GEP-NET : rare tumour connections. Pathophysiological aspects of gastroenteropancreatic neuroendocrine tumours

Kuiper, P.

Chapter 8

The IGF-matrilysin network in gastroenteropancreatic neuroendocrine tumours

P. Kuiper, I. Biemond, E.S.M. de Jonge-Muller,
C.B.H.W. Lamers, H.W. Verspaget

Department of Gastroenterology and Hepatology,
Leiden University Medical Centre, Leiden,
The Netherlands.

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Abstract

The Insulin-like Growth Factor (IGF) system plays an important role in the tumour development, growth, and spread of several cancers.

Matrilysin (MMP-7) has been implicated in tumour processes like invasion and metastasis. Recently, matrilysin was found to be able to cleave IGF binding proteins (IGFBPs), thereby increasing the bioavailability of IGFs.

The aim of the present study was to investigate the expression of IGF-1, IGFBP-3 and matrilysin in gastroenteropancreatic neuroendocrine tumours (GEP-NETs), and their relation to the pathogenetic factors of the tumours.

Tissue expression and levels of IGF-1, IGFBP-3, and matrilysin were analyzed by immunohistochemistry and ELISA, respectively.

IGF-1 and IGFBP-3 tissue levels were increased in tumours compared to associated normal tissue. This increased IGFBP-3 expression in tumours was related to a larger tumour size and the presence of metastases, whereas IGF-1 was not related to any clinicopathological parameter. Matrilysin expression was found to be down-regulated in tumours, and negatively correlated to the expression of IGFBP-3.

These findings suggest that IGFBP-3 plays a role in the pathogenesis of GEP-NETs, whereas matrilysin might indirectly be involved via regulation of this IGFBP-3 expression. Further studies are indicated to assess the contribution of this IGF-matrilysin network in the etiopathogenesis of GEP-NETs.

Introduction

Neuroendocrine tumours comprise a heterogeneous group of neoplasms, arising from enterochromaffin cells widespread distributed throughout the gastrointestinal and bronchopulmonary system[1,2]. In this study, we focus on gastroenteropancreatic neuroendocrine tumours (GEP-NETs), including the pancreatic neuroendocrine tumours (PNETs) and the gastrointestinal carcinoids (GI-NETs). Although slowly-growing, the majority of GEP-NETs are malignant and characterized by angioinvasion and the presence of metastases[3].

The insulin-like growth factor (IGF) system, composed of two IGF ligands (IGF-1 and IGF-2), three receptors and six binding proteins (IGFBPs), plays an important role in growth and development[4]. Furthermore, this system is involved in tumour cell processes like proliferation, survival and growth[5]. Increased levels of insulin-like growth factor-1 (IGF-1) have been reported to be related to the development of cancer of the breast, lung, colon and prostate[6-9]. In NETs, mRNA levels of several components of the IGF-system were found to be variable in different types of NETs[10]. Furthermore, increased expression of IGF-1 and its receptor IGF-1R in gastrinomas were found to be associated with higher tumour aggressiveness[11].

MMPs, or matrix metalloproteinases, constitute a family of more than 20 proteolytic enzymes, with similar protein sequences and domain structures, but diverse substrate specificities, which are involved in remodeling of the extracellular matrix under both physiological and pathological conditions[12]. Matrilysin, or MMP-7, belongs to the subgroup of stromelysins. Like other MMPs, matrilysin is secreted as a proenzyme, of which proteolytic removal of the 9 kDa prodomain from the N-terminus leads to activation of the enzyme. Uniquely, matrilysin is produced by epithelial rather than stromal cells[13]. Various studies have shown that matrilysin is significantly enhanced in cancer of the breast, prostate, lung, skin, and colorectum[14-19]. Furthermore, matrilysin expression has been related to the presence of lymph node metastases in gastric cancer patients[20].

In addition, several studies have shown that matrix metalloproteinases indirectly participate in controlling the levels of IGFs, through proteolytic cleavage of the IGFBPs which form complexes with the IGFs in the circulation[21-23]. For example,

Miyamoto *et al.* have recently shown that proteolysis of the IGFBP-3 by matrilysin increases the bioavailability of IGF-1, leading to enhanced cell survival[24]. In the present study, we aimed to evaluate the expression of matrilysin, IGF-1 and IGFBP-3 on GEP-NETs, to assess whether such a growth-activation cascade also exists in these tumours.

Material and methods

Patients

After surgical removal, tumour tissues were collected at the Department of Gastroenterology, Leiden University Medical Centre (LUMC), Leiden, and either frozen at -80 ◦C and/or embedded in paraffin for immunohistochemical staining.

Fifty-one homogenates (23 tumour samples and 28 normal samples) of 25 patients were available for the determination of tissue levels of matrilysin, IGF-1 and IGFBP-3. For immunostaining of IGF-1, IGFBP-3 and matrilysin, 44, 44 and 36 samples respectively, of 35 patients, were available.

GEP-NETs comprised pancreatic neuroendocrine tumours (PNETs) and gastrointestinal neuroendocrine tumours (GI-NETs), which were also referred to as 'carcinoids'. Clinicopathological information was obtained by evaluation of patients' medical files and pathology reports, when available. According to the classification of the World Health Organization for GEP-NETs, tumours were categorized into well-differentiated neuroendocrine tumour (NET), well-differentiated neuroendocrine carcinoma (NEC), or poorly differentiated NEC[25]. This study was performed according to the guidelines of the Medical Ethics Committee of the LUMC in compliance with the Helsinki Declaration.

Quantitative determination of matrilysin, IGF-1 and IGFBP-3 in tissue samples

Tissues were homogenized and protein concentrations were determined according to Lowry *et al.*[26]. Matrilysin, IGF-1 and IGFBP-3 levels were determined in tissue homogenates, using commercially available quantitative immunoassays (ELISA) for human matrilysin, IGF-1 and IGFBP-3, respectively, performed according to

the manufacturer's instructions (R&D Systems). Matrilysin, IGF-1 and IGFBP-3 levels were expressed per mg protein.

Immunohistochemistry

Immunohistochemistry was performed as follows. Tissues were fixed in formalin, embedded in paraffin and cut into 5 µm sections. After deparaffinisation and rehydration, endogenous peroxidases were blocked in methanol containing 0.3% H_2O_2 (Merck, Darmstadt, Germany). Antigen retrieval was performed by boiling in 0.01M citrate buffer pH 6.0 for 10 minutes. Slides were incubated overnight at room temperature (RT) with primary antibodies: monoclonal mouse anti-human MMP-7 (1.25 µg/mL), polyclonal goat anti-human IGF-1 (10 µg/mL), and polyclonal goat anti-human IGFBP-3 (5 ug/mL, all R&D Systems Europe, Abingdon, UK), diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). Incubation with goat-anti-mouse (for MMP-7) and rabbit-anti-goat (for all IGF-system components) immunoglobulins for 30 minutes at RT was followed by incubation with horseradish peroxidase (HRP)-streptavidin complex (all Dako, Glostrup, Denmark) for 30 minutes at RT. Cervix carcinomas were used as positive controls. Negative controls were included by omitting the primary antibodies. Representative photomicrographs were taken with an Olympus BX-51TF microscope equipped with a DP23-3-5 camera.

Immunohistochemical evaluation

Staining for matrilysin, IGF-1, and IGFBP-3 in tumour cells was scored semiquantitatively, according to a system proposed by Ruiter *et al.*[27]. As final score, the mean result of 2 independent individuals (P.K. and E.J.M.) was used. The percentage of tumour cells that stained positive were scored as follows: **0**, absent; **1**, 1–5% sporadic; **2**, 6–25% local; **3**, 26–50% occasional; **4**, 51–75% majority and **5**, 76–100% large majority. The intensity of tumour cell staining was scored as: **0**, no; **1**; weak; **2**, moderate and **3**, intense staining. A total score was calculated by adding the scores for percentage and intensity, resulting in values from 0 to 8.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences version 16 (SPSS) and GraphPad Prism version 5. Unpaired *t* test and one-way ANOVA were used to compare mean levels between various data sets. Pearson's correlation (*r*) was used to explore the relationship between two variables. Results are reported as mean ± standard error (S.E.). A p-value of <0.05 was considered statistically significant.

Results

Patient and tumour characteristics

The majority of the patients in this study were female (59%). Primary tumours of nine patients were localized in the pancreas, one in the duodenum, and six in the small bowel. Functional tumours were four insulinomas, two gastrinomas and one glucagonoma. The majority of tumours (95.7%) were well-differentiated. Tumours were classified according to the WHO classification into five well-differentiated NETs, seventeen well-differentiated NECs, and one poorly differentiated NEC. Carcinoid tumours were larger in size compared to pancreatic neuroendocrine tumours, mean size 4.9±1.2 cm vs. 2.7±0.6 cm, *P*=0.08. Metastases were seen in the majority of patients (76.5%); one patient with liver metastases, four patients with lymph node metastases, and eight patients with both liver and lymph node metastases. Angioinvasion was present in only 21.7% of the tumours.

Tissue levels of matrilysin, IGF-1 and IGFBP-3

IGF-1, IGFBP-3 and matrilysin tissue levels were measured from 25 patients with GEP-NETs (Table 1). Both IGF-1 and IGFBP-3 levels were found to be increased in tumours compared to (associated) normal tissues, only the latter being statistically significant, *P*<0.01. In contrast, tumour levels of matrilysin were somewhat lower compared to matrilysin levels in normal samples. Among the various types of GEP-NETs, i.e., carcinoids versus functional PNETs and non-functional PNETs, levels of IGF-1 and IGFBP-3 were not significantly different. Matrilysin levels, however, were significantly higher in non-functional PNETs compared to

functional PNETs and carcinoids, *P*=0.03. IGFBP-3 levels were somewhat higher in metastatic tumours compared to primary tumours, *P*=0.06. IGFBP-3 levels in primary tumours with and without metastases were not significantly different. Both IGF-1 and matrilysin levels were higher in primary tumours compared to metastases, and lower in primary tumour tissues of patients who had developed metastases than those without metastases, although not significant. IGF-1, IGFBP-3 and matrilysin levels were not related to other tumour parameters like tumour size or the presence of angioinvasion.

Table 1. Tissue levels of IGF-1, IGFBP-3 and matrilysin in GEP-NETs

	IGF-1 (pg/mg)		**IGFBP-3 (ng/mg)**		**Matrilysin (pg/mg)**	
	Mean±S.E.	**P-value**	**Mean±S.E.**	**P-value**	**Mean±S.E.**	**P-value**
Tissues						
Normals (n=28)	52.1±10.1		0.6±0.2		206.6±56.7	
Tumours (n=23)	85.4±19.3	0.12	6.9±1.6	**<0.01**	163.0±48.1	0.57
Tumour types						
Carcinoid (n=7)	51.4±12.7		3.5±2.1		46.7±14.9	
Functional PNET (n=12)	115.3±33.7		8.0±2.1		146.3±52.2	
Non-functional PNET (n=4)	54.9±27.3	0.28	9.5±5.5	0.35	416.8±192.7	**0.03**
Origin						
Primary (n=16)	96.6±26.7		5.0±1.7		196.5±67.2	
Metastasis (n=7)	59.6±16.3	0.39	11.2±3.0	0.06	86.4±26.3	0.30
Metastases						
Present (n=11)	79.1±29.3		4.7±2.1		120.6±57.6	
Absent (n=5)	135.2±57.2	0.35	5.65±2.8	0.81	363.4±160.4	0.09

Table 1. Mean values of IGF-1, IGFBP-3 and matrilysin tissue levels in relation to clinicopathological parameters. Significant p-values are in bold.

Immunohistochemical expression of matrilysin, IGF-1 and IGFBP-3

The results of the immunohistochemical evaluation is shown in Table 2 and Figure 1. For IGF-1, IGFBP-3 and matrilysin the cytoplasmic staining of tumour cells was generally strong. Remarkably, staining of matrilysin was stronger in tumour-associated normal tissue compared to tumour tissue in 61.5% of the samples, in particular pancreatic and hepatic NETs. IGF-1 and IGFBP-3 staining were

generally absent in normal tissue, and when present, the staining was less strong than in tumour tissue. Nuclear staining of tumour cells by IGF-1 was seen in 75% of the tumours. For IGFBP-3, membrane staining was found in more than 50% of the tumours.

Table 2. Immunohistochemical evaluation			
	IGF-1	***IGFBP-3***	***Matrilysin***
GEP-NETs	***n=44***	***n=44***	***n=36***
Mean total score	*6*	*6*	*7*
(range)	*(0-8)*	*(2-8)*	*(6-8)*
Staining present	***%***	***%***	***%***
Cytoplasm	*100*	*97.7*	*97.2*
Nucleus	*75.0*	*2.3*	*0*
Membrane	*0*	*52.3*	*0*
Normal tissue	***n=34***	***n=34***	***n=26***
Staining present	*38.2%*	*14.7%*	*84.6%*
N>T	*32.3%*	*8.8%*	*61.5%*

Table 2. Immunohistochemical staining results for IGF-1, IGF-2, IGF-1R, IGFBP-3 and matrilysin on tumour cells in 36<n<44 gastroenteropancreatic neuroendocrine tumours.

Staining scores for matrilysin did not differ between carcinoids and the two types of PNETs (all 7). For IGF-1 and IGFBP-3, staining scores in carcinoids and F-PNETs were both 6, whereas for NF-PNETs these were both 7.

Total staining scores were mutually correlated (Table 3). IGF-1 expression was significantly related to the expression of IGFBP-3. Interestingly, matrilysin expression was negatively correlated to the expression of IGF-1 and IGFBP-3, although only the latter being significant, P=0.02. Furthermore, staining scores were evaluated in relation to clinicopathological parameters, such as tumour size, angioinvasion, WHO classification, and the presence of metastases. Interestingly, a larger tumour was correlated to more IGFBP-3 staining in tumour cells (r=0.45, P=0.002). No other significant correlations were found. Finally, tumour levels of IGF-1, IGFPBP-3 and matrilysin were not significantly correlated with the immunohistochemical staining scores for these proteins (-0.143<r<0.413).

Table 3. Mutual correlations of immunostaining scores			
	IGF-1	***IGFBP-3***	***Matrilysin***
IGF-1		*r=0.44* ***P<0.01***	*r=-0.23* *P=0.15*
IGFBP-3	*r=0.44* ***P<0.01***		*r=-0.37* ***P=0.02***
Matrilysin	*r=-0.23* *P=0.15*	*r=-0.37* ***P=0.02***	

Table 3. Mutual correlations between immunohistochemical staining results for IGF-1, IGFBP-3 and matrilysin on tumour cells of gastroenteropancreatic neuroendocrine tumours. Bold p-values indicate a significant correlation.

Figure 1. Immunostaining of IGF-1, IGFBP-3 and matrilysin on GEP-NETs

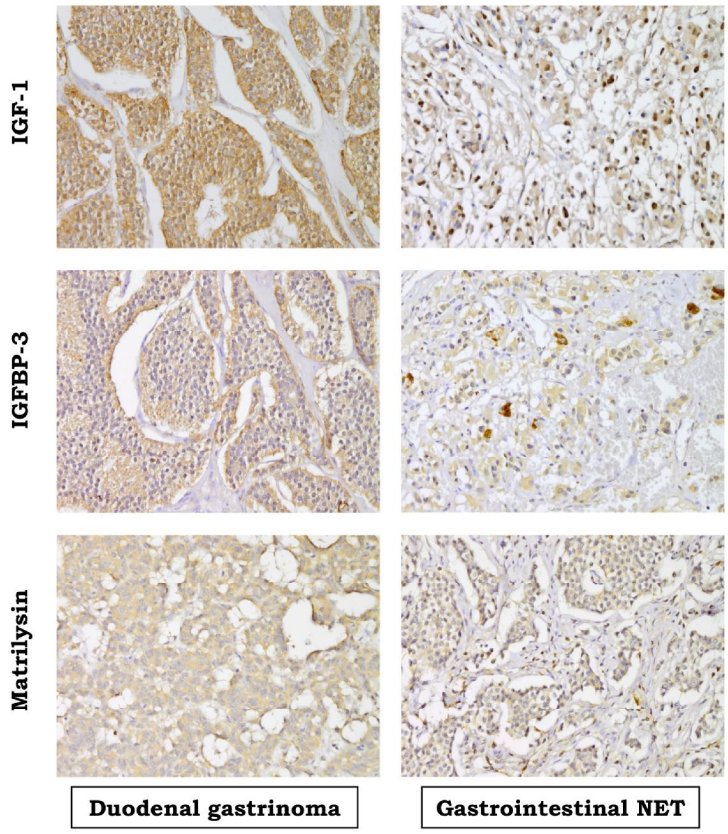

Figure 1. Immunohistochemical staining for a, b) IGF-1, c,d) IGFBP-3 and e,f) matrilysin on tumour cells of a duodenopancreatic gastrinoma (a, c, d) and gastrointestinal NET (carcinoid) (b, c, e). Magnification x100. In the insert of d) membrane staining of IGFBP-3 is shown at a higher magnification (x200).

Discussion

In this study, we examined the expression of IGF-1, IGFBP-3, and matrilysin in GEP-NETs, and find indications of an interrelated role for IGFBP-3 and matrilysin in the pathogenesis of these tumours.

The IGF system is of particular interest in cancer, as it is involved in many processes related to tumour growth[4]. IGF-1 has been described to have important functions in tumour development, such as the inhibition of apoptosis, the promotion of tumour growth, the inducement of transformation, and the promotion of metastasis in several cancers[28]. In gastrinomas, IGF-1 mRNA levels were found to be increased, and related to tumour growth, aggressiveness and curability[11]. IGF-1 is mainly present in the circulation, where it is bound to IGFBPs that act to protect IGF-1 from degradation by proteases[28,29]. IGFBP-3 is the most abundant IGFBP in the circulation[24]. The IGFBPs have both stimulating and inhibiting effects on IGFs. Gigek *et al.* described that when IGFBP-3 binds IGF-1, it inhibits its binding to one of the IGF receptors, thereby counteracting the actions of IGF-1[30]. In a study of Miyamoto *et al.*, a correlation between high levels of IGF-1 and low levels of IGFBP-3 was found[24]. Matrilysin has been shown to be involved in tumour cell invasion and the development of metastases[20]. In several cancers of the digestive tract, including gastric, oesophageal, pancreatic and colorectal cancer, matrilysin tissue levels were upregulated and related to malignant behaviour and a poor prognosis of the patients[20,31-34]. From previous studies it is known that matrix metalloproteinases are able to serve as proteinases for the various IGFBPs[21-23,35]. McGaig *et al.*, for example, have shown that *Helicobactor pylori*-associated epithelial-derived matrilysin cleaves IGFBP-5, thereby liberating IGF-2, which in turns stimulates epithelial cell proliferation, suggested to contribute to the progression to gastric cancer[36]. In addition, matrilysin was shown to be able to cleave IGFBP-3, thereby increasing the bioavailability of IGF-1 to cancer cells[24].

We evaluated IGF-1, IGFBP-3, and matrilysin in GEP-NETs to assess whether they are part of a similar growth activation process in these tumours.

Using ELISA, we measured tissue levels of matrilysin, IGF-1, and IGFBP-3 in the various neuroendocrine tumours of the gastroenteropancreatic tract. Both IGF-1 and IGFBP-3 tumour levels were increased compared to levels in normal tissue, although only the latter was significant. In addition, we found that IGFBP-3 levels were up-regulated in metastatic tumours samples compared to primary tumours. Furthermore, a higher IGFBP-3 staining was be indicative of a larger tumour size. Together, these findings suggest that IGFBP-3 might play a role in the tumourigenesis of GEP-NETs, independent of IGF-1. Although the expression of IGF-1 and IGFBP-3 were significantly correlated, IGF-1 expression in the tumour alone showed no association with any clinicopathological parameter. Similarly, Wulbrand *et al.* found no relation between IGF-1 expression and the presence of metastases in GEP-NETs, whereas Furukawa *et al.* previously showed that enhanced levels of IGF-1 in gastrinomas were related to tumour growth, aggressiveness and extent[10,11].

Remarkably, matrilysin levels were lower in tumours compared to associated normal tissues of patients with GEP-NETs. By immunohistochemical staining of matrilysin, we observed a similar pattern. Furthermore, tissue levels of matrilysin were lower in metastatic tumours and in metastases compared to primary tumours. Although a high matrilysin expression in tumours has been related to a more malignant phenotype and a poor prognosis in several cancer types, the results of our study suggest that matrilysin is not directly involved in the pathogenesis of GEP-NETs[14-20]. However, matrilysin might indirectly be related to malignant tumour behaviour, as a negative correlation between matrilysin and IGFBP-3 expression on tumour cells by immunohistochemistry was observed. So when the expression of matrilysin on tumour cells was high, a low expression of IGFBP-3 was found, and vice versa. Possibly matrilysin regulates the expression of IGFBP-3, thereby indirectly effecting the tumour's extent of malignancy. One explanation could be that matrilysin acts as a protease that cleaves IGFBP-3 present on the tumour cells. The observation that IGFBP-3 staining is also present on the membrane of tumour tissue supports this assumption. Further *in vitro* studies are required to determine IGFBP-3 levels in the medium of

gastroenteropancreatic neuroendocrine tumour cells, to reveal whether these cells secrete IGFBP-3, and if so, whether this process is mediated by matrilysin. In combination with serological analyses of GEP-NET patients these studies will elucidate whether matrilysin regulates secretion, complex formation and breakdown of IGFBP-3 in these tumours.

In summary, we found that the levels of IGF-1 and IGFBP-3 were increased in GEP-NETs, whereas matrilysin was decreased. Higher IGFBP-3 expression was related to the presence of metastases and a larger tumour size, which might indicative of a more malignant tumour. For matrilysin, an opposite trend was observed. Together, these findings suggest that IGFBP-3 plays a direct role in the pathogenesis of GEP-NETs, whereas matrilysin might indirectly be involved via regulation of this IGFBP-3 expression. Further studies are required to investigate this potential growth mechanism in more detail.

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