Expression of β-Catenin, MUC1 and c-Met in Diffuse-type Gastric Carcinomas: Correlations with Tumour Progression and Prognosis

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Abstract. *Previous studies on the immunoreactivity of β-catenin, MUC1 and c-met in gastric carcinomas regarding survival and clinico-pathological features led to contradictory results. Therefore, a series of 94 diffuse-type and mixed-type subcardial gastric carcinomas according to the Laurén classification were investigated to elucidate possible correlations with clinico-pathological and prognostic data. An immunohistochemical study was performed to detect the expression of β-catenin, MUC1 and c-met. Loss of membranous/cytoplasmic β-catenin expression in the tumour centre correlated with pT, loss at the invasion front with pTNM stage. MUC1 expression in the tumour centre correlated with lymph node metastasis and pTNM stage. c-Met did not show such associations. In multivariate survival analysis, loss of membranous/ cytoplasmic β-catenin expression as well as a strong MUC1 expression at the tumour invasion front represent independent predictors of a worse prognosis. On the other hand, c-met expression did not exhibit any prognostic value in this study.*

Despite a declining incidence in Western countries, gastric carcinoma represents still the second most common cause of cancer death worldwide. Carcinomas of the stomach are an heterogeneous group of tumours with a multifactorial carcinogenesis; however, infection with *Helicobacter pylori* seems to play an important role. Gastric tumours can be subdivided into early or advanced, by localisation and by histopathological aspects. The classification system by Laurén has important implications for surgical management and may help to understand the different molecular mechanisms of tumour development (1).

Diffuse- and intestinal-type gastric cancer exhibit different origins; the intestinal type is related to *Helicobacter pylori* infection and the diffuse type more often arises *de novo* and/or by genetic predisposition. Since the diffuse type is less related to environmental influences, its relative incidence increased and it occurs more often in younger patients.

Cell-cell adhesion in gastric cancer is mediated by E-cadherin in a zipper-like way. β-Catenin represents an important multifunctional protein that is expressed in membrane and cytoplasm as well as in the nucleus of epithelial cells. It plays two fundamental and contradictory roles: The cytosolic and membranous β-catenin is linked to E-cadherin and the actin cytoskeleton, working as a key component of cell-cell adhesive junctions. Changes in the phosphorylation levels of β-catenin have been shown to alter E-cadherin function. In the interplay with E-cadherin it exerts a restrictive effect on tumour growth. Nuclear β-catenin plays a crucial role in the so-called canonical Wnt/wingless pathway. It accumulates in the nucleus and acts as an transcriptional activator together with the LEF-1/TCF family, activating important genes responsible for cellular proliferation and differentiation (2).

Mucins are viscoelastic gels that exist in various organs. They are characterised by a peptide core and a dense O-glycosylation. MUC1, the first mucin that was investigated extensively, represents a transmembrane glycoprotein, which is expressed on the apical cell surface of various epithelia.

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Table I. *Clinical and pathological characteristics of the patients.*

Characteris	No. of cases (94 total)	%
Sex		
Male	46	48.9
Female	48	51.1
Laurén classification		
Diffuse type	88	93.6
Mixed type	6	6.4
Goseki classification		
Type II	9	9.6
Type III	5	5.3
Type IV	80	85.1
WHO classification		
Signet-ring cell	76	80.9
Mucinous	2	2.1
Unclassifiable	16	17.0
Depth of invasion		
pT1	19	20.2
pT2	48	51.1
pT3	22	23.4
pT4	5	5.3
Lymph node metastasis		
pN0	23	24.5
pN1	39	41.5
pN2	25	26.6
pN3	7	7.4
pTNM stage		
Stage I	27	28.7
Stage II	26	27.7
Stage III	30	31.9
Stage IV	11	11.7
Grading		
Grade II	3	3.2
Grade III	91	96.8

The biological functions are multiple, especially regarding the protection of epithelia by mucociliary clearance and cell-cell interactions involved in adhesive as well as antiadhesive mechanisms. Furthermore, MUC1 overexpression could induce the inhibition of the integrin-mediated adhesion of carcinoma cells to extracellular matrix components. On the other hand, MUC1 plays an important role in cell-cell signaling: fragments of the MUC1 cytosolic tail are associated with β-catenin in cytoplasm and nuclei. Thereby, an overexpression of MUC1 increased the level of nuclear β-catenin and coactivates the transcription of β-catenin Tcf-binding sites. Finally, MUC1 competes with E-cadherin for β-catenin, as reviewed earlier (3).

Hepatocyte growth factor (HGF) and its receptor c-met represent mediators for multiple biological activities such as mitosis (proliferation, invasion), morphogenesis, mitogenesis and angiogenesis. They play an important role in embryonic development, but are also active in adults. c-Met expression is stronger in diffuse-type compared to intestinal-type tumours, but besides some correlations to clinico-pathological features their role as prognostic markers remains unclear. c-Met overexpression possibly represents a very early event in tumourigenesis, ultimately leading to gastric cancer (4, 5).

It has been demonstrated that β-catenin, MUC1 and c-met play important roles in human carcinogenesis. Several studies investigated their expression in gastric carcinomas but contradictory results regarding correlations with clinico-pathological parameters as well as survival probability were reported. To date, there are no studies and only few subgroup analyses investigating exclusively the subcardial diffuse-type cancer according to Laurén. Because this type may be a entity of its own with regard to morphogenesis and progression, this study investigated 94 subcardial carcinomas of the diffuse type and mixed type (6,7).

Patients and Methods

Patients. A series of 94 subcardial diffuse- or mixed-type gastric cancers according to Laurén was derived from the files of the Institute of Pathology of the University of Cologne. Two pathologists performed the histopathological classification in a double blind fashion. All patients had undergone curative resection (R0 according to the UICC guidelines) between 1982 and 1991. No patient had received pre- or postoperative chemo- or radiotherapy. Patients who died within four weeks after surgical intervention (postoperative mortality) were excluded from the study. The clinical and pathological characteristics of the patients aged between 25 and 89 years (mean: 59) are summarised in Table I. The minimal observation time was 5.4 years (up to 9.6 years).

Immunohistochemistry. Immunohistochemical staining was performed applying the DAKO EnVision System/DAKO EnVision Doublestain System (DAKO, Hamburg, Germany) according to the manufacturer's instructions. c-Met was detected by the polyclonal antibody h-met (C-28): sc-161 (Santa Cruz Biotechnology, Heidelberg, Germany), MUC1 was detected by the monoclonal antibody HMFG-2 (Immunotech, Hamburg, Germany) and the β-catenin specific monoclonal antibody was purchased from Becton-Dickinson Transduction Laboratories (Heidelberg, Germany).

The formalin-fixed and paraffin-embedded tissues were cut and deparaffinised in xylene and rehydrated in decreasing ethanol solutions and water. Antigen retrieval (pretreatment) was performed in a microwave (2×4 min, 600W) using citrate buffer (pH 6.0). The endogenous peroxidase activity was blocked by 0.3% H_2O_2/methanol. MUC1 and β-catenin were visualised applying the EnVision Doublestain System (DakoCytomation, Hamburg, Germany) in order to detect the co-localisation of both antigens. Both primary monoclonal antibodies were incubated for 30 min at room temperature. Immunoreactivity of c-met was investigated using the EnVision+HRP System (DakoCytomation, Hamburg, Germany). The

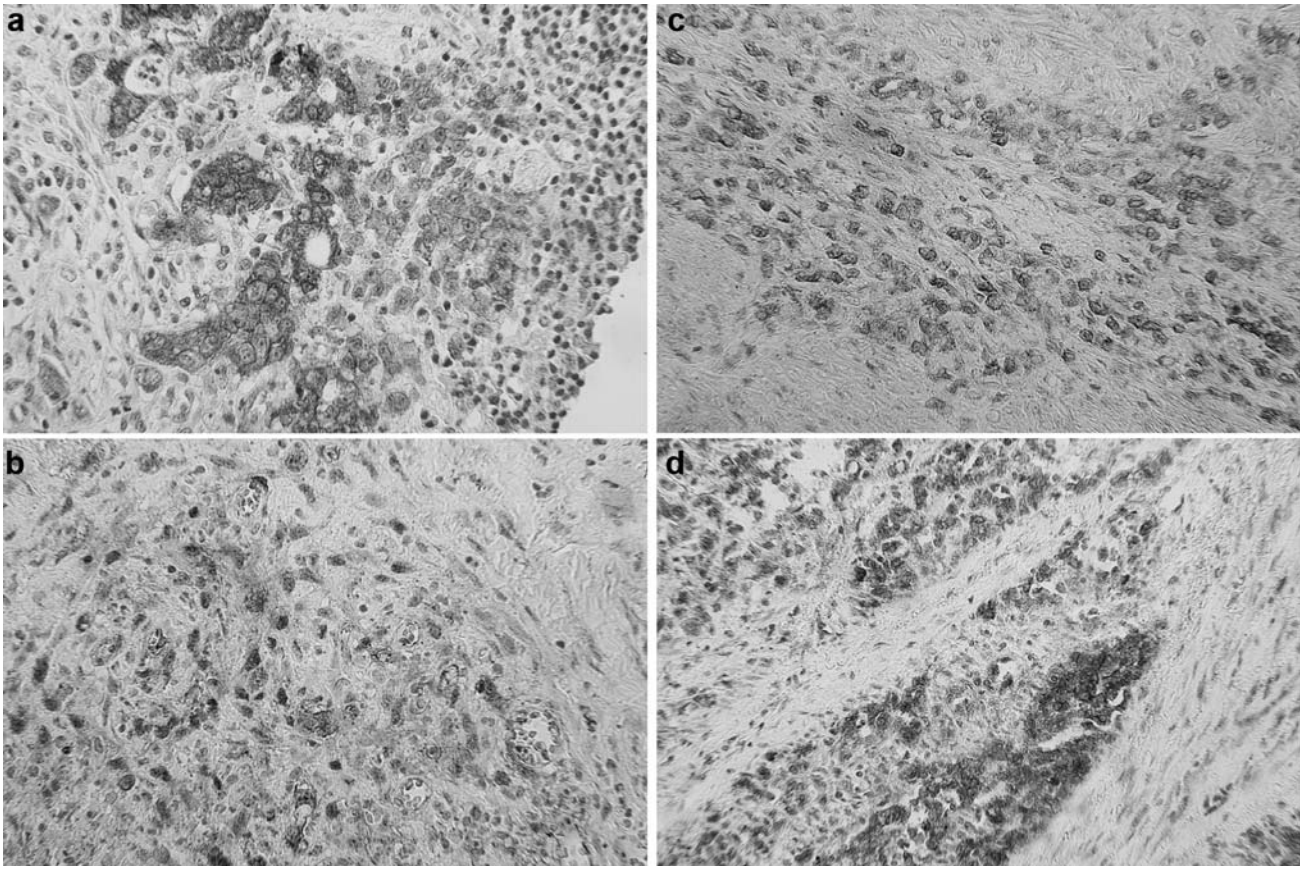

Figure 1. *Immunohistochemical localization of MUC1, β-catenin and c-met (a-c)MUC1 (red) color and β-catenin (brown) color could be co-localized in the tissue specimens. Both were detected in a membranous or cytoplasmic pattern. (d) c-Met expression showed a cytoplasmic expression pattern.*

monoclonal antibody was incubated overnight at 4˚C. The staining kits were applied according to the manufacturer's instructions. Following rinsing in aqua dest., the nuclei were counterstained with hematoxylin and the tissues were embedded in glycerol jelly.

Two pathologists performed the microscopic evaluation at a magnification of ×400 independently and in a blind fashion. A consensus was achieved in the cases of different opinions (<10%). Scoring for β-catenin and MUC1 at the invasion front and in the tumour center was done separately according to the percentage of cells showing a positive staining. For β-catenin the scoring was done for membranous/cytoplasmic and nuclear staining separately. Membranous/cytoplasmic and nuclear β-catenin as well as MUC1 immunoreactivity were regarded as positive, if 35% or more of the tumour cells were stained (Figure 1 a-c). On the other hand, c-Met expression was scored as positive, if 50% or more of the tumour cells were stained (Figure 1 d).

Statistical analysis. Correlations between the degree of staining and the subgroups according to the clinico-pathological classifications were calculated by Fisher's exact test (two-sided) whenever appropriate or chi-square test at a significance level of 5%. The overall univariate survival analysis was performed according to the Kaplan-Meier product-limit method and the significance of differences between the survival curves was determined using the log-rank test. Multivariate survival analysis was performed according to the Cox proportional hazard model. The results were considered to be statistical significant at p-value less than 0.05. All statistical analyses were conducted using the SPSS©11.5 statistical software program (Chicago, USA) for Windows.

Results

Immunoreactivity for each antigen (Figure 1) was correlated with clinico-pathological variables. A statistically significant association of membranous/cytoplasmic β-catenin staining was observed at the invasion front with the pN (p=0.043) and the pTNM stage (p=0.04). On the other hand nuclear β-catenin immunoreactivity at the invasion front exhibited a trend (but no significant) association with the depth of invasion.

MUC1 expression in the tumour center (Table II) correlated with pN stage (p=0.011) as well as pTNM stage (p = 0.035). In addition, it correlated with the classifications according to Laurén (p=0.005), Goseki (p<0.001) as well as WHO (p=0.003). Semiquantitatively scored c-Met expression (Table II) was not associated with any clinico-pathological variable under study.

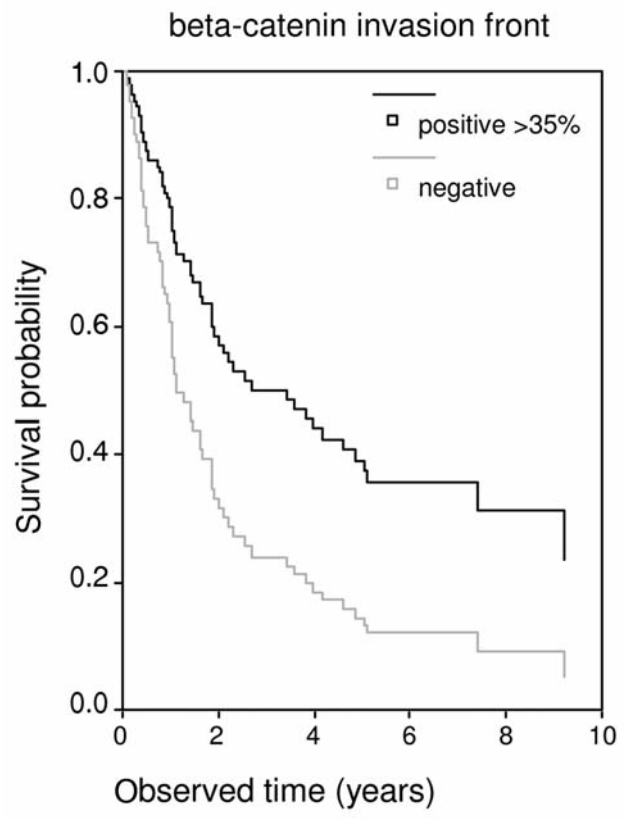

Figure 2. *Membranous/cytoplasmic β-catenin at the invasion front correlates with survival probability (Cox proportional hazards model).*

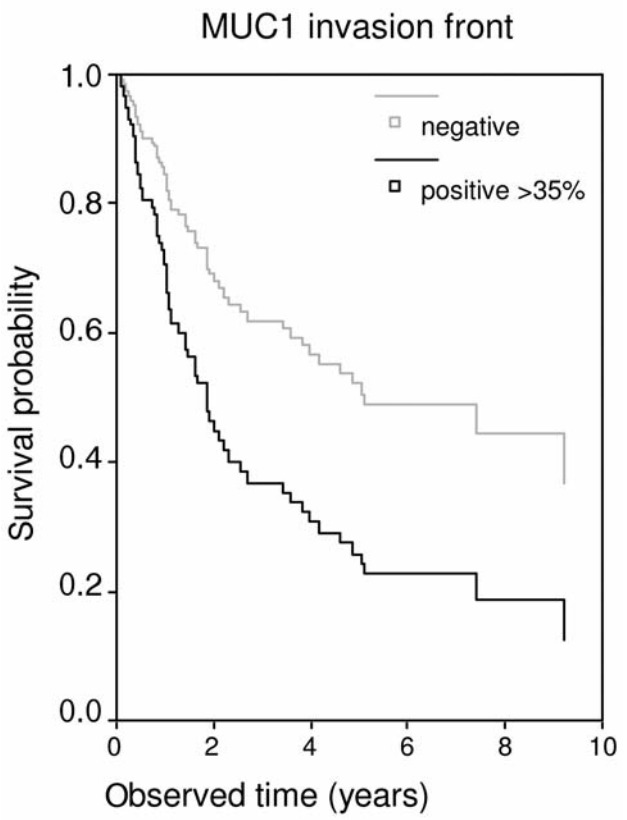

Figure 3. *MUC1 at the invasion front correlates with survival probability (Cox proportional hazards model).*

Univariate and multivariate survival analysis were performed in order to investigate the prognostic impact of c-met, MUC1 and β-catenin. In the univariate Kaplan-Meier analysis, there was a trend for a better prognosis in patients with a strong membranous/cytoplasmic β-catenin reactivity at the invasion front.

In multivariate survival analysis, which included age, sex, the classifcations according to WHO, Laurén and Goseki as well as the pTNM stage, a strong membranous/cytoplasmic β-catenin reactivity at the invasion front revealed as an independent predictor for a better prognosis (Figure 2, Table III). Additionally, a worse survival probability was observed for carcinomas exhibiting a strong MUC1 reactivity independently of the β-catenin expression at the invasion front. (Figure 3, Table III) In this series, neither nuclear β-catenin or c-met expression had any prognostic significance.

Discussion

The present study focused on the expression of β-catenin, MUC1 and c-met in subcardial diffuse- and mixed-type gastric cancer and their correlation with clinico-pathological features and survival probability. An association of membranous/cytoplasmic β-catenin expression was observed at the invasion front with pT, pN and pTNM stage, but not with other histopathological classification systems. Moreover, patients with decreased membranous/cytoplasmic β-catenin reactivity at the invasion front exhibited a worse prognosis. Although a trend was only found with the univariate Kaplan-Mayer survival analysis, it was revealed as an independent prognostic marker in multivariate survival analysis. In this context, a loss of normal membranous β-catenin expression in more aggressive tumours has been reported by several authors (8-10). As in the present study with regard to diffuse- and mixed-type gastric cancer, no correlation between nuclear β-catenin accumulation and 5-year survival was observed earlier for intestinal-type carcinoma (11). However, a shorter survival period in tumours accumulating nuclear β-catenin could be expected, since activation of the Wnt/β-catenin pathway is associated with tumour progression (12) and a worse prognosis associated with a strong nuclear β-catenin expression at the invasion front in colorectal adenocarcinoma (3). However the results remain contradictory, and other investigators

Table II. *Relationship between antigen expression and clinical – pathological variables.*

		β-catenin, membranous/ cytoplasmic		β-catenin, nuclear		MUC1		c-met
Characteristic	n	Tumour center	Invasion front	Tumour center	Invasion front	Tumour center	Invasion front	
Staining		Positive p	Positive p	Positive p	Positive p	Positive p	Positive p	Positive p
Laurén								
diffuse	88	27	26	82	76	52^{S}	73^{S}	45
unclassifiable	6	1	2	6	6	0	2	2
Goseki								
II	9	3	3	8	8	1^{S}	4^{S}	3
III	5	0	0	5	5	0	3	1
IV	80	25	25	75	69	51	68	44
WHO								
mucinous	2	0	0	1	1	2^{S}	2	1
signet ring cell	76	23	25	72	67	47	63	41
undifferentiated	16	5	3	15	14	3	10	5
Depth of invasion								
pT1	19	6	7	19	17	12	16	10
pT2	48	12	11	42	41	26	39	25
pT3	22	8	9	22	20	12	18	10
pT4	5	2	1	5	4	2	2	2
Nodal metastasis								
pN0	23	10	12^{S}	22	21	11^{S}	20	16
pN1	39	8	7	35	34	22	28	17
pN2	25	8	7	24	22	8	21	12
pN3	7	2	2	7	5	6	6	2
pTNM stage								
I	27	11	12^{S}	26	24	14^{S}	23	17
II	26	3	3	22	22	13	19	11
III	30	11	11	29	28	11	26	16
IV	11	12	2	11	8	9	7	3
Grading								
II	3	1	1	3	3	1	1	0
III	91	27	27	85	79	51	74	47

S: Significant.

reported that nuclear β-catenin represents an independent factor for better survival in ovarian cancer (13). A correlation between depth of invasion and nuclear β-catenin expression was also observed by others (11). Moreover Aihara (14) concluded that nuclear β-catenin is an independent factor for depth of invasion (and metastasis) in undifferentiated early gastric cancer (14). In contrast, several other investigators did not find such an association (10, 15, 16). A correlation of lymph node metastasis (and lymphatic vessel invasion) with atypical β-catenin expression was described previously (17), in accordance with the current data. These partly contradictory results might be attributed to the fact, that β-catenin plays different roles in tumour growth and differentiation. Membranous β-catenin represents an important mediator for cell adhesion, therefore a loss of expression may result in tumour growth, enhanced tumour cell migration and, finally, in a worse survival probability. On the other hand, nuclear β-catenin accumulation leads to an activation of several transcription factors. However, its role in gastric tumour biology remains unclear and should be investigated in future studies.

Table III. *Multivariate survival analysis.*

Variables	Hazard ratio	5%	95%	SE	*p*
pTNM staging	0.086	0.034	0.219	0.478	0.000
β-catenin membranous/ cytoplasmic at the invasion front	2.057	1.153	3.671	0.295	0.015
MUC1 at the invasion front	2.074	1.153	3.671	0.366	0.046

Currently, there is strong consensus regarding the correlation of MUC1 expression with histological subtypes of gastric cancer, which was examined for the first time by Ho *et al*. (18). In several studies it was demonstrated that intestinal-type carcinomas exhibit a strong MUC1 expression, whereas diffuse type exhibit only a weak reactivity (19-23). Additionally and corresponding to the current data, several investigators have found an association with pN and pTNM stage, as well as WHO, Laurén or Goseki classification (21, 24, 25). In the present study, a worse survival prognosis in patients with MUC1 (HMFG-2) positive diffuse-type adenocarcinomas of the stomach was observed independently of age, gender, WHO, Laurén and Goseki classification, the pTNM stage and membranous/cytoplasmic β-catenin expression. In accordance, in most previous investigations of gastric carcinomas, a worse prognosis was associated with an increased MUC1 expression (19, 20, 22, 23, 25-28). However, in most studies this effect was revealed only in univariate survival analyses. Contradictory results with regard to MUC1 protein expression may be due to its strong glycosylation and different epitope specificities of the various antibodies applied (29). However, in some studies monoclonal as well as polyclonal antibodies were used (20), others found a worse prognosis for MUC1 positive patients independently of their glycosylation stage (22). In addition, the composition of the patient series varies strongly, for example regarding the relation between early and advanced cancer or intestinal-*vs*. diffuse-type tumours.

An association of the c-Met protein expression in diffuse- and mixed-type gastric cancer with the presence with any clinico-pathological variables could not be found in the present study. These results are confirmed by several studies (30-34). In agreement with the current findings, most authors have not been able to find a significant correlation with advanced tumour stage or other clinico-pathological features like the pTNM stage or degree of differentiation (34-37). However, c-Met expression in gastric cancer tissue was increased as compared with healthy tumour-free gastric mucosa (38, 39). In accordance with other authors, a relationship between c-Met expression and survival probability could not be observed in the present study (32-34, 40, 41).

In conclusion, the loss of membranous/cytoplasmic β-catenin expression as well as an increase in MUC1 expression represent markers for tumour progression and worse prognosis in diffuse- and mixed-type gastric cancer. This could be explained by anti-adhesive and invasion-promoting characteristics of tumour cells, which may be induced by altered patterns of β-catenin and MUC1 expression. In this context, GSK3β seems to regulate interactions between β-catenin and MUC1 (42-44), which should be further investigated. In addition, the role of target genes activated by β-catenin should be thoroughly evaluated.

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