p53 Mutation and Protein Accumulation during Multistage Human Esophageal Carcinogenesis

William P. Bennett, Monica C. Hollstein, Robert A. Metcalf, Judith A. Welsh, A. He, Si-min Zhu, Inca Kusters, James H. Resau, Benjamin F. Trump, David P. Lane, and Curtis C. Harris

Abstract

Preinvasive lesions of squamous cell carcinoma are well defined morphologically and provide a model for multistage carcinogenesis. Since alterations in the p53 tumor suppressor gene occur frequently in invasive esophageal squamous cell carcinoma, we examined a set of preinvasive lesions to investigate the timing of p53 mutation. Surgically resected tissues from nine patients with esophageal squamous cell carcinoma contained precursor lesions which had not yet invaded normal tissues. Immunohistochemistry showed high levels of p53 protein in both preinvasive lesions and invasive carcinomas in six cases; sequence analysis of all invasive tumors identified p53 missense mutations in two cases. Preinvasive lesions from both tumors with mutations plus one wild-type tumor were microdissected and sequenced. In one patient there were different mutations in the invasive carcinoma (codon 282, CGG > TGG) and a preinvasive lesion (codon 272, GTC > T/GTC/mut). In a second case, an invasive carcinoma had a mutation in codon 175 (CGC > CAC/mut), and adjacent preinvasive lesions contained a wild-type sequence. A carcinoma and preinvasive lesion from the third case contained high levels of protein and a wild-type DNA sequence. Therefore, p53 mutation may precede invasion in esophageal carcinogenesis, and multifocal esophageal neoplasms may arise from independent clones of transformed cells. The timing of p53 protein accumulation is favorable for an intermediate biomarker in multistage esophageal carcinoma.

Introduction

Mutation in the p53 tumor suppressor gene is the most common genetic alteration in human cancer (reviewed in Ref. 1). Mutation occurs in all of the common cancers, and within any one type, one-half or more of the individual tumors may be affected. Increased levels of p53 protein are also found in 29–80% of malignant tumors but only rarely in benign tumors and normal tissues (2). Molecular mechanisms leading to excess protein accumulation include mutation with conformational change (3), complex formation with viral oncoproteins (4–7), and possibly aberrant expression of the p53 gene by cellular transcriptional regulators (8). The frequency and specificity of p53 protein accumulation suggest that elevated protein levels may be useful as an intermediate biomarker. This possibility has been the subject of recent debate among groups analyzing human tissues for evidence of malignancy (9, 10).
of amplification (15). In cases with identified mutations, the germ-line sequence of the affected exon was analyzed from nontumor tissues.

**Results and Discussion**

This study began when squamous precursor lesions were noted to contain elevated levels of p53 protein. In nine sets of preinvasive lesions and invasive tumors, the staining patterns were concordant; that is, in six pairs, both preinvasive lesions and tumors contained high levels of p53 protein, and in three pairs, both were negative. These findings suggested that p53 protein accumulation and/or mutation may occur before the development of invasion in esophageal SCC. Most of the progenitor lesions were next to invasive cancers, but in one instance, the tumor was distinct from the dysplastic area which appeared to be an independent neoplasm (HE90-72). This observation suggested that dietary or environmental agents caused widespread genetic damage (i.e., a “field defect”) leading to multiple independent neoplasms. Alternatively, a monoclonal proliferation may have spread through the mucosa or lymphatics to form multiple neoplasms derived from a common progenitor cell. To test these hypotheses, all six immunostain-positive, invasive tumors were sequenced in exons 5—8; nonsense mutations were found in two. Since a point mutation provides a molecular marker, microdissection and sequence analysis were conducted on the precursor lesions from those two tumors. An advanced third lesion (i.e., carcinoma in situ from case HE90-27) was also sequenced, although the adjacent tumor contained a wild-type sequence.

Noncancerous esophageal tissues from the first patient, HE90-72, contained extensive dysplastic mucosa, but invasive cancer was not detected in serial sections through 255 µm of the affected mucosa. In the available cross-section, the abnormal cells extended through a 0.9-cm-long segment of the mucosa. If this were the diameter of a round lesion, the area would be approximately 0.64 cm². In about 75% of the abnormal mucosa, only the lower and middle levels were occupied by cells with enlarged, darkly stained, irregularly shaped nuclei; this morphology is typical of moderate dysplasia (Fig. 1A). In the remainder of the affected mucosa, the irregular nuclei filled the mucosa in a manner typical of severe dysplasia; in some places, there was an abrupt transition between normal and abnormal mucosa (Fig. 1C). Immunohistochemical analysis showed high levels of p53 protein in the irregular nuclei, but the cells in the submucosa, superficial layers, and adjacent normal mucosa were unstained (Fig. 1, B and D). Microdissection and sequence analysis of two different areas, one moderately and one severely dysplastic, showed the same heterozygous G:C to T:A transition in the first position of codon 272, GTGval > T/GTG<sup>val</sup>val (Table 1 and Fig. 2). In contrast, analysis of tissues containing the invasive tumor from this patient demonstrated a heterozygous C:G to T:A transition in the first position of codon 282, CGG<sup>wt</sup> > TGG<sup>mut</sup> (Table 1 and Fig. 2). Persistence of the wild-type allele indicates that either the tumor contains normal and mutated copies of the p53 gene or that nonneoplastic tissues associated with the tumor contributed the normal allele. Histological review showed tumor-infiltrating muscle bundles composing the esophageal wall plus extensive inflammatory infiltrates and fibrous tissue. While microdissection can eliminate gross contamination by nonneoplastic tissues, it is most likely in this case that the wild-type allele was contributed by nonneoplastic tissues intermingled with the tumor. Normal tissues had wild-type sequences at both codons (Fig. 2). The finding of a p53 mutation in the preinvasive lesions demonstrates that mutation may occur before invasion, and the expansion of the cell population through a macroscopic region of the mucosa suggests that the mutation confers a growth advantage. Whether deletion of the remaining wild-type allele of p53 is sufficient to cause invasion is unknown. The timing of p53 mutation in this single example is similar to the molecular agenda for colorectal cancer (16), although one could argue that mutation at the stage of moderate dysplasia (i.e., HE90-72) is earlier than the late adenoma stage in the colorectal model.

In a second case, HE90-43, a homozygous sequence in exon 5 at codon 175 (CGC<sup>wt</sup> > CAC<sup>mut</sup>) was found in the invasive tumor, but no exon 5 mutation was found in the adjacent dysplastic mucosa. This base change indicated a G:C to A:T transition plus a deletion of the second allele in the invasive tumor. There were extensive precursor lesions including mild, moderate, and severe dysplasia, and both the invasive tumor and adjacent precursor lesions contained high levels of p53 protein. There are two main interpretations of these data: (a) The precursor lesions contain wild-type p53 sequence and the protein accumulated by a nonmutational mechanism (e.g., complex formation with viral or cellular oncoproteins); in this scenario, p53 mutation would correlate with invasion. (b) The precursor lesions (some within microns of the invasive tumor) contain p53 mutations in unexamined exons and may represent independent neoplasms.

In the third case (i.e., HE90-27), both the invasive tumor and the contiguous progenitor lesions contained high levels of p53 protein and wild-type DNA sequence in exons 5—8. This case illustrates a neoplastic pathway which is independent of p53 mutation in the highly conserved sequences. Although accumulation of p53 protein frequently reflects a nonconservative point mutation, exceptions to this rule have been observed (12, 17—20). Often only the highly conserved sequences in exons 5—8 are examined, but in some human tumor cell lines expressing detectable levels of p53 protein, exons 2—11 have been sequenced without finding a mutation (21, 22). Possible nonmutational mechanisms for p53 protein accumulation include (a) inactivation of an enzymatic pathway responsible for p53 protein degradation (23), (b) stabilization of wild-type protein through complex formation with a DNA tumor virus protein (4—7) or a cellular oncoprotein, (c) aberrant posttranscriptional modification conferring extended protein half-life, and (d) altered expression of the p53 gene by cellular transcriptional regulators (8). Regardless of the mechanism, it is notable that the excess protein provides a distinct marker of both the preinvasive lesion and the invasive tumor (Fig. 3).

These data indicate that p53 alterations may occur at different time points during the development of esophageal SCC. As illustrated by case HE90-72, p53 mutation can occur in an early stage of moderate dysplasia and may confer a growth advantage to the preinvasive cells. Loss of the remaining wild-type allele and/or accumulation of additional mutations may produce an invasive subclone within the original population. The second and third cases show that excess p53 protein may accumulate in the absence of either invasion or apparent p53 mutation. Further studies will be needed to define the predominant stage of p53 alteration in the pathogenesis of esophageal SCC; however, these findings suggest that the timing of p53 protein accumulation may be suitable for an intermediate biomarker.
Fig. 1. p53 immunohistochemical analysis in moderate to severe dysplasia (case HE90-72). A and B, serial sections of moderately dysplastic squamous mucosa. The lower and middle epithelial levels contain cells with enlarged, darkly stained, irregularly shaped, dysplastic nuclei (A; H&E). Immunohistochemical analysis shows
p53 MUTATION AND PROTEIN ACCUMULATION DURING CARCINOGENESIS

Table 1  p53 sequence analysis of preinvasive lesions and invasive tumors

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histology</th>
<th>Codon</th>
<th>Sequence</th>
<th>Amino acid</th>
<th>Histology</th>
<th>Codon</th>
<th>Sequence</th>
<th>Amino acid</th>
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<td>HE90-72</td>
<td>MSD</td>
<td>272</td>
<td>GTG</td>
<td>VAL</td>
<td>SCI</td>
<td>282</td>
<td>CCG</td>
<td>ARG</td>
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<td>HE90-43</td>
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<td>282</td>
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<td>ARG</td>
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<tr>
<td>HE90-27</td>
<td>CIS</td>
<td>WT</td>
<td></td>
<td></td>
<td>SCI</td>
<td>WT</td>
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</tbody>
</table>

* All precursor lesions and invasive tumors selected for analysis contained high levels of p53 protein.
* WT, wild type; MUT, mutant; MSD, moderate to severe dysplasia; CIS, carcinoma in situ.
* Heterozygous mutation showing two bands in the first base position.
* The invasive tumor was previously reported as HE90-15 in Ref. 12.

An intermediate biomarker should be present before or during the early stages of invasion to minimize the occurrence of false negative results. The timing of p53 mutation and protein accumulation has been studied best in colorectal adenocarcinoma. This cancer develops in several well-defined stages, and p53 mutation most frequently occurs between the stages of late adenoma and invasive carcinoma (16, 24). This timing is favorable for a tumor marker because a positive result would signal either a late adenoma or an invasive tumor; typically both are treated by surgical resection.

In addition to the colorectal model, recent studies indicate that alterations in the p53 gene and/or its protein product frequently occur in preinvasive lesions of the esophagus, skin, testis, and breast. In the esophagus, p53 mutations were found commonly in Barrett’s epithelium, which is considered a precursor to adenocarcinoma of that site (25). In human skin cancer, elevated levels of p53 protein were seen in some squamous dysplastic lesions as well as in 16% of cases of Bowen’s disease, a preinvasive form of epidermal SCC (26). In a murine model for epidermal carcinogenesis, p53 deletion and mutation were found in SCC but not in precursor papillomas (27). Immunohistochemical analyses have shown elevated levels of p53 protein in preinvasive lesions of the human testis (28) and breast (29, 30). These data suggest that in these tissues, p53 mutations and/or protein accumulation frequently occur near the time that cells in precursor lesions become malignant and invade surrounding tissues.

However, the timing of p53 alteration may be different in other tumors. For example, in bladder cancer, the incidence of p53 mutation is greater in high-grade and invasive tumors than in superficial and low-grade tumors. Similar observations have been made in low- and high-grade tumors of the brain and thyroid (31, 32). These findings suggest that, in some tissues, p53 mutation may contribute more to tumor progression than the conversion to malignancy. These data suggest that the agenda for p53 alteration may vary with tumor type and underscore the need for analysis of preinvasive lesions in a variety of tissues.

Multifocal Cancer. Esophageal cancer may develop in multiple sites either simultaneously or sequentially (33). Two theories have been advanced to explain these so-called synchronous and metachronous carcinomas. The monoclonal neoplasia theory holds that progeny from a single transformed cell may spread to produce multiple tumors (34–36), while the “field defect” model predicts that independent tumors develop from the genotoxic effects of carcinogens (37, 38). If a patient is treated for one SCC and later develops a second, then there are two possibilities, (a) the second SCC is a new, independent neoplasm (i.e., “field defect” model) or (b) the second SCC developed from an occult focus of the first cancer which survived the initial treatment (i.e., monoclonal neoplasia theory). The distinction between these mechanisms is important both for defining multitstage carcinogenesis and for refining cancer treatment protocols. For example, if the first tumor recurs, the treatment may have been ineffective, or progeny from the primary tumor may have spread through the mucosa or lymphatics to produce an occult tumor which survived the treatment regimen. Alternatively, the genotoxic effects of tobacco, alcoholic beverages, and certain dietary factors may have caused a “field defect” predisposing the entire esophageal mucosa to develop multiple independent cancers (39). In this setting, there might be multiple neoplasms in the early stages of development, and chemoprevention might be effective (see Comments in Ref. 40). The proof of these theories required demonstration of the clonal nature of cancer, which was not established until the advent of molecular genetics (34–36).

staining in the dysplastic nuclei, but the submucosa and superficial layers are unstained (B; CM-I; no counterstain). C and D, serial sections of severely dysplastic squamous mucosa (right) with abrupt transition to normal mucosa (left) (C; H&E). Immunostain shows p53 protein in the abnormal mucosa, but the normal mucosa is unstained (D; CM-I; no counterstain). ×200.
Recent analyses of multifocal hepatocellular carcinomas support the field defect model and the development of multiple, independent cancers within a single patient (41). The presence of different p53 mutations in two esophageal neoplasms from case HE90-72 also may be interpreted in this light, but the possibility remains that progeny from a single transformed cell later developed independent p53 mutations. Conversely, there is evidence supporting monoclonal neoplasia. For example, we recently examined a bronchial SCC which contained extensive dysplasia near the invasive cancer (42). Immunohistochemical analysis showed high levels of p53 protein in both dysplastic and invasive areas. Microdissection and DNA sequence analysis of three separate foci revealed the same p53 missense point mutation in preinvasive, microinvasive, and fully invasive lesions. Similar evidence has been found in multifocal carcinomas of the bladder (43), renal pelvis (44), and liver (41). In summary, these data support both the field defect and the monoclonal neoplasia models for carcinogenesis. Further studies are needed to determine the frequency of each in esophageal SCC and in other cancers.

Acknowledgments

We thank Ricardo V. Dreyfuss for expert photomicrography and Dorothea Dudek for editorial assistance.

References

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