The overexpression of c-met as a prognostic indicator for gastric carcinoma compared to p53 and p21 nuclear accumulation

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Abstract. The purpose of this study was to clarify the relationship of the immunohistochemical expression of c-met, p53 and p21 with clinicopathological parameters and prognosis in gastric carcinomas. We analyzed specimens from 114 gastric cancer patients (median age 64 years, range: 33-86) who underwent gastrectomy with lymphadenectomy. Specimens were categorized according to the tumor differentiation, based on UICC, WHO, Laurén, Ming and Goseki classifications. Specimens were examined immunohistochemically with antibodies against c-met, p53 and p21. The expression was evaluated semiquantitatively and correlated with the clinicopathological parameters. The c-met staining pattern was positive in 73.7%. P53 and p21 were positive in 86.8 and 67.5%, respectively. No significant correlation between c-met or p21 expression and the clinicopathological parameters was seen. A significant increase of p53 expression was observed in stage pT3 and -4. The overexpression of c-met and p53 was significantly associated with a poor prognosis in the univariate survival analysis. In the multivariate analysis this impact was maintained for c-met. P21 proved to be a significant prognostic factor in the multivariate analysis. Our data suggest that the overexpression of c-met and p21 may represent independent prognostic factors in gastric carcinoma.

Introduction

Gastric cancer is the second most common cancer in the world (1). Patient outcome varies but the prognosis is still dismal because of a high incidence of metastasis and recurrence (2). The TNM staging system for gastric cancer is widely used and it provides relevant prognostic information (3). Lymphatic and vascular invasion indicate a poor prognosis and are often observed in advanced cases (4,5). Patients with early gastric cancer have a better prognosis compared to those with advanced cancer stages (6).

Insight into genetic and molecular alterations involved in the development of gastric cancer has improved remarkably in recent years (2,7,8). Potential molecular and prognostic markers as well as new targets for cancer treatment have been proposed. However, a clear concept of molecular carcinogenesis has to be developed, and better understanding of the prognostic parameters is required. Moreover, individualized cancer treatment based on molecular targets may improve the survival of gastric cancer patients (2,9).

The progression of carcinoma and the development of metastasis result from a loss of growth control; invasion to the stromal tissue and vessels, resulting from complex multistage cascades; and networks, involving the modulation of oncogenes and/or tumor suppressor genes (10). In this context, molecular parameters and predictors for the progression and prognosis of gastric cancer were investigated in various studies. The oncogene c-met encodes a receptor tyrosine kinase which is activated by different endogenous ligands and regulates various cell signaling pathways pivotal for growth, differentiation and proliferation. The amplification and overexpression of c-met have been reported in gastric and many other carcinomas (2,11). A potential role of c-met as a prognostic marker for gastric carcinoma has been proposed (12). The molecular mechanisms regulating c-met gene transcription are largely unknown. A p53 binding site of the c-met promoter has been identified, suggesting that the c-met gene is one target of p53 gene regulation (13).

The tumor protein p53 represents the product of the tumor suppressor gene TP53, located on the short arm of chromosome 17. Of all human cancers, ~50% show loss of p53 or express an inactive, mutant protein (14). The allelic loss of p53 occurs in >60% of gastric carcinomas with tumor progression. There are still conflicting results in studies on the prognostic significance of p53 mutations in gastric cancer (15).

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One of the notable effectors of p53 is p21 which represents a general inhibitor of cyclin-dependent kinases thereby negatively controlling the cell cycle (16). In addition to its function as an inhibitor of cell cycle progression, a role for the promotion of cell survival and cell cycle progression has also been described. The expression of p21 is induced by the tumor suppressor gene, p53 and alterations of p53 may result in a loss of p21 expression. In contrast to p53, the mutation of p21 is rare (17). It is discussed that p53 and p21 alterations may be associated with specific pathological steps of gastric cancer development and contribute to tumor progression (18).

Previous attempts to unequivocally specify the prognostic significance of amplification, overexpression and mutation of the above-mentioned molecular markers for gastric carcinoma have been made. However, further studies are warranted to clarify the association of these molecular markers with clinicopathological classifications, tumor progression and prognosis (16).

In the present study, the expression of c-met, p53 and p21 in 114 gastric adenocarcinomas was investigated by immunohistochemistry. The expression of the molecular markers is correlated with patient characteristics, clinical and pathological variables as well as patient outcome.

Materials and methods

Patients. This prospective study includes a consecutive series of 114 patients with primary gastric adenocarcinoma obtained from the files of the Department of Visceral- and Vascular Surgery and the Institute of Pathology of the University of Cologne. One hundred and one patients (88.6%) underwent total gastrectomy and 12 patients (10.5%) sub-total gastrectomy. An extended lymphadenectomy (compartments I and II) was performed. An average of 39 lymph nodes was resected and analyzed for each patient. Surgery was curative (R0 resection) for 105 patients (92.1%). The remaining 9 patients (7.9%) underwent palliative gastrectomy, 6 with microscopic (R1) and 3 with macroscopic tumor residues (R2).

Tissue samples. Tumor samples were routinely fixed in 5% phospate-buffered formalin and embedded in paraffin. Serial sections (5 mm) were cut for routine staining and immunohistochemistry with hematoxylin and eosin. All slides were evaluated by two independent experienced pathologists (S.E.B. and U.D.). The tumors were documented according to the UICC, WHO, Laurén, Goseki and Ming classifications.

Immunohistochemistry and antibodies. The antibodies used were: c-met, Hepatocyte Growth Factor Receptor (clone 8F11, dilution 1:100), Novocastra, Newcastle, UK; p53, p53 (clone BP53-12-1, dilution: 1:100), Bio-Genex San Ramon, USA and p21, Waf1 Ab-1 [clone EA 10 (3), dilution 1:100], Oncogene, Boston, MA, USA.

Paraffin sections. Tissue specimens were deparaffinized according to standard histological techniques. Microwave antigen retrieval was applied in order to unmask all antigens (0.01 M citrate buffer, 650 W, 2x4 min). Endogenous peroxidase activity was blocked by 3% H_2O_2/methanol for 30 min at room temperature (RT). Non-specific binding sites were blocked by normal swine serum X 901 (Dako, Copenhagen, Denmark), diluted 1:20 (v/v) in Tris-buffered saline pH 7.2 (TBS) for 30 min at RT.

The immunohistochemical procedure. The immunomax method was applied as previously described (19). Primary antibodies were incubated overnight at 4˚C. Non-specific binding sites were blocked by rabbit serum and 2% casein PBS (1:5) as antibody solution buffer. The secondary antibody (biotinylated monoclonal rabbit anti-mouse: E0354, Dako, 1:300) was incubated for 30 min at RT. Biotin conjugation was performed by applying the ABC-HRP kit system (Dako) containing avidin and biotinylated horseradish peroxidase. Then slides were incubated in biotinylated tyramine (20 mg NHS Sulfo LC-Biotin in 0.5 ml DMSO and 6.4 g Tyramine Sigma, Munich, Germany) with TBS (1:50) and 0.03% H_2O_2 for 10 min at RT. Incubation with the ABC-AP kit (Dako) with streptavidin and biotin was performed for 30 min at RT. The reaction was visualized by applying neofuchsin as described. Counterstaining was performed with hematoxylin.

Semiquantitative analysis. All slides were evaluated by two pathologists who had no access to patient data and clinical status. Scoring was exclusively restricted to tumor cell staining, stromal staining was not considered. The degree of expression of all the markers was estimated by semiquantitative evaluation and described in %. The scores used were 0, 0-5; 1, 5-30; 2, 30-60 and 3, >60%. The cut-off points chosen for c-met tumors were positive with >30% cytoplasmatic and membraneous expression and for p53 and p21 positive with >5% nuclear expression.

Statistical analysis. Explorative, univariate data analysis was performed (SPSS Inc., Chicago, IL, USA). In order to evaluate correlations between the staining results and clinicopathological variables, the Chi-square test was applied at a significance level of 5%. A univariate survival analysis was performed according to the Kaplan-Meier approach. To analyze the predictive value of c-met, p53 and p21 compared to other known predictors, Cox's regression analysis was performed. The following variables were included in the conditional forward model: TNM-stage, gender and protein expression of c-met, p53 and p21. A supplementary multivariate analysis included all available parameters (including c-met, p53 and p21 expression) in a conditional forward model. The follow-up of surviving patients was at least 5 years. Two patients were unavailable for follow-up. Informed consent was obtained from all patients.

Results

Tissue specimens of 114 patients were investigated. The median age of all patients was 64.28 years (SD: 33-85), 67 patients were male and 47 were female (ratio, 1:38).

Expression of c-met, p53 and p21. C-met expression was observed on the cell membranes and in the cytoplasm of gastric carcinoma cells (Fig. 1A). Gastric adenocarcinomas (84,73.7%) displayed a positive staining reaction for c-met

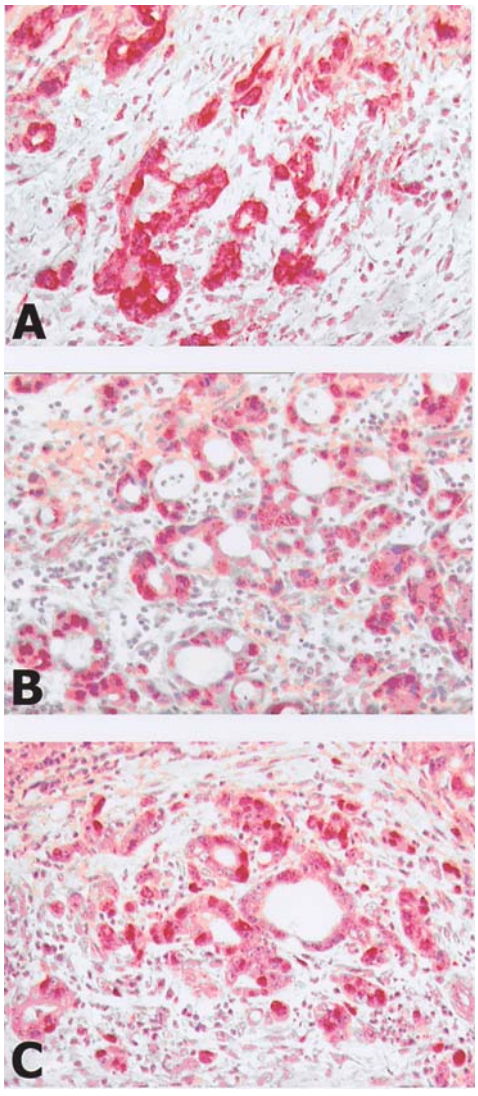

Figure 1. Immunohistochemical staining of gastric carcinoma. (A) Positive c-met staining with a membraneous and cytoplasmatic pattern. (B) Nuclear staining pattern for p53. (C) Nuclear staining pattern for p21 (A-C, 1:250).

(score 2: n=41 and score 3: n=43) while 30 (26.3%) specimens were negative (score 0: n=6 and score 1: n=24).

The nuclear expression of p53 was noted in 86.8% of the adenocarcinomas (n=99) and p53 negativity in 15 cases (13.2%). Nuclear staining for p21 was positive in 77 (67.5%) of the adenocarcinomas and negative in 37 cases (32.5%) (Fig. 1B and C).

Correlation of c-met, p53 and p21 with clinicopathological parameters. A significant correlation between the c-met expression and variables such as gender, age, WHO classification, tumor differentiation, Laurén, Goseki, Ming and TNM classification or UICC stage was not found (Table I).

No significant correlation between p53 expression and gender, age, WHO, Laurén and Ming classification, as well as the grade of differentiation was found (Table II). An increased expression of p53 was observed in advanced pT stages (p=0.010) as well as in advanced pTNM stages (p=0.002). A significant correlation of p53 expression and nodal status was not found (p=0.256).

Table I. C-met tissue status and clinicopathological parameters.

	Positive c-met staining (score 2-3)			
	N	c-met+	%	p
Sex				
Male	67	50	74.6	0.785
Female	47	34	72.3	
WHO				
Papillary	5	3	60.0	0.160
Tubular	67	53	79.1	
Signet-ring cell	36	22	61.1	
Mucinous	3	3	100.0	
Unclassified	3	3	100.0	
Differentiation				
Well	1	1	100.0	0.739
Moderate	32	22	68.8	
Poor	81	61	75.3	
Laurén				
Intestinal	44	34	77.3	0.705
Diffuse	55	40	72.7	
Mixed	15	10	66.7	
Goseki				
I	52	38	73.1	0.249
II	9	8	88.9	
III	20	17	85.0	
IV	33	21	63.6	
Ming				
Expanding	44	34	77.3	0.490
Infiltrative	70	50	71.4	
Tumor staging				
pT1	22	14	63.6	0.090
pT2	38	28	73.7	
pT3	44	37	84.1	
pT4	10	5	50.0	
Nodal status				
pN0	40	29	72.5	0.473
pN1	34	23	67.7	
pN2	18	13	72.2	
pN3	22	19	86.4	
Metastasis				
M0	95	71	74.7	0.568
M1	19	13	68.4	
UICC				
Ia	18	12	66.7	0.367
Ib	18	11	61.1	
II	21	18	85.7	
IIIa	13	11	84.6	
IIIb	8	7	87.5	
IV	36	25	69.4	

Table II. p53 tissue status and clinicopathological parameters.

	Positive p53 staining (>10% positive tumor cells)			
	N	p53+	%	p
Sex				
Male	67	42	89.4	0.505
Female	47	57	85.1	
WHO				
Papillary	5	4	80.0	0.315
Tubular	67	61	91.0	
Signet-ring cell	36	28	77.8	
Mucinous	3	3	100.0	
Unclassified	3	3	100.0	
Differentiation				
Well	1	1	100.0	0.745
Moderate	32	27	84.4	
Poor	81	71	87.7	
Laurén				
Intestinal	44	38	86.4	0.721
Diffuse	55	47	85.5	
Mixed	15	14	93.3	
Goseki				
I	52	46	88.5	0.317
II	9	9	100.0	
III	20	18	90.0	
IV	33	26	78.8	
Ming				
Expanding	44	38	86.4	0.905
Infiltrative	70	61	87.1	
Tumor staging				
pT1	22	15	68.2	0.010
pT2	38	32	84.2	
pT3	44	42	95.5	
pT4	10	10	100.0	
Nodal status				
pN0	40	32	80.0	0.256
pN1	34	29	85.3	
pN2	18	17	94.4	
pN3	22	21	95.5	
Metastasis				
M0	95	81	85.3	0.265
M1	19	18	94.7	
UICC				
Ia	18	11	61.1	0.002
Ib	18	17	94.4	
II	21	16	76.2	
IIIa	13	13	100.0	
IIIb	8	8	100.0	
IV	36	34	94.4	

No statistical correlation between the p21 expression and clinicopathological parameters was found (Table III).

Survival analysis. The median overall survival of the patients was 31.55 months. A Kaplan-Meier analysis showed a significant correlation between the time of survival and UICC stage ($p<0.0001$), pT, pN and pM stage ($p<0.0001$) as well as the Laurén classification ($p=0.03$).

The univariate survival analysis and protein expression. A univariate analysis, using the Kaplan-Meier method, showed a significantly lower survival probability for c-met positive compared to c-met negative carcinomas ($p=0.0035$) (Fig. 2). Kaplan-Meier analysis was subsequently performed on clinicopathological features in order to assess the influence of stratification. The prognostic significance of c-met expression was most important in the cases of diffuse type carcinoma (Laurén) ($p=0.0278$) and infiltrative carcinoma according to Ming ($p=0.0061$).

The univariate analysis, using Kaplan-Meier, showed a significantly higher median survival time for patients with p53-negative adenocarcinomas compared to those with p53-positive tumors ($p=0.0499$). For p53-positive tumors the median survival time was 39.14 months, for p53-negative tumors it was 55.17 months (Fig. 3). The stratification of the clinicopathological parameters showed a significantly higher survival of patients with p53 negative carcinomas and diffuse type carcinoma according to Laurén ($p=0.0306$) as well as infiltrative carcinoma according to the Ming classification ($p=0.0453$).

A significant difference in survival probability between p21 negative and positive groups was not found in the univariate Kaplan-Meier analysis ($p=0.0578$). However, the median survival time of p21-negative patients (50.28 months) was higher than that of the positive patients (37.2 months) (Fig. 4).

Multivariate survival analysis. Significant prognostic factors resulting from the univariate analysis were included in the multivariate survival analysis in addition to c-met, p21 and p53 expression. The pTNM stage, c-met ($p<0.001$) and p21 ($p=0.008$) expression proved to be independent prognostic factors. A supplementary multivariate analysis including all available parameters was conducted. This analysis demonstrated a prognostic significance for T, N and M stages ($p<0.001$) and c-met ($p<0.049$) and p21 ($p=0.010$) (Table IV).

Discussion

Gastric carcinoma is a heterogeneous disease, biologically and genetically (2,8). Further characterization of the various molecular genetic pathways should elucidate the role of independent prognostic factors and afford opportunities to discriminate subgroups with different biological behaviour (20). More specific and effective therapies could be developed according to the tumor characterization with regard to independent molecular prognostic factors (2).

A good correlation of c-met expression between the mRNA and the protein level has been confirmed in the literature by several authors (11,21). C-met has been reported to be overexpressed in 18-68% of gastric cancer tissues, and c-met

Table III. p21 tissue status and clinicopathological parameters.

	Positive p21 staining (>5% positive tumor cells)			
	N	p21+	%	p
Sex				
Male	67	43	64.2	0.360
Female	47	34	72.3	
WHO				
Papillary	5	2	40.0	0.301
Tubular	67	46	68.7	
Signet-ring cell	36	25	69.4	
Mucinous	3	3	100.0	
Unclassified	3	1	33.3	
Differentiation				
Well	1	1	100.0	0.713
Moderate	32	23	71.9	
Poor	81	53	65.4	
Laurén				
Intestinal	44	30	68.2	0.992
Diffuse	55	37	67.3	
Mixed	15	10	66.7	
Goseki				
I	52	35	67.3	0.449
II	9	4	44.4	
III	20	14	70.0	
IV	33	24	72.7	
Ming				
Expanding	44	30	68.2	0.908
Infiltrative	70	47	67.1	
Tumor staging				
pT1	22	16	72.7	0.064
pT2	38	26	68.4	
pT3	44	32	72.7	
pT4	10	3	30.0	
Nodal status				
pN0	40	28	70.0	0.824
pN1	34	21	61.8	
pN2	18	12	66.7	
pN3	22	16	72.7	
Metastasis				
M0	95	64	67.4	0.929
M1	19	13	68.4	
UICC				
Ia	18	13	72.2	0.955
Ib	18	11	61.1	
II	21	15	71.4	
IIIa	13	8	61.5	
IIIb	8	6	75	
IV	36	24	66.7	

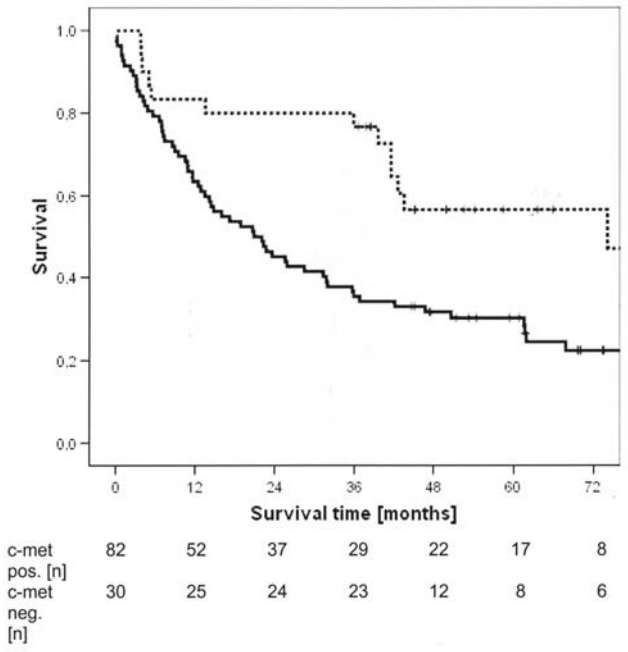

Figure 2. Survival analysis of patients with c-met positive (—) and negative carcinomas (---).

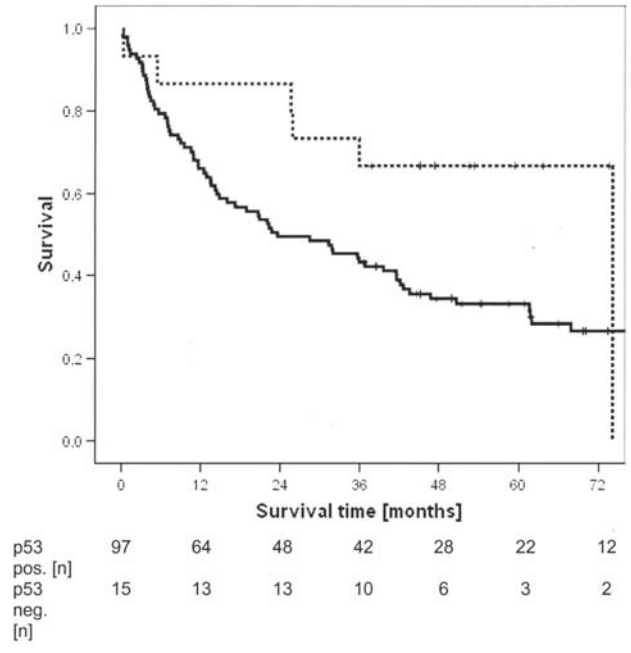

Figure 3. Survival analysis of patients with p53 positive (—) and negative carcinomas (---).

expression correlated to advanced tumor stages (3,11,12,21,22). C-met amplification has been reported to occur in a higher percentage of diffuse type gastric carcinomas compared to intestinal type cancers (23). Nakajima *et al* (12) described c-met overexpression as an independent prognostic factor for gastric carcinoma indicating a significantly poorer survival. Amemiya *et al* found a significantly higher frequency of c-met expression in stage IV gastric cancer with liver metastasis

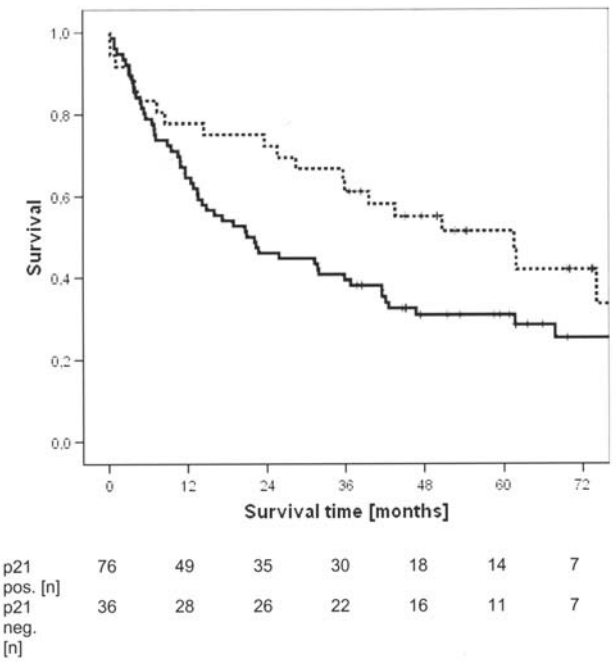

Figure 4. Survival analysis of patients with p21 positive (—) and negative carcinomas (---).

than in stage IV carcinoma without liver metastasis, proposing the role of c-met as a useful indicator of liver metastasis (11). Huang *et al* described a correlation between the depth of tumor invasion and c-met expression, but did not find an association with lymph node, liver or peritoneal metastasis (3). In contrast to others we did not find a correlation between c-met protein expression and tumor stage according to the TNM classification, neither a higher rate of c-met positive carcinomas in diffuse type carcinoma compared to intestinal type. However, in agreement with Nakajima *et al* (12), Huang *et al* (3) and Carneiro *et al* (24) we found a statistically significant correlation between c-met expression and a poor prognosis, confirming the role of c-met as independent prognostic factor. According to our data, the prognostic significance of c-met was most important in diffuse type gastric carcinomas (infiltrating type), as well as in carcinomas with advanced stages (pT4).

Of note is that the HGF promoter was reported to be transcriptionally activated by p53. These findings suggest that wild-type p53 plays a role in controlling the expression of c-met and thus ultimately leading to the regulation of cell growth and differentiation (13).

The tumor suppressor gene TP53 controls the cell cycle and prevents genetic mutations for carcinogenesis (14). It is frequently mutated in gastric carcinoma and the mutant protein has a longer half-life and a higher stability than the wild-type protein. P53 mutations are known to occur in 30-50% of gastric carcinomas (25,26). However, the increase in p53 staining is not always due to a mutated gene, since the overexpression and inhibition of p53 mRNA degradation is a normal mechanism that slows down the cell cycle (27). The immunohistochemical expression of p53 ranges from 13-54% (15,18,28). In our study, 86.8% of the carcinomas expressed p53 which correlated significantly with advanced T stage.

Table IV. Multivariate regression analysis.

	p	Hazard ratio (95%CI)
pT	0.000	
pT1-pT2	0.001	7.7 (2.2-26.8)
pT-pT3 and 4	0.000	17.4 (5.0-60.9)
pN	0.036	
pN0-pN1	0.563	0.8 (0.4-1.6)
pN0-pN2	0.790	1.1 (0.5-2.4)
pN0-pN3	0.030	2.2 (1.1-4.6)
pM	0.000	3.8 (2.0-7.1)
c-met	0.038	1.9 (1.0-35)
p21	0.008	2.1 (1.2-3.6)

However, there is controversy about the correlation with p53 expression and clinicopathological variables. Some authors observed an association between stage and p53 expression (15,18,29) while others did not confirm such a relationship (30,31).

There are conflicting results concerning the prognostic significance of p53 expression. In Western countries, a poor prognostic effect of p53 overexpression was described (15,18), whereas in Japanese studies p53 overexpression was not related to a poor prognosis. In our study, the univariate survival analysis showed a significantly longer survival in patients with p53 negative carcinomas. However, in the multivariate regression analysis, p53 expression was not confirmed as an independent prognostic factor. Similar results were obtained by Lee *et al* (15) and Pinta-de-Susa *et al* (32). A critical downstream effector of p53 is the protein encoded by p21. P21 acts as a potential inhibitor of cyclin-dependent kinases (33). Thus, the p21 gene is thought to play a central role in tumor suppression. Alterations of the p21 expression have been observed in a wide variety of human carcinomas. In gastric carcinomas p21 expression has been reported in 32-75% (34). Loss of the p21 expression correlated with advanced stage, lymph node metastasis and a poor survival (16,35). In our study, 67.5% of the carcinomas were positive for p21. We found no correlation between the low p21 expression and advanced tumor stage or lymph node metastasis. However, we observed a better prognosis for patients with p21-positive compared to negative carcinomas. This correlation was significant in the multivariate survival analysis, strengthening the importance of p21 as an independent prognostic factor.

In conclusion, we demonstrated that the immunoreactivity of c-met and p21 are independent prognostic factors in gastric cancer. The identification of c-met expression may be an additional tool in identifying subgroups of more aggressive gastric carcinomas that may benefit from multimodal treatment.

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