

Short CommunicationGenetic and Immunohistochemical Analyses of p53 Independently Predict Regional Metastasis of Gastric Cancers¹

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Abstract

Either *p53* gene mutation or immunohistochemical detection of p53 protein has not been consistently shown to have prognostic significance in human cancers, including gastric carcinomas. One hypothesis to explain this inconsistency is that some *p53* mutations and p53 protein accumulation are not indicative of tumor progression. To test this hypothesis, we categorized p53 status in 105 gastric carcinomas according to types of mutations, numerical scores of immunohistochemical staining (IHC), or combinations thereof. The p53 status was then correlated with metastasis to liver or peritoneum. Gastric cancers with no *p53* mutations were significantly less likely to metastasize than tumors with mutations. Intermediate IHC scores were inversely associated with metastasis. A substantial number of gastric cancers (31 of 105) showed positive p53 immunostaining without detectable mutations (p53–/IHC+), which suggested an accumulation of wild-type p53 protein, and also a significantly lower risk for metastasis. After adjusting for depth of invasion and lymph node involvement, the p53–/IHC+ combination predicted low metastatic risk better than either p53– or IHC+ with intermediate scores. These findings suggest that an accumulation of wild-type p53 protein occurs in gastric cancer cells and represents a stress-response mechanism that lowers metastatic potential.

Introduction

Mutations of the *p53* tumor suppressor gene can result in a loss of function of the wild-type gene product to regulate cell cycle

progression and apoptosis (programmed cell death; Refs. 1, 2). *p53*-knockout mice that lack functional p53 are prone to certain tumors and are less responsive to chemo- or radiotherapy (1, 2), which suggests that *p53* mutation is an indicator of poor cancer prognosis. Most but not all mutant p53 proteins have a prolonged half-life and accumulate in tissues and can be directly detected by IHC³ (3). Therefore, p53 protein accumulation is commonly interpreted as indicative of the presence of *p53* mutations.

The prognostic value of *p53* mutations or p53 protein accumulation has not been consistently demonstrated in many human neoplasms, including gastric cancer (4, 5). Both positive and negative findings have been reported in studies with various sample sizes, which indicates that inadequate statistical power alone, as suggested by an early observation (6), cannot entirely explain the discrepancy. It has been shown that *p53* mutations vary in their biological effects (7). Some mutated p53 proteins behave like the wild-type counterpart, whereas others are correlated with tumor aggressiveness. In addition, an accumulation of p53 protein may not necessarily indicate the presence of a *p53* mutation, because not all mutated p53 proteins yield positive immunohistochemical staining, and wild-type protein can also accumulate in response to stress stimulants, such as DNA damage (1, 4). Because of the biological variability of *p53* mutations and the diverse causes of p53 protein accumulation, either parameter alone may fail to predict prognosis consistently.

In this study, we categorized p53 changes into various groups according to types of mutations, scores of immunohistochemical staining, or combinations thereof, and then correlated these changes with the presence of regional metastases of gastric cancers. The possible biological functions and implications for regional metastasis are discussed.

Materials and Methods

Subjects. One hundred and five gastric carcinoma patients (41 females, 64 males; mean age, 62 years; range, 34–76 years) were selected from a population-based case-control study conducted in central Italy, as described previously (8). Formalin-fixed paraffin-embedded tissues were retrieved from archives at the Department of Pathology, University of Florence, Italy. Demographic and clinicopathological information was abstracted from our population-based database, and staging was carried out for each case by the TNM classification at the time of surgery. For this study, “metastasis” refers to hepatic and/or peritoneal spread from the primary site. No systematic documentation of metastasis to other sites was available. Data on *p53* gene mutations were obtained from a previous study (8).

p53 IHC. Dewaxed 5- μ m formalin-fixed paraffin-embedded sections were subjected to an antigen retrieval method by

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³ The abbreviations used are: IHC, immunohistochemistry/immunohistochemical staining; p53+, *p53* (gene) mutation; p53–, without detectable *p53* mutations; p53–/IHC+, positive p53 immunostaining without detectable mutations.

Table 1 Associations of p53 changes by mutation types and/or scores of protein accumulation to regional metastasis of gastric cancers

p53 changes ^a	No metastasis	Regional metastasis	Odds ratio	P ^b
p53-	63	9	1.0	
p53+	22	11	3.5	0.014
p53-	63	9	1.0	
p53+/nonhot spot	13	5	2.7	0.131
p53+/hot spot	9	6	4.7	0.019
p53-	63	9	1.0	
p53+/nonmissense	4	2	3.5	0.210
p53+/missense	18	9	3.5	0.022
p53-	63	9	1.0	
p53+/CpG	14	5	2.5	0.159
p53+/non-CpG	8	6	5.3	0.013
IHC+ (1/2/3) ^c	23	0	1.0	
IHC+ (4) ^c	23	6	13.0 ^d	0.020 (0.028) ^e
IHC- (0) ^c	39	14	17.2 ^d	0.003 (0.007) ^e
p53-/IHC+	31	0	1.0	
p53-/IHC-	32	9	18.4 ^d	0.003 (0.008) ^e
p53+/IHC+	15	6	26.4 ^d	0.002 (0.003) ^e
p53+/IHC-	7	5	46.2 ^d	<0.001 (<0.001) ^e

^a Hot spot, mutations at codons 175, 248, and 273; missense, one base substitution resulting in change of encoded amino acid; CpG, G:C to A:T transitions at CpG sites; IHC+, positive immunohistochemical stain as an indicator of protein accumulation; IHC-, negative stain.

^b $P < 0.05$ is considered to be statistically significant.

^c Scores of IHC are in parentheses.

^d The odds ratios were computed with an addition of 0.5 to each cell in the contingency table.

^e P in parentheses was computed with Fisher's exact test.

microwave boiling in the presence of lead thiocyanate solution according to the manufacturer's instructions (BioGenex, San Ramon, CA). After blocking endogenous peroxidase activity with normal goat serum, sections were incubated at room temperature for 30 min with a 1:1000 dilution of the polyclonal antibody, CM-1 (Novocastra Lab., Newcastle, United Kingdom), against wild-type and mutant forms of p53 protein. For signal detection, the avidin-biotin complex procedure (Vectastain Elite ABC kit) was used according to the manufacturer's direction (Vector Laboratory Co., Burlingame, CA). As a negative control, the same procedure was followed except that the primary antibody was replaced with PBS. The neoplastic areas were examined for immunohistochemical staining by one investigator (Y-H. S), blinded to the p53 mutation status. Only the nuclear immunostain was considered as positive for p53 protein accumulation. The level of protein accumulation was scored as 0 (no detectable immunostain), 1 (few nuclei), 2 (up to 10% nuclei), 3 (10–50% nuclei), and 4 (>50% nuclei). The numerical scoring was confirmed by a second independent examination, blinded to the initial score.

Statistical Analysis. Associations between metastasis of gastric cancers and p53 changes, according to types of mutations, scores of immunohistochemical staining, and combinations thereof, were examined by logistic regression contingency table analyses (9, 10). Probabilities were computed using the likelihood ratio χ^2 statistic or Fisher's exact test as appropriate. When one of the cells in the contingency table was zero, a standard correction with an addition of 0.5 to each cell in the table was applied to calculate the odds ratio (11, 12). Multivariate logistic regression analysis was used to determine whether p53 changes, scores of immunohistochemical staining, or combinations thereof, contributed significantly as independent prognostic indicators of metastasis.

Table 2 Multivariate analysis of independence of p53 changes to predict regional metastasis^a

p53 changes ^b	P
p53+ versus p53-	0.041
p53+/nonhot spot versus p53-	0.207
p53+/hot spot versus p53-	0.050
p53+/nonmissense versus p53-	0.298
p53+/missense versus p53-	0.058
p53+/CpG versus p53-	0.136
p53+/non-CpG versus p53-	0.119
IHC (0/4) versus IHC+ (1/2/3)	0.003
IHC+ (4) versus IHC+ (1/2/3)	0.011
IHC- (0) versus IHC+ (1/2/3)	0.002
Other p53/IHC versus p53-/IHC+	<0.001
p53-/IHC- versus p53-/IHC+	<0.001
p53+/IHC+ versus p53-/IHC+	<0.001
p53+/IHC- versus p53-/IHC+	<0.001

^a Adjusted for depth of invasion and lymph node involvement.

^b High-risk versus low-risk of regional metastasis.

Results

Hepatic and/or peritoneal regional metastases of gastric carcinomas were observed in 20 of 105 patients. The relation of p53 changes to the presence of metastasis is shown in Table 1. Gastric cancers having p53 mutations of any type (p53+), mutations at "hot spot" regions (codons 175, 248, and 273, predominant in the current study), missense-type mutations, and mutations at non-CpG sites significantly increased the risk for metastasis in comparison with tumors without detectable p53 mutations (p53-). Elevated metastatic risks were also observed for tumors carrying non-hot spot mutations (other than codons 175, 248, and 273), nonmissense (deletion, nonsense, or intronic) alterations, or G:C to A:T transitions at CpG sites, but the risks did not reach statistically significant levels.

Table 1 also relates the changes in p53 protein level by immunohistochemical analysis to metastasis of gastric cancers. After examining various combinations of IHC scores, it was found that IHC+ of intermediate scores (IHC scores of 1, 2, and 3) showed the lowest risk of metastasis. The highest score of IHC (IHC score, 4) and the zero IHC score were significantly associated with metastasis as compared with the intermediate IHC scores. It is noteworthy that 10 (34%) of 29 tumors with the highest IHC score showed no concurrent p53 mutations. In contrast to the highest IHC score, tumors with scores of 1, 2, or 3 had a low frequency (2 of 23) of p53 mutations. Among 53 tumors with a zero IHC score, 12 tumors (23%) carried mutations in the p53 gene, including missense in 7, nonsense in 2, intronic in 1, and deletion in 2.

When p53 changes were categorized by both mutation and protein status, no metastases were observed in 31 tumors having p53 protein accumulation but without detectable p53 mutations (p53-/IHC+; Table 1). When this group was considered as the referent, metastatic risk was elevated in tumors that were either p53-/IHC-, p53+/IHC+, or p53+/IHC-. The estimated risks relative to p53-/IHC+ ranged from 18.4 to 46.2 and were highly significant.

The prognostic values of various p53 changes were also examined by multivariate analysis (Table 2). After adjusting for depth of invasion and lymph node involvement, the association of metastasis with p53+, IHC scores, and p53/IHC combinations remained significant. The computed significance of p53/IHC combinations as independent prognostic factors was much more prominent than that of p53 mutation status or IHC scores alone. Other demographic and clinicopathological data were not

significantly associated with metastasis in the univariate analysis and, therefore, were not included in the multivariate analysis.

Discussion

Determinations of p53 changes as prognostic variables in human cancers have yielded inconsistent results, whether based on mutations by gene analysis or protein accumulation by IHC (4). The present study indicates that the types of p53 changes may be crucial in relation to the likelihood of regional metastasis of gastric cancers.

In general, gastric cancers having p53 mutations were at increased risk for metastasis in both univariate and multivariate analyses, which is consistent with a previous report of a very poor survival rate for gastric cancers with p53 mutations (13). When mutation types were taken into account, the risk was further magnified for tumors carrying mutations at hot spots (codons 175, 248, and 273) and at non-CpG sites. The association of metastatic risk with hot-spot mutations of the p53 gene is consistent with the enhancement of tumorigenic potential by these mutations in animal and *in vitro* experiments (7, 14). The higher risk associated with non-CpG versus CpG mutations is intriguing, particularly because we previously reported that gastric cancers with non-CpG mutations are related to dietary factors and tobacco smoking (15), which suggests a molecular mechanism linking life-style variables to both the etiology and the progression of tumors.

To our surprise, intermediate levels of p53 protein accumulation (IHC scores of 1, 2, and 3) were associated with a lower risk of metastasis in the current study. This finding is consistent with the improved prognosis reported for gastric tumors with intermediate levels of p53 protein accumulation in a study from Finland (16). The zero risk for metastasis in our study may be attributed in part to the lack of p53 gene mutations in 21 of the 23 tumors with intermediate IHC scores. The high percentage of tumors having IHC score of 1, 2, or 3 but no detectable p53 gene mutations cannot be dismissed simply as false-negative analyses of the p53 gene, because sensitivity of the current SSCP protocol is quite high (17), and the mutation rate outside exons 5–8 is less than 10% (18). Instead, this may reflect an accumulation of wild-type p53 protein. A relationship of the putative wild-type p53 protein accumulation to favorable prognosis is further supported by the combined evaluation of gene and protein status, in which no metastases were observed in 31 p53-/IHC+ tumors. It is noteworthy that the additional 10 tumors without p53 gene mutations had an IHC score of 4, which suggests that the accumulation of wild-type p53 protein in gastric tumors, if confirmed, is not a rare phenomenon.

After adjusting for depth of invasion and lymph node involvement, we found that p53 mutations of any type (p53+), the lowest or highest level of p53 protein accumulation (IHC score of 0 or 4, respectively), and p53/IHC combinations other than p53-/IHC+ independently predict regional metastasis of gastric cancer. The p53-/IHC+ combination, probably attributable to wild-type p53 protein accumulation, seems to be an excellent independent predictor of low metastatic risk.

It is known that wild-type p53 protein plays a critical role in cell cycle arrest at the G₀-G₁ and G₂-M phases and in apoptosis (1, 2). Lacking wild-type p53 protein, cells continue to proceed to the next replication cycle, leading to uncontrolled growth. Many studies have shown that the accumulation of wild-type p53 protein can be induced by certain stresses, such as hypoxia (19), oncogene activation (20), and changes in the nucleotide pool (21), which are commonly observed in primary

tumors. Up-regulation of wild-type p53 protein in tumors may indicate the last step of defense before metastasis. If our findings are confirmed in a larger series sample, appropriate treatments could be formulated according to p53 status as determined by the combination of mutational and immunohistochemical analyses. In the future, surgery may be adequate for tumors showing p53-/IHC+, treatments to activate wild-type p53 protein level may be useful for tumors with p53-/IHC-, and p53 gene therapy may be considered for tumors with p53 mutations.

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