

Combined p53/Bax mutation results in extremely poor prognosis in gastric carcinoma with low microsatellite instability

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Abstract

Gastric cancer is highly refractory to DNA-damaging therapies. We therefore studied both gene mutation and protein expression of p53 and Bax in a cohort of 116 patients with gastric cancer who underwent R0-resection with a curative intent. Bax mutation was independent from severe microsatellite instability (MSI), that is, global mismatch repair deficiency as determined by analysis of BAT-25/BAT-26 microsatellite markers. Thus, Bax-frameshift mutation is a feature of tumors with low MSI. In contrast and as expected, no p53 mutations were observed in the microsatellite instable tumors. p53 Mutation or p53 overexpression did not have an impact on disease prognosis. p53-inactivation was, however, associated with an extremely poor prognosis in the subgroup of patients with Bax-mutated tumors. Thus, we show for the first time that the combined mutation of p53 and Bax, two key regulators of the mitochondrial apoptosis pathway, results in an extremely aggressive tumor biology and poor clinical prognosis.

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Keywords: Bax; p53; mutation; gastric carcinoma; DNA mismatch repair (MMR) deficiency; microsatellite instability (MSI); prognosis

Abbreviations: MSI, microsatellite instability; MMR, mismatch repair; BH, Bcl-2 homology domain; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; SI, staining index; SSCP-PCR, single-stranded conformational polymorphism-polymerase chain reaction

Introduction

Gastric cancer has a poor prognosis and remains the second common cause of cancer-related deaths worldwide. Although the incidence of gastric cancer is declining, the mortality because of stomach cancer is ranking fourth among different cancers in the European Union and accounts for 7% of cancer-related death in males and 5% in females.^{1–3} The only chance for cure is early and complete surgical resection including adjacent lymph nodes,⁴ which results in Europe in a 5-year survival rate of roughly 20%. This reflects the fact that gastric cancer recurs in 80% of cases despite 'curative' gastrectomy and displays an extreme resistance to cytotoxic, DNA-damaging anticancer therapies. This highlights the need for a better understanding of disease pathogenesis and progression to improve therapeutic outcome.

We have shown previously that the analysis of apoptosis signaling pathways is a suitable approach to identify patients with a good or extremely poor prognosis. In this line, we were the first to describe a defect in the expression of the proapoptotic Bax protein in breast cancer,⁵ compared with the nonmalignant breast epithelium. Re-expression of Bax in breast cancer cell lines reduced tumor growth in nude mice.⁶ The complete loss of Bax results in resistance to some but not all apoptosis stimuli including anticancer drugs.^{7–9} Likewise, overexpression of the Bax homolog Bak or the BH3-only protein Nbk/Bik counteracts acquired drug resistance.¹⁰

Furthermore, we and others showed that reduced Bax expression is a negative prognostic factor and correlates with a shorter overall survival in patients with breast,¹¹ ovarian,¹² colorectal,^{13,14} esophageal¹⁵ and pancreatic cancer.¹⁶ In childhood acute lymphoblastic leukemia (ALL), the loss of Bax protein expression is associated with chemoresistant relapse and a defective activation of the caspase cascade.¹⁷

The tumor suppressor gene p53 is a transcriptional activator of the Bax gene¹⁸ and both genes act in the same signaling cascade to trigger the mitochondrial apoptosis pathway.^{19–21} In fact, we observed that the deregulated expression of wild-type p53 protein in follicular thyroid carcinoma coincides with increased expression of the p53 target genes p21^{CIP/WAF-1} and Bax.²² Nevertheless, the loss of Bax is associated with a poor prognosis independent of the p53 gene mutation status in metastatic or locally advanced colorectal cancer.^{13,14} Inactivation of Bax by mutation has been shown to be restricted to tumors with defects in DNA mismatch repair (MMR).^{14,23} Loss of Bax expression occurs, however, in consequence of disturbed transcriptional regulation in the vast majority of cancers.^{5,7,14,15,17,24}

Thus, no concise data were so far available regarding the consequences of p53 and Bax inactivation by mutation on

disease prognosis in gastric and other gastrointestinal tumors. In this study, we therefore examined the inactivation of the p53 and Bax gene in a group of 116 patients with R0-resected, sporadic gastric cancer to determine the influence of these regulators on tumor biology and disease prognosis.

Surprisingly and unlike the situation in other tumors, we observed that loss of Bax expression is paradoxically associated with better prognosis in gastric cancer. This was even more evident when the tumors were analyzed for Bax G(8) tract frameshift mutations as underlying cause for the loss of Bax protein expression. Thus, the presence of such Bax-frameshift mutation identifies patients with a better disease prognosis. This also suggests that such tumors may share, apart from a different tumor biology, a different mode of pathogenesis as suggested previously for MMR-deficient tumors. Nevertheless, the analysis of the microsatellite markers BAT-25 and BAT-26 that identify with high specificity tumors expressing high microsatellite instability (MSI), that is, global MMR deficiency, showed that Bax mutations do not cluster in BAT-25/BAT-26 microsatellite instable tumors. In conclusion, inactivation of Bax by frameshift mutation occurs in tumors with low MSI, which supports our hypothesis of a different tumor biology of this population. Moreover, this is the first report to demonstrate that the additional inactivation of p53 in these Bax-frameshift-mutated tumors is associated with a very aggressive clinical course and an extremely poor prognosis. Altogether, this indicates that additional genetic events such as p53 inactivation may represent a key event in tumor progression in tumors with disturbed MMR, especially in those tumors displaying inactivating mutations in the Bax gene.

Results

Bax expression and mutation

We analyzed tumor samples of patients who underwent R0-resection (tumor-free resection margins upon pathology review) of sporadic gastric carcinoma with a curative intent. Clinicopathological data are shown in Table 1. At the end of the follow-up period, 33 from 116 (28.4%) patients survived. Median follow-up after gastric resection for the 33 censored patients was 60.9 months (range 26–131). For the whole group, the median overall survival was 28.6 months.

In contrast to our findings in other disease entities we observed that loss of Bax expression was related to a slightly better prognosis in this cohort of patients with gastric cancer (Figure 1a, log-rank (Mantel–Cox) test: $P=0.19$; not significant).

Previous reports demonstrated that Bax protein expression may be impaired in gastrointestinal cancers with MMR deficiency because of a frameshift mutation in a G(8) tract in exon 3 of the Bax gene. For the analysis of Bax-frameshift mutations, we performed a fragment-length analysis of PCR products encompassing the G(8) tract as previously described.¹⁴ We analyzed 99 out of 116 tumors for the presence of Bax-frameshift mutations. In the remaining samples, quality of PCR-products was not sufficient for the fragment-length analysis. Out of 99, 18 (18.2%) tumors showed a frameshift

Table 1 Clinicopathological characteristics for all patients ($n=116$) and the subgroup of Bax-mutated patients ($n=18$)

	All patients <i>n=116</i>	Bax-frameshift mutated <i>n=18</i>
Male/female	69/47	7/11
Age (in years)		
Median	63.9 years	70.6 years
Range	(25–91 years)	(34 years)
<i>T-stage of primary tumor</i>		
T1	12	4
T2	50	9
T3	49	5
T4	5	0
<i>Lymph node status</i>		
N0	53	9
N1	25	5
N2	38	4
<i>Grading</i>		
G1	11	2
G2	55	10
G3	50	6
<i>Histological type</i>		
Adenocarcinoma	73	12
Signet-ring cell carcinoma	33	6
Mixed	10	0
<i>UICC stage</i>		
Ia	10	3
Ib	28	6
II	25	4
IIIa	24	2
IIIb	17	3
IV	12	0
<i>Lauren classification stage</i>		
Diffuse	40	6
Intestinal	65	10
Mixed	11	2
<i>Localization</i>		
Cardia	30	3
Fundus	7	1
Corpus	40	4
Antrum	39	10

mutation in the G(8) tract of Bax gene, leading to the formation of a truncated, nonfunctional Bax protein. Only deletions and no insertions in the G(8) tract were observed. Most Bax-frameshift-mutated tumors presented as localized stage tumors (72% in UICC stages I and II), and 56% were localized in the antrum (χ^2 test and Fisher's exact test: $P>0.05$). Figure 1b shows that patients with tumors carrying a Bax-frameshift mutation have a better overall survival. In gastrointestinal carcinomas, such Bax G(8) frameshift mutations are known to be caused by MMR deficiency. Thus, the paradoxically better clinical course in Bax-mutated tumors is well in line with the known overall better prognosis of patients with MMR-deficient tumors.^{25,26} Figure 1c confirms that Bax mutation was associated with lower Bax expression in the tumors (Mann–Whitney U -test: $P=0.0079$).

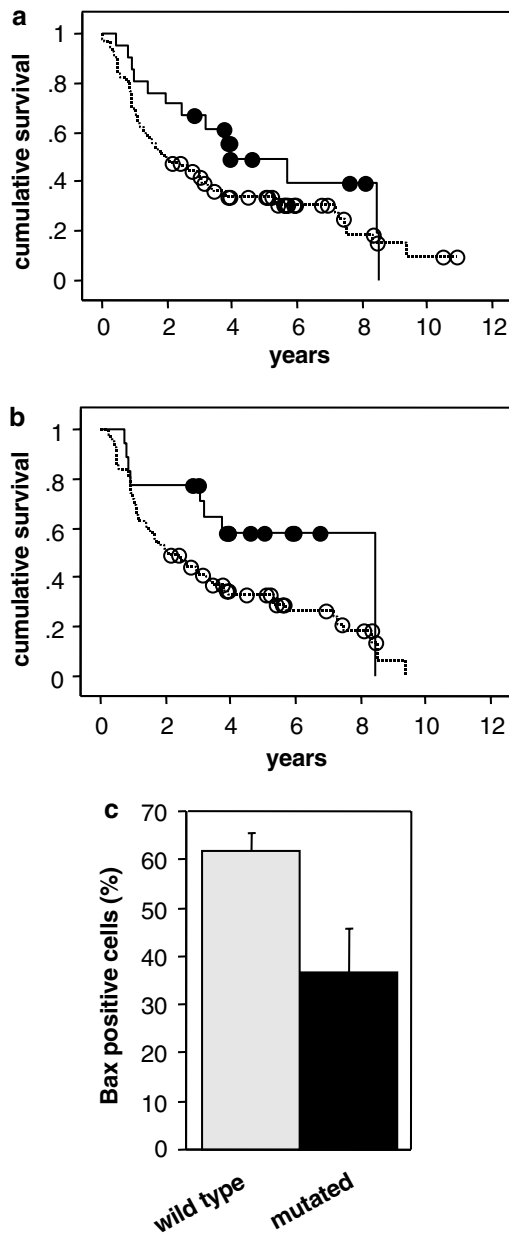


Figure 1 Bax expression and mutation in gastric cancer. Kaplan–Meier analysis of overall survival. (a) Patients were grouped in Bax positive (black circles, continuous line) and Bax negative (open circles, broken line; log-rank (Mantel–Cox) test: $P=0.19$) or (b) Bax wild type (open circles, broken line) versus Bax mutated (black circles, continuous line; frameshift mutation in the G8 tract of exon 3; log-rank (Mantel–Cox) test: $P=0.048$). (c) Bax mutation is associated with lower percentage of Bax protein expressing cells (Mann–Whitney U -test: $P=0.0079$; mean values \pm S.E.M. are shown)

p53 status and Bax expression and mutation

Apart from defining the impact of Bax mutation on Bax expression, we were interested in the role of p53, a transcriptional activator of the Bax gene and upstream activator of mitochondrial apoptosis.¹⁸ Analysis of the p53 gene by single-stranded conformational polymorphism-polymerase chain reaction (SSCP-PCR) analysis of the exons 5–8 detected p53 mutations in 24 of 110 primary tumors (21.8%).

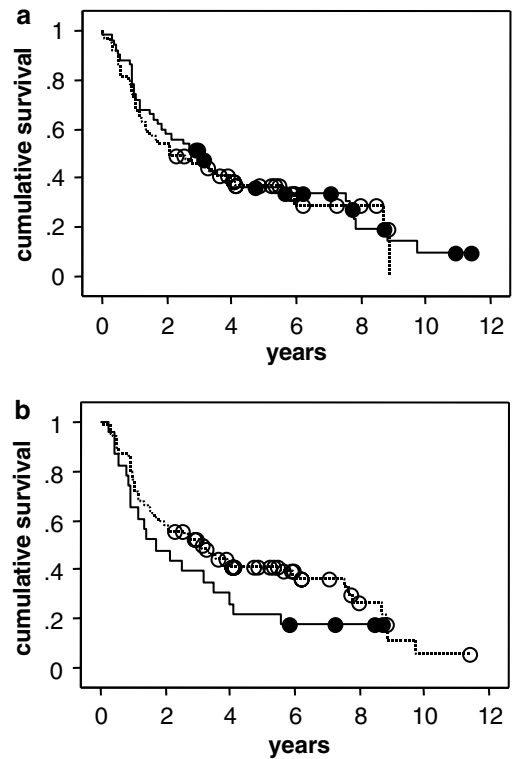


Figure 2 p53 Overexpression and p53 mutation in gastric cancer. Kaplan–Meier analysis of overall survival. (a) Patients were grouped in p53 positive (black circles, continuous line) and p53 negative (open circles, broken line; log-rank (Mantel–Cox) test: $P=0.71$) or (b) p53 wild type (open circles, broken line) versus p53 mutated (black circles, continuous line; log-rank (Mantel–Cox) test: $P=0.075$)

In the remaining cases, tumor DNA was not of sufficient quality to reliably analyze the samples for p53 SSCP-PCR upon two occasions. Altogether, 26 mutations were detected in the tumors: nine in exon 6, nine in exon 7, eight in exon 8. Two tumors exhibited mutations in two exons. In addition, overexpression of p53 was analyzed by immunohistochemistry. As shown in Figure 2a, there was no significant correlation between overall survival and p53 expression levels (log-rank test: $P=0.71$). Likewise, the presence of p53 mutations did not significantly discriminate between good or poor disease prognosis (Figure 2b). Patients with p53-mutated tumors showed a slightly worse overall survival as compared with the wild-type group, which, however, failed to reach significance (log-rank test: $P=0.075$). There was no significant correlation between p53 and Bax mutation (Table 2; χ^2 test: $P>0.99$) indicating that both events are independent. As described previously in other tumor entities,^{7,14,15} there was no correlation between p53 mutation and the level of Bax protein expression, which corroborates that additional factors, apart from p53, regulate this proapoptotic factor (Mann–Whitney U -test: $P>0.99$).

p53 mutation in Bax wild-type versus mutated tumors

In contrast to the negative findings when assessing p53 mutation in the whole patient cohort, we observed a significant

Table 2 Relation of Bax and p53 mutation*

	Bax wild type	Bax mutation**	Total
p53 wild type	63	14	77
p53 mutation***	17	4	21
	80	18	98

χ^2 test: $P > 0.999$ *Tumor DNA was suitable for both analyses in 98 of the 116 patients**Bax-frameshift mutations were detected by fragment-length analysis of the G(8) tract in exon 3***P53 mutations were analyzed by SSCP-PCR of exons 5–8

impact of p53 mutation on survival in the Bax-mutated patients (Figure 3a). Patients with tumors carrying both a mutation of the Bax gene and a p53 mutation showed an early relapse after surgery and rapidly succumbed to the disease. In contrast, patients with Bax-mutated but p53 wild-type tumors had an excellent prognosis and survival reaches a plateau of survival at 76% at 4 years after surgery. Figure 3b shows that p53 does not have any impact on survival in the Bax wild-type cohort. In addition, the small difference in overall survival as seen in Figure 2b is not present when comparing the p53 status in the Bax wild-type cohort.

Analysis for MSI

The microsatellite markers BAT-25 and BAT-26 identify with high specificity tumors expressing high-frequency MSI.^{27–29} To determine the linkage of Bax-frameshift mutations to high MSI and global MMR deficiency, we performed a PCR fragment-length analysis for BAT-25 and BAT-26. Informative results were obtained in 113 of the 116 tumors and eight tumors (6.9%) were identified as microsatellite instable, which is in line with frequencies for BAT-25/BAT-26 microsatellite instable tumors as reported by other groups.^{28,30} No p53 mutations could be detected in these eight tumors confirming that both events, p53 mutation and MMR deficiency, are related to different pathways of tumorigenesis and tumor progression.³¹ The BAT-25/BAT-26 microsatellite instable tumors showed a slightly better overall survival, which, however, did not reach the level of statistical significance (Cox–Mantel log-rank test, $P = 0.26$). Bax-frameshift analysis and BAT-25/BAT-26 mutation data were both available for 97 of the 116 patients. Contingency table testing revealed that Bax mutations do not cluster in the subgroup of tumors displaying high MSI (χ^2 test: $P > 0.99$; Table 3). This indicates that Bax mutations are related either to a mutator genotype with low MSI or may even arise sporadically, independent of MMR deficiency.

Discussion

The disruption of apoptotic pathways is a critical event in tumorigenesis and disease progression. In previous studies, we demonstrated that the loss of Bax in consequence of disturbed transcriptional control and protein expression is related to an impaired response to anticancer therapy and poor prognosis in esophageal and colorectal carcinoma.^{13–15}

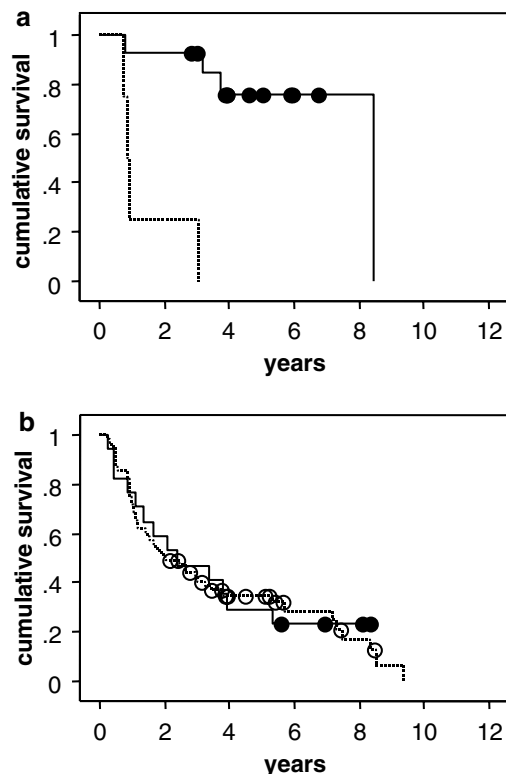


Figure 3 Combined analysis of Bax and p53 mutation. Patients were grouped in p53-mutated (open circles, broken line) versus p53 wild-type tumors (black circles, continuous line). (a) Kaplan–Meier analysis of patients with Bax-frameshift mutated tumors (log-rank (Mantel–Cox) test: $P < 0.0001$). (b) Kaplan–Meier analysis of patients with Bax wild-type tumors (log-rank (Mantel–Cox) test: $P = 0.96$). Log-rank (Mantel–Cox) test for all four groups (3 degrees of freedom): $P = 0.004$

Table 3 Relation of Bax and global MSI*

	Bax wild type	Bax mutation **	Total
MSI negative	74	16	90
MSI positive ***	6	1	7
	80	17	97

χ^2 test: $P > 0.999$ *Tumor DNA was suitable for both analyses in 97 of the 116 patients**Bax-frameshift mutations were detected by fragment-length analysis of the G(8) tract in exon 3***Global mismatch repair deficiency/MSI was determined by fragment-length analysis of BAT-25 and BAT-26 microsatellites

Gastric cancer is extremely refractory to cytotoxic anticancer therapies. In comparison to other gastroenterological tumors, prognosis is especially poor and relapse after surgery is common. Nevertheless, the role of disruption of Bax and other apoptosis regulators in this disease entity remained poorly defined. To this end, we retrospectively analyzed the expression and mutation of Bax and of p53, a transcriptional activator of the Bax gene, in 116 patients with gastric cancer, who underwent tumor resection with curative intent.

To our surprise, we found that gastric carcinoma patients with loss of Bax expression paradoxically presented with a

slightly better prognosis. Mutational analysis of Bax revealed that loss of Bax occurs in consequence of a frameshift mutation in the G(8) tract of the Bax gene being significantly associated with a better overall survival. This finding would be well in line with a MMR-deficient genotype of these patients, which in turn, is known to be associated with a better disease prognosis. The analysis of global MMR deficiency by testing for frameshifts in the BAT-25 and BAT-26 microsatellites revealed, however, that Bax-frameshift mutations are not related to a highly microsatellite instable genotype reflecting global MMR deficiency. This suggests that Bax-frameshift mutations may occur sporadically also in tumors with low MSI, which could explain the lower frequencies of Bax-frameshift mutations (25–60%) previously described in BAT-25/BAT-26 microsatellite instable tumors.^{28,32} Bax-frameshift mutations may reflect a high spontaneous mutation rate, the mitotic history of the tumor and the clonal expansions occurring during tumorigenesis and tumor progression.³³ In addition, such mutations in the Bax gene may confer a selection advantage, which led to the hypothesis that inactivation of Bax in MMR-deficient tumors represents a late event related to disease progression.²³ Given their good prognosis, such tumors could share not only a different tumor biology but also a different disease pathogenesis. Thus, in view of our present data, Bax-frameshift mutation could be a key feature of tumors with low MSI.

In contrast, concomitant mutation of p53 is rarely observed in MMR-deficient gastric cancer.²⁸ This is supported by our present observation that none of the BAT-25/BAT-26 microsatellite instable tumors displayed a p53 mutation. During gastric carcinogenesis, p53 disruption is known to be associated with progression from intestinal metaplasia to dysplasia.^{34,35} Sporadic gastric carcinomas frequently show alterations of the p53 gene, either by mutation or allelic loss. This is in clear contrast to the low frequency of p53 inactivation in microsatellite instable colorectal^{36,37} and gastric carcinomas.²⁸ Thus, it is not surprising that no clear data are available regarding the role of p53 mutation in mismatch-deficient tumors of the gastrointestinal tract.

The present study is the first report to show that the combined inactivation of p53 in Bax-mutated tumors results in early relapse and disastrous survival. In contrast, tumors with Bax-frameshift mutation but p53 wild type have a rather good prognosis. Notably, inactivation of p53 in Bax wild-type tumors did not affect prognosis. Contingency table testing demonstrates that mutation of p53 and Bax are statistically independent events. The reasons for this are unknown. Possibly, the single inactivation of either Bax or p53 can be compensated for because of the activities of redundant homologs, that is, Bak or p63/p73. The concomitant inactivation of two key regulators acting consecutively could, however, be sufficient to functionally disrupt apoptosis signaling.

In this line, it is not surprising that the role for p53 as negative prognostic indicator in gastric cancer has been discussed controversially.^{38–41} Inactivation of p53 may result in an aggressive type of gastric cancer with early infiltration of the submucosa.⁴² In the present study, both isolated p53 mutation and p53 overexpression were not associated with differences in disease prognosis. Thus, in consideration of our

data, the deleterious effect of inactivation of p53 as described in several studies in gastric cancer might be an epiphenomenon, which depends on the inactivation of Bax, as shown in this study, or disruption of other regulatory genes.

Our findings thereby support the notion that gastric carcinomas are biologically heterogeneous and may arise in part through different mechanisms. Future studies will reveal if such good or poor prognostic subgroups may also benefit from aggressive adjuvant cytotoxic therapies.

Materials and Methods

Patients

A total of 116 patients with curatively intended R0-resection of gastric carcinoma from a single institution (Department of General and Vascular Surgery, University Hospital, Frankfurt, Germany) were analyzed. Surgery was performed between 1986 and 1996. No patient received preoperative chemo- or radiotherapy. Actual follow-up data concerning survival were assessed with the help of the patients' general care physicians and the official administration department. Clinicopathological informations are given in Table 1.

Immunohistochemistry for Bax and p53

For protein detection by immunohistochemistry, 4 μ m slices of paraffin-embedded tissue were stained as described previously.^{14,15,24} Murine monoclonal antibodies against Bax (clone YTH-2D2, Trevigen Inc., MD, USA, dilution 1 : 750) or p53 (clone DO-7, Dako, Denmark, dilution 1 : 75) were employed. A blinded approach was used for slide analysis. Four high-power fields (\times 400) were evaluated for percentage of positive cells (0–100% in 5% steps for Bax and p53) and staining intensity (negative to strongly positive, categorized in integer values from 0 to 3). From the staining intensity and the percentage of positive cells, we calculated the product, which is expressed as staining index (SI). In addition, we compared the SI as measure for protein expression with the 'percentage of stained tumor cells' in all analyses.

Mutation analysis of p53

DNA was extracted from 30 μ m slices of paraffin-embedded tissue. Extraction of genomic DNA was done after deparaffination (*n*-octane) and rehydration using the Invisorb Spin Tissue Kit (Invitex, Berlin, Germany). For storage, the DNA was eluted in 10 mM Tris-HCl/0.1 mM EDTA buffer (pH 8.7). p53 Mutations in the DNA binding region were detected by SSCP-PCR (single-strand conformational polymorphism) analyses. Precise description and primer sequences for exons 5–7 of the method are given elsewhere.¹⁴ For exon eight the primers were modified (sense: CCT TAC TGC CTC TTG CTT C and antisense: GGC ATA ACT GCA CCC TTG G, 94°C followed by 35 cycles at 94°C for 30 s, 55°C for 20 s, 72°C for 20 s). Briefly, exons 5–8 of the DNA binding domain of the p53 gene were amplified, and for SSCP analysis, 5 μ l of the amplified fragments were diluted in 7 μ l loading buffer (82% formamide, 10 mmol/l NaOH, 50 mmol/l EDTA, bromophenol blue, xylene cyanole dye). The samples were denatured at 95°C for 5 min and cooled on ice. The denatured fragments were analyzed on a 10% nondenaturing polyacrylamide gel at 500 V and 50 mA for 2 h at 10°C for exons 5, 7 and 8 and at 22°C for exon 6 in a Multiphor electrophoresis chamber (Pharmacia, Freiburg, Germany) and were subsequently visualized by silver staining.

Bax-frameshift mutations

A 94 bp fragment of the Bax exon 3 encompassing the G(8) tract was amplified by PCR using primer sequences and cycling conditions as described previously.¹⁴ Taq polymerase (Invitex, Berlin, Germany) was used and the reversed primer was labeled with the ABI fluorescence dye HEX. PCR fragment length was analyzed on a ABI 310 Sequencer (Perkin Elmer Cetus, Weiterstadt, Germany) and compared to an internal size standard. As positive control the human colon carcinoma cell line LoVo was used, which carries mutations in both Bax alleles: one shows an insertion (G(9)), the other a deletion (G(7)) in the G(8) tract.²³ The human colon carcinoma cell line SW 620 served as wild-type control. In dilution experiments, the sensitivity (cutoff: 10% mutated cells) and in blinded experiments the specificity (100%) of the fragment-length analysis were confirmed.

Analysis for MSI

BAT-25 and BAT-26 microsatellites were analyzed by fragment-length polymorphism analysis as described³⁰ to identify patients with high-frequency (global) MSI.²⁷

Statistical analysis

Overall survival was estimated by the Kaplan–Meier product-limit method, starting from the time of surgery. The survival curves were compared by the means of the Mantel–Cox log-rank test. As 'normal values' for gastric cancer are not known for Bax and p53 protein expression, we employed the same cutoff points as employed in a previous study, that is, 10% positive cells.¹⁴ Expression-values above or equal to cutoff points were considered as positive, those below the cutoff values were considered as negative staining. In parallel, we analyzed the staining indices for Bax and p53 as described above which yielded results comparable to the analysis of the percentages of positively stained tumor cells.

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