

Prognostic Impact of Protein Overexpression of the Proto-oncogene *PIM-1* in Gastric Cancer

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Abstract. *Background:* PIM kinases are mediators of cytokine signalling pathways in hematopoietic cells and contribute to the progression of certain types of leukemia and solid tumor. Here the prognostic impact of proto-oncogene *PIM-1* was analyzed in gastric carcinoma. *Patients and Methods:* Cancer tissues of 117 patients with potentially curative (R0) resections for gastric cancer were immunohistochemically stained for *PIM-1*. *Results:* Cytoplasmic immunoreactivity for *PIM-1* in tumor (46%) was higher ($p=0.003$) compared to that in gastric glands (16%) and foveolae (1%). *PIM-1* immunoreactivity in gastric carcinoma correlated with tumor grading ($p<0.05$) and Laurén category ($p<0.02$). Overexpression of *PIM-1* in gastric glands ($p<0.001$) was associated with formation of lymph node metastases ($p=0.035$) and survival ($p=0.04$). Multivariate analysis of *PIM-1* expression in gastric glands confirmed its association with prognosis. *Conclusion:* Up-regulation of *PIM-1* oncogene might be a tumor marker for gastric cancer. The correlation of *PIM-1* overexpression in gastric glands with formation of lymph node metastases and survival proposes a prognostic role in gastric cancer.

Although a linear decrease of incidence has been observed for gastric cancer for the Western world during recent years, gastric carcinoma as well as carcinomas of the gastroesophageal junction are the most frequently tumor-caused deaths worldwide. Despite curative resection, even

patients with stages II-III of gastric cancer have a poor 5-year survival with a high risk of local recurrence and distant metastasis (1, 2).

This places a high priority on elucidating the molecular mechanisms underlying the disease with the aim of developing novel and effective therapeutic strategies to target this malignancy, as well as to identify molecular markers for early diagnosis and monitoring of therapy. *PIM-1* was originally found as a primary integration site for Moloney murine leukemia virus and has emerged as a potential diagnostic marker in prostate cancer (3). It belongs to a family of serine/threonine protein kinases and is associated with transcriptional regulation of cell cycle proteins (4) and involved in the control of cell growth, differentiation and apoptosis (5). A number of cytoplasmic and nuclear proteins are phosphorylated by PIM kinases and may act as their effectors in normal physiology and in disease (6). *PIM-1* also associates with protein complexes necessary for mitosis (7). Two *PIM-1* proteins are produced from the same gene, via an alternative upstream CUG initiation codon, a 44 kDa and a shorter 34 kDa form that both contain the characteristic kinase domain and play a role in drug resistance (8, 9). A report on its crystal structure indicates that *PIM-1* is a constitutively active kinase. *PIM-1* is widely distributed in tissues, with the highest expression found in hematopoietic tissues and testes (10), enhancing cellular survival (11, 12). *PIM-1* also appears to be involved in tumorigenesis of solid tumors. Microarray expression profiling identified *PIM-1* overexpression in human prostate tumor. Expression of *PIM-1* protein was associated with poor survival of patients with prostate cancer (3), whereas in oral squamous cell carcinoma no correlation was found (13). For gastric cancer, Chen *et al.* proposed a model for prediction of survival consisting of CD36, signalling lymphocyte activation molecule (SLAM) and *PIM-1* expression (14).

PIM-1 protein expression in gastric cancer was analyzed by immunohistochemistry in the present study. Expression and localization were analyzed for their association with the clinical parameters pathogenesis and prognosis.

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Patients and Methods

Patients. A total of 117 consecutive patients with potentially curative resections for gastric cancer between 1996 and 2000, with a median age of 65 years (min. 32, max. 85), 70 men and 47 women, were included in the study. The clinical and pathological characteristics of the patients are summarized in Table I. Of these patients, 105 (89.7%) underwent gastrectomy with D2 lymphadenectomy with curative intent, and 12 (10.3%) underwent subtotal gastric resection with D2 lymphadenectomy. Tumor specimens were collected from all patients. A mean number of 39 lymph nodes were resected per patient. R0 resection was achieved in 106 (90.6%) patients. Lymph node metastases (N+) were found in 75 (64.1%) specimens, and distant metastases (M1) were seen in 24 (21%) patients. Follow-up of surviving patients was at least 5 years. Informed consent was obtained from each patient according to the local Ethics Committee.

Immunohistochemistry. PIM-1 protein was detected by the polyclonal antibody Anti-PIM-1 (Abgent, San Diego, CA, USA) raised against a synthetic peptide selected from C-terminal amino acids 298-313 of the protein.

Five µm sections of the paraffin-embedded tissues were cut and deparaffinized according to standard histological techniques. Subsequently, a high-sensitivity immunohistochemical staining was performed applying the DAKO EnVision System (DakoCytomation, Hamburg, Germany) according to the manufacturer's instructions. In brief, pretreatment for antigen retrieval was performed in a microwave using 10 mM citrate buffer, pH 6.0, for 3x5 min at 700 W. Endogenous peroxidase activity was blocked by immersing the slides in 0.03% hydrogen peroxide in methanol for 5 min. Subsequently, sections were incubated with primary antibody anti-PIM-1 (polyclonal ab; Abgent, San Diego, USA), diluted 1:50 with 10 mM phosphate-buffered saline (PBS), pH 7.4, at 4°C overnight. After washing twice in Tris-buffered saline (TBS) sections were incubated with avidin-horse radish peroxidase-conjugated secondary antibody (goat anti-rabbit) of the Envision-kit for 30 min at room temperature. After washing (2x5 min in TBS) the sections were stained by the chromogen 3-amino-9-ethylcarbazol for 30 min and then rinsed with H₂O. Nuclei were counterstained with hematoxylin. The staining procedure without primary antibody was used to develop a negative control, a PIM-1 expressing gastric normal tissue specimen was applied as positive control. The staining results were monitored by concurrently treating negative and positive controls.

Evaluation of immunostaining and statistical analysis. The microscopic evaluation was performed at a magnification of x400 independently and in a blind fashion by two pathologists (U.D.) and (S.E.B.)

Analysis of PIM-1 immunostaining was performed using a scoring system based on the fraction of positive cells and the staining intensity in the specimens. The whole tissue of each section was evaluated. PIM-1 status was defined as score 0: no expression <5%; score 1: low expression of 5-35%; score 2: medium expression of 35-65%; and score 3: strong expression >65% positive cells. Due to the limited number of patients, score 1 and 2 were grouped for graphic presentation. For statistical analyses, scores were grouped depending on the correlation analyzed, with foveolae or tumor parameter, respectively.

Associations between the degree of staining and the subgroups according to the clinical and pathological classifications were calculated by Chi² test. Related samples were compared using the

Table I. Clinicopathological characteristics of patients according to PIM-1 expression in gastric carcinoma.

Parameter	n=117	PIM-1 immunoreactivity score (%)			p-Value	
		%	0-1 <35%	2 35-65%		3 >65%
pT category^a						
pT1	22	19	22	21	57	0.93
pT2	35	31	28	18	54	
pT3	50	41	27	21	52	
pT4	10	9	30	10	60	
pN category^b						
pN0	43	36	26	21	53	0.67
pN+	74	64	28	16	56	
pM category^c						
pM0	93	79	25	21	54	0.24
pM1	24	21	37	9	54	
R category^d						
R0	108	93	26	20	54	0.24
R1	9	7	43	0	57	
Grading						
G1/G2	36	31	10	20	70	<0.05
G3/G4	81	69	34	17	49	
Laurén						
Intestinal	43	37	14	19	67	<0.02
Diffuse	58	50	44	16	40	
Mixed	16	13	0	21	79	

Histopathological ^atumor category, ^blymph node category and ^cmetastasis category according to the Union Internationale Contre Le Cancer 5th edition 1997, ^dresection category. Score 0-1, none-low; 2, medium; 3, strong PIM-1 immunoreactivity.

Wilcoxon signed-rank test with two-sided assumption. Survival curves were calculated by the Kaplan-Meier method and the significant difference between PIM-1 status was evaluated by means of the log-rank test. P-values <0.05 were considered significant. All statistical calculations were performed using the statistical software package release 12.0 (SPSS Inc, Chicago, IL, USA).

Results

Immunohistochemical staining patterns of PIM-1 in foveolae, gastric glands and tumor cells. PIM-1 expression was examined by immunohistochemistry in gastric carcinomas and corresponding normal epithelium (Figure 1). PIM-1 expression was detected in more than 86% of gastric cancer specimens. PIM-1 was predominantly present in the cytoplasm in both neoplastic and non-neoplastic cells. In only 2% of the samples was PIM-1 immunoreactivity shown in the nucleus as well. Normal foveolae showed no or low PIM-1 expression (high expression in <1%). Gastric glands exhibited stronger staining (high expression in 16%) and gastric carcinoma the strongest staining (high expression in 46%).

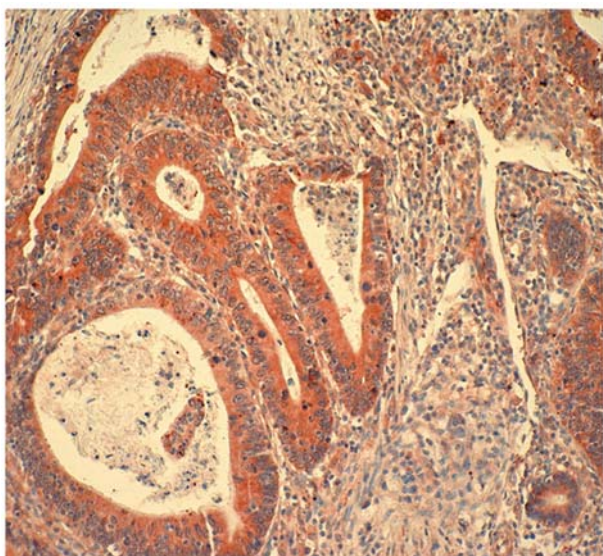


Figure 1. Immunohistological localization of PIM-1 in gastric cancer. Strong positive brownish staining for PIM-1 in cytoplasm of tumor cells can be seen, while there is a lack of staining of stroma cells ($\times 100$).

Table II. Distribution of PIM-1 protein in foveolae, gastric glands and tumor cells from 117 patients with gastric cancer.

Cell type	PIM-1 immunoreactivity score			p-Value
	0 <5%	1 5-35%	2-3 >35%	
Foveolae	19 (20%)	61 (70%)	7 (8%)	
Gastric glands	8 (9%)	30 (35%)	49 (5%)	<0.001 ^a
Tumor	15 (30%)	14 (12%)	76 (62%)	0.003 ^b

PIM-1 protein up-regulation in ^agastric glands compared to foveolae, ^btumor cells compared to foveolae. Score 0, none; 1, low; 2-3, medium-strong PIM-1 immunoreactivity.

The expression status is summarized in Table II. PIM-1 expression was stronger in tumor compared to that in gastric glands ($p=0.003$) and stronger in gastric glands compared to foveolae ($p<0.001$). The distribution of PIM-1 expression in the different cell types is presented in Figure 2.

Association of PIM-1 immunoreactivity with clinicopathological parameters. PIM-1 immunoreactivity in foveolae, gastric glands and tumor cells was tested for association with various clinicopathological parameters. The p -values are listed in Table I. There was a significant correlation between PIM-1 immunoreactivity of tumor cells and tumor grading ($p<0.05$) as well as Laurén classification ($p<0.02$). Increased PIM-1 protein expression in gastric glands was significantly ($p=0.035$) associated with the formation of lymph node metastases (Table III).

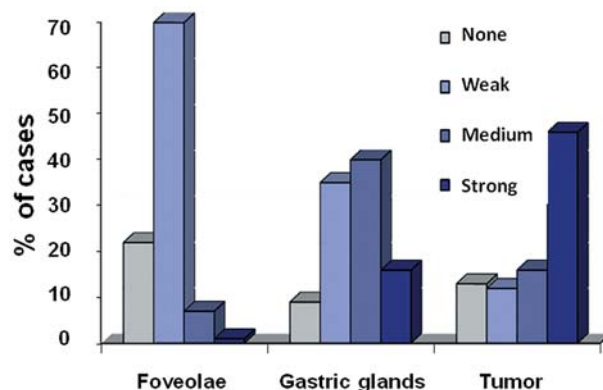


Figure 2. Immunohistochemical detection of PIM-1 in gastric carcinoma and non-neoplastic cells. Expression: none <5%; low 5-35%; medium 35-65%; strong >65% PIM-1 positive cells.

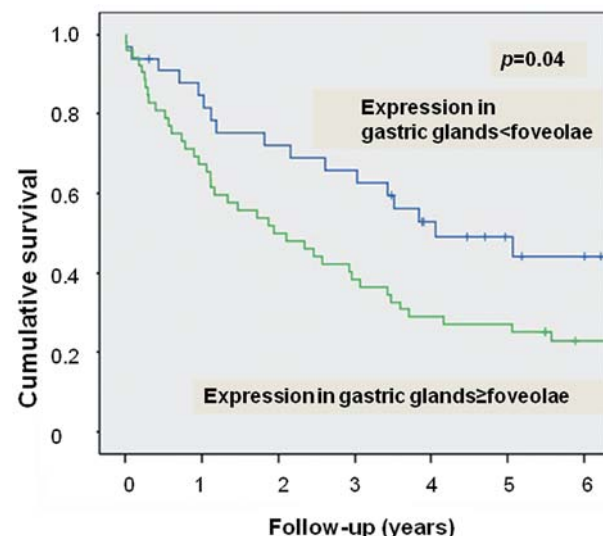


Figure 3. Prognostic impact of PIM-1 overexpression in gastric glands compared to foveolae. Comparison of Kaplan-Meier curves including 117 gastric cancer patients. There was a significant difference between group 1: PIM-1 expression in gastric glands < expression in foveolae (upper curve) and group 2: PIM-1 expression in gastric glands \geq expression in foveolae (lower curve). Overexpression of PIM-1 protein in gastric glands as compared to foveolae was significantly associated with poorer prognosis.

Analysis of the prognostic relevance of PIM-1. The immunoreactivity of PIM-1 was tested with regard to its possible prognostic importance according to the univariate survival analysis by Kaplan-Meier. PIM-1 expression in foveolae, gastric glands and tumor cells alone did not show any significant correlation with patients' survival probabilities. However, an association between PIM-1 up-regulation in gastric glands and poorer survival ($p=0.04$) was detected (Figure 3). Moreover, multivariate analysis

Table III. Clinicopathological characteristics of patients according to elevated PIM-1 status in gastric glands compared to foveolae.

Parameter	PIM-1 immuno-reactivity (%)				p-Value
	n=117 (%)		Gastric glands >foveolae	Gastric glands ≤foveolae	
pT category^a					
pT1	22	19	63	37	0.091
pT2	35	31	42	58	
pT3	50	41	69	31	
pT4	10	9	62	38	
pN category^b					
pN0	43	36	51	49	0.035
pN+	74	64	70	30	
pM category^c					
pM0	93	79	58	42	0.088
pM1	24	21	69	31	
R category^d					
R0	108	93	61	39	0.531
R1	9	7	43	57	
Grading					
G1/G2	36	31	63	37	0.987
G3/G4	81	69	62	38	
Laurén					
Intestinal	43	37	58	42	0.051
Diffuse	58	50	53	47	
Mixed	16	13	85	15	

Histopathological ^atumor category, ^blymph node category and ^cmetastasis category according to the Union Internationale Contre Le Cancer 5th edition 1997, ^dresection category.

demonstrated prognostic impact of up-regulation of PIM-1 protein expression in gastric glands for tumor stage and formation of metastases (Table IV).

Discussion

This is the first report describing significant overexpression of PIM-1 protein in gastric cancer by immunohistochemistry. We detected a significant increase of PIM-1 immuno-reactivity in gastric cancer, indicating that PIM-1 could be important for the tumorigenesis.

Our results confirm previous data of overexpression of PIM-1 in human carcinomas including oral, prostate, head and neck cancer (13, 15, 16). For gastric cancer, mRNA overexpression data of PIM-1 combined with CD36 and SLAM as a prognostic model have been published (14).

Its overexpression in gastric cancer supports the assumption that proto-oncogene PIM-1 promotes cell cycle progression. Up-regulation of PIM-1 contributes to cellular proliferation, anti-apoptosis activity, differentiation and genomic instability by subverting the mitotic spindle

Table IV. Multivariate survival analysis of PIM-1 expression in gastric glands vs. foveolae.

	Hazard ratio	95% Confidence interval for hazard ratio		p-Value
		Lower	Upper	
pT1-pT2 ^a	3.8	1.3	10.5	0.012
pT1-pT3	7.8	2.9	21.3	<0.001
pT1-pT4	4.2	1.3	13.8	0.020
pM0-pM1 ^b	2.7	1.4	5.3	0.003
PIM-1 protein 0-1 ^c	1.7	1.0	3.1	0.069

Histopathological ^atumor category and ^bmetastasis category according to the Union Internationale Contre Le Cancer 5th edition 1997; ^cPIM-1 expression in gastric glands compared to foveolae (0=expression in gastric glands<expression in foveolae, 1=expression in gastric glands≥expression in foveolae).

checkpoint in prostate epithelial cells (17). Overexpression of PIM-1 may enhance cellular survival by protecting cells from apoptosis. A decrease in its expression would shift the balance towards apoptosis. Cells deficient for all PIMs were found to be defective in response to proliferative signals (18). On the other hand, PIM-1 overexpression dysregulates cyclin D1 protein expression, which contributes to the development of polyploidy by delaying cytokinesis (19).

Interestingly, we also found up-regulation of PIM-1 protein in gastric glands. This overexpression was significantly associated with poorer prognosis, whereas PIM-1 up-regulation in cancer did not correlate with survival. Moreover, up-regulation of PIM-1 in gastric glands correlated with the formation of lymph node metastases, which could be one reason for the association of PIM-1 protein expression with survival.

Chiang *et al.* also did not find any correlation between PIM-1 expression in oral squamous cell carcinoma and survival (13). There was no correlation detected between the level of PIM-1 expression and clinicopathological features of prostatic and oral squamous cell carcinoma (13, 15). We did detect a correlation between PIM-1 overexpression in cancer with grading and Laurén classification.

In the present study, immunoreactivity was observed in the majority of gastric cancer tissues, mostly expressed in a cytoplasmic pattern. Only 2% of PIM-1 staining was detected in the nucleus. Inov *et al.* have shown that nuclear localization of PIM-1 is essential for regulation of the proto-oncoprotein MDM2 that counteracts the p53 tumor suppressor (20).

In conclusion, our data confirm overexpression of PIM-1 in gastric tumor cells and show an association with tumor grading. PIM-1 up-regulation in neoplastic gastric tissues

might play an important role in gastric cancer and might serve as a diagnostic tumor marker. Due to the identified significant correlation with the formation of lymph node metastases and survival, PIM-1 overexpression seems to be a prognostic parameter in gastric cancer.

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