterozygosity of 15% in other genes. Hollstein and coworkers²² reported p53 mutations in five of 14 cases (36%) of squamous cell carcinoma of the esophagus from southern France. Casson and colleagues²³ from the M.D. Anderson Cancer Center in Houston, Texas, found only one p53 mutation in 10 (10%) cases of squamous esophageal cancer. Our report of 10 of 15 tumors (67%) with p53 mutations is the highest rate of such mutations reported to date.

For a protein to perform its function, it must fold to a specific conformation, the structure of which is determined by the amino acid composition. If one compares the p53 sequence among many species, one finds that four domains are highly conserved.²⁴ These domains likely represent the biologically active¹² site or at least are necessary for proper conformational folding of the protein. Thus any significant change would disrupt the critical steric relationships and, consequently, alter its function and stability. In this study, six of 10 tumors with mutations had at least one mutation within one of these domains. Among the four other tumors, one produced a

stop codon and the other three had mutations that have previously been described in human esophageal tumors.

Another related finding is the nature of the amino acid changes. Eleven of the 13 total mutations had changes affecting the charge, size, or reactivity of the amino acid side-chain. Such nonconservative changes are necessary to alter the conformation of a protein. Thus these single amino acid changes in p53 may produce significant alteration in the protein's function and stability. Indeed, the half-life of wild-type p53 is 20 minutes, whereas a single mutation characteristically increases the half-life to approximately 12 hours.²⁵

A great deal of interest has been generated in whether specific mutations can implicate the etiology of a tumor. All 15 patients in this study had a significant history of tobacco use. Tobacco smoke contains a variety of mutagens that may result in a characteristic pattern of gene mutations.²² Recent data show that smoking may be a principal cause of p53 mutations. In patients with head and neck cancer, 78% of smokers had p53 point mutations compared with 14% of nonsmokers.⁸ Overexpression, a marker of p53 mutation, was detected by the CM-1 polyclonal antibody in 33% of lung adenocarcinomas. This correlated well with the patients' smoking history, because all cells with overexpression came from smokers (23/58, 40%), whereas cells without overexpression came from nonsmokers (0/12, 0%).⁹ Moreover, the sites and types of mutations do not occur randomly.²⁶ G to A transitions are often found in patients with histories of tobacco use. Five of the 13 mutations in our study were G to A transitions. This transition has been suggested to be the result of DNA alkylation of deoxyguanosine to O⁶-methyl-deoxyguanosine, which pairs with thymidine instead of cytosine. This may be induced by methylating nitrosamines, which are present in significant amounts in tobacco smoke.²²

The CpG dinucleotide is a frequent site of spontaneous mutations in which 5-methylcytosine deaminates to thymidine.²⁷ This event is thought to be responsible for the high frequency of transitions that are seen at this site and seems to account for many germ-line mutations that cause human genetic disease.²⁸ Two of the eight black patients with mutations had a mutation at a CpG dinucleotide site. Squamous cell carcinoma of the esophagus is disproportionately prevalent in black men in the South Carolina low country. At this time, however, no specific reason for this has been elucidated. A prevalence of germ-line p53 mutations could increase the likelihood for the development of cancer. This would partially explain the racial difference seen in this region.

As in all studies, p53 mutations are not found in all of the specimens. Technical concerns may have prevented us

from detecting a p53 mutation. Sampling error is possible because of the heterogeneous nature of biopsied tissue. Despite the significant number of tumor cells within each specimen, there are still many normal cells whose p53 may provide the source for falsely negative results. In addition, a mutation could have occurred outside the analyzed sequence. Although 98% of all previously described mutations have occurred within the analyzed region, it is possible that a specimen could have a significant mutation outside the region.

Clearly, not all squamous cell carcinomas of the esophagus necessarily have p53 mutations. Several reasons may explain this. First, the target sequence for the p53 protein may, in fact, undergo mutation such that wild-type p53 is unable to bind it and perform its suppressor function.²⁹ Second, a cellular or viral oncogene product may serve as an inhibitor of function of wild-type p53. This is best described in cervical carcinoma, where the E6 product of the human papilloma virus inhibits p53 function.³⁰ Third, another molecular event may produce a similar phenotypic change that provides sufficient alteration to permit tumorigenesis. Recently a p90 protein (mdm-2) has been found that co-immunoprecipitates with p53. It is proposed that this protein functions by binding with oligomers of p53 and together they bind nuclear DNA, producing the regulatory function.³¹ Among these tumors (human sarcomas) that had mdm-2 gene amplification, no mutations of p53 were found.³² Thus amplification of the mdm-2 protein has the similar phenotypic effect of a p53 mutation.³³

In summary, this study shows a large percentage of p53 mutations in patients with squamous cell carcinoma of the esophagus from the South Carolina Lowcountry. The frequency of these mutations is greater than those from other regions where the disease is prevalent. The specific mutations and their resultant amino acid changes support a role for p53 in the process of esophageal cancer tumorigenesis. Further studies are indicated to determine if specific mutations correlate with prognosis, as is now being elucidated for other tumors with p53 involvement.³³

We are grateful to James Norris, PhD, for his support and assistance in producing this study.

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Discussion

Dr. Harvey I. Pass (*Bethesda, Md.*). Have you done studies of the surrounding tissue, along with the tumor, with regard to p53 expression?

Dr. Gates. We have done a monoclonal antibody test looking at the surrounding tissue both in the immediate area and 10 cm away from the tumorous tissue. What we found is that these

monoclonal antibody studies suggest that some of the normal cells have the p53 mutations. This observation supports a field defect in p53, which has been suggested by other studies.

Dr. Martin F. McKneally (*Toronto, Ontario, Canada*). Can you discuss the larger picture of what you were attempting to do apart from defining the molecular events?

Dr. Gates. I think the larger picture is improved therapeutic strategies, therapies such as what Dr. Roth will be doing in Houston, where a complementary strand of DNA will be placed in the cells, which will bind the mutant p53 and thereby inhibit its function in cellular replication. Also, understanding that different tumors have different p53 mutations and that not all tumors have p53 mutations will be relevant for the management of these patients.

Dr. Valerie W. Rusch (*New York, N.Y.*). How many of these specimens had you put in cell culture? Since you are using RT-PCR, it seems unnecessary to put any specimens in cell culture, a technique that could alter your results.

Second, did you examine p53 alterations at the protein level? In lung cancers a greater number of alterations have been found at the protein than the RNA level.

Dr. Gates. In answer to your first question, we have entirely departed from cell culture techniques, and this appears to be working much better with more reliable results. In answer to the second question, no, we have not done any protein studies, per se, looking for alteration in p53 configuration, half-life, or structure. This could certainly prove to support our contention that p53 mutations are involved in esophageal carcinogenesis, and such studies will be addressed further in the future.

Dr. Mark B. Orringer (*Ann Arbor, Mich.*). Dr. Rusch has made an important point that I would only reemphasize, that PCR techniques are more reliable than cell culture for demonstrating the genetic abnormalities in patients with thoracic malignancies. I also want to point out that the subspecialty of general thoracic surgery has clearly established a leadership role within thoracic surgery in using molecular biologic techniques to better define our patient population and potentially apply the genetic findings to clinical practice. The p53 oncogene is but one of these recently identified in patients with esophageal carcinoma. Others are now being demonstrated as we start to map which oncogenes may be important in both esophageal and lung cancer. In our laboratory, for example, we have also found that *N*-aminopeptidase and sucrase isomaltase are common oncogenes in esophageal adenocarcinoma. Such genetic characterization of these tumors may potentially provide prognostic information once we have better correlated survival with specific oncogenes. As we expand on the use of this information that Dr. Gates has presented, in the near future we can envision sending biopsy tissue of esophageal cancer both to the pathologist for routine histologic evaluation and also to the tumor biology laboratory, where mapping of the oncogenes of each tumor will reveal which cancers have the better prognosis. Perhaps as we learn to link monoclonal antibodies to these proteins, we will have a new weapon against systemic metastases. We are entering a new and exciting era of general thoracic surgery, and I congratulate Dr. Gates and his associates for a very fine piece of work.