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Barrett's Esophagus

The Significance of p53 in Clinical Practice

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Objective

The authors provide an updated review the molecular biology of the p53 tumor suppressor gene with reference to its role in the malignant degeneration of Barrett's esophagus.

Summary Background Data

Appreciation of the function of the tumor suppressor gene p53 has given new insight into regulation of the cell cycle, and the gene appears to play an important role in many solid tumors. Esophageal adenocarcinoma is increasing in frequency in the western world at an alarming rate and is unique because there is a clear metaplasia (Barrett's mucosa)/dysplasia/carcinoma sequence. p53 malfunction arises as an early event in this carcinogenic process and has been demonstrated in patients with nondysplastic Barrett's metaplasia. The possible causes of p53 malfunction in this setting are discussed. The most reliable method for the detection of p53 mutations is DNA sequencing. p53 immunohistochemistry appears too insensitive to act as a reliable marker for the presence of a mutation and cannot be used as a reliable marker for the future development of cancer.

Conclusions

High-grade dysplasia within Barrett's mucosa remains the best clinical predictor of adenocarcinoma. The mutational spectrum observed in these tumors should provide clues to their etiology.

Barrett's esophagus is an acquired condition in which the squamous epithelium of the distal esophagus is replaced by a metaplastic columnar epithelium characterized by the presence of goblet cells. It represents a peculiar form of healing that can occur at any time in patients with reflux esophagitis. Barrett's esophagus is a premalignant lesion because it is the initiating factor of a metaplasia-to-dysplasia-to-carcinoma sequence. It offers an ideal opportunity for the investigation of the genesis of esophageal carcinoma. Endoscopic innovations make the esophagus easily accessible for inspection and biopsy. The premalignant lesion is sampled for diagnosis but not removed, in contrast to the case of colonic adenomas. This permits follow-up of the premalignant tissue. Particularly disturbing is the increase in the frequency of Barrett's adenocarcinoma in the United States during the last 15 years.[1,2] The incidence of Barrett's adenocarcinoma is rising faster than that of any other tumor and in many

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centers, esophageal adenocarcinoma outnumbers squamous carcinoma.[3]

Appreciation of the function of p53 is a major advance in the understanding of the molecular biology of cancer, and there are multiple reports on p53 in Barrett's esophagus in the scientific literature. Because of the rapid advances in the field of molecular biology, many clinicians are unaware of the progress that has been made at the molecular level. The aim of this article is to discuss recent findings regarding the nature and function of p53 and to outline the potential role of p53 in the malignant transformation of Barrett's esophagus.

BACKGROUND

What Is p53?

The p in p53 stands for protein, and 53 represents its molecular weight in kilodaltons. p53, when used alone, refers to the protein whereas the gene that codes for this protein is referred to as "the p53 gene." The normal gene is referred to as the wild-type, in contrast to the mutant gene that results from a mutation. Similarly, the normal protein is the wild-type and the abnormal protein, the mutant. p53 was first discovered in 1979. Little attention was paid to p53 until 1989, when point mutations in p53 were detected in colorectal cancers. Since then, there has been an explosion in interest in p53, which culminated in it being named molecule of the year by *Science* in 1993. Vogelstein discovered that p53 plays a central role in many critical tumorigenic processes, whereas Harper and Elledge outlined the role of p53 in the regulation of the normal cell cycle.[4] These two breakthroughs link the basic biology of the cell cycle to the process of tumorigenesis.[4] p53 mutations are the most common mutations found in cancer, with roughly one half of all cancers having a p53 mutation. In most patients with cancer, the p53 mutations are acquired; in contrast, one of the hereditary cancer syndromes is due to a germ-line mutation in the p53 gene (Li-Fraumeni syndrome).[5]

The Role of p53 in Normal Cells

To understand the role of p53 in normal cells, it is necessary to understand some basic concepts—the cell cycle, an oncogene, a tumor suppressor gene, and transcription factors. Cell division consists of four phases called M, G1, S, and G2. After mitosis (the M phase) cells enter the first gap phase (G1). Next is the phase of DNA synthesis (S phase), and finally, the G2 phase, before mitosis. During the cell cycle, there are checkpoints that regulate cell division.[6] If certain criteria are not met, the cell cycle stops. One such checkpoint is at the G1-S transition, before the cell replicates its DNA. Errors in the regulation of the cell cycle appear to play a major role in the transformation of a "normal" cell to a "tumor" cell. p53 regulates the cell cycle, and loss of this regulation is one of the reasons that p53 malfunction contributes to many cancers.

Wild-type, *i.e.,* normal p53, behaves as a tumor suppressor gene. Tumor suppressor genes are segments of DNA that normally are present in healthy cells and suppress tumorigenesis. Oncogenes are segments of DNA normally present in the healthy cell and serve essential functions in regulation of cellular growth. Excess activity of these genes promotes tumorigenesis. There is a balance between the tumorigenic effect of oncogenes and the antitumorigenic effect of tumor suppressor genes. Excess activity of oncogenes or loss of the function of tumor suppressor genes upsets this balance in favor of tumorigenesis.

A transcription factor is a protein that acts as a component of the "transcription machine" that copies DNA into RNA.[7] Transcription factors act as switches that turn on transcription of DNA if all the other components of the "transcription machine" are present. The p53 protein is a transcription factor for many genes. Recognition of a gene for induction of transcription by p53 depends on the presence of a specific DNA sequence in the upstream regulatory region of the target gene. Expression of genes that contain this specific sequence will be induced by binding of p53 to this regulatory region. This is how p53 exerts many of its effects. Examples of genes that p53 acts as a transcription factor for include WAF1 (which encodes p21), GADD45 (growth arrest and DNA damage inducible), Bax, and the oncogene Mdm-2 (murine double minute). Consequently, the main function of p53 in normal cells is "guardian of the genome." It also plays a role in angiogenesis.

Guardian of the Genome

It is not known how the p53 gene is turned on, but DNA damage appears to be the stimulus. When a cell sustains damage to its DNA, p53 attempts to repair the DNA (Fig. 1). If damage is severe and repair impossible, then the cell is killed (apoptosis).

Three molecular mechanisms by which p53 accomplishes its "guardian" function have been discovered.

G1-S Checkpoint

One of the genes that p53 acts as a transcription factor for is WAF1, whose product (p21) regulates cyclin-dependent kinases. Cyclin-dependent kinases are enzymes involved critically in control of the cell cycle. Binding of p21 to cyclin-dependent kinases leads to slowing of the cell cycle at the G1-S transition. This is a checkpoint in

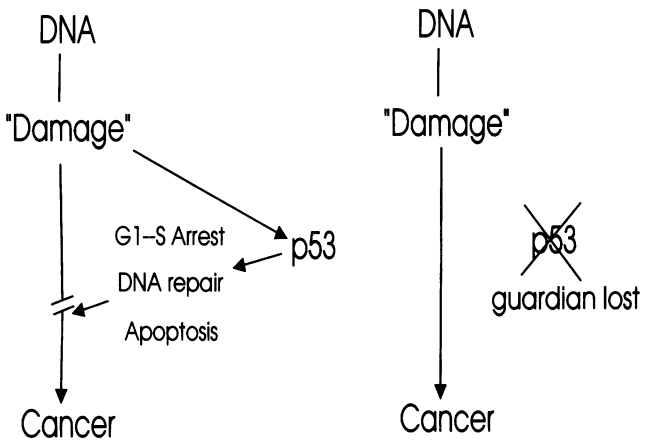

Figure 1. p53 is the guardian of the genome. When DNA is damaged, p53 is turned on. p53 then stops the cell cycle at the G1-S checkpoint and stimulates DNA repair. If damage is severe, p53 induces apoptosis. This prevents the cell cycling in the presence of DNA damage. When the guardian is lost, the cell continues cycling with increasing genomic instability and progression of tumorigenesis.

the cell cycle to allow the cell to rest and repair damaged DNA before DNA synthesis.

Increase in DNA Repair

Besides increasing time for DNA repair by slowing the cell cycle at the G1-S transition, p53 stimulates DNA repair by interacting with proliferating cell nuclear antigen and ERCC3. Proliferating cell nuclear antigen is required for copying and repair of DNA, whereas ERCC3 is an excision repair enzyme that participates in the removal of damaged DNA. Binding of p53 to ERCC3 promotes its excision activity. p53 affects proliferating cell nuclear antigen by acting as a transcription factor for p21 and GADD45.[8,9] GADD45 complexes with proliferating cell nuclear antigen and stimulates its activity whereas binding of p21 prevents replication of long stretches of DNA but allows replication of short stretches. By its interactions with p21, ERCC3, GADD45, and proliferating cell nuclear antigen, p53 turns on the DNA repair machinery.

Induction of Apoptosis

If the DNA damage is severe, p53 pushes the cell into programmed cell death. p53 interacts with two of the main mediators of apoptosis, the proteins Bcl2 (B cell lymphoma) and Bax. Bax activity augments apoptosis and Bcl2 suppresses it. p53 has been shown to have effects on both Bcl2 and Bax expression. In the case of Bcl2, p53 reduces expression, and in the case of Bax, p53 acts as a transcription factor to induce expression of Bax.[10] It is unknown why p53 expression sometimes results in slowing of the cell cycle at the G1-S checkpoint and sometimes results in apoptosis.

Angiogenesis

To sustain itself, a tumor must induce the growth of new blood vessels. This angiogenesis is one of the hallmarks of cancer. Patients with the Li-Fraumeni syndrome inherit a mutation in one of the alleles of the p53 gene. Investigators working on cultured fibroblasts from patients with Li-Fraumeni syndrome discovered that loss of the wild-type p53 gene decreased expression of thrombospondin 1, a potent inhibitor of angiogenesis.[11,12] They also noted that wild-type p53 can stimulate the endogenous thrombospondin 1 gene and increase expression of thrombospondin 1. Loss of p53 function results in increased angiogenesis due to a lack of thrombospondin 1.

How Does p53 Fail?

In normal cells, there are two alleles of the p53 gene present, one on each of the short arms of chromosome 17 (17p). The normal genotype is denoted, $17p^{+}/17p^{+}$. Both alleles are expressed normally. To lose the protective function of a tumor suppressor gene, the effect of both alleles must be eliminated. The presence of one normal allele is sufficient to prevent tumorigenesis. A mutant allele would produce a mutant phenotype, promoting tumorigenesis, if either the other allele was deleted or replaced by a copy of the mutant. The mutant alleles phenotype would be expressed in a recessive manner. Possible causes of functional loss of an allele include mutation ($17p^{p53}$), deletion of a segment of a chromosome ($17p^{-}$), and deletion of the chromosome (17^{-}) (Table 1). The effect of each allele is removed in two discrete events, considered the two step hypothesis.[13] The most common

Table 1. GENOTYPES AND CORRESPONDING PHENOTYPES FOR p53 DYSFUNCTION

Genotype			
Allele	**Allele**	**Phenotype**	**Event**
$17p^{+}$	$17p^{+}$	Normal	—
$17p^{p53}$	$17p^{+}$	Normal*	Mutation
$17p^{p53}$	$17p^{p53}$	Tumor	Somatic recombination
$17p^{p53}$	$17p^{-}$	Tumor	Deletion
$17p^{p53}$	17^{-}	Tumor	Nondysjunction, chromosomal loss
$17p^{-}$	$17p^{-}$	Tumor ? lethal	
$17p^{-}$	17^{-}	Tumor ? lethal	
17^{-}	17^{-}	Tumor ? lethal	

* This genotype may result in a tumor phenotype.
$17p^{+}$ = wild-type allele; $17p^{p53}$ = mutant allele; $17p^{-}$ = deletion of the short arm of chromosome 17; 17^{-} = deletion of the whole of chromosome 17. Deletions of large parts of DNA may be fatal.

way to lose the effect of both alleles is to destroy the function of one by a mutation (the first step) and to remove the second allele entirely from the other chromosome (the second step). The deleted allele may be replaced by a copy of the mutant allele, or the cell may be left with only one allele. Another rare possibility for the second step is that the cell sustains a second mutation to the remaining normal allele.

Mutation in One Allele

Point mutations are mutations in which one nucleotide is substituted for another. Eighty percent of the mutations seen in the p53 gene are missense point mutations, *i.e.*, the genetic code is altered so that one amino acid is substituted for another and an abnormal protein results. Most point mutations in the p53 gene occur in the DNA that codes for amino acids in the DNA-binding part of the protein. This interferes with the interaction of p53 with DNA and causes the loss of p53's ability to function as a transcription factor. Normal p53 protein has a short half-life and is removed rapidly from the nucleus. If the half-life is extended, as it usually is in mutant p53, protein accumulates in the nucleus.

Other types of mutation are more rare. In a nonsense point mutation, nucleotide substitution results in a stop sequence that prematurely arrests DNA transcription so that a truncated protein is produced. The truncated protein is unlikely to be detected with immunohistochemistry. Frame-shift mutations result from insertions or deletions of one or more nucleotides that cause a shift in the reading frame of the DNA so that there is a marked change in amino acid sequence. This type of mutation also causes false-negative results on immunohistochemistry.

The aforementioned mutation may arise spontaneously, may be due to defective DNA replication or repair, or may be due to the effect of a carcinogen. The mutational spectrum observed in a particular cancer can provide information on the cause of the cancer.

Loss of the Other Allele

The zygote, from which all cells are descended, derives one allele of each somatic gene from the mother and the other allele from the father. These alleles are often different. A cell that has two different alleles is termed heterozygous. If a cell has two identical alleles, then the cell is homozygous. If one of the two alleles is lost and the cell has only one allele, then the cell is hemizygous. If normal somatic cells are heterozygous and tumor cells arising from them have either two identical alleles or only one allele, the net result is the same—there is a loss of the heterozygous state. Most tests do not differentiate between these two alternatives, they just show that there has been loss of heterozygosity, or allelic loss. If, in a tumor, allelic loss occurs more commonly at a particular location than at other locations, it is probable that this allelic loss leads to clonal selection of cells with that loss, *i.e.*, the allelic loss contributes to the tumorigenic process. The cause of allelic loss is thought to be a gross chromosomal event, such as mitotic recombination or abnormal segregation at mitosis.

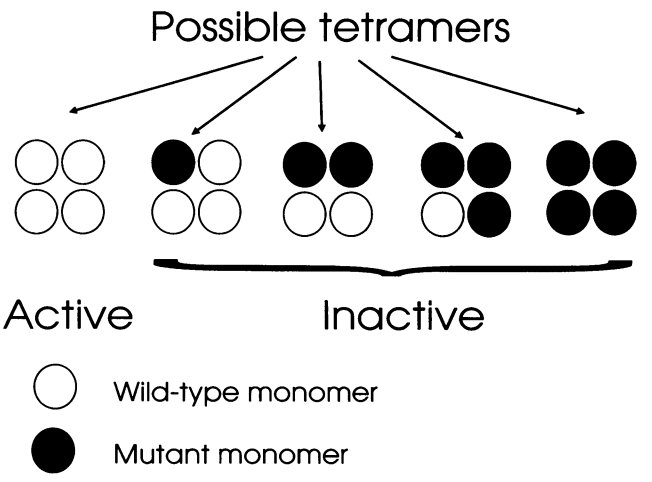

Figure 2. The dominant negative effect. p53 oligomerizes to form active complexes. If one member of the complex is mutant, this inactivates the rest of the complex. Even in the presence of wild-type p53 mutant, p53 can have a tumorigenic effect.

Eighty-six percent of colon cancers with a 17p allelic deletion have a p53 mutation in the remaining allele. When both parental copies of the p53 gene are present in the tumor, the rate of mutation is only 17%.[14] Does this mean that the p53 mutation does not contribute to tumorigenesis in cancers without allelic loss? The short answer is no; a p53 mutation in one allele without loss of the other allele may still cause loss of normal p53 function. Identification of 17p mutations without allelic loss in breast tumors[15,16] is evidence that this finding is not isolated to colonic tumors. p53 proteins oligomerize (join together) *in vitro,* suggesting that a single p53 protein unit, termed a monomer, must complex with other p53 monomers to be active (Fig. 2). If one p53 member of the complex is abnormal, the whole complex will not function correctly. Thus combination of mutant and wild-type p53 in a cell would lead to inactive p53 complexes. Mutant p53 often is stabilized so that it has a prolonged half-life. If the mutant and wild-type alleles are transcribed at the same rate, mutant p53 will accumulate because of the prolonged half-life. Most p53 monomer complexes would then contain mutant p53 and be inactive. This type of effect is termed dominant negative.[17] The dominant negative effect has been shown to occur with the combination of wild-type and mutant p53 *in vitro.*[18,19] Even though most cells with a mutated p53 also have loss of the nonmutated allele, some do not. The presence of both the wild-type and mutated forms would be expected

to result in loss of activity of the wild-type protein because of the dominant negative effect.

Thus, wild-type p53 behaves as a recessive tumor suppressor gene, and mutant p53 often behaves as a dominant oncogene.

What Happens When It Fails?

Failure of p53 results in loss of its normal functions. Cells lose their G1-S checkpoint and the DNA repair promoting effects of p53. This results in abnormal cells being allowed to progress in the cell cycle with increasing genomic instability and progression of tumorigenesis. Experiments on genetically engineered mice with removal of the p53 gene (knockout mice) demonstrate that there is a failure of genotoxic agent-induced apoptosis. This has obvious clinical relevance, suggesting that tumors with defective p53 function may be more resistant to radiation and chemotherapy than tumors with normal p53 function. This may a partial explanation why p53 malfunction has been shown to be associated with an adverse prognosis in some cancers. Loss of p53 function occurs when cells are in transition from the precancerous state to overt cancer. It is probable that loss of p53 function is causative in this transition. Individuals with a germline mutation in the p53 gene (Li-Fraumeni syndrome) are prone to developing a variety of cancers.[5] This syndrome is characterized by early onset breast cancer, childhood sarcomas, and a variety of other cancers. Carriers of the mutation have a 50% chance of having cancer develop by 30 years of age, and 90% by 65 years of age. Genetically engineered knockout mice with deletion of the p53 gene are born healthy, but after several weeks tumors develop, and by 6 months of age, all have tumors or are dead.

Molecular Epidemiology

The pattern of mutation in the DNA of the p53 gene gives clues about the underlying cause of the mutation.[20–23] The "fingerprint" of a carcinogen is left in the DNA molecule so that an analysis of the pattern of mutations in a particular tumor will give clues to the underlying cause. Certain patterns of mutation have been linked to known carcinogens and another pattern may indicate a "spontaneous" mutation. Mutations in DNA may be classified into insertions, deletions, and point mutations where there is, respectively, insertion, deletion, or substitution of nucleotides. Most of the mutations found in the p53 gene are point mutations. Ignoring which DNA strand is affected, there are six possible point mutations that can occur. If there is an equal number of each nucleotide in the DNA of the p53 gene, then if mutations occur randomly, there should be an equal number of each of the six possibilities. In addition, it would be expected that random mutations should occur scattered about the nucleotides of the p53 gene and not concentrated in "hot spots." Analysis of mutations in the p53 gene from many different cancers show that the pattern is not random. Certain carcinogens have an affinity for a particular site in the DNA molecule, and others have an affinity for changing one particular nucleotide to another. A good example of a carcinogen having an affinity for a particular site in the p53 gene is hepatocellular carcinoma. Tumors from high and low endemic areas of hepatocellular carcinoma have been analyzed for p53 mutations. The known risk factors in the high endemic areas are hepatitis B infection and dietary exposure to the mold toxin aflatoxin B1. The most common finding in tumors analyzed from such high-risk areas is a mutation in the third base pair of codon 249 of the p53 gene. This is in striking contrast to the findings in tumors from low-risk areas, where mutations are scattered throughout the DNA of the p53 gene. Investigation has shown that the pattern of mutation in high-risk areas is due to the action of aflatoxin B1,[23] with a possible selective growth advantage of hepatocytes containing the specific mutation in a liver chronically infected with hepatitis B.

Carcinogens implicated in lung cancer have an affinity for changing one particular nucleotide to another. In this situation, exposure to carcinogens in tobacco is characterized by a transversion from a G:C base pairing to a T:A pairing.[23] This mutation can occur anywhere in the DNA molecule where G:C bases are located. A similar pattern may occur in esophageal carcinomas, particularly squamous and possibly adenocarcinoma, because these tumors also have been linked to smoking.[24]

In other situations, the pattern of mutation appears to be the "spontaneous" deamination of 5-methyl cytosine to thymine. This deamination occurs at CpG dinucleotides, where the cytosine is methylated. As a result, what was a C:G base pairing becomes a T:A pairing with a change in the genetic code. This pattern of mutation has been described best in colon cancer. Transitions from a C:G pairing to a T:A pairing also may be due to the effects of a carcinogen, particularly alkylating nitrosamines.[25–27] These transitions do not show a predilection for CpG dinucleotides. N-nitroso compounds may play an etiologic role in several human cancers,[28] including esophageal cancer.

It is anticipated that accumulation of data on the pattern of mutations seen in esophageal adenocarcinoma will point the finger at the underlying cause of the mutations. If the mutational pattern is similar to that observed in other human cancers, animal cancer models or experimental cancer systems, it is likely that the cancers in question share a similar etiology.

Table 2. PREVALENCE OF POSITIVE p53 IMMUNOREACTIVITY IN DIFFERENT EPITHELIA FROM BARRETT'S ESOPHAGUS

Reference	Year	Barrett's (+/N)	LGD (+/N)	HGD (+/N)	CA (+/N)
Ramel[32]	1992	1/21	2/13	5/11	8/15
Jankowski[33]	1992	1/15	—	—	7/15
Flejou[34*]	1993	0/7	0/3	5/5	6/7
Younes[35]	1993	0/53	4/44	5/9	7/8
Casson[36]	1994	3/10	—	—	6/10
Jones[37]	1994	7/73	12/20	7/7	7/10
Flejou[38*]	1994	—	—	—	41/62
Hamelin[39*]	1994	0/6	0/3	3/5	11/17
Hardwick[40]	1994	0/20	1/3	10/21	16/30
Rice[41]	1994	0/28	0/27	18/26	14/23
Krishnadath[42]	1995	3/50	12/43	8/9	20/24
	Total	15/283	31/156	61/93	143/221
	%	5.3	19.9	65.6	64.7

* This group has published several times, and it is possible that results for the same patients appear in the different studies.
LGD = low-grade dysplasia; HGD = high-grade dysplasia; CA = invasive cancer.

p53 AND BARRETT'S ESOPHAGUS

The first reports of p53 mutations in esophageal cancers came from Hollstein and Harris in 1990,[29] who reported on cell lines and tissue samples taken from patients with esophageal squamous carcinoma. They subsequently reported p53 mutations in 20 of 48 esophageal squamous cell carcinomas in samples taken from Normandy and Uruguay,[30] areas with a high prevalence of esophageal squamous carcinoma. Casson et al.[31] identified a single p53 mutation in 14 esophageal adenocarcinomas in 1991.

p53 Immunoreactivity in Barrett's Esophagus

Wild-type (*i.e.*, normal) p53 protein has a short half-life and is undetectable on immunohistochemistry. Most mutations in p53 result in a protein with a prolonged half-life, which then accumulates in the cell. This accumulation is the basis for the immunohistochemical detection of p53 protein overexpression. There are many clinical reports on p53 immunohistochemistry in Barrett's esophagus. It is relatively cheap, quick, and easy compared with other techniques. The chances of the technique becoming clinically applicable are greater than for more difficult investigations, such as DNA sequencing, because results are obtained rapidly and the technique is available in most pathology laboratories.

Rates of p53 positivity are highest in patients with high-grade dysplasia and cancer (Table 2).[32–42] In cases in which tumor shows p53 protein overexpression, the adjacent dysplastic epithelium often overexpresses p53.

Theoretically, p53 protein overexpression detected on immunohistochemistry is an indirect method of detecting a p53 gene mutation. Some investigators screen tissues for p53 protein overexpression using immunohistochemistry and then look for gene mutations in those specimens with positive immunohistochemistry.[43] There are two reports on p53 gene mutations and immunohistochemistry on matched specimens from Barrett's adenocarcinomas. A review of these shows that there is significant discordance between a genetic mutation and p53 protein overexpression. Using the polymerase chain reaction followed by single-strand conformation polymorphism (SSCP), Casson et al.[36] looked for point mutations in ten patients with Barrett's metaplasia alone (group B) and ten patients with Barrett's metaplasia and carcinoma (group A). All samples also were examined for p53 protein overexpression using immunohistochemistry. The best practice is to confirm suspected DNA mutations observed on SSCP by complementary methods to prevent false-positive results. The investigators neglected this step, so their results must be interpreted with caution. However, they did repeat the experiment several times with a similar result, and an abnormal mobility shift on SSCP is positive evidence of a DNA mutation. Six of the samples from patients with cancer in group A were p53 positive and only two of these showed mobility shifts in keeping with a point mutation on SSCP. In group B, three of the ten patients with Barrett's metaplasia alone were p53 positive, and two of these had an abnormal SSCP. Five patients in group B had an abnormal SSCP, and three of these did not demonstrate positive p53 immunoreactivity. This study suggests that not all p53 mutations cause p53 protein overexpression; absence of p53 protein overex-

pression does not preclude mutation. It also suggests that p53 protein overexpression may exist without a p53 mutation.

Further evidence for a discrepancy between p53 protein expression and p53 mutations comes from the report of Hamelin et al.,[39] who examined the surgical specimen of resected esophageal adenocarcinoma from 17 patients. The patients had immunohistochemistry and polymerase chain reaction, followed by denaturing gradient gel electrophoresis and DNA sequencing for those who showed a mobility shift in keeping with mutation. The findings of a p53 mutation on DNA analysis were in keeping with p53 protein overexpression on immunohistochemistry in the majority of patients. One patient with adenocarcinoma was p53 positive on immunohistochemistry without a detectable mutation in the p53 gene. Five patients had a detectable mutation without evidence of excess expression of the p53 protein. Two of these mutations were nonsense, two were splicing mutations, and the other was a frame-shift caused by an eight base-pair insertion. These mutations produce either a truncated protein or one that bears little resemblance to the wild-type protein.

In a large review of 84 studies examining p53 malfunction in various tumors[23] with concomitant immunohistochemistry for p53 protein overexpression and DNA sequencing for p53 gene mutations, 36% of tumors contained a p53 mutation and 44% had positive staining on immunohistochemistry. The sensitivity of immunohistochemistry in predicting a mutation from these studies was 75%, with a positive predictive value of 63%. Immunohistochemistry is not accurate enough to screen for mutations but may be clinically useful because it is quick and relatively easy compared with DNA sequencing.

From these studies, we conclude:

- Immunohistochemistry may show positive staining for the p53 protein in cells that do not have p53 mutations. Overexpression of the p53 protein does not correlate with p53 mutation. Other factors, such as DNA damage, failure to breakdown p53 protein, or loss of signals that turn off p53, may increase p53 protein expression.
- A p53 mutation may exist without protein overexpression. This was shown in work on cultured fibroblasts from patients with Li-Fraumeni syndrome (germ-line mutation in p53 gene).[5] In addition, nonsense mutations result in a stop sequence so that a truncated protein is produced. Thus, not all mutations of the p53 gene result in overexpression of the p53 protein. If the protein is not overexpressed, it cannot be detected by immunohistochemistry.
- When antibodies against p53 are used to detect p53 overexpression, there are several problems. The antibody may bind nonspecifically to other antigens, giving false-positive results. Different antibodies bind differently so that results will vary depending on the antibody used. Certain types of mutation will result in a protein that will not bind to an antibody against the wild-type protein. For example, a frame-shift mutation will result in a highly abnormal protein that bears little resemblance to the wild-type protein. No known antibody or combination of antibodies will detect all p53 mutations.

Table 3. FREQUENCY OF p53 MUTATIONS IN BARRETT'S ADENOCARCINOMAS*

Reference	Year	Tissue	N	Mutations	%
Casson[31]	1991	Archival	14	1	7
Neshat[44]	1994	Flow cytometry	14	6	43
Hamelin[39]	1994	Frozen	17	15	88
Schneider[45]	1994	Fresh	21	8	38
Gleeson[46]	1995	Frozen	16	11	69
		Total	82	41	50

* All used polymerase chain reaction based amplification of at least exons 5–8 of the p53 gene with mutational confirmation using DNA sequencing.

Frequency of p53 Mutations in Barrett's Adenocarcinoma

In a review of more than 2500 tumors or cell lines analyzed for p53 mutations,[23] the five cancers with the most frequent p53 mutations were, lung (56%, N = 899), colon (50%, N = 960), esophageal (45%, N = 279), ovarian (44%, N = 386), and pancreatic (44%, N = 170). Based on tumors from a large number of patients in each group, the frequency of mutations ranged from 44% to 56% for the different cancers. There have not been many reports on p53 mutations in Barrett's adenocarcinomas. Different studies have different methods of tissue preparation, polymerase chain reaction protocols, and extent of DNA analysis, which makes comparison between studies difficult. Five studies that used polymerase chain reaction of at least exons 5 through 8 and confirmed potential mutations indicated by mobility shifts on gels with DNA sequencing are summarized in Table 3.[31,39,44–46] The overall number of patients reported to date is much less than that reported for other tumors. Further studies are awaited, but it can be seen that the prevalence of p53 mutations varies from 7% to 88% among these five studies. Work from the Washington group to be described subsequently suggests that p53 mutations may be quite common in Barrett's adenocarcinomas.

Using SSCP and confirming potential mutations by

DNA sequencing, Casson et al.[31] identified one p53 mutation in 14 esophageal adenocarcinomas and another mutation in 10 squamous cancers. Surprisingly, Casson found p53 mutations in four of seven specimens of Barrett's epithelium taken from the 14 patients with adenocarcinoma. The Barrett's epithelium showed only minimal or no dysplasia. The same mutation was present in several different areas from the Barrett's epithelium, but not in the cancer from the same patient. The finding of the same mutation in several different areas of Barrett's epithelium suggests that there is a clonal expansion of the cells with the mutation. This report is unusual because mutations usually are found more commonly in cancers than in premalignant tissues.

Moore et al.[43] found a mobility shift on SSCP, suggesting p53 gene mutations in 6 of 11 patients with adenocarcinoma and in 1 of 5 patients without cancer (this patient had dysplasia).

Hamelin et al.,[39] using polymerase chain reaction followed by denaturing gradient gel electrophoresis and DNA sequencing, showed mutations in 15 of 17 surgically resected patients with adenocarcinoma. One of these mutations was in the consensus splice donor sequence in intron 5 and one was in the consensus splice acceptor region in intron 6; these two mutations would be missed by the primers normally used to investigate the p53 gene. The authors also evaluated five specimens with high-grade dysplasia alone and found that five had p53 mutations. When a p53 mutation was present in the specimen containing high-grade dysplasia, the same mutation was present in the cancer specimen. None of five nondysplastic specimens evaluated showed a p53 mutation or positive p53 immunohistochemistry.

Gleeson et al.[46] sequenced mutations in the p53 gene in 11 of 16 patients with esophageal adenocarcinoma. In three patients in whom a mutation was identified, dysplastic epithelium adjacent to the tumor was available for analysis. The dysplastic epithelium harbored the same mutation that was found in the tumor. In one of these patients, benign Barrett's epithelium was analyzed and did not contain a mutation.

Pattern of p53 Mutations in Barrett's Adenocarcinoma

An analysis of 36 mutations in Barrett's adenocarcinomas published in the literature[31,39,44,46,47] shows an interesting pattern (Table 4). The number of tumor specimens from patients with esophageal adenocarcinoma that have been analyzed for p53 mutations and reported to date still is small compared with other types of tumors. The emerging pattern differs from that seen in other tumors because there is a marked preponderance of G:C to A:T transitions, a pattern that has been linked to the effect of N-nitroso carcinogens.[25–27] However, most of these transitions occur at CpG dinucleotides, in keeping with "spontaneous" mutations caused by the deamination of 5-methyl cytosine to thymidine. There is intense investigation into the possible causes of this type of transition at CpG dinucleotides.[48–51] Fifty-eight percent of these 36 mutations were G:C!A:T transitions at CpG dinucleotides. Other tumors with a high prevalence of this type of mutation are colon 47% (N = 960), endometrial 37% (N = 224), and gastric 35% (N = 314).[23]

Evolution of p53 Abnormalities in Barrett's Esophagus

In elegant experiments using cell flow cytometry, the Washington group showed that DNA aneuploidy occurs frequently in Barrett's adenocarcinomas. Aneuploidy also is common in high-grade dysplasia but is rare in low-grade/indefinite dysplasia and "ordinary" specialized intestinal metaplasia[52] (Table 5). This experiment showed that genomic instability (aneuploid cell populations) can occur before histologic evidence of cancer. Aneuploidy was present in 50% of dysplastic Barrett's epithelium and in all cases of Barrett's adenocarcinoma.

This group subsequently showed that p53 protein expression is high in aneuploid cell populations from high-grade dysplasia and adenocarcinoma patients[32] (Table 6). This study shows the point in the progression of Barrett's metaplasia to adenocarcinoma that aneuploid cell populations and p53 protein overexpression emerge. The presence of three patients with overexpression of p53 protein before the high-grade dysplasia stage, one with Barrett's metaplasia and two with indefinite/low-grade dysplasia, suggests that p53 mutations occur before the development of aneuploidy. However, the numbers reported are small and conclusions must be viewed with caution. The authors defined "normal" p53 protein content on the basis of their findings in Barrett's specialized epithelium. Therefore, it is not surprising that 5% of the patients with specialized intestinal epithelium had p53 protein overexpression. The p53 protein expression was higher in specialized epithelium than in gastric fundic epithelium from the same patient. This could indicate that p53 is being turned on in the Barrett's epithelium due to DNA damage, or it may reflect differences in the proportion of cells in the different stages of the cell cycle.

In collaboration with the Baltimore group, Blount and associates next investigated the frequency and temporal relationship of 17p and 5q allelic loss in 29 patients with high-grade dysplasia or adenocarcinoma.[53] Twenty-one of the 29 patients had two or more aneuploid cell populations. Fourteen were informative for DNA polymorphisms on 17p and 5q. All 14 patients had loss of heterozygosity on 17p. In the many different aneuploid cell

Table 4. P53 MUTATIONAL SPECTRUM IN ESOPHAGEAL ADENOCARCINOMA

N	G:C → A:T	G:C → T:A	G:C → C:G	A:T → G:C	A:T → T:A	A:T → C:G	Other	CpG*
36	24	2		4			6	21
%	66	6		11			17	58

* Percentage of mutations that were G:C → A:T transitions at CpG dinucleotides.

populations from these patients, only one did not demonstrate 17p allelic loss. In contrast, 5q allelic loss was more rare. These findings suggest that 17p allelic loss is common in Barrett's adenocarcinoma and precedes 5q loss.

What is the temporal relationship between 17p allelic loss and the development of aneuploidy? The high prevalence of 17p allelic loss in aneuploid cell populations suggests that 17p allelic loss precedes or occurs simultaneously with aneuploidy. If it is possible to find 17p allelic loss in nonaneuploid cell populations, this would indicate that the allelic loss occurs before aneuploidy. In 11 patients with 17p allelic loss in aneuploid cell populations from high-grade dysplasia or cancer, 10 had 17p allelic loss in diploid cell populations from premalignant Barrett's epithelium.[54] Moreover, the same allele was lost in the diploid cells from the premalignant epithelium as was lost in the aneuploid cells. Not all of the sorted diploid cell populations had 17p allelic loss, and several from the same patient in different samples had both alleles. This demonstrates that 17p allelic loss precedes aneuploidy. To investigate the association between 17p allelic loss and p53 mutations, the authors sequenced exons 5 through 9 of the p53 gene in three of the patients. All three had p53 mutations, suggesting that p53 mutation precedes 17p allelic loss. In addition, the same mutation was found in diploid cells as was found in aneuploid cells.

The results of these studies suggest the following sequence of events in the evolution of Barrett's adenocarcinoma (Table 7). A diploid cell with two wild-type p53 alleles sustains a mutation in one p53 gene. After this, the other allele is lost. This is followed by increasing genomic instability and the emergence of aneuploid cell populations. This may represent the most common sequence of events in Barrett's adenocarcinoma. Aneuploidy precedes the development of tumor and is seen in virtually all tumors, 50% of dysplastic samples and almost never in nondysplastic tissue. Loss of heterozygosity on 17p antedates aneuploidy and has been observed in diploid cell populations from nondysplastic epithelium. p53 mutations have not been observed without loss of heterozygosity on 17p in samples from nondysplastic Barrett's epithelium, dysplasia, or cancer. Some investigators have reported a loss of heterozygosity on 17p without a detectable mutation. This is rare; both abnormalities frequently coexist. p53 mutation and loss of heterozygosity first arise in nondysplastic epithelium, and this is followed by progression in tumorigenesis with emergence of aneuploid cell populations and invasive cancer.

Table 5. ANEUPLOIDY IN BARRETT'S ESOPHAGUS[52]

Histologic Diagnosis	No. of Patients	Aneuploidy
Esophagitis	18	0
Negative for dysplasia	34	1
Indefinite or low-grade dysplasia	4	0
High-grade dysplasia	4	2
Adenocarcinoma	7	7

CLINICAL RELEVANCE OF p53

The easiest way to examine p53 in tumors is to look for intranuclear protein accumulation using immunohistochemistry. Detailed DNA analysis to look for mutations in the p53 gene currently is suitable only for research. Most of the published work on p53 in Barrett's esophagus is based on immunohistochemical staining of p53 protein, but it must be emphasized that immunohistochemistry is only a marker for loss of p53 function, and there may be significant false-positive and false-negative rates.[23] Two possible uses for p53 protein overexpression are 1) to predict future progression to cancer and 2) to determine whether tumors that are p53 positive are associated with a poor prognosis after resection. It is unlikely that positive immunohistochemistry will be useful in predicting which cases of Barrett's metaplasia will progress to cancer. The test has a low sensitivity, with roughly 65% of patients with invasive carcinoma being p53 positive[55] (Table 2). Currently, the predictive value of p53 positivity in nondysplastic Barrett's epithelium is unknown. Casson et al.[36] reported on patients with Barrett's epithelium and little

Table 6. P53 PROTEIN EXPRESSION IN BARRETT'S ESOPHAGUS[32]

Source of Specimen	No. of Patients	No. (%) with p53 > 28%	Mean ± SEM p53 Protein Expression	
			Diploid	Aneuploid
Fundic gland mucosa	20	0 (0)	7.2 ± 0.7	—
Negative for dysplasia	21	1 (5)	12.5 ± 1.7	—
Indefinite or low-grade dysplasia	13	2 (15)	16.4 ± 2.8	—
High-grade dysplasia	11	5 (45)	13.0 ± 2.8	29.9 ± 5.4
Adenocarcinoma	15	8 (53)	6.9 ± 1.3	37.5 ± 5.8

SEM = standard error of the mean.

or no dysplasia; three of ten had positive p53 immunostaining. In one of these patients, dysplasia developed increasingly, and the patient had an esophagectomy; there was no evidence of *in situ* or invasive cancer in the resected specimen. The remaining two patients had been followed for a median of 8 years, and cancer had not developed. In two more of these ten patients, high-grade dysplasia developed increasingly without positive p53 immunostaining, both had an esophagectomy, and neither had *in situ* or invasive cancer in the resected specimen. Hardwick et al.[40] reported three patients with high-grade dysplasia at their initial presentation in whom adenocarcinoma that was positive for p53 subsequently developed. The patients developed carcinoma at 6-, 11-, and 12-month follow-up. The dysplastic epithelium overexpressed p53 in two of the patients at presentation, whereas in the third patient, p53 overexpression developed while the patient was treated with omeprazole (20 mg daily). Progression of the neoplastic process occurred in this patient, despite good symptom relief and apparent regression of the columnar lined segment. Younes et al.[35] did a retrospective follow-up study in 24 patients who had evidence of dysplasia (indefinite, low, or high) on at least one biopsy with at least 9 months follow-up. Three patients progressed to high-grade dysplasia in this study; previous biopsies showed indefinite/low-grade dysplasia in one patient and nondysplastic specialized intestinal metaplasia in two patients. Both patients who progressed from nondysplastic epithelium showed positive p53 immunohistochemistry at the indefinite/low-grade dysplasia stage. In one of these patients, blocks from nondysplastic epithelium were unavailable for evaluation of p53 immunohistochemistry. In the other patient, the previous nondysplastic epithelium was negative for p53 protein overexpression. The patient who progressed from indefinite/low-grade dysplasia to high-grade dysplasia without prior biopsies showing nondysplastic epithelium did not have positive p53 immunohistochemistry. In this retrospective analysis, the development of positive p53 immunohistochemistry first appeared at the stage of indefinite/low-grade dysplasia and preceded the development of high-grade dysplasia. This suggests that in some patients, when there is doubt about the degree of dysplasia, the presence of positive p53 immunohistochemistry should prompt early extensive repeat biopsy to search for high-grade dysplasia. However, negative p53 immunohistochemistry in this setting does not mean that the patient will not progress to high-grade dysplasia.

Thus, currently, the best predictor of progression of Barrett's epithelium to cancer remains high-grade dysplasia. Accurate interpretation of dysplasia requires an experienced pathologist. There is a marked interobserver variation in the assessment of lower grades, but agreement is better for high-grade dysplasia. It is unlikely that p53 immunoreactivity can act as an adjuvant predictor for the presence of high-grade dysplasia. The fate of nondysplastic specialized intes-

Table 7. POSSIBLE EVOLUTION OF CHANGES IN BARRETT'S ESOPHAGUS

	Mutation	→	Allelic Loss*	→	Aneuploidy
Diploid	Diploid		Diploid		Aneuploid
$17p^{+}/17p^{+}/17p^{+}$	$17p^{p53}/17p^{+}$		$17p^{p53}/(17p^{-}$ or $17p^{p53})$		$17p^{p53}/(17p^{-}$ or $17p^{p53})$

* Allelic loss may be due to a deletion of the remaining wild-type allele or may be due to conversion of the wild-type allele to a copy of the mutant allele.
$17p^{+}$ = wild-type p53 allele; $17p^{p53}$ = mutant allele; $17p^{-}$ = deletion of the short arm of chromosome 17.

tinal epithelium that shows positive p53 immunohistochemistry is unknown, and many cases of high-grade dysplasia do not overexpress p53 (Tables 2 and 6).

It has been our experience[56] and the experience of others[57–59] that many patients undergoing surgery for high-grade dysplasia have occult tumors in the resected specimens. In our series, 7 of 11 patients referred with the diagnosis of high-grade dysplasia had adenocarcinoma in the resected specimen. The tumor was intramucosal in five patients, submucosal in one patient, and reached the muscularis propria in one patient who had ulceration of the tumor. None of these patients had lymph node metastases. The postoperative mortality rate in these patients was 0%, and they were all alive and well at median follow-up of 12 months (range, 4–66 months). Aggressive repeat biopsy of these patients before surgery revealed only two of the seven adenocarcinomas found in the surgical specimen. Because of these results, we advocate surgical resection for patients with high-grade dysplasia, unless the patient is unfit for surgical intervention.

Retrospective studies have linked p53 immunoreactivity with a poorer prognosis. This has been show in carcinomas of the lung, breast, stomach, cervix, and prostate, and most recently, in bladder tumors.[60] p53 status is being investigated to see if it is useful marker in the management of node-negative breast cancers.[61,62] p53 status appears to have no prognostic value in ovarian[63] and colonic[64] cancers. The reports on prognosis related to p53 immunoreactivity in Barrett's adenocarcinoma are few at present. Flejou et al.[38] reported on p53 immunoreactivity in 62 consecutive esophagogastrectomies for adenocarcinoma developing in Barrett's esophagus. Forty-one were p53 positive and 21 were p53 negative. The two groups were comparable with respect to clinical features and to pathologic staging features. Over a median follow-up of 28 months (range, 0.4–135 months), there was no significant survival difference between patients who were p53 positive and those who were p53 negative. Vijeyasingam et al.[65] examined the effect of p53 immunohistochemistry on prognosis in 30 5-year survivors after esophageal resection for cancer and compared these with 30 matched nonsurvivors. Twenty-four of the 60 patients in this study had adenocarcinoma. p53 status did not influence prognosis. In a smaller group of patients, Casson et al.[36] also did not observe an adverse effect on prognosis. Duhaylongsod et al.[66] reported on the effect of positive p53 immunohistochemistry in 42 patients treated preoperatively with chemotherapy and irradiation. Positive p53 immunoreactivity did not correlate with disease-free survival. Because p53 immunohistochemistry and mutational analysis may be performed on archival pathology specimens, it is possible that a collaboration between different centers could lead rapidly to the recruitment of sufficient subjects to definitively analyze the role p53 plays in prognosis of esophageal adenocarcinoma.

Within the spectrum of gastroesophageal reflux disease, there is a subset of patients in whom Barrett's esophagus develops; some of these patients progress through dysplasia to adenocarcinoma. Constituents of the gastric juice which refluxes into the esophagus may play an etiologic role in this process. Of the possible contenders, duodenal content has attracted the most attention. In a rat model of esophageal carcinoma, in rats given carcinogen alone, esophageal squamous cancer develops in a time- and dose-dependent fashion. However, when rats undergo surgery to permit the reflux of duodenal content into the esophagus, the administration of the carcinogen results in a greater yield of tumors, with most of the additional tumors being adenocarcinomas.[67–70] There is clinical evidence that patients with Barrett's esophagus have increased esophageal exposure to duodenal content.[71–73] These observations raise the possibility that reflux of duodenal content into the esophagus may play a role in the development of Barrett's esophagus and subsequent adenocarcinoma. One explanation is that this is due to toxic chemicals eliminated by the liver through the bile. These xenobiotics are most concentrated in the bile, and mucosae exposed to such contaminated bile will have a high exposure to these toxins. Another possibility is the Ames hypothesis,[74] whereby mitogenesis increases mutagenesis. Injury to the esophageal mucosa from exposure to duodenal content causes an ongoing cycle of injury and repair. This mechanism is thought to explain squamous cancer that arises in a burn scar (Marjolins ulcer) or in adenocarcinoma that occurs in lung at the site of healed granulomas.

Components of duodenal juice that have been studied extensively as promoters of esophageal adenocarcinoma are bile salts. Aspiration studies have shown that bile salts reflux into the esophagus[75] of patients with gastroesophageal reflux disease, in concentrations in excess of 200 mol/L.[76] In a perfused rabbit model, Harmon's group[77,78] has shown that bile salts cause disruption of the esophageal mucosal barrier. On repetitive exposure, the bile salts can enter the mucosal cell, disrupt the mitochondria, and can result in cell death or injury at luminal concentrations less than 200 mol/L.[79] At our institution, Hill et al. (unpublished observations, 1984) have shown that bile salts increase the radiation-induced transformation frequency in the C3H 10T1/2 mouse fibroblast cell line. However, bile salts have not been shown to be mutagenic by themselves in the salmonella mammalian microsome mutagenicity test.[80] This test has not been performed in various pH environments, a factor that may be important in the mutagenicity of bile salts. Experimental data to date show that components of duodenal content are synergistic or are co-mutagens in the development of adenocarcinoma

in the rat esophagus. The mechanism as to how this occurs is debatable, but what appears to be clinically important is prevention of duodenal juice from refluxing into the esophagus. This can be accomplished by surgically re-establishing the gastroesophageal antireflux barrier. Chronic acid suppression therapy may be detrimental because duodenal contents have free access to the esophagus through the alkalinized environment of the stomach.

SUMMARY

The study of the p53 gene has increased remarkably our understanding of the pathogenesis of cancer. Loss of p53 function plays a major and common role in the transition of Barrett's metaplasia to dysplasia to cancer. As yet, there is no evidence that identifying loss of p53 function is important in the outcome of patients with Barrett's esophagus and esophageal adenocarcinoma. It is uncertain whether positive p53 immunoreactivity staining for p53 protein has any clinical value in the assessment of these patients. It would be beneficial to determine if positive immunostaining is helpful in the management of patients with Barrett's esophagus who histologically are benign or show indefinite or low-grade dysplasia. Increased understanding carries the potential of identifying an environmental factor that may be removed, or at least not potentiated by the chronic regurgitation of gastroduodenal juice, to prevent this dreaded complication of gastroesophageal reflux disease.

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