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Comparative study of the immunohistochemical expression of metalloproteinases 2, 7 and 9 between clearly invasive carcinomas and "in situ" trophoblast invasion

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Matrix metalloproteinases (MMPs) are endopeptidases considered to participate in the transient invasive property of trophoblastic cells during embryo implantation and placentation. The same molecules play an important role in the invasive and metastatic potential of cancer cells. The aim of this study was to compare the immunohistochemical expression of MMP2, 7 and 9 between clearly invasive carcinomas and "in situ" trophoblast invasion in an effort to illuminate their distinct roles in uncontrolled and controlled invasion.

We performed an immunohistochemical analysis of 45 clearly invasive carcinomas of various organs (colorectal, gastric, breast, pulmonary, renal) and 40 first trimester gestation specimens (before the 9th week of gestation). The markers expression was evaluated semiquantitavely, seperately in cancer parenchymal and gestational trophoblastic cells as well as cancer stromal and decidual cells, according to a percentage scale (0 %, <10%, 10-50% and >50% of cells) and according to staining intensity (0, +, ++, +++).

MMP9 was expressed more often in the malignant parenchymal as well as in the malignant stromal component of carcinomas than in the trophoblastic (p=0, 0118) and decidual (p=0,017) component of gestations respectively. Although all carcinomas and almost all gestation specimens stained for MMP2 and MMP7, the immunostaining for both molecules was statistically more extensive and intense in trophoblasts and decidual cells by comparison to cancerous elements.

In conclusion, although there seems to be a direct link between cancer invasion and MMP9 immunohistochemical expression, the role of MMP2 and MMP7 appears to be more complicated underlining the complexity of the mechanisms involved in cancer spreading.

Key words: metalloproteinases, invasive carcinomas, trophoblast invasion

The success of reproduction relies primarily on the ability of the developing embryo to establish an intimate connection with its mother, through a process of embryonic attachment and implantation that allows the embryo to penetrate deeply into the maternal decidua and to invade the endometrial spiral arteries [1]. A series of synchronized and strictly regulated molecular and biochemical interactions are required for successful implantation, including degradation and remodeling of the extracellular matrix by various enzymes [2]. The fact that trophoblastic cells from first trimester exhibit behaviors similar to these of cancer cells, such as migration and invasion, is really impressive. Although placental trophoblastic cells behave like metastatic cancer cells, they are only transiently invasive. Specifically, their invasion is limited to the first trimester and spatially to the endometrium and the proximal third of the myometrium [1]. The invasive phenotype of placental cells has been directly linked to their ability to produce extracellular matrix metalloproteinases (MMPs) [3].

The MMPs are a family of more than twenty proteases that can virtually process all extracellular matrix components [4–6]. Their proteolytic activity allows them to participate in many steps of tumor progression such as involvement in the early stages of tumor growth and development, alteration of cell adhesion , degradation of basic membranes-extracellular matrix and angiogenesis [7].

MMP2 and MMP9 (also known as gelatinases) were initially viewed only as essential proteases for basal membrane-invasive events , but the finding that MMP2 is also produced by mesenchymal cells and MMP9 by inflammatory cells (macrophages and neutrophils) at the tumor site has attributed to them the

role of pathological angiogenesis regulators [8, 9]. MMP2 (Gelatinase A) is expressed by various cell types including fibroblasts, keratinocytes, endothelial cells, chondrocytes, osteoblasts and monocytes. MMP9 (Gelatinase B) is produced by normal alveolar macrophages, polymorphonuclear leukocytes, osteoclasts, keratinocytes and other. Gelatinases degrade type IV, V, VII, X, XI and XIV collagens , gelatin, elastin, proteoglycan core proteins, myelin basic protein, fibronectin, fibrillin 1 and precursors of TNF α and IL1 β [10].

MMP7 (matrilysin) is the smallest MMP and is mainly expressed by epithelial cells (and less by stromal cells). It is expressed by normal glandular epithelial cells in endometrium, small intestinal crypts, skin and airways and by malignant epithelial cells in tumors of the gastrointestinal tract, prostate and breast.In addition to a wide range of ECM components including fibronectin, laminin, nidogen, type IV collagen and proteoglycans, MMP7 cleaves β4 integrin [10].

Although the underlying mechanisms of trophoblast invasion-placentation and cancer have been studied, no adequate comparative data exist on the expression of the above mentioned MMPs in these two phenomena. The aim of our study was to compare the expression of MMP2, 7 and 9 between a physiological process with conrolled invasion (implantation-placentation) and an abnormal one with uncontrolled invasion (cancer) in an effort to assess any differential expression of these molecules in these two biologically distinct phenomena. The elucidation of the mechanisms that cancer uses to spread could contribute to the development of more effective anticancer drugs, targeting specific molecules with a key function in the process of true invasion.

Materials and methods

From 2003 to 2008, tissue samples from 45 patients with various carcinomas of advanced stage (all carcinomas had positive lymph nodes) and grade (comprising 15 samples of colorectal carcinomas, 8 samples of gastric carcinomas, 8 samples of breast carcinomas, 7 samples of lung carcinomas and 7 samples of renal cell carcinoma) and 40 samples from surgical, non- spontaneous abortion curettages (before the 9th week of gestation) were obtained in the Histopathology Department of 417 N.M.T.S Veterans Hospital and in the First pathology Department of the Medical School of National and Kapodistrian University of Athens. None of the patients with carcinoma had received radiotherapy or chemotherapy before surgery. Formalin fixed, paraffin embedded samples of all 45 carcinomas and 40 curettages were studied .Two pathologists reviewed all tissue samples and confirmed diagnosis and histological characteristics.

Immunohistochemistry. a) Antibodies. Ready to use for immunohistochemistry staining monoclonal antibody against human MMP9(92Kda Collagenase IV, Thermo Scientific, Runcorn,UK) , ready to use for immunohistochemistry staining monoclonal antibody against human MMP-2 (72kDa Collagenase IV, Thermo Scientific , Runcorn ,UK) and purified monoclonal antibody against human MMP-7 (Matrilysin,Ab-1,Clone ID2, Runcorn ,UK) were used as primary antibodies. The immunogenic component was a synthetic peptide derived from the near C-terminal of human MMP-9 protein and a synthetic peptide derived from the near C-terminal of human MMP-2 protein. As far as for MMP7 the immunogenic component was recombinant human matrilysin (PUMP-1). All the antibodies we used recognize both latent and active forms of MMPs.

b) Immunohistochemical technique. Tissues were immediately fixed in formalin (10%) and then processed as paraffin blocks. Sections of formalin fixed tissues, 4 μm thick were deparaffinated in xylene and rehydrated through a graded series of ethanol solutions.Sections were immunostained using the Bond Max automated immunoistohemistry system (Leica Microsystems) with Bond Polymer Refine Detection. The automated procedure was based on a novel controlled polymerization technology that prepared polymeric HRP-linker antibody conjugates. The detection system avoided the use of streptavidin and biotin and therefore eliminated non-specific staining as a result of endogenous biotin. Bond Polymer Refine Detection consisted of the following steps: incubation of the specimen with hydrogen peroxide to quench endogenous peroxidase activity, application of the primary antibody, application of post primary polymer penetration enhancer containing 10% animal serum in Tris-buffered saline and 0, 09% ProClin 950, and application of a poly-HRP anti-mouse/rabbit IgG reagent that localized the primary antibody. The substrate chromogen was 3,3 diaminobenzidine (DAB) and the counterstain was hematoxylin. For MMP9 and MMP2 tissue sections boiled in 10mM citrate buffer, pH 6.0 for 15 min followed by cooling at room temperature.MMP9 and MMP2 were used as ready to use prediluted antibodies and MMP7 was used at a concentration of 8μg/ml for 30 minutes (dilution of 1/25) at room temperature.

c)Analysis of immunohistochemistry. Each case was evaluated blindly by 2 independent investigators. Immunoreaction was assessed semiquantitavely and qualitatively according to the evaluation system used by Dominique Trudel et al [11] and a similar system used by O.Graesslin et al [12]. The immunostaining of MMP9, MMP2 and MMP7 was comparatively evaluated in cells of the cancer parenchyma and trophoblastic cells as well as in the cells of malignant stroma and decidual cells. The number of cells expressing the marker was assessed using a semiquantitative 4 grade scale (0%,<10%,10-50% and >50%). The intensity of the staining was evaluated using a 4 grade scale (0,+,++,+++).Since no differences in staining between synchiotrophoblastic and cytotrophoblastic cells were noticed , staining was assessed in both cell populations simultaneously. Discordant results were reviewed by both investigators and a consensus was reached.

d)Statistical analysis. We performed the chi-square test for categorical variables .For the chi square test we used the software package Graphpad Prism 5. P values below 0, 05 were considered statistically significant.

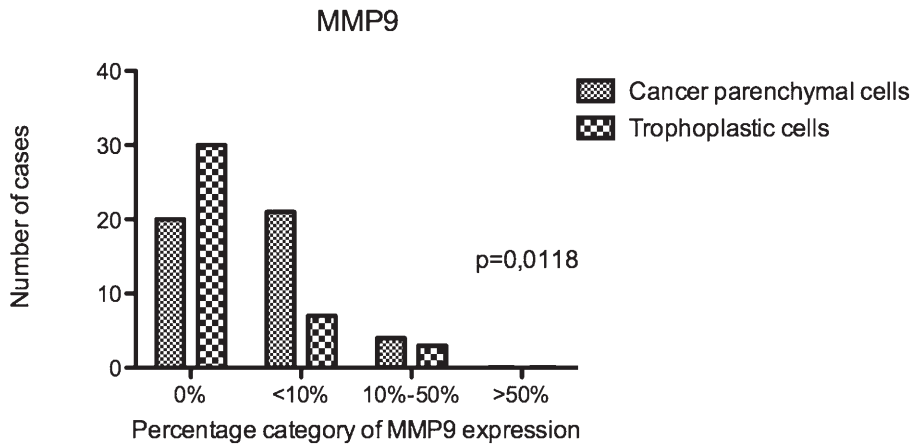

Figure 1. MMP9 immunostaining of cancer parenchymal and gestational trophoblastic cells in the various percentage categories.MMP9 immunostaining was more extensive in carcinomas than gestation.

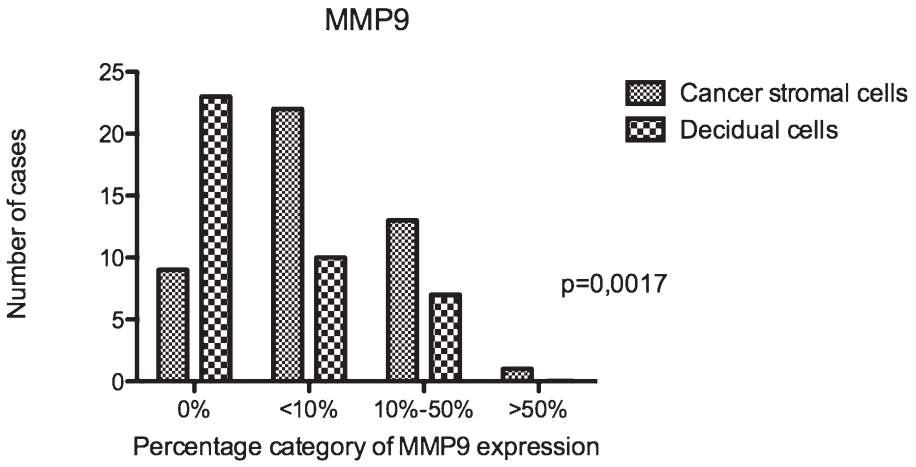

Figure 2. MMP9 immunostaining of cancer stroma and decidua in the various percentage categories. MMP9 was more extensive in carcinomas than gestation.

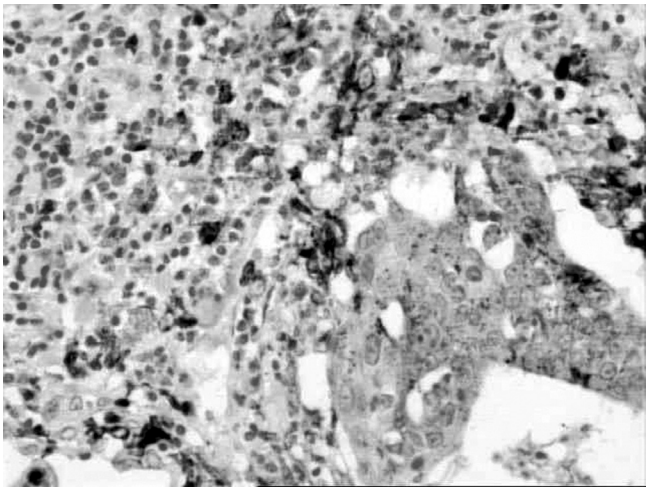

Figure 3. MMP9 immunostaining in the cancerous elements (parenchymal and stromal) of a colorectal carcinoma. (Immunoperoxidase stain, x 40)

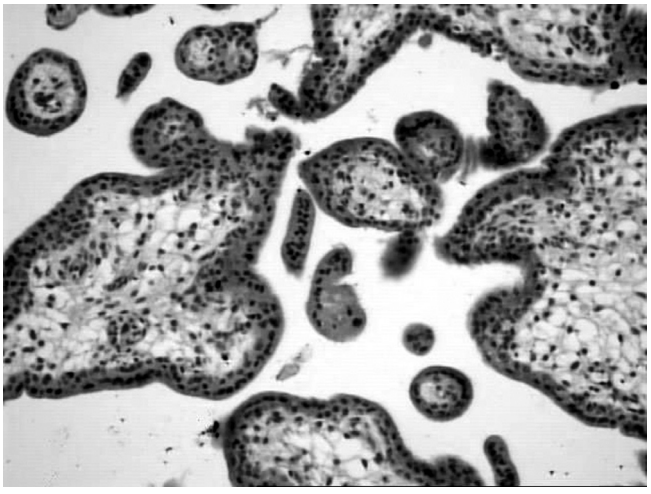

Figure 4. MMP9 cytoplasmic immunostaining in trophoblastic cells. (Immunoperoxidase stain, x 10)

Table 1.Intensity of MMP9 staining according to cell type in cancer and gestation.

MMP9 staining intensity category	Carcinomas (Parenchymal cells) N (%)	Gestation (Trophoblastic cells) N (%)	Carcinomas (Stromal cells) N (%)	Gestation (Decidual Cells) N (%)
0	20 (44%)	30 (75%)	9 (20%)	23 (57,5%)
+	15 (33%)	8 (20%)	0 (0%)	14 (35%)
++	8 (19%)	1 (2,5%)	13 (29%)	2 (5%)
+++	2 (4%)	1 (2,5%)	23 (51%)	1 (2,5%)
	p=0,001		p<0,001	

Table1. It is obvious that intensity of MMP9 staining is greater in cancer epithelial cells and cancer stromal cells than trophoblasts(p=0,01) and decidua (p<0,001) respectively.

Results

MMP9. The comparison of MMP9 labeling results between cancer and gestation in the various percentage categories is shown in Figures 1 and 2.

Briefly, MMP9 expression was higher in the parenchymal and stromal cells of carcinomas (Figure 3) than in the trophoblasts (Figure 4) and decidua of gestations. Immunostaining was observed in the parenchymal cells of 25 carcinomas (55, 5%) and in the stromal cells of 36 carcinomas (80%), while it was observed in the trophoblasts of 10 gestations (25%) and in the decidua of 17 gestations (42, 5%).

The results of MMP9 staining intensity are summarized in Table 1.The intensity of immunostaining was greater in the stroma of carcinomas (23 cases or 51% with intensity +++). In many carcinomas (especially colorectal carcinomas) the stromal staining was more intense along the invasive border of the tumor.

MMP2. The comparison of MMP2 labeling results of cancer and gestation in various expression percentage categories is shown in Figures 5 and 6.

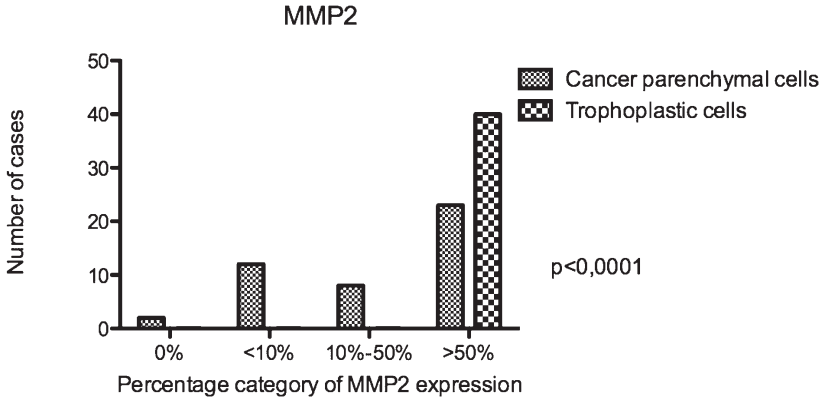

Figure 5. MMP2 immunostaining of cancer parenchymal cells and trophoblasts in the various percentage categories. MMP2 predominates in trophoblasts in the >50% category.

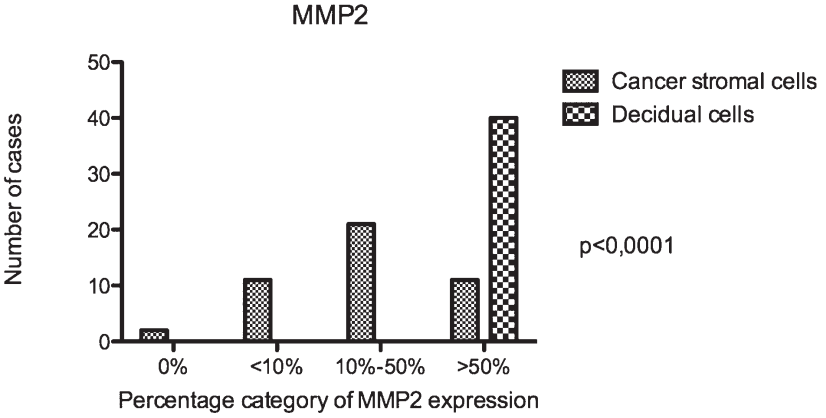

Figure 6. MMP2 immunostaining of cancer stroma and decidua in the various percentage categories.MMP2 predominates in decidua in the >50% category.

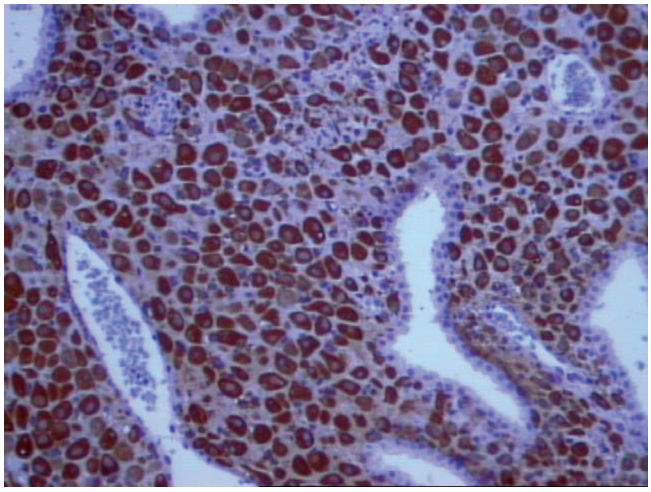

Figure 7. MMP2 decidual immunoreactivity (Immunoperoxidase stain, x 10)

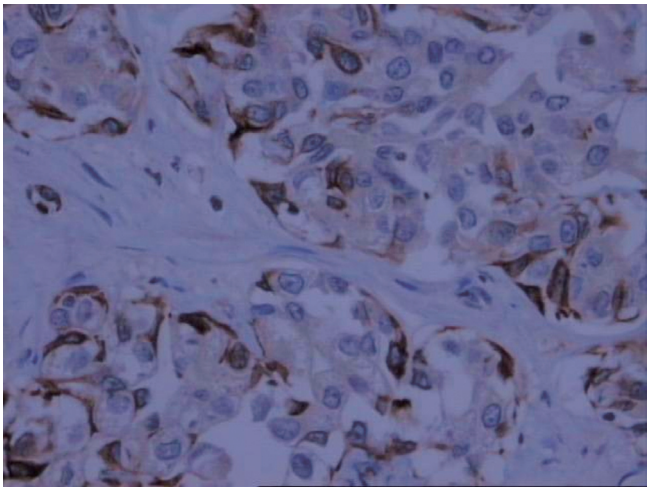

Figure 8. MMP2 immunostaining in the parenchymal and stromal cells of a colorectal adenocarcinoma. (Immunoperoxidase stain, x40)

Table 2. Intensity of MMP2 staining according to cell type in carcinomas and gestation.

MMP2 staining Intensity category	Carcinomas (Parenchymal cells) N (%)	Gestation (Trophoblastic cells) N (%)	Carcinomas (Stromal cells) N (%)	Gestation (Decidual cells) N (%)
0	2 (4%)	0 (0%)	2 (4%)	0 (0%)
+	18 (40%)	0 (0%)	15 (33%)	0 (0%)
++	19 (42%)	0 (0%)	18 (40%)	0 (0%)
+++	6 (51%)	40 (100%)	10 (22%)	40 (100%)
	p<0,0001		p<0,0001	

Table2. MMP2 staining was more intense in trophoblasts and decidua than cancer epithelium (p<0,0001) and cancer stroma (p<0,0001) respectively.

Although all gestation specimens and almost all (except for two) carcinomas stained for MMP2, there was a predominance of gestations in the >50 % expression percentage category (p<0, 0001) (Figures 5 and 6). Briefly, that means that MMP2 was found in more trophoblastic and decidual (Figure 7) cells than cancer parenchymal (Figure 8) and stromal cells. All gestations stained for MMP2 homogeneously. Trophoblastic and decidual cells stained for MMP2 in >50% of their cells while the immunostaining pattern in carcinomas was heterogeneous (51% of carcinomas expressed MMP2 in >50% of their malignant parenchymal cells, 18% in 10-50% of their parenchymal cells and 27% in <10% of their parenchymal cells).

The results of MMP2 staining intensity are summarized in Table 2.The intensity of immunostaining both in trophoblastic and decidual cells was strong (+++) in all the cases.On the other hand MMP2 expression was weaker in carcinomas. Only 14% of and 22 % of carcinomas stained strongly (+++) in their parenchymal and stromal cells respectively. Expression of MMP2 wasn't noticed in inflammatory cells.

MMP7. The comparison of MMP7 labeling results in the various percentage categories in cancer and gestation is shown in Figures 9 and 10.

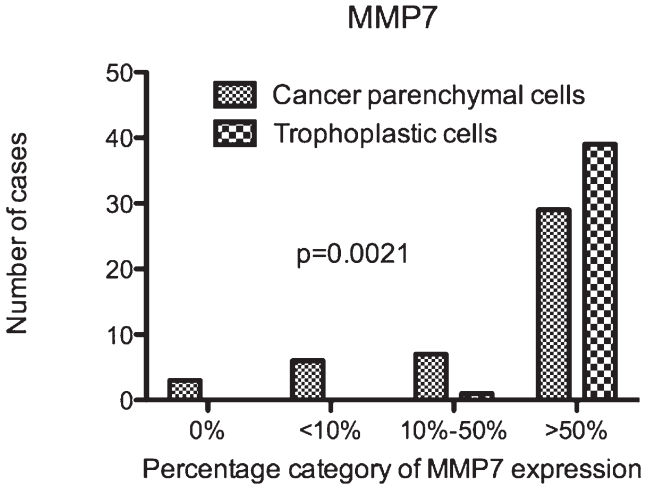

Figure9 . MMP7 immunostaining of cancer parenchyma and trophoblasts in the various percentage categories. MMP7 predominates in trophoblasts in the >50% expression percentage category.

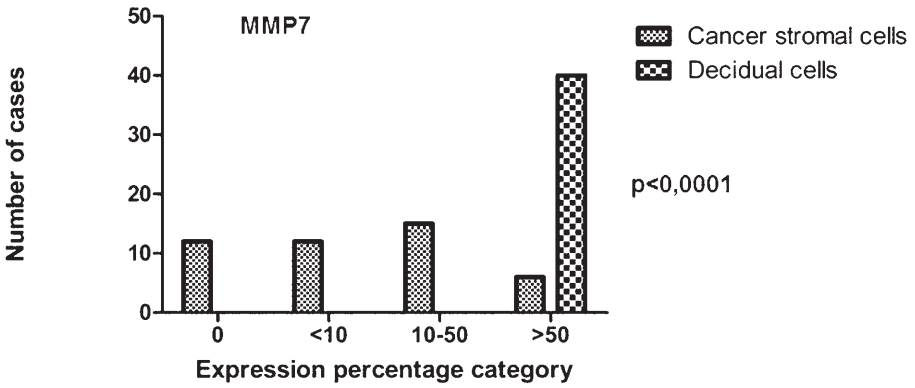

Figure 10. MMP7 immunostaining of cancer stroma and decidua in the various percentage categories.MMP7 predominates in decidua.

In a similar way to MMP2 , all gestations and almost all carcinomas (except for 3) stained for MMP7. A statistically significant difference at the level of p=0, 0021 was observed in MMP7 immunostaining between trophoblastic (Figure 11) and cancer parenchymal cells (Figure 12), with trophoblastic cells predominating in the >50% expression percentage category . P was smaller than 0, 0001 between MMP7 expression in cancer stromal cells and decidual cells in gestation, with decidual cells predominating in the >50% percentage category. Briefly the above mean that MMP7 was found in more trophoblastic and decidual cells than cancer parenchymal and stromal cells.

The results of MMP7 staining intensity are summarized in Table 3.The intensity of staining was weaker both in cancer epithelial cells (32 cases or 71% with staining +) and stromal cells (26 cases or 58% with staining +) than in trophoblastic cells (36 cases or 90% with staining ++ or +++) and decidual cells (35 cases or 87,5% with staining ++ or +++) respectively.

Discussion

Some of the results of our experiment were expected, while other seemed paradoxal at first sight. In order to interprete them we may have not taken into consideration the differences between the microenviroment of each organ. All our malignant specimens were clearly invasive and metastatic , so in any case we considered cancer in general as a highly invasive -metastatic phenomenon. The aim of our study was to compare the expression of MMP2, 7 and 9 between a clearly invasive, metastatic phenomenon(cancer) and a physiological "in situ process" that normally does not metastasize(trophoblast invasion) in

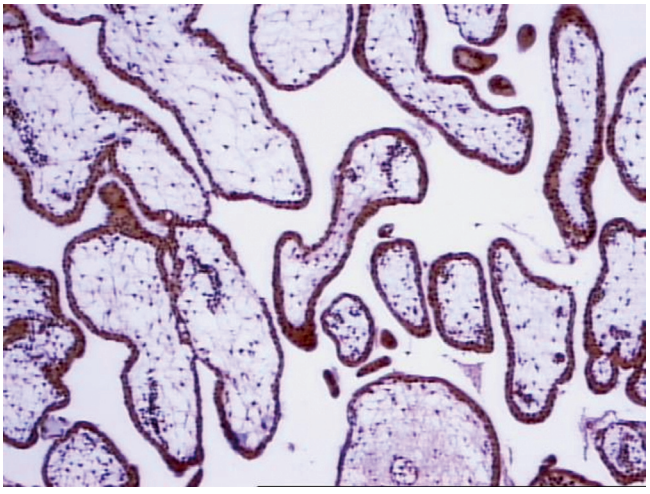

Figure 11. MMP7 immunostaining in trophoblasts. (Immunoperoxidase stain, x 10)

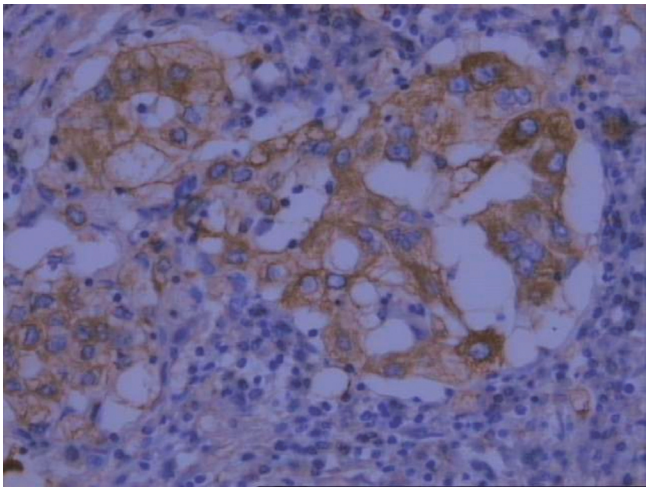

Figure 12. MMP7 immunostaining in a lung adenocarcinoma. (Immunoperoxidase stain, x40)

Table 3. Intensity of MMP7 staining according to cell type in carcinomas and gestation

MMP7 staining intensity category	Carcinomas (Parenchymal cells) N %	Gestation (Trophoblastic cells) N %	Carcinomas (Stromal cells) N %	Gestation (Decidual cells) N %
0	3 (7%)	0 (0%)	13 (29%)	0 (0%)
+	32 (71%)	4 (10%)	26 (58%)	5 (12,5%)
++	7 (15%)	21 (52,5%)	6 (13%)	28 (70%)
+++	3 (7%)	15 (37,5%)	0 (0%)	7 (17,5%)

p<0,0001 p<0,0001

Table 3.Intensity of MMP7 staining was greater in gestation trophoblasts and decidua than cancer epithelium(p<0,0001) and stroma(p<0,0001) respectively.

order to assess any differential expression of these molecules in these two distinct phenomena.

Our results showed MMP9 to be more often expressed in the parenchyma and stroma of carcinomas than in the trophoblastic and decidual cells.This makes MMP9 an important molecule for extensive invasion and comes to agreement with many studies that report increased MMP9 expression in various high stage and grade carcinomas [13-16].In particular a study with monoclonal antibodies to MMP9 in a group of gastric cancers (n=74) revealed MMP9 in 70 per cent of the cases studied [13]. MMP9 mRNA expression in colorectal tumors (n=74) as determined by northern hybridization was increased [14] and a high staining for MMP9 was noted in 113 (62%) out of 212 cases with non small cell lung carcinoma [15]. Finally, immunohistochemical staining for MMP9 expression has been found to be significantly high in lymph node-positive breast carcinomas [16].

Furthermore, the result that MMP9 is less expressed in a normal process that doesn't penetrate uncontrollably the neighbouring tissues may also highlight the invasive potential of this particular molecule. It seems that mother's body keeps the MMP9 production at low levels until the ninth week of gestation, through various ways,probably in order to control the MMP9 induced invasion. Xu et al [17] and Staun-Ramet et al [18] confirm that MMP9 is not the main gelatinase during the first nine weeks of gestation (in contrast to MMP2). Fata et al report progesterone as a negative regulator of MMP9 transcription and similar role has been argued for IL10β [19].

Another point worth reporting is that an obviously increased MMP9 expression was observed in inflammatory cells surrounding the cells of carcinomas, especially at the site where tumor cells were penetrating stroma. This comes to agreement with data that consider MMP9 produced by inflammatory cells (mainly macrophages) to participate in pathological angiogenesis, probably mobilizing VEGF sequestered in extracelllular matrix [20]. Nothing similar was observed in the specimens of gestation .

Although all gestations and almost all carcinomas stained for MMP2 ,the immunostaining pattern in gestation specimens was absolutely homogeneous (with the total amount of gestations expressing it in more than 50% of their trophoblastic and decidual cells), while MMP2 staining in carcinomas was heterogeneous and diverse. On a first look that could make MMP2 more important for time and space "limited" invasion (gestation) than for extensive spreading (cancer), but at the same time we cannot ignore that MMP2 was expressed in almost all carcinomas (even if in smaller amount of cells and with lower intensity). Furthermore, data from literature emphasize the invasive potential of MMP2 and its increased expression in carcinomas in contrast to the normal tissue or benign neoplasms. For instance, MMP2 knocked out mice show reduced formation of metastases [21] and MMP2 was expressed in 94 per cent (n=74) in a group of gastric carcinomas [13]. Bramhal et al suggested that the aggressive phenotype of pancreatic carcinoma might be related to overexpression of MMP2 [22] and Stearns and Steams found that expression of activated MMP2 was undetectable in normal prostate, benign prostate hyperplasia and prostate cancer of low grade Gleason score , while it was detected in prostate cancer of high Gleason score and lymph node metastases [23].

Since the importance of MMP2 in extensive and metastatic invasion is well documented , our result of MMP2 predominance in first trimester gestation lead us to the following assumptions:

a) increased immunoreactivity of MMP2 in gestation specimens does not necessarily provide evidence of activated MMP2 presence. It is known that all metalloproteinases are secreted in latent forms which require activation by proteolytic cleavage and immunohistochemistry cannot distinguish between active and latent form of MMPs. But probably this isn't the case as studies report increased MMP2 enzymic activity in first trimester gestation [17,18].
b) the activity of MMP2 in gestation is strictly controlled through various factors in contrast with cancer. It is known that even when activated, MMPs are not necessarily available for tissue degradation,since a family of endogenous TIMPS proteins (tissue inhibitors of metalloproteinases) act to restrain their action by binding MMPs active forms with a 1 to 1 stoichiometry [24] .It seems that in gestation, the coordinate expression of MMPs and TIMPs might be important for the degradatrion of matrix proteins in a controlled fashion [17].
c) factors that participate in MMP2 activity regulation in gestation play different roles in cancer. For instance:

- MT MMP1 (membrane type metalloproteinase 1,a metalloproteinase anchored in cell membrane) and TIMP2(tissue inhibitor of metalloproteinases 2) are of critical importance in the regulation of MMP2 activation in trophoblast invasion converting MMP2 from its latent form to the active one [24]. Data generated by Agnes Noel's laboratory indicate a major role of MT MMP1 in tumor angiogenesis [20]. The overexpression of MT MMP1 in different cancer cell lines was associated with enhanced in vitro invasion and increased in vivo growth and vascularization [25]. Furthermore, increasing evidence show that TIMP2 in cancer is a multifunctional protein and its antiapoptotic effect may favor tumor development [26].
- **D**ecidua derived TGFβ (transforming growth factor β) is considered to be a regulator of trophoblastic invasion through induction of TIMPs (endogenous tissue inhibitors of metalloproteinases) [24].On the opposite, in carcinomas, tumor cells release TGFβ, among other soluble growth factors and chemokines (such as VEGF-A and TNFa) that create a permissive microenviroment for incoming circulating cancer cells [27].In colorectal carcinomas, TGFβ accelerates epithelial mesenchymal transformation (a complex process by which epithelial cells lose their strong intercellular adhesion and their basolateral polarity to gain front-end back-end polarity, and the ability to migrate through the extracellular matrix) modulating a critical step in colon carcinogenesis [28].

Finally, the homogeneous predominant expression of MMP7 in trophoblastic cells in comparison to cancer parenchymal cells, could be attributed to the distinct role that MMP7 appears to play in gestation in contrast to cancer. Although we couldn't find many bibliographical data about the exact role of MMP7 in implantation and placentation, this molecule seems to have a regulatory role in trophoblast invasion, processing molecules such as uPA and TNFa precursor, that lead to increased TIMP production and consequent ceasing of trophoblast invasion [24]. On the contrary, MMP7 in carcinomas cleaves matrix components in the cellular microenviroment resulting in degradation of basement membrane structures, as well as degradation of adhesion molecules such as E cadherin resulting in loss of epithelial cell to cell junctions and increased cellular invasion [29].

In conclusion, extracellular matrix metalloproteinases are key molecules for both placentation and cancer invasion. MMP9 seems to be strongly connected to the invasive potential of cancer. On the other hand the role of MMP2 and MMP7 appears to be more complicated, underlining the complexity of the mechanisms involved in cancer spreading. While placentation is a perfectly synchronized process that remains under the strict control of various inhibitors, in cancer the same or different regulatory factors probably augment uncontrollable invasion. Thus, functional drugs against specific targets could be quite promising in the future of cancer therapy.

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