ORIGINAL ARTICLE

Prognostic significance of $p21^{WAF1/CIP1}$, $p27^{Kip1}$, p53 and E-cadherin expression in gastric cancer

Armando Gamboa-Dominguez, Stefan Seidl, Edgardo Reyes-Gutierrez, Christine Hermannstädter, Leticia Quintanilla-Martinez, Raymonde Busch, Heinz Höfler, Falko Fend, Birgit Luber

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See end of article for authors' affiliations

Correspondence to: Dr Birgit Luber, Institut für Allgemeine Pathologie und Pathologische Anatomie, Trogerstr. 18, 81675 München, Germany; luber@lrz.tu-muenchen.de

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Background: Gastric carcinoma is characterised by numerous genetic and epigenetic alterations that influence cell cycle progression, apoptosis and DNA repair. These alterations include down-regulation of the cyclin-dependent kinase (CDK) inhibitors $p21^{WAF1/CIP1}$ and $p27^{Kip1}$, and mutations of the tumour suppressor protein p53 and the cell adhesion molecule E-cadherin. Combined evaluation of the prognostic significance of these alterations has not been reported in Mexican Mestizo patients.

Aims: To evaluate $p21^{WAF1/CIP1}$, $p27^{Kip1}$, p53 and E-cadherin protein expression, including mutant E-cadherin variants with deletion of exon 8 (*del 8*) or 9 (*del 9*), in gastric cancer from Mexican patients.

Methods: Immunohistochemistry for the above-mentioned markers, including mutation-specific E-cadherin antibodies, was carried out in 69 gastric carcinomas; expression levels were correlated with histotype, tumour stage and prognosis.

Results: Expression of $p21^{WAF1/CIP1}$ alone or in combination with $p27^{Kip1}$ or in the absence of p53 was associated with favourable prognosis. Staining of *del 8* and *del 9* E-cadherin was found exclusively in patients negative for p53 and positive for $p21^{WAF1/CIP1}$, suggesting that the $p21^{WAF1/CIP1}$ regulatory function of p53 was intact.

Conclusion: Combined evaluation of the prognostic significance of cell cycle regulators and E-cadherin should be performed. Even though patients negative for p53 and positive for $p21^{WAF1/CIP1}$ have a favourable prognosis, it may have a negative influence on prognosis if they acquire in addition E-cadherin mutations which have been shown previously to be associated with poor survival.

Gastric carcinoma is one of the most frequent malignancies worldwide and one of the leading causes of cancer mortality in Mexico, with a higher prevalence of diffuse versus intestinal type gastric carcinoma in low-income Mestizo descendants.[1] In contrast, subtype distribution is comparable to that in western countries in medium-income patients.[2] These differences might result from dietary or environmental influences. The question whether gene expression patterns of gastric carcinomas of Mexican origin is different from tumours of European or Asian countries remains open. Common genetic and epigenetic alterations in gastric cancer are mutations in the tumour suppressors E-cadherin and p53.[3] In addition, $p21^{WAF1/CIP1}$ and $p27^{Kip1}$, CDK inhibitors of the CIP/Kip family that control the G1-S transition, are frequently down-regulated. Although a multitude of studies has examined the prognostic significance of the markers mentioned above, results are controversial, and little is known about their prognostic relevance in high risk groups, such as Mexican Mestizo patients.

Decrease of $p21^{WAF1/CIP1}$[4–6] and $p27^{Kip1}$ expression has frequently been associated with poor prognosis in gastric cancer,[7–9] while some authors failed to detect a prognostic significance of both markers.[10 11] In gastric cancer, the prognostic value of p53 is under discussion, since most of the studies show an association of p53 with patient survival,[12–15] while other investigations do not support these findings.[4] E-cadherin participates in cell cycle regulation by up-regulating $p27^{Kip1}$.[16] Somatic E-cadherin mutations occur predominantly in diffuse type gastric carcinomas.[17–22] The most common mutations in gastric cancer are splice-site mutations and in-frame deletions located in exons 8 or 9.[22 23] We have recently shown that abnormal E-cadherin expression on the protein level in gastric adenocarcinomas from Mexican Mestizo patients has low impact on patient survival.[24] However, patients carrying deletions of exons 8 or 9, observed in 5.3% of tumours in this series, had a worse prognosis than patients without these alterations.[24]

In the present study, we examined the expression and prognostic significance of $p21^{WAF1/CIP1}$, $p27^{Kip1}$, p53 and E-cadherin in a Mexican series of gastric adenocarcinomas.

MATERIALS AND METHODS

Patient selection

Patients with a diagnosis of gastric adenocarcinoma who had undergone partial or total gastrectomy from 1982 to 2001 in the Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, with available clinical information and follow-up were included in the study. A blinded review of the slides was made by two pathologists (AG-D, I Becker) and a diagnosis according to Laurén's classification of gastric adenocarcinoma was established.[24 25] Mexican Mestizo patients with available paraffin material and a morphological diagnosis of poorly differentiated intestinal, mixed or diffuse-type adenocarcinoma in which UICC (International Union against Cancer) staging criteria could be applied, were included.[26]

Immunohistochemical analysis

Antibodies against p53 (clone DO-7, M7001, dilution 1:20) and Ki-67 (clone MIB-1, M7240, dilution 1:50) were purchased from DakoDiagnostika (Hamburg, Germany) Staining was carried out on automated immunostainers (Nexes, Benchmark, Ventana, Tucson, Arizona, USA) with the recommended reagents. Antigen

Abbreviations: CDK, cyclin dependent kinase; *del 8* E-cadherin, E-cadherin with deletion of exon 8; *del 9* E-cadherin, E-cadherin with deletion of exon 9; TNM, tumour node metastasis

Table 1 Clinicopathological features of 69 patients with gastric cancer

Age (y)		
Mean	59.2	
Median	62.0	
SD	15.4	
Range	14–86	
	n	%
Gender		
Female	35	50.7
Male	34	49.3
Tumour location		
Antrum	22	31.9
Corpus/fundus	25	36.2
Gastro-oesophagic junction	11	15.9
More than one zone	11	15.9
Histotype (Laurén)		
Intestinal	28	40.6
Diffuse	39	56.5
Mixed	2	2.9
Stage (UICC)		
IA	2	2.9
IB	1	1.4
II	15	21.7
IIIA	17	24.6
IIIB	12	17.4
IV	22	31.9
Residual disease		
R0	54	78.3
R1	15	21.7

UICC, International Union against Cancer

retrieval was performed in CC1 buffer from Ventana in a water bath (1 h, 100°C). A manual staining protocol was used for $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ staining with the following antibodies and dilutions: $p21^{WAF1/CIP1}$ antibody (DakoDiagnostika, clone SX118, M7202, dilution 1:20), $p27^{Kip1}$ antibody (clone F-8, sc-1641, Santa Cruz Biotechnology, Heidelberg, Germany, dilution 1:10). Staining was carried out with LSAB-DAB from Dako. Ten representative high power fields were counted for each antibody staining and the percentage of positive tumour cells was calculated. Immunohistochemical analysis of the E-cadherin expression profile and of mutant E-cadherin variants (*del 8* and *del 9*) was carried out as previously described.[24] In brief, antibody clone 36 against E-cadherin (Transduction Laboratories, Lexington, Kentucky, USA, dilution 1:1000) and the mutation-specific antibodies E-cad delta 8-1 and E-cad delta 9-1, recognising *del 8* or *del 9* E-cadherin, have been used.[27 28] For qualitative analysis of the E-cadherin staining pattern, three different staining patterns were analysed: 1, normal; 2, abnormal (including *atypical* with partial membrane staining and cytoplasmic staining and *heterogeneous* staining with positive and negative tumour cells in the same slide); or 3, negative staining. Tumours were considered as *del 8* or *del 9* positive when membranous staining was detected in tumour cells, without quantifying the percentage of positive tumour cells.

Statistical analysis

Evaluation was performed by three pathologists (AG-D, SS, FF) who were unaware of clinical features and survival. Statistical analyses were performed using Fisher's exact, Kruskal–Wallis or χ^2 tests when appropriate. Kaplan–Meier survival time analysis was used to correlate the investigated markers with pT, pN, and pM status with clinical evolution. Cox regression analysis was performed correlating the investigated markers with prognosis. A two sided p value <0.05 was considered to be statistically significant.

RESULTS

Table 1 shows the clinicopathological features of 69 Mexican Mestizo patients with gastric cancer.

The 69 cases were investigated immunohistochemically for expression of $p21^{WAF1/CIP1}$, $p27^{Kip1}$, p53, Ki-67 and E-cadherin (including mutant E-cadherin variants with deletions of exons

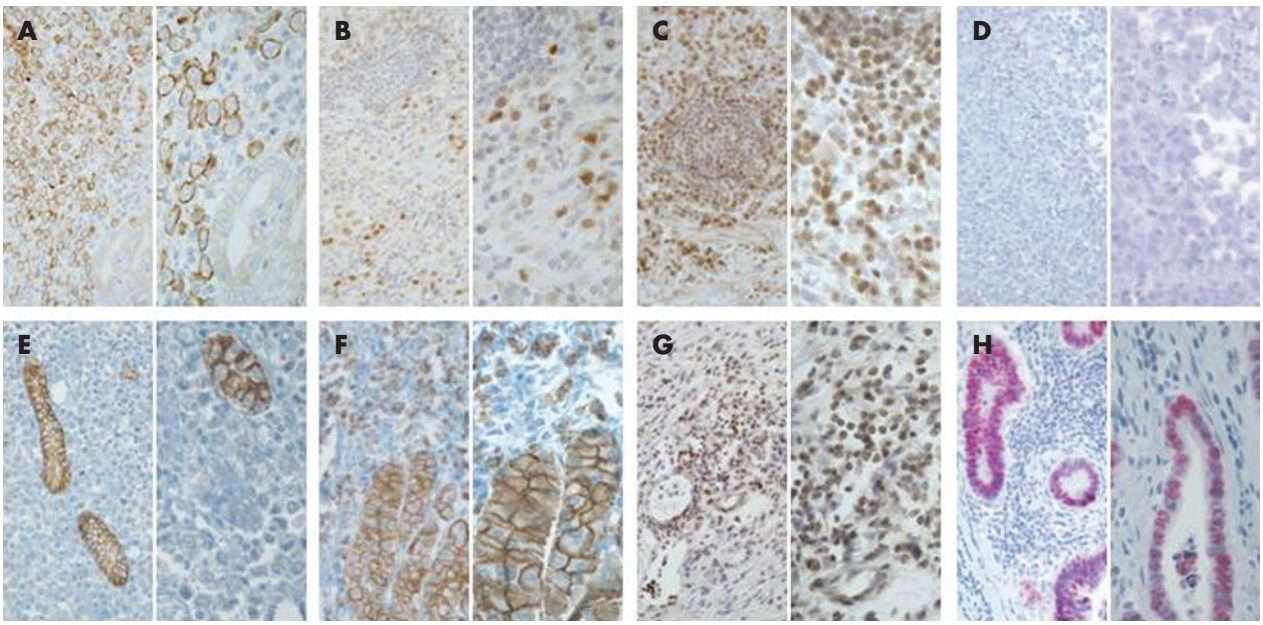

Figure 1 Immunohistochemical staining for *del 9* E-cadherin, p53, $p21^{WAF1/CIP1}$, and $p27^{Kip1}$ in a diffuse type gastric cancer. (A) Staining with delta 9-1 antibody recognising E-cadherin lacking exon 9. Note negativity of residual normal glands. (B) Nuclear expression of $p21^{WAF1/CIP1}$ was detected in 30% of gastric carcinoma cells and not in lymphocytes. (C) Nuclear $p27^{Kip1}$ staining was present in 50% of tumour cells and in lymphocytes. (D) Complete absence of p53 staining. (E, F) Negative (E) or abnormal heterogeneous (F) E-cadherin expression in diffuse type gastric cancer cases is shown. (G) Absence of $p27^{Kip1}$ expression in neoplastic cells in an example of a diffuse type gastric cancer. Note reactivity of lymphocytes. (H) Positive p53 staining in 60% of tumour cells was detected in an intestinal type gastric carcinoma. Original magnification: left panel ×200, right panel ×400.

Table 2 Relationship between the percentage of $p21^{WAF1/CIP1}$, $p27^{Kip1}$, p53 or Ki-67 positive tumour cells and clinicopathological parameters

		Median (range) in %			
	n	$p21^{WAF1/CIP1}$	$p27^{Kip1}$	p53	Ki-67
Histotype (Laurén)					
Intestinal	28	15.0 (0–50)	9.5 (0–60)	5.0 (0–80)	40.0 (10–70)
Mixed	2	19.5 (9–30)	30.0 (0–60)	45.0 (0–90)	32.5 (15–50)
Diffuse	39	20.0 (0–70)	32.5 (0–65)	17.5 (0–90)	20.0 (0–55)
p value		0.712	0.004	0.578	0.001
Tumour invasion					
pT 1–2	6	25.0 (13–70)	20.0 (0–50)	0.0 (0–40)	20.0 (10–65)
pT 3–4	63	20.0 (0–70)	20.0 (0–65)	15.0 (0–90)	25.0 (0–70)
p value		0.195	0.874	0.218	0.844
Perigastric lymph node status					
pN0	18	30.0 (0–70)	35.0 (0–65)	20.0 (0–70)	15.0 (5–55)
pN1–2	51	15.0 (0–70)	20.0 (0–60)	10.0 (0–90)	25.0 (0–70)
p value		0.086	0.621	0.482	0.022
Distant metastases					
pM0	56	20.0 (0–70)	15.0 (0–65)	15.0 (0–90)	22.5 (0–70)
pM1	13	10.0 (0–60)	20.0 (9–60)	9.5 (0–80)	25.0 (10–70)
p value		0.225	0.091	0.940	0.829

p values were obtained by Kruskal–Wallis test.

8 or 9). Figure 1 shows an example of a gastric cancer sample with tumour cell staining for *del 9* E-cadherin (A), $p21^{WAF1/CIP1}$ (B) and $p27^{Kip1}$ (C) and absence of p53 staining in neoplastic cells (D). In addition, examples of tumours lacking E-cadherin expression (E) or with abnormal E-cadherin expression (F) in neoplastic cells as well as a case without $p27^{Kip1}$ expression in neoplastic cells (G) and a p53 positive tumour (H) are depicted.

The percentage of positivity was correlated with the clinicopathological parameters histotype and tumour node metastasis (TNM) stage (table 2). $p27^{Kip1}$ expression was significantly associated with histotype ($p = 0.004$), with a higher median expression level in diffuse (32.5%) compared to intestinal type gastric carcinoma (9.5%). Ki-67 expression was also significantly correlated with histotype, with a median positivity of 40.0% in intestinal versus 20.0% in diffuse type gastric cancer ($p = 0.001$). These results indicate an inverse correlation between $p27^{Kip1}$ and Ki-67 expression. Ki-67 staining was correlated with the perigastric lymph node status ($p = 0.022$), but not with pT stage, or distant metastasis formation. No association between $p27^{Kip1}$ reactivity and TNM stage was identified. For $p21^{WAF1/CIP1}$ and p53, no correlation was detectable with histotype or TNM status.

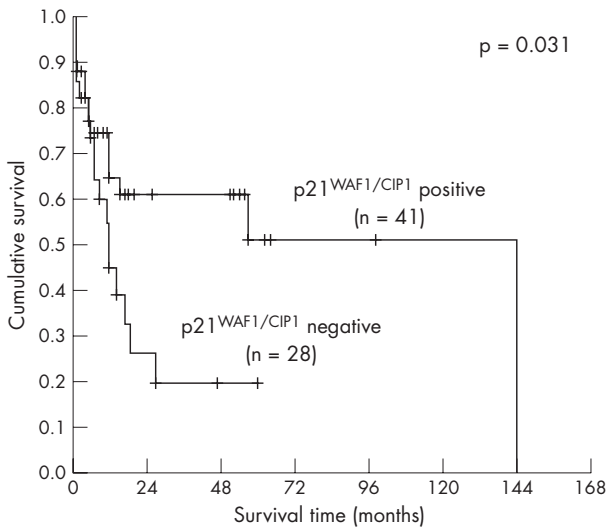

Figure 2 Survival impact of $p21^{WAF1/CIP1}$ expression. Kaplan–Meier survival curve for the 69 patients with gastric carcinoma stratified according to $p21^{WAF1/CIP1}$ expression status in gastric carcinoma cells. A cut-off of 15% was used.

The optimal cut-offs separating positive and negative cases were searched by correlating $p21^{WAF1/CIP1}$, $p27^{Kip1}$ or p53 expression with patient survival using the log rank test. Cut-offs used in the literature did not discriminate well within our Mexican patient collective, explaining the requirement of a statistical search for the best cut-offs. Tumours were considered as positive when they expressed >15% $p21^{WAF1/CIP1}$, >35% $p27^{Kip1}$ or >30% p53. Of the 69 carcinomas examined, 41 (59.4%) showed positive tumour cell staining for $p21^{WAF1/CIP1}$, 23 (34.3%) for $p27^{Kip1}$ and 24 (34.8%) for p53.

The Kaplan–Meier method was used to correlate $p21^{WAF1/CIP1}$, $p27^{Kip1}$ and p53 expression alone and in combination with patient survival. The mean and median of the overall patient follow-up were 19.3 or 11.0 months, respectively (range 1–144 months, SD 25.5 months). A significant correlation with survival was detected for $p21^{WAF1/CIP1}$ (fig 2, log-rank 0.031), but not for $p27^{Kip1}$ (fig 3) or p53 (fig 4). When a combination of p53 negative *and* $p21^{WAF1/CIP1}$ positive cases was compared with p53 positive *or* $p21^{WAF1/CIP1}$ negative cases, a significant association with survival was observed (fig 5, log-rank 0.028). Patients expressing either $p21^{WAF1/CIP1}$ *or* $p27^{Kip1}$ had a significantly better prognosis than patients negative for both CDK inhibitors (fig 6, log-rank 0.011).

The prognostic value of $p21^{WAF1/CIP1}$, $p27^{Kip1}$, and p53 was also investigated in combination with E-cadherin expression because E-cadherin plays a role in growth regulation and E-cadherin mutations are associated with poor prognosis.[24] Four cases with *del 8* or *del 9* reactivity were included (5.8%), two cases with *del 8*, and two cases with *del 9* E-cadherin (three cases were of diffuse and one of mixed type). All cases with exon 8 or 9 deletion were p53 negative (maximal 5% p53 positive tumour cells) and $p21^{WAF1/CIP1}$ positive (table 3, $p = 0.009$). No significant association between absence of p53 staining and presence of $p21^{WAF1/CIP1}$ was detected with abnormal E-cadherin expression (clone 36 staining), although a trend was also observed here ($p = 0.075$). Cases with normal E-cadherin expression were either p53 positive *or* $p21^{WAF1/CIP1}$ negative, but never p53 negative *and* $p21^{WAF1/CIP1}$ positive.

A multivariate analysis using Cox's proportional hazard model was used to correlate expression of $p21^{WAF1/CIP1}$,

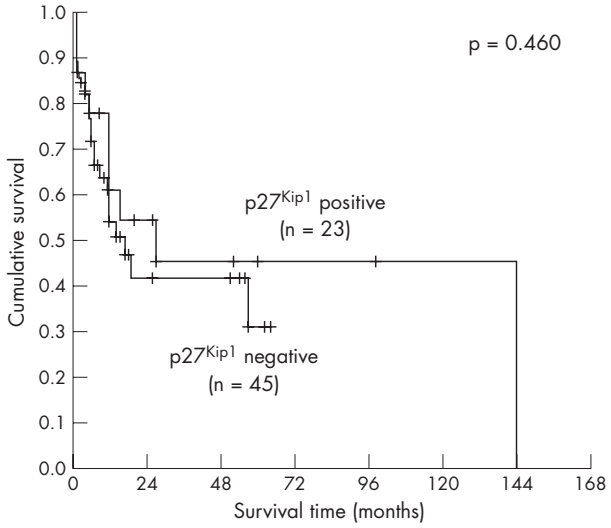

Figure 3 Survival impact of $p27^{Kip1}$ expression. Kaplan–Meier survival curve for 68 patients with gastric carcinoma stratified by $p27^{Kip1}$ expression in tumour cells. A cut-off of 35% was used.

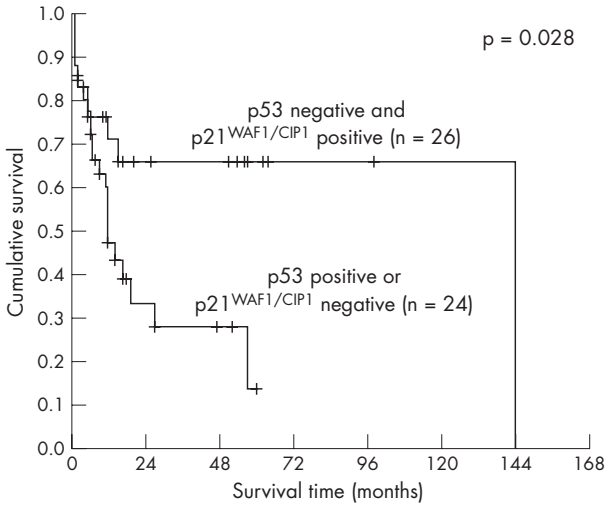

Figure 5 Survival impact of a combination of p53 and $p21^{WAF1/CIP1}$ expression. Kaplan–Meier survival curve for 68 patients with gastric carcinoma divided into two groups by absence of p53 staining in combination with positivity for $p21^{WAF1/CIP1}$ (group 1) and p53 positivity in combination with a lack of $p21^{WAF1/CIP1}$ (group 2).

$p27^{Kip1}$, p53, Ki-67, E-cadherin (including *del 8* and *del 9* E-cadherin), with prognosis. $p21^{WAF1/CIP1}$ in combination with $p27^{Kip1}$ ($p21^{WAF1/CIP1}$ or $p27^{Kip1}$ positive versus $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ negative) was significantly correlated with survival and is an independent predictor of patient survival ($p = 0.019$). Residual disease status and stage were significantly correlated with each other ($p = 0.001$). Even if the residual disease status was included into the multivariate analysis, only the combination of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ expression resulted in statistical significance.

DISCUSSION

In this study, we obtained evidence that combined evaluation of several markers important for cell proliferation in a Mexican series of gastric carcinomas is of predictive value for the estimation of patients' survival. Expression of $p21^{WAF1/CIP1}$ alone or in combination with $p27^{Kip1}$ or in the absence of p53 was associated with favourable prognosis. E-cadherin was also investigated, because it acts as a growth suppressor[16] and E-cadherin mutations have been shown to be associated with poor survival.[24] Staining of *del 8* and *del 9* E-cadherin was found exclusively in patients negative for p53 and positive for $p21^{WAF1/CIP1}$, suggesting that the $p21^{WAF1/CIP1}$ regulatory function of p53 was intact. These data suggest a selection for mutations in either the E-cadherin or the p53-$p21^{WAF1/CIP1}$ pathway. Of note, the investigated cohort was of relatively small size, limiting the conclusions that can be drawn, especially with regard to the cases with *del 8* and *del 9* reactivity.

Our finding of a prognostic value of $p21^{WAF1/CIP1}$ in gastric carcinoma is in accordance with previous publications.[4 15] $p27^{Kip1}$ was described as an independent prognostic factor in gastric carcinomas of Asia and Europe,[7 29–31] but there are also reports that loss of $p27^{Kip1}$ does not predict patient survival.[32] A

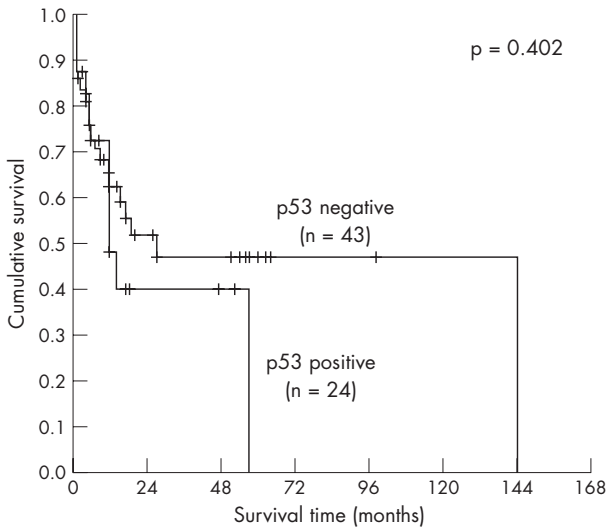

Figure 4 Survival impact of p53 expression. Kaplan–Meier survival curve for 67 patients with gastric carcinoma stratified by p53 expression in neoplastic cells. A cut-off of 30% was used.

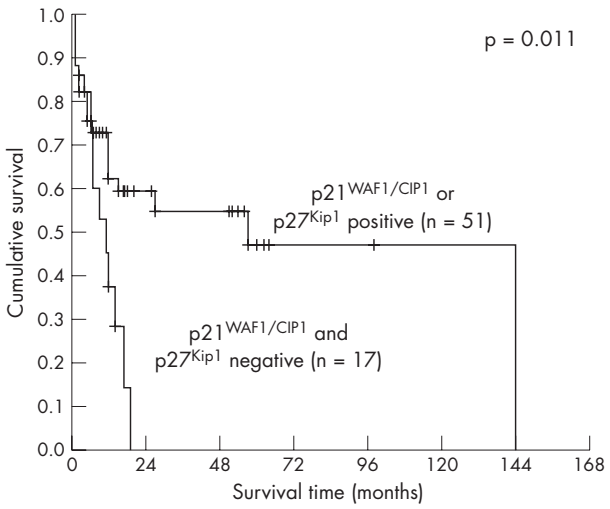

Figure 6 Survival impact of a combination of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ expression. Kaplan–Meier survival curve for 68 gastric carcinoma patients stratified into two groups by positivity for either $p21^{WAF1/CIP1}$ or $p27^{Kip1}$ staining (group 1) and p53 positivity in combination with absence of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ (group 2).

Table 3 Correlation of E-cadherin expression with p53 and $p21^{WAF1/CIP1}$ expression

	E-cadherin (clone 36)			E-cadherin *del 8/9* reactivity	
	Normal	Abnormal	−	+	−
p53 − and $p21^{WAF1/CIP1}$ +	0	24	2	4	22
p53 + or $p21^{WAF1/CIP1}$ −	6	30	6	0	42
		p=0.075		p=0.009	

+, positive; −, negative; p values were obtained by χ^2 test.

combination of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ with expression of at least one of the CDK inhibitors was of prognostic value in a Japanese tumour collective.[29] In our patient collective, expression of both cell cycle regulators $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ was not correlated with each other, while such an association was found in this study of Japanese tumours.[29]

Our result of no prognostic significance of p53 is independent of the chosen cut-off, because statistical significance was not reached using cut-offs of 10%, 20%, or 30%. Expression of $p21^{WAF1/CIP1}$ in combination with a lack of p53 expression was significantly associated with prognosis which is in concordance with a previous report.[15] Moreover, we observed that all tumours harbouring E-cadherin mutations in exons 8 or 9 were negative for p53 and positive for $p21^{WAF1/CIP1}$. Our present finding is in accordance with our previous investigation of p53 expression in gastric carcinoma from European patients where we show that p53 accumulation occurs more frequently in tumours without E-cadherin mutations compared to patients harbouring E-cadherin mutations determined by sequence analysis.[33]

Taken together, our data indicate that cell cycle regulators should be investigated in combination with the E-cadherin mutation status. Even though Mexican Mestizo patients negative for p53 and positive for $p21^{WAF1/CIP1}$ have good prognostic factors, it may have a negative influence on prognosis if they acquire in addition E-cadherin mutations which have been shown to be associated with poor survival.[24]

Take-home messages

- Gastric carcinoma is characterised by numerous alterations including down-regulation of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ as well as genomic mutations of p53 and E-cadherin.
- Combined evaluation of the prognostic significance of $p21^{WAF1/CIP1}$, $p27^{Kip1}$, p53 and E-cadherin protein expression, including mutant E-cadherin variants with deletion of exon 8 (*del 8*) or 9 (*del 9*), was performed in 69 gastric carcinomas from Mexican patients.
- Expression of $p21^{WAF1/CIP1}$ alone or in combination with $p27^{Kip1}$ or in the absence of p53 was associated with favourable prognosis.
- Staining of *del 8* and *del 9* E-cadherin was found exclusively in patients negative for p53 and positive for $p21^{WAF1/CIP1}$.
- Combined evaluation of the prognostic significance of cell cycle regulators and E-cadherin should be performed.

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Authors' affiliations

A Gamboa-Dominguez, E Reyes-Gutierrez, Department of Pathology, Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, Mexico City, Mexico
S Seidl, C Hermannstädter, H Höfler, F Fend, B Luber, Technische Universität München, Klinikum rechts der Isar, Institut für Allgemeine Pathologie und Pathologische Anatomie, München, Germany
L Quintanilla-Martinez, GSF-Forschungszentrum für Umwelt und Gesundheit, Institut für Pathologie, Neuherberg, Germany
R Busch, Technische Universität München, Klinikum rechts der Isar, Institut für Medizinische Statistik und Epidemiologie, München, Germany

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