p53 in esophageal adenocarcinoma: A critical reassessment of mutation frequency and identification of 72Arg as the dominant allele

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Abstract. p53 alterations have been implicated in the progression of Barrett's esophagus to esophageal adenocarcinoma. However, the wide range of reported p53 alteration frequencies in esophageal adenocarcinoma makes using p53 as a marker of malignant transformation of Barrett's esophagus problematic. To determine the utility of p53 in Barrett's esophagus monitoring, the frequency of p53 alteration was critically reassessed using esophagectomy specimens of 40 cases of esophageal adenocarcinoma, including 10 with Barrett's esophagus and high-grade dysplasia, 8 with low-grade dysplasia and 7 with no dysplasia. DNA was extracted from tumor cells isolated by laser capture microdissection to maximize the assay sensitivity and mutations in exons 4-8 of *p53* were determined by PCR direct sequencing. Mutations in *p53* were identified in 75% (30/40) of the esophageal adenocarcinoma. p53 protein overexpression, detected by immunohistochemistry, was found in 58% (23/40) of the esophageal adenocarcinoma, 60% (6/10) of Barrett's esophagus with high-grade dysplasia, 12% (1/8) of Barrett's esophagus with low-grade dysplasia, and 0% of Barrett's esophagus without dysplasia. In addition to the mutations, a predominance of the 72Arg allele (89% homozygous) was found over the 72Pro allele in this series. *p53* mutation frequency in this study was higher than reported in most of the literature and DNA sequencing detected more p53 alterations than immunohistochemical staining. However, p53 appeared to be a late marker in the neoplastic transformation, and no p53 change was found in ~25% of the adenocarcinoma. We concluded that p53 is insufficient as a single marker for Barrett's esophagus monitoring but may be useful as part of a panel due to its high specificity.

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Introduction

The *p53* gene has been implicated in many human solid tumors where its mutation results in the loss of wild-type p53 function as a tumor suppressor gene (1,2). Previous studies have demonstrated *p53* gene mutations and protein accumulation in esophageal adenocarcinoma (EAC) and its presumed precursor Barrett's esophagus (BE). The reported frequency of *p53* mutation in EAC shows a wide range of 7-82% (3-16). Immunohistochemistry (IHC) for p53 has also been used as an indirect method of detecting *p53* mutation and p53 immunoreactivity has been reported in 53-87% of EAC (5,9,12,14,17-20). Mutations in *p53* have also been found in BE with high-grade and low-grade dysplasia, also with variable frequencies (3-7,9,10,12,15). In parallel, p53 protein accumulation has been detected by IHC in 55-100% of BE with high-grade dysplasia (HGD), 0-71% of BE with low-grade dysplasia (LGD) but not in metaplastic BE (12,17-20). These results suggested that *p53* gene alterations contribute to the development of EAC and are likely to precede the development of invasive carcinoma in patients with BE. For this reason, evaluation of *p53* mutation and/or over-expression has been proposed as a potential marker that might be useful in the clinical monitoring of patients with BE (7,12,15).

For this monitoring purpose, however, the wide variation in the prevalence of p53 alteration reported in different studies is problematic. A previous study (16) demonstrating mutations in only 18% of EAC, for instance, implies that p53 has no value in this regard, which also challenges the theory that p53 plays a major role in the progression to carcinoma. To determine whether p53 can be a useful marker for BE monitoring, it is thus critical to reassess and document the frequency of *p53* alteration in EAC, followed by similar studies in HGD and LGD if warranted. The aim of this study was thus to accurately determine the frequency of *p53* mutation in resected specimens of EAC, using laser capture microdissection (LCM) and direct sequencing to maximize the sensitivity for detection. IHC was also performed to evaluate EAC and adjacent BE with dysplasia, and the results were correlated with mutation status to determine whether IHC can be a valid alternative in detecting p53 changes for this setting.

Materials and methods

Tissue collection. Esophagogastrectomy specimens of 40 cases of EAC were identified from the surgical pathology files of Weill Medical College of Cornell University, New York, NY. Blocks containing carcinoma and adjacent BE were selected. Adjacent to the carcinoma, BE with HGD was identified in 10 cases, BE with LGD was identified in 8 cases and BE without dysplasia was identified in 7 cases. Sections (5 μm) from formalin-fixed paraffin-embedded blocks were prepared for IHC and LCM for DNA analysis. The procurement of tumor samples for this study was approved by our institutional review board.

LCM and DNA extraction. LCM was performed using an Arcturus AutoPix LCM system. The paraffin section was deparaffinized and stained with hematoxylin and eosin. Approximately 2000-4000 cells were collected from EAC through microdissection per case from a single 5-μm section. The DNA extraction was performed using a PicoPure DNA extraction kit (Arcturus), following the manufacturer's protocol. The final DNA preparation measured 50 μl, and 5 μl was used for each 25-μl PCR.

PCR amplification and mutation analysis. Nested PCR was utilized for amplification of exons 4-8 of the *p53* gene. The primer sequences used were as previously described (21). The first PCR was performed for 35 cycles in a 25-μl reaction, each cycle consisting of 95˚C denaturing (15 sec), 60˚C annealing (1 min) and 72˚C extension (1 min). The nested PCR was performed in a 100-μl reaction under the same conditions, using 1 μl of the 1:10 diluted first PCR product as a template. PCR products were sequenced bidirectionally using the Big Dye Terminator chemistry and Applied Biosystems Automated 3730 DNA analyzer (Biotechnology Resource Center, Cornell University, Ithaca, NY) and analyzed using Mutation Surveyor™ software. All mutations were confirmed by duplicated PCR analysis.

Immunohistochemistry. Sections (5 μm) adjacent to those used for DNA extraction were cut from each case. Slides were deparaffinized by baking at 60˚C, then by placing in xylene solution. Endogenous peroxidase was inactivated with 4.5% hydrogen peroxidase in methanol. After antigen retrieval in autoclave at 105˚C for 5 min with Dako Cytomation target retrieval solution, sections were incubated for 1 h with the primary p53 antibody (Biogenex, 1:50 dilution). This was followed by incubation in EnVision system labeled polymer (anti-mouse), DAB, and hematoxylin counterstaining. Intensity of nuclear staining was graded as 0, 1^+, 2^+, or 3^+. Percentage of tumor nuclei staining was categorized as negative, focal (0-10%), intermediate (11-50%) and diffuse (51-100%). More than 10% nuclear staining of the carcinoma was considered positive for overexpression.

Results

p53 mutation analysis. *p53* mutation was analyzed in EAC. HGD and LGD were not analyzed due to their small foci and insufficient DNA yields. Exons 5-8 were successfully

Table I. Location of mutations in the *p53* gene, with nucleotide and amino acid changes and immunohistochemistry (IHC) results.

Case	*p53* mutation analysis			Amino acid change	IHC
	Exon	Codon	Nucleotide change		
1	4	36	G->A	P->P	+
	4	110	C->T	R->C	
	7	253	C->T	T->I	
6	7	248	C->T	R->W	+
9	7	242	G->T	C->F	+
11	8	282	C->T	R->W	+
12	5	176	G->A	C->Y	+
13	7	248	C->T	R->W	+
15	4	89	C->T	P->S	+
16	4	34	C->T	P->L	+
	5	167	C->T	Q->X (termination)	
	7	248	C->T	R->W	
	8	279	G->T	G->W	
17	5	177	C->A	P->T	+
18	7	249	G->C	R->T	+
22	5	154	G->A	G->S	+
	7	249	A->G	R->G	
26	8	273	G->A	R->H	+
30	6	214	A->G	H->R	+
31	4	241	T->G	F->V	+
32	7	248	G->A	R->Q	+
35	4	162	C->T	P->S	+
	6	213	A->G	R->R	
36	6	216	T->G	V->G	+
37	5	153	C->T	P->S	+
	7	248	C->T	R->W	
38	5	151	C->T	P->S	+
40	8	282	C->T	R->W	+
2	5	155	C->T	T->T	-
7	5	155	C->T	T->T	-
	6	213	A->G	R->R	
4	5	151	C->T	R->C	-
	5	154	C->T	G->G	
	7	236	C->A	Y->X (termination)	
	8	274	G->A	V->I	
10	4	45	T->C	L->P	-
	6	213	C->T	R->X (termination)	
39	4	119	C->A	A->A	-
	6	196	C->T	R->X (termination)	
25	5	155	C->deletion	Frameshift	-
5	4	58	C->T	P->L	-
	4	102	C->T	T->I	
	5	175	C->T	R->C	
	5	177	C->T	P->P	
	8	274	G->A	V->I	
19	4	76	C->T	A->V	-
	4	98	C->T	P->S	
20	4	74	C->T	A->V	-
	5	175	G->A	R->H	
29	6	198	G->A	E->K	-

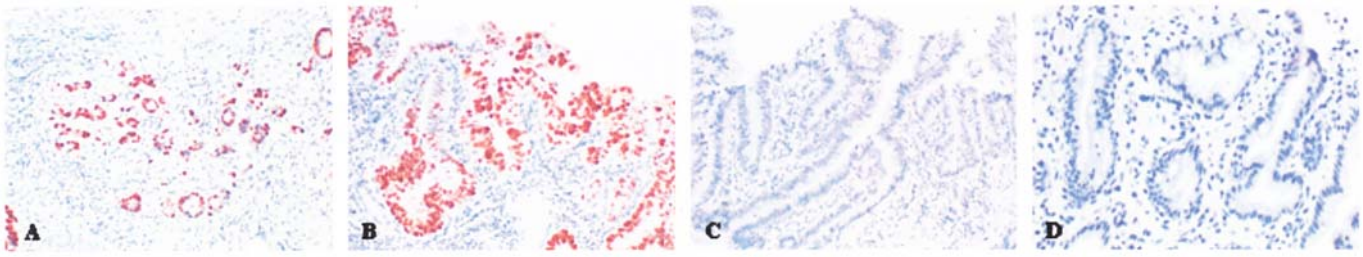

Figure 1. Diffuse, intense p53 nuclear immunoreactivity in esophageal adenocarcinoma (A) and in Barrett's esophagus (BE) with high-grade dysplasia (B). No p53 reactivity in BE with low-grade dysplasia (C) and in BE without dysplasia (D).

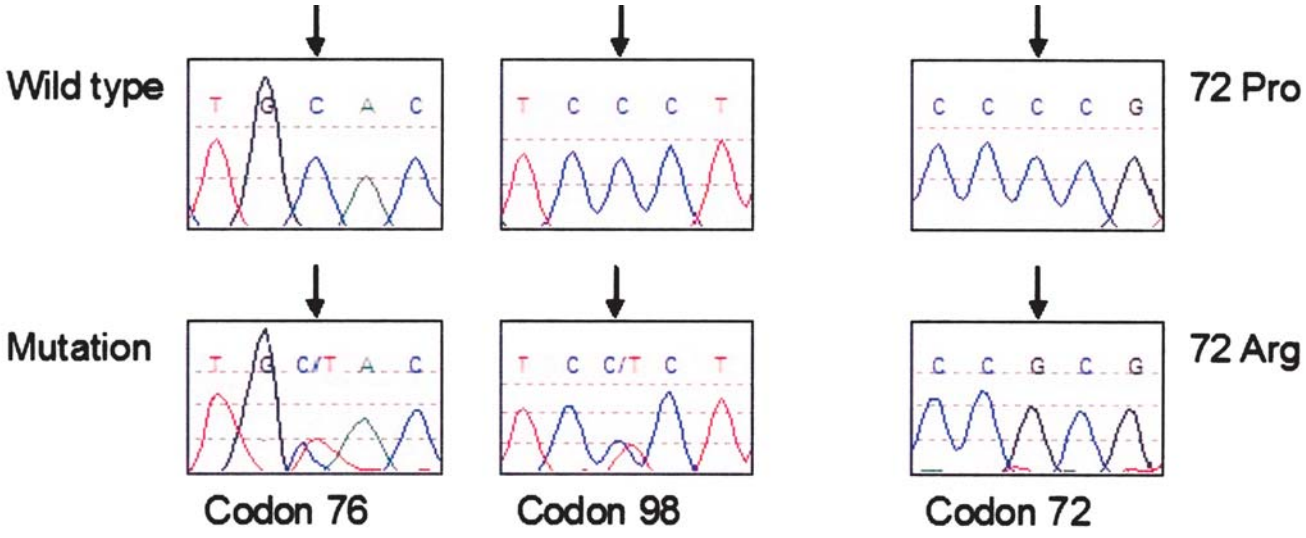

Figure 2. Representative *p53* mutations and allelic polymorphism. DNA sequencing chromatograms of case 19 (mutation-positive, IHC-negative) were shown in the bottom panel, showing two mutations in exon 4 (codons 76 and 98). Both mutations involved one allele, with one wild-type allele remaining. This case was also homozygous for 72Arg (CGC) polymorphism, versus the other common allele 72Pro (CCC).

amplified and sequenced in all 40 EACs. Exon 4 was analyzed in 37/40 cases, with PCR amplification failing in 3 cases, presumably due to the larger (353 bp) amplicon size of this exon. Mutations in the *p53* gene were identified in 75% (30/40) of EAC (Table I). In 12 of these cases, more than one mutation was found, bringing the total number of mutations to 51. The mutations were distributed as follows: 27% (14/51) in exon 4, 27% (14/51) in exon 5, 14% (7/51) in exon 6, 20% (10/51) in exon 7 and 12% (6/51) in exon 8. The 51 mutations consisted of 37 missense mutations, 4 nonsense mutations, 9 silent mutations and 1 deletion; 82% (42/51) were G:C to A:T base transitions and 14% (7/51) were G:C to T:A base transversions. Of the 30 cases with *p53* mutations, the mutation resulted in amino acid substitutions in 77% (23/30), termination in 13% (4/30), no amino acid change in 6% (2/30), and frameshift in 3% (1/30) of cases. Fig. 2 shows an example of *p53* gene mutation detected in EAC DNA.

p53 protein overexpression. Nuclear expression for p53 by IHC was detected in 58% (23/40) of EAC (Fig. 1A). In 22 of the 23 cases, >10% of the tumor showed nuclear reactivity with 2-3^{+} intensity; the last case showed less intense (1^{+}) reactivity, but in >10% of cells. In cases with adjacent BE with HGD, 60% (6/10) were reactive for p53 (Fig. 1B). Only one of 8 (12%) cases with BE with LGD overexpressed p53 (Fig. 1C). All cases of BE without dysplasia were negative for p53 IHC (Fig. 1D).

Table II. Relationship between *p53* mutation and protein expression.

		p53 IHC expression		
		+	-	Total
p53 mutation analysis	+	20	10	30
	-	3	7	10
	Total	23	17	40

Correlation between p53 mutation and protein expression. Of the 30 EAC with *p53* mutation, 20 also showed p53 expression by IHC (Table II). Of the 10 cases showing *p53* mutation but no overexpression, 4 were missense mutations, 3 were terminations, 2 were silent mutations and one was a deletion. On the other hand, mutations were not detected in three cases that overexpressed p53.

Identification of a dominant 72R allele in exon 4. In addition to the mutations, a previously reported (26) single-nucleotide polymorphism (SNP) was observed in exon 4. This SNP (C versus G) results in p53 protein with either arginine (72R) or proline (72P) at codon 72. Of 40 cases, exon 4 was successfully amplified and sequenced in 37 cases; 33 (89%) were found to be homozygous for 72R, 3 were homozygous for 72p, and only one was heterozygous for 72P/72R. The calculated allelic frequency that encodes 72R is thus 0.905 in this population.

Discussion

The reported frequencies of *p53* gene mutation and p53 immunoreactivity vary considerably for EAC and BE. In our series, DNA sequence analysis detected *p53* mutations in 75% (30/40) of EAC. This frequency is higher than the 18% found in a study that also used LCM and DNA sequencing (16), and higher than the 43% (150/350) calculated from a total of 14 studies in the literature (3-16). This discrepancy can be attributed to multiple reasons. First, earlier studies did not use LCM to study a pure population of tumor cells and mutations could have been underestimated due to DNA contamination by non-neoplastic cells. Second, many studies (3,5,7,8,10,11-15) used indirect methods such as SSCP, electrophoresis or RFLP to screen for *p53* mutations before sequence analysis. The initial screening may have missed some mutations, resulting in false-negative results. Third, our study included sequencing for exon 4, which has previously been studied in only one other report (13). Twenty-seven percent of the mutations were found in exon 4, which is different from previous data that showed >90% of *p53* mutations in exons 5-8 in solid tumors (2). Although the reason for this is unclear, we believe that the possibility of false-positive results due to PCR infidelity is minimal, as the mutation peaks were of significant amplitude in all cases, as would be expected when tumor cell populations with minimal non-neoplastic contamination were analyzed.

Eighty-two percent of the mutations identified were G:C to T:A base transitions, some at CpG dinucleotides, indicating likely mutational 'hot spots' in EAC. Endogenous mutagenic mechanisms, such as deamination of 5-methylcytosine to thymidine followed by a faulty repair procedure, have been suggested to account for these mutations (22). A similar predominance of these transitions occurred in other studies of EAC (4-7,15), colon (2) and gastric (23) carcinomas while transversion mutations in *p53* are more common in smoking-related squamous cell carcinomas of the esophagus (24,25). These observations are consistent with the notion that different mechanisms underlie the development of esophageal carcinomas of squamous or glandular origin.

One interesting finding was that we found more than one *p53* mutation in a significant number of cases. As a tumor suppressor gene, the conventional notion is that one *p53* gene is often lost by deletion whereas the second allele is inactivated by mutation. Our finding, in contrast, would suggest that p53 inactivation by mutations in both alleles might occur more often in EAC than was previously documented. Alternatively, these multiple mutations may have accumulated in the same allele, reflecting the genetic instability of the tumor genome and emergence of new mutations during tumor progression. Cloning and other experiments to further evaluate this issue would be of interest and are ongoing.

The prevalence of p53 protein accumulation in EAC is also variable, ranging from 57-83%. In the current series, p53 nuclear accumulation was detected in 58% of EAC, comparable with the frequency in the literature. A close correlation between missense mutation and p53 protein expression was seen, as was recognized previously (5,9,12). Excluding cases with frameshift mutations and nonsense mutations that would not be detectable by IHC, true discordant cases included 4 mutation-positive/IHC-negative and 3 IHC-positive/mutation-negative cases. Thus, the disconcordance rate is 18% (7/40), which is similar to data reported previously (5,12) and shows that utilizing immunohistochemical p53 protein expression as a surrogate marker for *p53* gene mutation is suboptimal.

Studies have reported a close association between p53 overexpression in adenocarcinoma and in adjacent BE with HGD (5,12,17-20). In the present study, p53 protein expression was seen in 60% of BE with HGD, but rarely in LGD, suggesting *p53* mutation as a late rather than early event in this transformation process.

Our study demonstrated *p53* gene mutations in ~75% of adenocarcinoma of the esophagus, detectable by IHC at ~60% of EAC and BE with HGD. Although this confirms the importance of p53 in this neoplastic pathway, this level of sensitivity is insufficient as a screening test for triaging patients for endoscopic follow-up. However, given the specificity of this genetic change in neoplasm and an estimated sensitivity of approximately 50% in HGD, p53 overexpression detected by IHC could still be a potentially useful marker, particularly if used as one of a panel of markers. For that purpose we have evaluated several additional potential markers, including p16, cyclin D1, and bcl-2. However, none of them appeared to provide additional sensitivity and/or specificity (data not shown), and future studies to identify better markers would be needed.

Of interest was our finding of the allele that encodes 72R (versus 72P) in exon 4 as the predominant SNP in this group of patients, with the calculated allelic frequency of 0.905. This polymorphism has been found to vary in allelic frequency depending on race and latitude. The frequency of the 72R allele was found to be high in Swedish and Finns (0.83 and 0.76), intermediate in American whites (0.66), and low in American blacks and African blacks (0.39 and 0.37) (26). Although the frequency of 72R in the patient population of our hospital is unknown, one would estimate it to be between 0.37 and 0.66 based on the US population study above, and the 0.905 observed frequency in these EAC patients thus represents an overrepresentation of this allele. This SNP has been shown to be biologically important, with the 72R form being implicated as more pro-apoptotic than the 72P form of p53 (27,28). However, whether the 72P or 72R allele is the more adverse genotype is controversial. The 72P allele has been associated with a higher risk for lung cancer (29), nasopharyngeal carcinoma (30) and earlier development of colorectal cancer in patients with the HNPCC syndrome (31). In contrast, the 72R allele has been associated with increased risk for bladder (32) and gastric cardia (33) cancer. In addition, it has also been reported that squamous cell carcinoma that retained 72R appeared to be more responsive to chemo-

radiotherapy (34). In this regard, this dominance of 72R in EAC could potentially be of both biological and therapeutic significance. It is unclear at present whether the 72R allele is a predisposing factor for BE (intestinal metaplasia) or for malignant transformation in patients with BE, and a large-scale study of patients without BE, with BE, and with BE and EAC would be necessary to further explore the biological significance of this finding.

In conclusion, this study showed a higher frequency of p53 mutations in EAC than was reported in most previous literature, but only as a late event in carcinogenesis, and 72R allelic polymorphism was found to be of potential biological significance in the development of BE and/or EAC. However, based on the observed frequency one would conclude that p53 alteration, detected either by DNA sequencing or by immunohistochemistry, is not by itself an adequate marker for patient selection in BE monitoring.

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