Maspin Expression in Adenocarcinoma of the Ampulla of Vater: Relation with Clinicopathological Parameters and Apoptosis

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Abstract. *Aim: Maspin is a unique serine proteinase inhibitor which has tumor suppressor activity. The aim of the present study was to investigate the role of maspin in ampullary adenocarcinomas, its correlation with apoptosis and its value as a prognostic marker. Patients and Methods: Twenty-three cases of ampulla of Vater adenocarcinoma were collected from archival material. For each sample, maspin, M30, p53 and Mib1 immunohistochemical reactivity were evaluated and results compared with clinicopathological parameters. Results: A statistical relation was found between nuclear maspin and M30 (Spearman's* $\varrho=0.46$, $p=0.02$*), and p53 (Kruskal-Wallis=0.03); a trend was found between nuclear maspin and pT (Kruskal-Wallis=0.09), and pM (Mann-Whitney=0.08) and pN status (Fisher's mid-point test:* $p=0.070$*). Conclusion: The present study evaluated the role of maspin in ampullary adenocarcinomas and for the first time demonstrated its association with apoptosis, tumor growth and metastasis.*

Mammary serine protease inhibitor (maspin) has been identified by subtractive hybridization as a candidate tumor suppressor protein in normal mammary epithelial cells (1). The maspin gene maps to human chromosome 18q21.3-q23, whose cDNA consists of 2,584 nucleotides encoding for a 42-kDa peptide (2). Tissue distribution studies have shown maspin expression in normal mammary epithelial cells, and in placenta, prostate, thymus, testis, oral cavity, small intestine, skin and cornea (3, 4). A number of findings support the inhibitory effects of maspin on tumors: levels of maspin expression show an inverse correlation with the progression of various types of carcinoma (5-11); maspin has also been shown to inhibit angiogenesis by blocking it in both *in vitro* and *in vivo* models (3). Although the mechanisms underlying its biological activity are largely unresolved, maspin seems to act on the cell membrane both directly and indirectly, affecting cell adhesion and inhibiting cell motility and invasion (12), and it is one of the few p53-targeted genes involved in tumor invasion and metastasis. The main mechanism of decreased maspin expression in mammary and prostatic cancer cells is thought to be *via* transcriptional down-regulation, rather than loss or rearrangement of the maspin gene (13). Maspin was recently shown to be involved in apoptosis, probably modulating its effect through regulation of Bcl2 family proteins (14). In a recent study, Latha *et al.* (15) demonstrated that a fraction of maspin translocates to the mitochondria and that this translocation is due to opening of the permeability transition pore, which in turn causes loss of transmembrane potential, thus initiating apoptotic degradation.

Apoptotic events may be favourable prognostic markers and the induction of apoptosis may be a promising treatment for cancers refractory to conventional therapies. In the present study, the possible relation of maspin with apoptosis was evaluated in ampullary adenocarcinomas.

M30 is an antibody which binds to a caspase-cleaved, formalin-resistant epitope of cytokeratin 18 cytoskeletal protein. The immunoreactivity of the M30 antibody is confined to the cytoplasm of apoptotic cells (16). The mitotic activity of neoplastic cells is presumed to be a parameter of worse prognosis; MIB-1 identifies the Ki-67 antigen present in the nuclei of cells in all phases of the cell cycle except G_0. Despite intuitive thoughts to the contrary, no correlation has yet been found between Ki-67 expression and prognosis in pancreatic and ampullary adenocarcinomas (17-21).

Recent evidence of maspin overexpression in pancreatic carcinoma and gastric adenocarcinoma opens up a new

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perspective on the function of maspin in human cancer (13, 22). In 2003, Fitzgerald *et al.* (23) found that although the maspin gene is not expressed in normal human pancreas, its expression is acquired during human pancreatic carcinogenesis. They concluded that human pancreatic carcinoma cells acquire maspin expression through epigenetic derepression of the maspin locus and, in so doing, appear to co-opt a normal cellular mechanism for its regulation. Ohike *et al.* (24) immunohistochemically studied maspin in a series of 57 pancreatic ductal adenocarcinomas and found that maspin high-expressers predominantly showed a statistically significant low histological grade.

Among pancreatic adenocarcinomas, ampullary adenocarcinomas have a better clinical course and an earlier time of detection, probably because of their onset of symptoms. Although two histological types are identified, intestinal and pancreatico-biliary, some mixed tumors can be found (20). Many carcinomas of the intestinal type express the immunohistochemical marker profile of intestinal mucosa (keratin 7–, keratin 20+, MUC2+). Those of the pancreatico-biliary type usually show the profile of pancreatico-biliary duct mucosa (keratin 7+, keratin 20–, MUC2–). The five-year survival rates of carcinoma of the ampulla of Vater and pancreatic carcinoma are very different, being 73.3% and 16.2%, respectively (median survival time 66.0 and 14.0 months). The overall 5-year survival for surgically treated ampullary carcinomas principally depends on T stage and lymph node status, varying from 64.4-58.8% to 33.3-20.8% respectively (25-27).

The aims of the present study were first to investigate the role of maspin in ampulla of Vater adenocarcinomas, immunohistochemically determining the expression of maspin and its correlation with apoptosis, in a cohort of patients using samples consecutively collected from archival material and, second, to evaluate maspin as a prognostic marker. We also examined the expression of p53 and Mib-1, and evaluated their correlation with maspin expression.

Patients and Methods

Patients. From 1996 to 2005, a total of 23 ampulla of Vater adenocarcinomas were collected from the archives of the Section of Pathology, University of Padova. Twenty-one patients underwent pylorus-preserving duodeno-cephalo-pancreasectomy in the Department of Surgery, University of Padova. In two cases, biopsies only were collected from inoperable patients. According to the TNM Classification of Malignant Tumours of the International Union Against Cancer (28), the pathological staging of primary ampullary lesions (pT) was T2 in 6 cases, T3 in 10 cases, and T4 in 5 cases; and pathological node staging (pN) was N0 in 10 and N1 in 11 cases. For the two biopsy cases, the clinical stage was used. Mean follow-up time was 19.8 months. Seven patients were lost to follow-up. All tissues were fixed in 10% formalin and embedded in paraffin wax.

Immunohistochemistry. From each of the 23 tissue blocks, 5-micron sections were cut for immunohistochemical characterization. The sections were pre-treated in a microwave oven (750 W) for 20 min in a citrate buffer (10 mM, pH 6.0). For each sample, the reactivity of maspin (mouse monoclonal antibody, clone G167-70, diluted 1:500; BD-Biosciences, PharMingen International, San Diego, CA, USA), p53 (mouse monoclonal antibody, clone DO-1, pre-diluted; Immunotech, Marseilles, France), Ki-67 (mouse monoclonal antibody, clone MIB-1, diluted 1:100; DAKO, Glostrup, Denmark), M30 CytoDEATH (mouse monoclonal antibody, clone M30, diluted 1:250; Roche, Mannheim, Germany), keratin 20 (mouse monoclonal antibody, clone Ks20.8, diluted 1:50; DAKO), keratin 7 (mouse monoclonal antibody, clone OVTL, diluted 1:100; Biogenex, San Ramon, CA, USA) and MUC2 (mouse monoclonal antibody, clone CCP58, prediluted; Biogenex) was evaluated by incubation. Sections were pre-incubated with protein block (Novocastra Laboratories Ltd., Newcastle-upon-Tyne, UK) for 5 min then stained with the selected antibodies. Post-primary block (Novolink Polymer Detection System, Novocastra) was applied to the specimens for 20 min, which were then washed with PBS (pH 7.0) for 3 min and incubated with Novolink Polymer for 20 min.

The Automate Staining System (Biogenex) was used. Color was developed using 3,3'-diaminobenzidine (DAB) (DAKO) for 4 min. Sections were counterstained with Meyer's hematoxylin.

Positive controls were placental tissue for M30 antibody, normal intestine for MUC2 and normal breast for maspin. Primary antibody was substituted with PBS in negative controls.

Image analysis (I.A.) determinations. Maspin, p53, M30 and Mib1 expression were evaluated with the CIRES workstation image analysis system (Zeiss, Jena, Germany), consisting of a conventional light microscope (Axioskop model, Zeiss) connected to a 3CCD color video camera (KY-F55BE JVC, Japan). Images were captured by a frame grabber (Kontron, Eching, Germany) and then analyzed. The frame grabber and image analysis (I.A.) program, operating on-line with the camera, were hosted by a PC. During all measurement sessions, illumination was kept constant at a fixed value and the stray light effect was reduced by Koehler's illumination setting (29).

In each case, the same selected non-overlapping fields were evaluated, choosing those at the worst grade of differentiation, at x200 magnification, counting a minimum of 600 cells, expressing the result as the percentage of positively labeled cells out of the total. The percentages of specifically stained cells over 5% were considered positive for p53 and over 10% as positive for Mib-1, with only nuclear staining being considered positive.

Data analysis. Given the small number of cases, interdependence between variables was assessed by applying the following non-parametric statistical tests: Spearman's ϱ correlation coefficient, Fisher's exact test, Fisher's mid-point test, Kruskal-Wallis test for trend and the Mann-Whitney test. Spearman's ϱ test was applied to evaluate the correlation between nuclear maspin and M30, and between nuclear maspin and Mib-1; the Mann-Whitney test was used to evaluate the association between maspin positivity and pM status; the Kruskal-Wallis test was used to evaluate the association between maspin and p53 positivity, and between p53 and histological grade; and Fisher's exact and mid-point tests were used to evaluate the association between maspin staining and histological grade, pT status, pN and pM status. A value of $p<0.05$

Figure 1. *Three patterns of maspin staining. A) prevalently nuclear pattern: most neoplastic cells stain at the nucleus, the cytoplasm is only occasionally and weakly stained. B) nuclear/cytoplasmic staining: more than 10% of nuclei and most cytoplasm of neoplastic cells show staining. C) cytoplasmic but not nuclear staining in neoplastic cells. D) duodenal epithelium adjacent to the tumor, negative for maspin. (Maspin antibody A-C: x200; D: x50).*

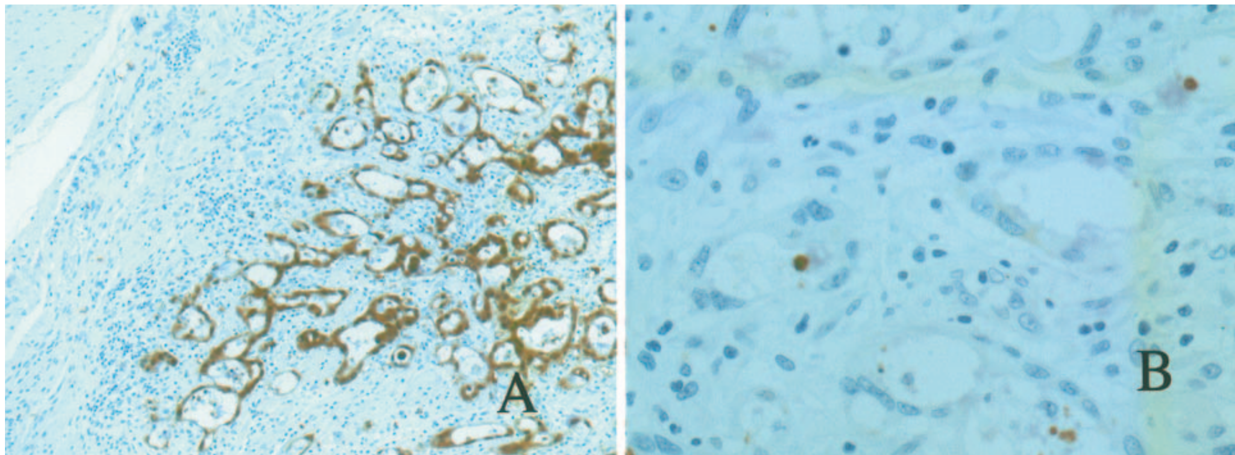

Figure 2. *Example of same matched neoplastic areas chosen for evaluation of examined antibodies. A) neoplastic area from a poorly-differentiated adenocarcinoma of ampulla of Vater, stained with maspin. B) same area stained with M30 antibody, for apoptotic count. (A: Maspin antibody, x100; B: M30 antibody, x400).*

Table I. *Immunohistochemical analyses and clinical data for the 23 cases of ampullary carcinoma.*

Case no.	Sex/ Age	Maspin % stained nuclei	Maspin staining pattern	M30 %	MUC2 %	CK7	CK20	Mib1 %	p53 %	Grade	pT	pN	pM	Clinical outcome follow-up in months
1	f/47	20	nuc	10	90	–	+	50	90	2	2	0	0	NED/39
2	m/49	0	neg	10	0	+	–	10	0	1	2	0	1	Deceased 2001/6
3	m/70	20	nuc-cyt	10	5	+	+	1	40	2	4	0	0	Lost
4	m/68	10	nuc-cyt	3	1	–	–	5	0	2	2	0	0	NED/22
5	f/54	0	neg	0	0	+	–	10	0	2	3	1	0	Lost
6	m/72	10	nuc-cyt	1	0	+	–	40	0	2	3	0	0	Lost
7	f/69	10	nuc-cyt	7	20	+	+	60	90	3	2	0	0	NED/15
8	m/78	30	nuc-cyt	10	0	+	+	20	10	2	4	1	0	Deceased §/3
9	m/85	0	neg	20	20	+	+	40	0	2	3	1	0	Lost
10	m/62	10	nuc-cyt	10	5	+	+	3	60	2	2	0	0	Lost
11	m/66	0	neg	2	0	+	+	1	0	2	3	0	1	M+ 2003/48
12	m/66	0	neg	1	1	+	–	30	10	2	3	0	0	NED/48
13	m/51	30	nuc-cyt	15	1	+	+	50	0	2	4	1	0	NED/36
14	f/66	0	cyt	1	1	+	+	35	0	2	3	1	0	Lost
15	m/53	5	cyt	8	0	+	+	10	30	3	3	1	0	Deceased* /1
16	m/69	0	neg	3	0	+	–	60	0	2	4	1	1	Deceased 2003/30
17	m/75	5	neg	10	0	+	–	70	0	2	3	1	0	NED/12
18	m//59	2	cyt	1	20	+	+	30	70	2	2	0	0	Deceased 1997/16
19	m/56	0	cyt	1	0	+	–	80	90	3	4	1	1	Deceased 2001/5
20	m/71	10	nuc-cyt	2	0	+	–	0	0	2	3	1	1	Deceased 2004/5
21	f/81	10	nuc-cyt	2	20	+	+	35	10	2	4	0	0	Lost
22	m/45	15	nuc-cyt	10	0	+	–	40	15	2	3	1	1	M+ 2005/20
23	m/44	30	nuc	6	0	+	–	20	20	2	4	1	0	NED/12

§: deceased, unrelated disease; *: deceased, post-surgical complications; NED: no evidence of disease.

was considered to be significant; p-values in the range $0.10>p\geq0.05$ were considered to indicate a statistical trend. The STATA 8 statistical package (StataCorp, College Station, TX, USA) was used for all evaluations.

For maspin reactivity, a double evaluation was performed, the first considering the percentage of nuclear positivity on 100 neoplastic cells. The second considered the distribution pattern, scored as: negative, prevalently nuclear (when cytoplasm reactivity was <10%); nuclear-cytoplasmic (when both nuclei and cytoplasm were positive, with more than 10% of nuclear positivity); and cytoplasmic. For statistical evaluation of the maspin staining pattern, we combined nuclear and nuclear/cytoplasmic distributions, considering the presence of nuclear maspin decisive in any case (Figure 1A-C).

Only cytoplasmic positivity was considered for M30, in both clearly apoptotic and non-apoptotic cells, and was expressed as the percentage of positivity on 100 cells. The same fields were exactly matched in specimens, selecting the tumor area at the worst grade of differentiation; all the above-mentioned antibodies were evaluated in the same selected area (Figure 2 A, B). For keratin 20 and keratin 7, immunohistochemical positivity was considered in the same tumor fields. The percentage of positivity on 100 cells was evaluated for MUC2.

To assess the reproducibility of I.A. against conventional determinations based on light microscopy, two pathologists (S.B. and A.P.) blindly scored 50% of the total and randomly selected slides, using the same strategy (except for I.A.), irrespective of protein type. The results of both evaluation methods correlated quite well: Spearman's coefficient 91%, $p<0.001$.

Results

Table I lists the results of immunohistochemical analyses and clinical data. Eight tumors showed positivity for both CK7 and CK20 in the same fields, while one did not display positivity for either. MUC2 positivity was randomly associated with CK expression.

Normal duodenal epithelium adjacent to the papilla of Vater did not stain for maspin (Figure 1D), whereas the epithelium overlying or close to the tumor displayed a weak cytoplasmic reaction.

A statistical relationship was found between the percentages of positive nuclear maspin on 100 neoplastic cells and of positive M30 neoplastic cells (Spearman's $\varrho=0.46$,

p=0.02), while there was an inverse correlation between nuclear maspin staining (nuclear/nuclear-cytoplasmic) and p53 expression (Kruskal-Wallis test: p=0.03).

A trend was found for an association between nuclear maspin staining and pT status (Kruskal-Wallis=0.09), and pM status (Fisher's mid-point test: p=0.072), between the nuclear maspin pattern and pN status (Fisher's mid-point test: p=0.070), and between nuclear maspin expression (% of positive nuclei) and pM status (Mann-Whitney test: p=0.08).

No statistical association was found between maspin (considered as a percentage of nuclear positivity or staining pattern) and Mib1 expression (Spearman's test: p=0.7 and p=0.6, respectively), or between the maspin staining pattern and pT status (Fisher's exact test: p=0.7).

No other statistical associations were found between histological grade, M30, p53 and Mib1 expression and the clinicopathological parameters examined.

Discussion

Ampulla of Vater adenocarcinomas differ from ductal adenocarcinomas in both clinical and pathological features. Ampullary adenocarcinoma has a significantly better outcome compared with pancreatic cancer after curative resection, mainly depending on T and N staging. Increasing evidence suggests that prognostic differences are influenced by different tumor biology. Prenzel *et al.* (30) found that the growth factor receptor genes *c-erbB-1* and *c-erbB-2* are differentially regulated in both tumors, adding further evidence that pancreatic adenocarcinoma is biologically different from that of the papilla of Vater.

Maspin has been implicated in tumor growth and metastasis and, more recently, apoptosis (31, 32). Maspin-induced apoptosis has been studied to determine its possible therapeutic use in human malignancies, *e.g.* prostate and breast carcinomas (33, 34).

Zhang *et al.* (14) reported that maspin-expressing tumor cells increase the rate of apoptosis when they are treated with staurosporine or subjected to serum starvation. They found that maspin-mediated apoptosis is partially blocked by caspase-8 and -9 inhibitors, is accompanied by changes in the Bcl-2 family proteins, and that maspin-expressing tumor cells have reduced anti-apoptotic protein Bcl-2 and increased pro-apoptotic protein Bax. The link between maspin and Bax up-regulation explains the loss of maspin-expressing tumor cells in invasive breast and prostate carcinomas. The tumor-suppressive mechanism of maspin suggests that it may be used as a modifier for apoptosis-based cancer therapy (35).

Watanabe *et al.* (36) evaluated the use of adeno-associated virus (AAV, serotype 2) vector-encoding maspin for *in vivo* gene therapy for human prostate cancer. They also demonstrated that the percentage of apoptotic cells in AAV-maspin-mediated maspin-expressing cells was significantly high compared with that in AAV-GFP-mediated GFP-expressing cells.

The maspin protein is a monomer present as a secretory, cytoplasmic, nuclear and cell surface-associated protein (10, 37).

Increasing evidence has shown that the sub-cellular location of maspin has a different role in regulating cellular homeostasis and may be associated with distinct tumor progression pathways. In particular, the nuclear location seems to be the strongest predictive parameter related to disease-free survival (5, 9, 10, 38) while the cytoplasmic location has been associated with a high S-phase cell fraction and aneuploidy (39).

In the present study, the nuclear presence of maspin (evaluated as the maspin staining pattern) showed a correlation with nodal (p=0.07) and metastatic status (p=0.072), whereas nuclear maspin (%) showed a correlation with M30 (p=0.02) and p53 (p=0.03), and a trend for an inverse association with metastatic status (p=0.08) and pT staging (p=0.09).

We found that nuclear maspin positivity, together with different staining patterns, may have prognostic implications, since nuclear and nuclear/cytoplasmic maspin patterns correlate inversely with lymph-node metastasis, absence of distant metastases, and low p53 staining.

In the present study, for the first time, apoptotic events were found to be numerically related to nuclear maspin expression in paraffin-embedded ampullary adenocarcinoma samples.

The different immunohistochemical profiles for CK7, CK20 and MUC2 expressed in the present series of ampullary carcinomas were not found to be related to prognosis or to be related with maspin expression. Our results are in agreement with previous studies in which the pancreatico-biliary, intestinal or mixed type of tumor differentiation did not influence the clinical outcome (20). However, it is interesting to note that we matched the same areas in the slides exactly and the above described double positivity was observed in the same neoplastic cells.

The present report is a preliminary study conducted on a relatively small number of cases and a larger sample population is needed to confirm our data, but this is the first time that maspin expression in ampullary adenocarcinoma has been related to histopathological parameters and, numerically, to apoptotic events.

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