University of Szeged
Department of Obstetrics and Gynecology

Harvard Medical School
Brigham and Women's Hospital

Matrix metalloproteinases and angiogenetic factors in gestational trophoblastic diseases and normal placenta

PhD Thesis

by
Margit Singh MD

Supervisor
Gábor Németh MD and Vilmos Fülöp MD

Szeged

2012

Publications related to the thesis

I. Margit Singh, David Kindelberger, Zoltan Nagymanyoki, Shu-Wing Ng, Charles M. Quick, Kevin M. Elias, Hidemi Yamamoto, Raina Fichorova, Vilmos Fulop, Ross S. Berkowitz: Matrix metalloproteinases and their inhibitors and inducer in gestational trophoblastic diseases and normal placenta. Gynecologic Oncology 2011; 122(1):178-82.
IF: 3,76

II. Margit Singh, David Kindelberger, Zoltan Nagymanyoki, Shu-Wing Ng, Charles M. Quick, Kevin M. Elias, Hidemi Yamamoto, Raina Fichorova, Vilmos Fulop, Ross S. Berkowitz: Vascular endothelial growth factors and their receptors and regulators in gestational trophoblastic diseases and normal placenta. Journal of Reproductive Medicine 2012 - accepted
IF: 0,88

III. Margit Singh, Gábor Németh, Vilmos Fülöp, Ross S Berkowitz, Attila Pál: Matrix metalloproteinases and their inhibitors and inducer in gestational trophoblastic diseases and normal placenta. Hungarian Journal of Gynecologic Oncology 2011; 16:71-74.
IF:-

IV. Margit Singh, Gábor Németh, Vilmos Fülöp, Ross S Berkowitz, Attila Pál: Vascular endothelial growth factors and their receptors and regulators in gestational trophoblastic diseases and normal placenta. Hungarian Journal of Gynecology 2012 - accepted
IF:-

V. Vilmos Fulop, Ivan Szigetvari, Janos Szepesi, Gyorgy Vegh, **Margit Singh**, Ross S. Berkowitz: The Clinical Epidemiology and Management of GTN in Hungary in the last 34 years. Journal of Reproductive Medicine 2012 - accepted
IF: 0,88

Matrix metalloproteinases and angiogenetic factors in gestational trophoblastic diseases and normal placenta

INTRODUCTION

Molar pregnancy and gestational trophoblastic neoplasms (GTN) comprise a group of interrelated diseases originating from the placenta. [1] Hippocrates's description of dropsy uterus was probably the first note about the gestational trophoblastic disease around 400 BC. Gestational trophoblastic diseases (GTD) were associated with high mortality and morbidity before the development of early detection and introduction of chemotherapy 50 years ago. GTN comprise a range of conditions characterized by abnormal proliferation and invasion of placental trophoblasts and include invasive mole, placental site trophoblastic tumor, and choriocarcinoma. [1] The inciting event is typically a complete or partial molar pregnancy, but GTN can occur after any gestational event. Most GTN involve only local uterine invasion, but GTN is one of the few highly curable human tumors recently, even in the setting of advanced disease and widespread metastases. [2]

1.Gestational trophoblastic diseases

Partial and complete molar pregnancy, invasive mole, placental site trophoblastic tumor (PSTT) and choriocarcinoma cover the spectrum of GTN. The incidence of GTN varies in different regions of the world. In Europe and North America, hydatiform moles affects approximately 1 in 40 pregnancies, choriocarcinoma rate is 1 per 40.000 pregnancies. In contrast, the incidence of choriocarcinoma is 9.2 and 3.3 in 40.000 pregnancies in Southeast Asia and Japan. This variation can be explained by hospital versus population based data but the high incidence in some populations also can be attributed to socioeconomic and nutritional factors. The rate of complete molar pregnancy increases with decreasing consumption of dietary carotene and animal fat. Women older than 40 years of age have 5 to 10 fold greater risk of having a complete molar pregnancy. [1-3]

TABLE 1. Clinicopathologic features of gestational trophoblastic diseases

Gestational trophoblastic disease	*Pathologic features*	*Clinical features*
Hydatidiform mole, complete	46,XX (mainly); 46,XY Absent fetus/embryo Diffuse swelling of villi Diffuse trophoblastic hyperplasia	15-20% trophoblastic sequelae hCG often >100,000 mIU/mL Medical complications
Hydatidiform mole, partial	Triploid (69, XXY; 69, XYY; 69 XXX) Abnormal fetus/embryo Focal swelling of villi Focal trophoblastic hyperplasia	<5% trophoblastic sequelae hCG usually <100,000 mIU/mL Rare medical complications
Invasive mole	Myometrial invasion Swollen villi Hyperplastic trophoblast	15% metastatic–lung/vagina Most often diagnosed clinically, rather than pathologically
Choriocarcinoma	Abnormal trophoblastic hyperplasia and anaplasia Absent villi Hemorrhage, necrosis	Vascular spread to distant sites–lung/brain/liver Malignant disease
PSTT	Tumor cells infiltrate myometrium with vascular/lymphatic invasion Intermediate cells/absent villi Less hemorrhage and necrosis Tumor cells stain positive for hPL	Extremely rare hCG levels less reliable indicator Relatively chemoresistant Mainly surgical treatment

Note: *hCG*: human chorionic gonadotropin; *hPL:* human placental lactogen; *PSTT:* placental site trophoblastic tumor. [3]

Hydatidiform moles are either complete or partial on the basis of karyotype, gross morphology and histopathology (Table 1). The presentation of complete hydatidiform mole (CHM) has changed dramatically over the last 30 years because of the early diagnosis via ultrasonography and hCG measurement. Vaginal bleeding remains the most common presenting symptom. Other classic signs such as excessive uterine enlargement, theca lutein cyst, hyperemesis, preeclampsia, hyperthyroidism and respiratory insufficiency are less commonly seen. Partial hydatidiform mole (PHM) presents as a missed or incomplete abortion and the diagnosis is generally only considered after histologic review of the specimens. When molar pregnancy is diagnosed early in the first trimester, it is difficult to distinguish CHM from PHM and hydropic abortion. The absence of nuclear staining of $p57^{KIP2}$, paternally imprinted and maternally expressed gene, confirms the complete androgenic nature of the chromosomes and the diagnosis of CHM. Complete molar pregnancies are well recognized to have the potential for local invasion and distant spread. After evacuation of complete moles, local uterine invasion occurs in 15% of patients and metastases in 4%. [3,4,5]

Gestational choriocarcinoma may be preceded by a hydatidiform mole (50%), a spontaneous abortion (20%), a normal term pregnancy (20-25%) or an ectopic pregnancy (2%). [3]

The frequency of placental site trophoblastic tumour (PSTT) is less well known. PSTTs grow more slowly, metastasise later, more commonly involve lymph-nodes, and produce less hCG than do choriocarcinomas. PSTTs arise also after any type of pregnancy. PSTT usually follows a normal term pregnancy, abortion or molar pregnancy, presenting months or years after a term gestation with irregular vaginal bleeding, amenorrhea, nephrotic syndrome, and rarely, polycythemia or virilization. These tumors are frequently resistant to chemotherapy, and although PSTT is usually confined to the uterus at the time of diagnosis, risk of later metastasis to the lungs and vagina is high. Involvement of the liver, kidney, lymph nodes, brain, skin, spleen, and stomach has also been reported. Gestational choriocarcinoma and PSTT do not contain chorionic villi. Choriocarcinoma (CCA) is composed of sheets of both anaplastic cyto- and syncytiotrophoblast but PSTT is composed of mononuclear intermediate trophoblast. [3,4]

The International Federation of Gynecology and Obstetrics (FIGO) has adopted an anatomic staging system for GTN (Table 2).

TABLE 2. FIGO Anatomic Staging for GTN

Stage I	Disease confined to the uterus
Stage II	GTN extends outside the uterus but is limited to the genital structures (adnexa, vagina, broad ligament)
Stage III	GTN extends to the lungs with or without genital involvement.
Stage IV	All other metastatic sites

Metastatic GTN occurs in 4% of patients after evacuation of CHM and infrequently after other pregnancies. GTN usually metastasizes as CCA because of its propensity for early vascular invasion with widespread dissemination. Nonmetastatic disease typically presents with persistently elevated hCG levels, irregular vaginal bleeding, uterine subinvolution, and/or theca lutein cysts. As trophoblastic tumor can erode into uterine vessels or through the myometrium, vaginal hemorrhage or intra-abdominal bleeding can occur. The most common metastatic sites are the lung (80%), vagina (30%), brain (10%) and liver (10%). Because trophoblastic tumors are perfused by fragile vessels, metastases are often haemorrhagic. Patients may present with hemoptysis, intraperitoneal bleeding from hepatic rupture, or acute neurologic changes. It is rare to diagnose hepatic or cerebral metastases without pulmonary or vaginal involvement. [3,5]

The optimal management of GTN requires a thorough assessment of the extent of disease prior to initiation of treatment. This assessment should include a history and physical examination; measurement of hCG level; hepatic, thyroid, and renal function tests; and a baseline complete blood cell count. Pelvic ultrasound may be useful to detect extensive uterine involvement and may identify patients who would benefit from a hysterectomy to reduce tumor burden and potentially limit the exposure to chemotherapy. To evaluate possible metastases, a chest x-ray should be obtained. If this is negative, a computed tomography (CT) scan of the chest also may be performed. Asymptomatic patients with a negative chest CT are very unlikely to have liver or brain metastases and, as a result additional imaging, can be omitted. [4,5]

Patients with GTN are stratified into “high risk” and “low risk” categories according to the prognostic scoring system of the World Health Organization (Table 3). [6]

TABLE 3. Modified World Health Organization Prognostic Scoring System as Adapted by FIGO

Scores	0	1	2	4
Age	<40	>40	—	—
Antecedent pregnancy	Mole	Abortion	Term	
Interval months from index pregnancy	<4	4-7	7-13	>13
Pretreatment serum hCG (IU/L)	<1,000	<10,000	<100,000	>100,000
Largest tumor size (including uterus)	—	3-<5 cm	5 cm	
Site of metastases	Lung	Spleen/kidney	Gastrointestinal	Liver/brain
No. of metastases	—	1-4	5-8	>8
Previous failed chemotherapy			Single drug	2 or more drugs

When a molar pregnancy is diagnosed, the preferred method of management for patients who desire to preserve their fertility is suction curettage. After evacuation, all patients must be followed with hCG measurements to assure remission. For patients who do not wish to preserve their fertility, hysterectomy is a reasonable management option. Although hysterectomy eliminates the risk of local invasive disease, it does not prevent metastases. Before surgery, medical conditions such as preeclampsia, electrolyte imbalances, hyperthyroidism, and anemia need to be evaluated and addressed. [1,5]

The vast majority of low risk patients can achieve remission with single agent chemotherapy using methotrexate or actinomycin D (ACT-D). High risk patients and low risk patients resistant to single agent therapy require combination chemotherapy with EMA-CO (etoposide, methotrexate, ACT-D, cyclophosphamide and vincristine) or MAC (methotrexate, ACT-D and cyclophosphamide). [1] EMA-CO primary treatment with adjuvant surgery and radiotherapy may achieve high rates (more than 90%) of remission in women with metastatic high risk gestational trophoblastic tumors. [1,4,7-9] PSTT is relatively unresponsive to chemotherapy. Cytoreductive surgery followed by multiagent chemotherapy has been reported as successful in the treatment of PSTT. Adjuvant chemotherapy and radiation were used in such patient because of extrauterine spread. Multiagent chemotherapy regimens, including etoposide, methotrexate, actinomycin D (EMA), MAC, EMACO, and etoposide, methotrexate, actinomycin D/etoposide and cisplatin (EMA/EP) have been used successfully in advanced, recurrent, and metastatic PSTT. [3,4]

All patients with stage I, II, and III GTN should be monitored with weekly hCG tests until normal for 3 consecutive weeks and then monthly for 12 months. During this time of follow-up patients need to use effective contraception. Patients with stage IV disease are followed similarly but for 24 months. The concept of false positive hCG tests is critical to remember when monitoring patients with a molar gestation or GTN. The risk of recurrence after initial remission varies by stage of disease: 2.9% for stage I disease, 8.3% for stage II, 4.2% for stage III, and 9.1% for stage IV. [1]

The optimal placental perfusion has a crucial role during the successful pregnancy. GTN is associated with unrestricted invasion. In contrast, fetal intrauterine growth restriction and preeclampsia are associated with insufficient invasion. Trophoblastic tumor biology stems from its placental origin. Placental tissue contains a heterogeneous population of cells, including villous syncytiotrophoblasts, cytotrophoblasts, extravillous trophoblasts and intermediate trophoblasts (Table 4). [10] The placenta is a special fetal allograft that is embedded in the maternal tissue. While the villous trophoblast does not exhibit invasive behavior, the extravillous trophoblast displays an invasive capacity similar to malignant cells. [11,12] During normal pregnancy, extravillous trophoblast cells migrate from the basement membrane of anchoring villi and invade deeply, reaching the myometrium. [9] This process of invasion involves enzymatic degradation of the extracellular matrix (ECM). [13,14] Many of the biological properties including invasiveness and angiogenesis that are relevant to implantation and placentation have key role also in tumor growth.

TABLE 4. Morphological and immunohistochemical characters of trophoblast cells

	Cytotrophoblast cell	**Extravillous trophoblast cell**	**Syncytiotrophoblast cell**
Nucleus	1	1 or more	more
Mitotic activity	High	Low	no
Cell morphology	Round	Various	irregular
Cytoplasm	thin, light	thick, eosinophil	thick
Cytokeratin	High	High	high
hCG	No	Low	high
hPL	No	High	Low
MIB1, TP53	High	Low	No
Hormonal secretion	No	No	estrogen, progesterone

Although the precise molecular genetics of molar pregnancies and GTN have not been determined, multiple growth factors and oncogenes have been investigated. Matsuda and colleagues noted homozygous deletions in the 7p12-q11.23 region on chromosome 7 in seven of eight choriocarcinoma cell lines. Mutations in a gene located on a chromosoma 19q13.4 may be responsible for these biparental, recurrent molar pregnancies. Molar pregnancies and choriocarcinoma have been characterized by overexpression of c-myc, c-erbB2, p53 and bcl-2. Choriocarcinoma and CHM have higher levels of epidermal growth factor receptor too. Extracellular proteinases such as matrix metalloproteinases e.g. MMP-1 and -2 have been noted to be overexpressed in CCA and may contribute to the aggressive invasiveness of this diseases. [1,15]

2.Matrix metalloproteinases

Matrix metalloproteinases (MMPs), a family of zinc-containing endopeptidases, were first described almost half a century ago. To date over 25 members of the MMP family have been reported and divided into four groups according to their substrate specificities: collagenases, gelatinases, stromelysins and membrane-type MMPs. Individual MMPs are referred to by their common names or according to a sequential numeric nomenclature reserved for the vertebrate MMPs (Table 5). [16]

In addition, MMPs are often grouped according to their modular domain structure (Figure 1). [16]

TABLE 5. The vertebrate MMPs

MMP type	Common name	Domain organization[a]
MMP1	Collagenase-1	B
MMP2	Gelatinase A	C
MMP3	Stromelysin-1	B
MMP7	Matrilysin	A
MMP8	Collagenase-2	B
MMP9	Gelatinase B	C
MMP10	Stromelysin-2	B
MMP11	Stromelysin-3	D
MMP12	Macrophage metalloelastase	B
MMP13	Collagenase-3	B
MMP14	MT1-MMP	E
MMP15	MT2-MMP	E
MMP16	MT3-MMP	E
MMP17	MT4-MMP	F
MMP18	Collagenase-4 (*Xenopus*)	B
MMP19	RASI-1	B
MMP20	Enamelysin	B
MMP21	XMMP (*Xenopus*)	G
MMP22	CMMP (chicken)	B
MMP23		H
MMP24	MT5-MMP	E
MMP25	MT6-MMP	F
MMP26	Endometase, Matrilysin-2	A
MMP27		B
MMP28	Epilysin	D

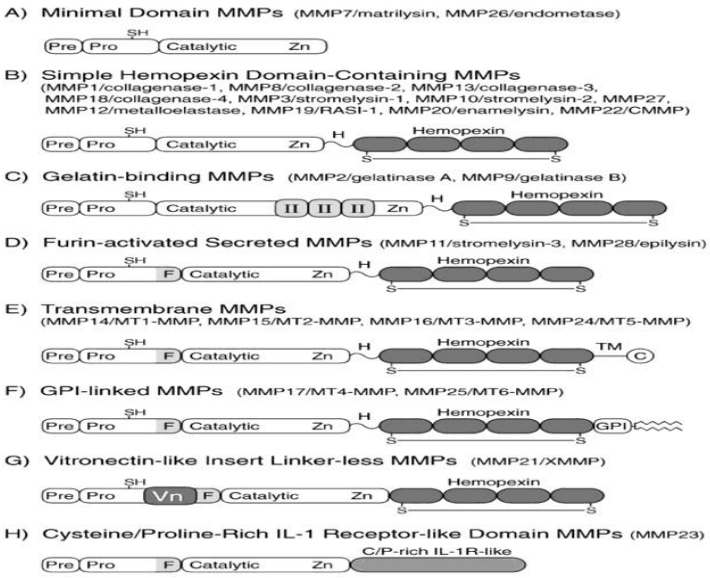


Figure 1. Domain structure of the MMPs. Pre, signal sequence; Pro, propeptide with a free zinc-ligating thiol (SH) group; F, furin-susceptible site; Zn, zinc-binding site; II, collagen-binding fibronectin type II inserts; H, hinge region; TM, transmembrane domain; C, cytoplasmic tail; GPI, glycophosphatidyl inositol-anchoring domain; C/P, cysteine/proline; IL-1R, interleukin-1 receptor. The hemopexin/vitronectin-like domain contains four repeats with the first and last linked by a disulfide bond. [16]

Various processes are modulated by MMPs including cell growth, inflammation, invasion, metastatic niche, angiogenesis and adipogenesis. (Figure 2)

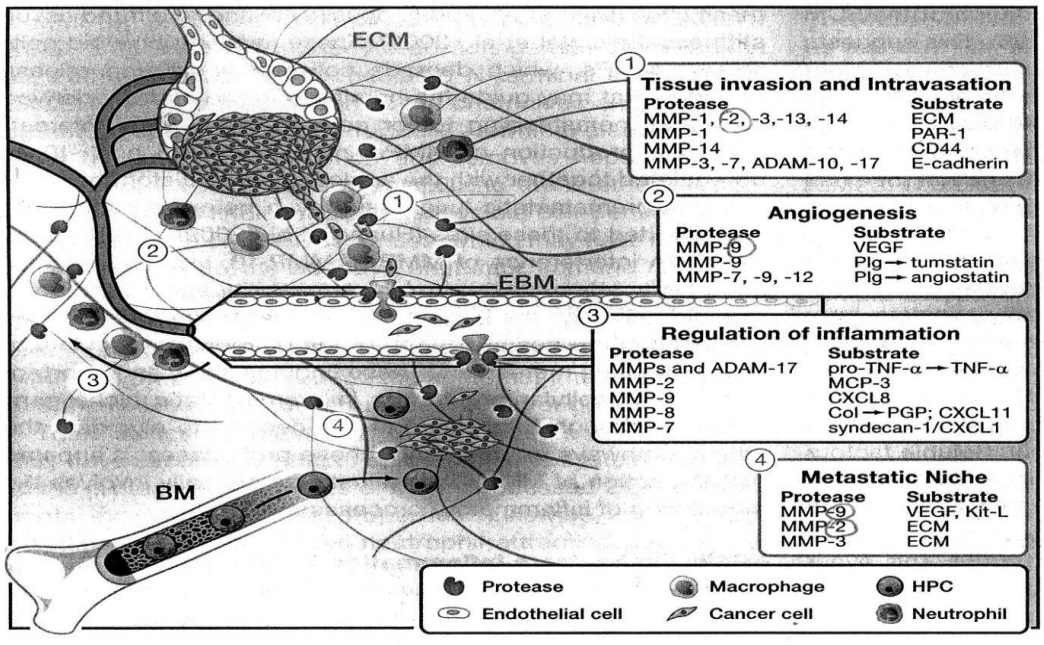


Figure 2. Multiple functions of MMPs in the tumor microenvironment [17]

MMPs mediate many of changes in the microenvironment during tumor progression. They represent key role in the molecular communication between tumor and non malignant stroma. The complexity of the tumor microenvironment allows for a variety of regulatory cascades that determine the functions of the diverse MMPs expressed. The proteolytic activity of MMPs is highly regulated. [16] This occurs through modulation of gene expression, post-translational modification, and co-localization of tissue specific enzyme inhibitors. The enzymatic activity of MMPs is reversibly arrested by tissue inhibitors of metalloproteinases (TIMP-1,-2,-3,-4) in a 1:1 stoichiometric fashion locally in the tissue, but irreversibly arrested by alpha-2 macroglobulin in the blood. [17]

In addition, all MMPs are secreted as zymogens and require local factors to become active. MMP expression is regulated by several cytokines and growth factors, including interleukins, interferons, EGF, KGF, NGF, basic FGF, VEGF, PDGF, TNF-α, TGF-β, and CD147, the extracellular matrix metalloproteinase inducer. The activity of MMPs is stimulated by pro-inflammation (e.g. IL-6) and inhibited by anti-inflammation cytokines (e.g. IL-10). [16,17] (Figure 3)

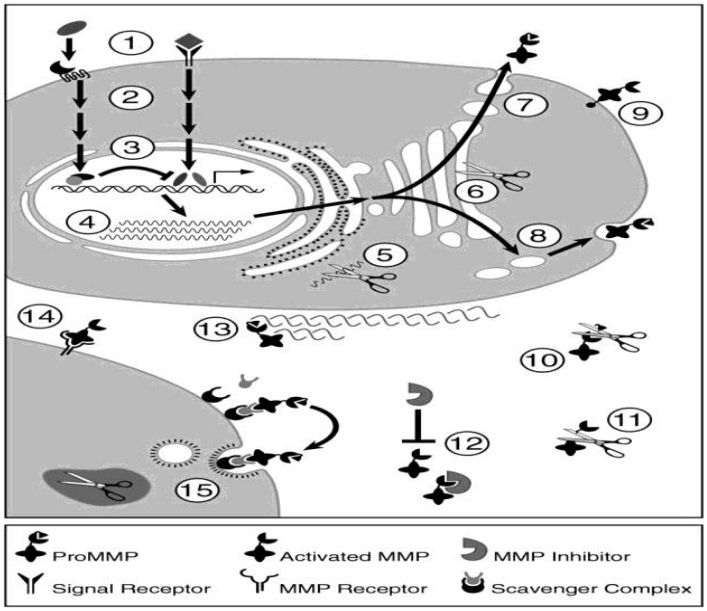


Figure 3. Regulation of the MMPs. MMP regulatory mechanisms include inductive and suppressive signaling (1), intracellular signal transduction (2), transcriptional activation and repression (3), post-transcriptional mRNA processing (4), mRNA degradation (5), intracellular activation of furin-susceptible MMPs (6), constitutive secretion (7), regulated secretion (8), cell surface expression (9), proteolytic activation (10), proteolytic processing and inactivation (11), protein inhibition (12), ECM localization (13), cell surface localization (14), and endocytosis and intracellular degradation (15). [16]

CD147 has ability to induce matrix metalloproteinase expression by neighboring fibroblasts, leading to tumor cell invasion. CD147 is highly expressed by various cancer cells and plays a crucial role in several processes including tumor invasiveness, metastasis, cellular proliferation, in VEGF production and multi-drug resistance. [18,19]

MMPs are an important class of proteases involved in the normal trophoblastic invasion of maternal tissues. [13,14,20] MMPs have ability to break down the proteins of the ECM, thereby giving the ECM an opportunity to renew and to transform. The metabolism of the ECM plays a role in many pathological processes: including inflammation and cancer. [17]

Tumor cell invasion through tissue and intravasation into blood vessels requires basement membrane degradation (e.g. cleavage of type IV collagen by MMP-2,-3,-9), and ECM remodeling and downregulation of cellular adhesion (e.g. cleavage of E-cadherin by MMP-3,-7). [17] The MMPs have the ability to break down other proteins besides the ECM as well. The common substrates of many MMPs have been identified (Table 6), but there are a couple of newfound MMPs (e.g. MMP-21,-28), whose role and substrate are still unknown. [16]

MMPs have the ability to activate other MMPs e.g. MMP-7 activates MMP-2 and MMP-9 and MMP-14 activate MMP-2. (Figure 4) [16]

TABLE 6. Common matrix metalloproteinase substrates

MMP substrates	**MMP**														
	1	**2**	**3**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**16**	**18**	**19**	**26**
ECM Proteins:															
Aggrecan	+	+	+	+	+	+	+		+	+	+				
Collagen I	+	+	−	+	+	−			+	+	+		+	+	−
Collagen II	+				+	−				+	+				−
Collagen III	+	+	+	−	+	−	+			+	+	+			
Collagen IV	−	+	+	+	−	+	+	−	+	−				+	+
Collagen V	−	+	+	−	−	+	+			−					
Collagen VI	−	−	−			−				+					
Collagen VII	+	+	+												
Collagen VIII	+														
Collagen IX	−	−	+							+					
Collagen X	+	+	+	−						+					

Collagen XI	+	+	+			+				−					
Collagen XIV	−		−	−		+				+					
Decorin	−	+	+	+		+									
Elastin	−	+	+	+		+	+		+						−
Entactin/Nidogen	+	+	+	+					+		+				
Fibrillin		+	+			+			+	+	+				
Fibronectin	+	+	+	+	−	−	+		+	+	+	+		+	+
Fibulins		+		+											
Gelatin I	+	+	+	+		+	+		+	+	+			+	+
IGFBPs	+	+	+					+							
Laminin	+	+	+	+		+		−	+		+				−
Link Protein	+	+	+	+		+	+								
Myelin Basic	+	+	+	+		+			+						
Osteonectin		+	+	+		+				+					
Tenascin	+	+	+	+		−								+	
Vitronectin	+	+	+	+		+			+		+				
Other Proteins:															
α1-AC	+	+	+			−									
α2-M	+	−	+		+	+		+	+	+	+				
α1-PI	+	+	+	+	+	+		+	+		+				+
Casein	+	−	+	+		+	+	−		+				+	−
C1q	+	+	+		+	+				+					
E-cadherin			+	+											
Factor XII					−				+	+	+				
Fibrin	+	+	+			+					+				
Fibrinogen	+	+	+	+	+	+	+		+	+	+				+
IL1α	−	−	−			−									
IL1β	+	+	+			+									
ProMMP2											+	+			
ProTGFβ		+				+									
ProTNFα	+	+	+	+		+			+		+				
Plasminogen		+	+	+		+			+						−
Substance P		+	+		+	+									
T kininogen		−	+			−									

Note: The symbols indicate whether the indicated protein is (+) or is not (−) digested by the indicated MMP. Abbreviations: α1-AC, α1-antichymotrypsin; α2-M, α2-macroglobulin; α1-PI, α1-proteinase inhibitor.

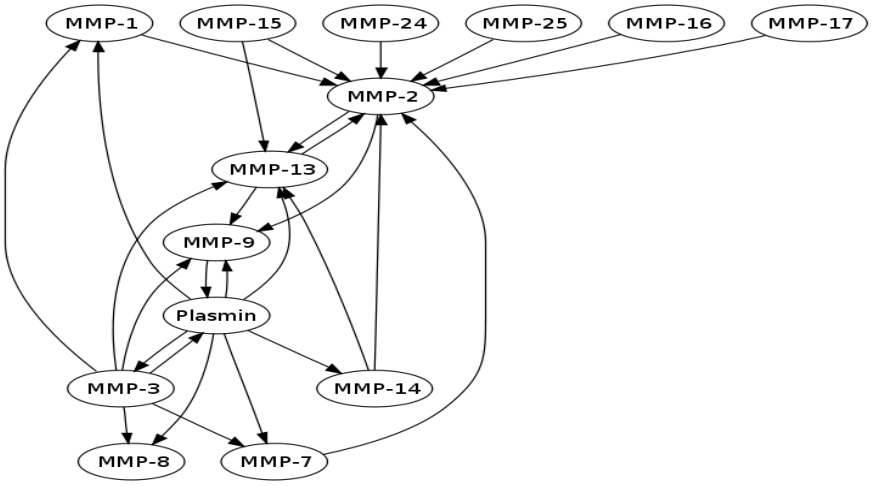


Figure 4. Mutual activation of MMPs

MMPs have been implicated in cancer for more than 40 years and the notion that MMP-mediated ECM degradation leads to cancer cell invasion and metastasis has been a guiding principle in MMP research. Cancer cells must cross multiple ECM barriers as they traverze the epithelial basement membrane and interstitial stroma, enter blood vessels or lymphatics, and extravasate to form metastatic deposits at a distant site. MMPs are thought to facilitate invasion and metastasis by degrading structural ECM components. MMPs are generally present in greater amounts and activated more often in and around malignant cancers than in normal or premalignant tissues, with the highest expression taking place in areas of active invasion at the tumor-stroma interface. [16, 17]

3. Angiogenetic factors

Angiogenesis has been demonstrated to be important in both placental development and the pathogenesis of neoplasia. Understanding the process of angiogenesis has led to advancing our knowledge of tumor biology and has resulted in novel therapies. GTN is characterized by increased vascularity hence the biology of gestational trophoblastic diseases may be substantially influenced by angiogenesis. [21, 22]

The tumor cell's ability to form new vessels, the process of angiogenesis, is a critical step in tumor development allowing tumors to grow beyond 1–2 mm in diameter. [21,22] By the overexpression of growth factors, these cells have the ability to acquire a new vasculature from existing vessels. [23,24] Studies have also demontrated an essential role of vascular endothelial growth factor (VEGF) in embryonic vasculogenesis and angiogenesis in the

mouse. [25,26] VEGF plays a crucial role in the formation of blood vessels that lead to tumor growth by its proliferative and chemotactic effects on the endothelial cells. [23] VEGF overexpression has been observed in many different tumors including lung, colon, breast, ovarian and cervical cancer. [27,28,29]

MMPs can contribute to angiogenesis in at least three ways: They can enable endothelial cell migration through surrounding tissues by disrupting ECM barriers, they can promote it by liberating sequestered angiogenic factors, or they can defy it by generating anti-angiogenic breakdown products (Figure 5). [16]

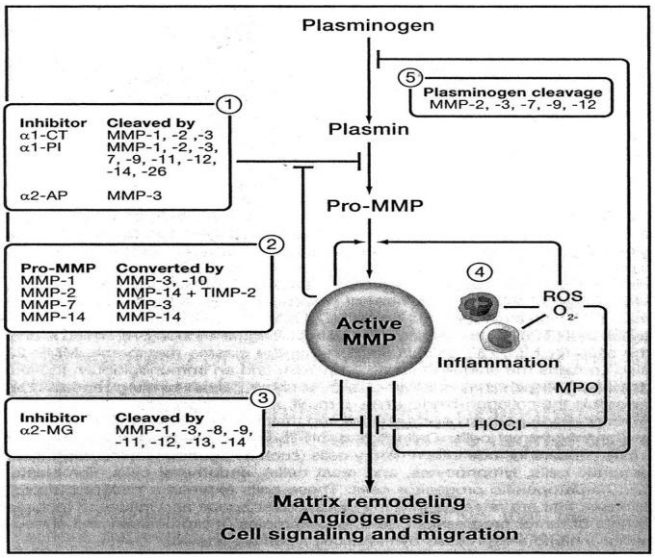


Figure 5. Proteolytic cascades regulate MMP function [17]

Isoforms of VEGF (VEGF-A,-B,-C,-D,-E) and placental growth factor (PLGF), the members of the Vascular Endothelial Growth Factor family, can induce angiogenesis and lymphangiogenesis since they are potent mitogens and differentiation factors for vascular and lymphatic endothelium. VEGF overexpression has been observed in many different tumors including lung, colon, breast, ovarian and cervical cancer. [23,24] VEGF-A (VEGF) has been well studied and can stimulate endothelial cell mitogenesis, migration, vasodilatation and increase microvascular permeability. [30] All members of the VEGF family have ability to bind to tyrosine kinase receptors called: VEGFR-1 (flt-1), VEGFR-2 (KDR) and VEGFR-3 (flt-4). VEGFR-1 is a key receptor in developmental angiogenesis but its function is not well-defined. VEGF-A,-B and PLGF are ligands for VEGFR-1. [31] Almost all of the known effects of VEGF-A including microvascular permeability, vascular endothelial cell proliferation and migration are mediated by VEGFR-2. [30] VEGFR-3 promotes

lymphangiogenesis and is found only in lymphatic endothelial cells in the adult. VEGF-C and VEGF-D, but not VEGF-A, can bind to this receptor. [32] (Figure 6)

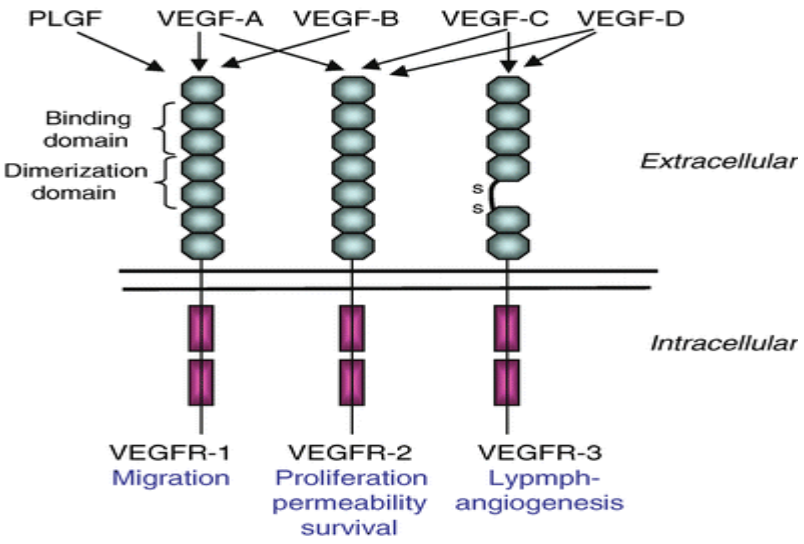


Figure 6. VEGF isoforms and possible bindings sites at VEGFR-1, VEGFR-2, and VEGFR-3. Whereas VEGFR-1 and -2 are mainly involved in angiogenesis, lymphangiogenesis is promoted by binding of VEGF-C to VEGFR-3 [51]

Hypoxia is the key regulator of VEGF expression. [23] A range of cytokines and growth factors have been shown to regulate and correlate with VEGF expression. Interleukin-6 (IL-6) has been shown to regulate VEGF levels in a variety of tissues. Higher VEGF expression has been identified in a cervical cancer cell line after addition of exogenous IL-6. [33] Positive correlation has been observed between serum levels of IL-6 and VEGF in patients with breast and advanced metastatic cancer. [34,35] Extracellular matrix metalloproteinase inducer (CD147) also has the ability to increase tumor cell expression of VEGF. [36,37]

Angiopoietins are a family of angiogenic growth factors mostly specific for the vascular endothelium. Angiopoietins have been shown to function as ligands for the Tie2/Tek vascular endothelial-specific receptor. Angiopoietin 1 (Ang1) is involved in the stabilization of mature vessels by promoting interaction between endothelial cells and its supporting cells such as pericytes and smooth muscle cells. Angiopoietin 2 (Ang2) is thought to play a role at sites of vascular remodeling by disrupting the constitutive stabilizing action of Ang-1. Angiopoietin receptors, Tie2/Tek, were thought to be expressed exclusively by endothelial cells. [38]

The purpose of this study was to extend our present understanding of invasiveness and angiogenesis in gestational trophoblastic diseases by profiling a panel of matrix

metalloproteinases and angiogenetic factors expression as well as their inhibitors and regulators in normal placenta and gestational trophoblastic diseases.

MATERIALS AND METHODS

I. Immunohistochemistry

1.Collection of tissues and clinical data

Paraffin-embedded gestational age-matched normal placenta (n=10), partial molar (n=10), complete molar (n=10), choriocarcinoma (n=10), placental site trophoblastic tumor (n=10) slides were supplied by the Division of Women's and Perinatal Pathology of the Brigham and Women's Hospital, Boston, USA. All tissues and clinical information were obtained under the approval of the Brigham and Women's Hospital Institutional Review Board.

2. Histopathologic examination

Conventional histologic sections were made of paraffin-embedded tissues and routinely stained with hematoxylin-eosin (HE). The diagnosis of partial and complete molar pregnancy, choriocarcinoma and placental site trophoblastic tumor was based on standard histopathologic criteria as determined by a gynecologic pathologist.

3. Immunolocalization of MMPs, TIMPs, MMP inducer and regulators

Immunolocalization of MMP-7,-14,-21,-28, CD147, TIMP-3,-4, IL-6, VEGFR-1,-2 and -3 in gestational tissues was performed on partial moles (n=10), complete moles (n=10), choriocarcinomas (n=10), placental site trophoblastic tumors (n=10) and gestational age matched normal placentas (n=10). Immunolocalization of MMP-1,-2,-3,-9,-13, TIMP-1 and VEGF, Angiopoietin-1 and -2 was performed on choriocarcinomas (n=10) and placental site trophoblastic tumors (n=10) using the method as previously reported [16]. In brief, tissue sections (paraffin-embedded) were deparaffinized, dehydrated, and antigen retrieval was performed in Antigen Unmasking Solution (Vector, Burlingame, CA, USA) with pressure cooker at 120 C for 20 min. In case of MMP-2 and MMP-3 we didn't perform antigen retrieval. Tissue sections were washed 2 times with 1x tris buffer saline (TBS) and incubated with 1% H2O2 for 20 minutes to block endogenous peroxidase activity. After blocking,

sections were incubated with the following dilutions of the primary antibodies MMP-1 1:200 (Novus, Littleton, USA), MMP-2 1:50 (Abcam, Camridge, USA), MMP-3 1:10 (Abcam, Cambridge, USA), MMP-7 1:250 (Novus, Littleton, USA), MMP-9 1:50 (Abcam, Cambridge, USA), MMP-13 1:200 (Abcam, Cambridge, USA), MMP-14 1:100 (Novus, Littleton, USA), MMP-21 1:250 (Abcam, Cambridge, USA), MMP-28 1:100 (Novus, Littleton, USA), CD147 1:75 (Abcam, Cambridge, USA), TIMP-1 1:50 (Calbiochem, San Diego, USA), TIMP-3 1:250 (Millipore, Billerica, USA) and TIMP-4 1:250 (Abcam, Cambridge, USA), IL-6 1:800 (Abcam, Cambridge, USA), CD34 1:1000 (Abcam, Cambridge, USA), alpha SMA 1:75 (Abcam, Cambridge, USA), VEGFR-3 1:100 (Abcam, Cambridge, USA), VEGFR-2 1:250 (Abcam, Cambridge, USA), VEGFR-1 1:250 (Abcam, Cambridge, USA), Ang-1 1:20 (R&D system, Minneapolis, USA) and Ang-2 1:200 (Novus, Littleton, USA), overnight at 4 C or with VEGF 1:750 (Abcam, Cambridge, USA) for one hour in room temperature. Tissue section were washed 2 times with 1x TBS, and incubated with secondary antibody and Vectastain ABC reagent (Vector, Burlingame, CA, USA). Diaminobenzidine (DAB) was used to visualize the positive signal. Positive and negative controls were performed. Antibody CD34 was used to identify vascular endothelial cells and alpha SMA to identify the smooth muscle cells of blood vessels.

4. Evaluation of immunohistochemical findings

Immunohistochemical staining was evaluated independently by two experienced pathologists in a blinded fashion using a semi-quantitative scoring system. Cytoplasmic staining of syncytiotrophoblast, cytotrophoblast, extravillous trophoblast, trophoblastic tumor cells and decidual stromal cells was categorized as negative (0), weakly positive (1+), moderately positive (2+) or strongly positive (3+). A strongly positive result was recorded when more than 50% of the cells exhibited strong staining. Similarly, a weakly positive result was recorded when less than 25% of the cells showed strong staining. A moderately positive staining result (2+) was recorded when between 25% and 50% of the cells of interest showed strong staining.

II. Electrochemiluminescence assay

Assay for MMP-1,-2,-3,-9, VEGFand VEGFR-2 protein levels

Human choriocarcinoma JEG-3 (ATCC HTB-36) trophoblastic cell line along with a normal placental cell line 3A-sub E (ATCC CRL-1584) were grown in Eagle's Minimum Essential Medium (ATCC, Manassas, Virginia, USA) supplemented with 50 ml standard fetal bovine serum, 200mM L-glutamine, 10000 units penicillin and 10 mg/ml streptomycin. Human choriocarcinoma cell line Jar (ATCC HTB-144) was grown in RPMI-1640 medium (ATCC, Manassas, Virginia, USA) supplemented with 50 ml standard fetal bovine serum, 200mM L-glutamine, 10000 units penicillin and 10 mg/ml streptomycin. All cell lines were maintained in a humidified 37^{o}C 5% CO_2 incubator. Cells were seeded at 5x10^4 cells/well in 96-well tissue culture plates (FALCON, Becton Dickson, USA) and incubated for 24h for Jar and 48h for the normal placenta and JEG-3 cell lines. At 100% confluence supernatants were collected. Lysates were prepared using lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 5mM EDTA, 10% glycerol, 1 % NP-40, 0.5% sodium deoxychlorate, 0.1% SDS, 0.2 mg/ml PMSF, 0.01mg/ml leupeptin, and 0.01 mg/ml peptastain A). Protein concentrations were determined using microBCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA).

Concentrations of MMP-1,-2,-3,-9, VEGF and VEGFR-2 were measured in neat cell culture supernatants and cell lysates using electro-chemiluminescence assays (ECL) on Meso Scale Discovery (MSD) Sector2400 imaging instrument (Gaithersburg MD). The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations (pg/ml). This assay system has been previously described and has been validated against the traditional ELISA method [39]. MMP-1,-3,-9 were assayed together as a three-plex and MMP-2 was assayed alone. The linearity ranges were 0.61-2500 pg/mL for the three plex and 122-500,000 pg/mL for the MMP-2 assay. VEGF and VEGFR-2 were assayed separately too. The linearity ranges were 100,000-24 pg/ml for VEGF assay and 15-0.00037 pg/mL for the VEGFR-2 assay. The results were also normalized against cell numbers used in preparing the cell lysates, which was measured using a MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] cell proliferation assay kit (Roche Diagnostics, Indianapolis, IN, USA) as described in the manufacturer's manual.

Statistical analysis

Categorical immunostaining data and the drug resistance assay were compared using RxC contingency tables. The results of electro-chemiluminescence assays were compared using the

non-parametric Mann-Whitney test (Minitab version 13.1, State College, PA, USA). Statistical significance was considered at the 0.05 level.

III. Measurement of cell proliferation using MTT assay

Human choriocarcinoma JEG-3 (ATCC HTB-36) trophoblastic cell line along with a normal placental cell line 3A-sub E (ATCC CRL-1584) were grown in Eagle's Minimum Essential Medium (ATCC, Manassas, Virginia, USA) supplemented with 50 ml standard fetal bovine serum, 200mM L-glutamine, 10000 units penicillin and 10 mg/ml streptomycin. Human choriocarcinoma cell line Jar (ATCC HTB-144) was grown in RPMI-1640 medium (ATCC, Manassas, Virginia, USA) supplemented with 50 ml standard fetal bovine serum, 200mM L-glutamine, 10000 units penicillin and 10 mg/ml streptomycin. All cell lines were maintained in a humidified 37^{o}C 5% CO_2 incubator. Cells were seeded at $8x10^4$ cells/well in 96-well tissue culture plates (FALCON, Becton Dickson, USA) and incubated for 24h for the normal placenta, Jar and JEG-3 cell lines. The cells were incubated with increasing concentration (0-10-20-50-100-200-300-500 microM) of methotrexate, etoposide and carboplatin for other 24h. The results of drug treatment, the cell number was measured using a MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] cell proliferation assay kit (Roche Diagnostics, Indianapolis, IN, USA) as described in the manufacturer's manual.

RESULTS

1.Results of immunohistochemistry

Table 7 reviews the expression of MMP-7,-14,-21 and -28 in normal placenta, partial mole, complete mole, choriocarcinoma and PSTT. The cytotrophoblast cells in normal placenta exhibited significantly stronger staining for MMP-7 than cytotrophoblast cells in complete mole ($p<0.01$). Choriocarcinoma showed stronger intensity of staining for MMP-7 than the extravillous trophoblast cells in partial mole ($p<0.05$). There was no difference in the expression of MMP-7 between choriocarcinoma and PSTT.

The syncytiotrophoblast cells in normal placenta had significantly stronger expression of MMP-14 than the syncytiotrophoblast cells in partial mole ($p<0.05$). Syncytiotrophoblast cells of complete mole also exhibited significantly stronger staining for MMP-14 than the

syncytiotrophoblast cells in partial mole ($p<0.05$). There were no significant differences in expression of MMP-14 in extravillous trophoblast cells among normal placenta, partial and complete mole. Choriocarcinoma showed significantly stronger staining for MMP-14 than PSTT ($p<0.05$). Expression of MMP-14 in PSTT was the weakest compared with the extravillous trophoblast cells in normal placenta, partial and complete mole ($p<0.01$, $p<0.01$, $p<0.05$) (Table 7).

TABLE 7. Immunostaining of Placentas, Partial and Complete Moles, Choriocarcinomas and PSTTs for MMP-7, MMP-14, MMP-21 and MMP-28 (Mean level of Expression, Range 0-3)

Type of sample		MMP-7	MMP-14	MMP-21	MMP-28
Normal Placenta (n=10)	CT	2.4	2.8	3.0	0.9
	ST	2.6	2.9	3.0	0.6
	ET	1.0	2.8	2.4	0.5
	SC	2.0	2.5	3.0	0
Partial Mole (n=10)	CT	2.0	2.4	3.0	2.6
	ST	2.0	2.2	3.0	1.8
	ET	0.3	2.8	1.9	1.6
	SC	2.0	2.5	3.0	1.6
Complete Mole (n=10)	CT	1.5	2.7	3.0	2.3
	ST	1.3	2.4	2.4	1.6
	ET	0.7	2.6	2.3	2.0
	SC	2.0	2.5	3.0	2.0
Choriocarcinoma (n=10)		2.0	2.6	3.0	2.6
PSTT (n=10)		1.0	1.4	3.0	1.2

Note: ST: syncytiotrophoblast, CT: cytotrophoblast, ET: extravillous trophoblast, SC: stroma cell, PSTT: Placental site trophoblastic tumor

The syncytiotrophoblast cells in normal placenta and partial mole have significantly stronger staining for MMP-21 than syncytiotrophoblast cells in complete mole ($p<0.01$, $p<0.01$). Choriocarcinoma and PSTT exhibited significantly stronger staining for MMP-21 than extravillous trophoblast cells in normal placenta, partial and complete mole ($p<0.05$, $p<0.05$, $p<0.01$) (Table 7).

Choriocarcinoma had significantly stronger staining for MMP-28 than extravillous trophoblast cells in normal placenta ($p<0.01$). Choriocarcinoma also exhibited significantly

stronger staining for MMP-28 than PSTT ($p<0.05$). MMP-28 expression in stromal cells in normal placenta was significantly lower than in stromal cells in partial and complete mole ($p<0.01$, $p<0.01$) (Table 7) (Figure 7).

Table 8 reviews the expression of TIMP-3,-4, CD147 and IL-6 in normal placenta, partial mole, complete mole, choriocarcinoma and PSTT. Stronger TIMP-3 immunoreactivity was found in PSTT than choriocarcinoma ($p<0.05$) (Table 8). Extravillous trophoblast cells in normal placenta and complete mole had significantly greater expression of TIMP-3 than choriocarcinoma ($p<0.01$, $p<0.01$). TIMP-3 expression in stromal cells in partial mole was significantly lower than in stromal cells in normal placenta and complete mole ($p<0.01$, $p<0.01$).

TABLE 8. Immunostaining of Placentas, Partial and Complete Moles, Choriocarcinomas and PSTTs for TIMP-3, TIMP-4, CD147 and IL-6 (Mean level of Expression, Range 0-3)

Type of sample		TIMP-3	TIMP-4	IL-6	CD147
Normal Placenta (n=10	CT	2.8	2.2	2.8	3.0
	ST	2.9	2.3	2.7	3.0
	ET	2.7	2.6	2.3	2.8
	SC	2.8	2.9	2.7	0.6
Partial Mole (n=10)	CT	2.0	2.3	2.0	1.4
	ST	1.4	2.7	2.4	2.3
	ET	1.5	2.9	1.2	0.5
	SC	1.7	3.0	2.1	1.0
Complete Mole (n=10)	CT	2.8	2.1	2.4	1.6
	ST	2.9	2.8	2.4	2.3
	ET	2.5	2.4	1.5	0.8
	SC	2.9	2.3	2.0	1.0
Choriocarcinoma (n=10)		1.0	1.2	0.2	2.6
PSTT (n=10)		2.0	2.4	1.4	1.0

Note: ST: syncytiotrophoblast, CT: cytotrophoblast, ET: extravillous trophoblast, SC: stroma cell, PSTT: Placental site trophoblastic tumor

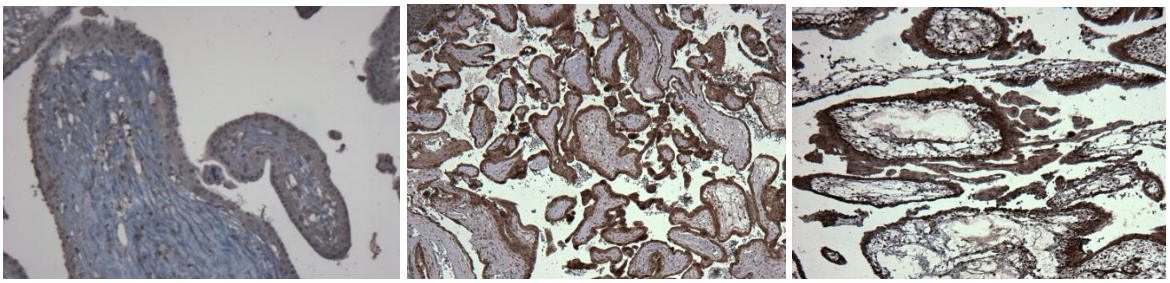
Extravillous trophoblast cells in normal placenta, partial and complete mole had significantly stronger expression of TIMP-4 than choriocarcinoma ($p<0.01$, $p<0.01$, $p<0.05$). PSTT also showed significantly stronger staining for TIMP-4 than choriocarcinoma ($p<0.05$). TIMP-4

expression in stromal cells in partial mole was significantly lower than in stromal cells in complete mole (p=0.001) (Table 8).

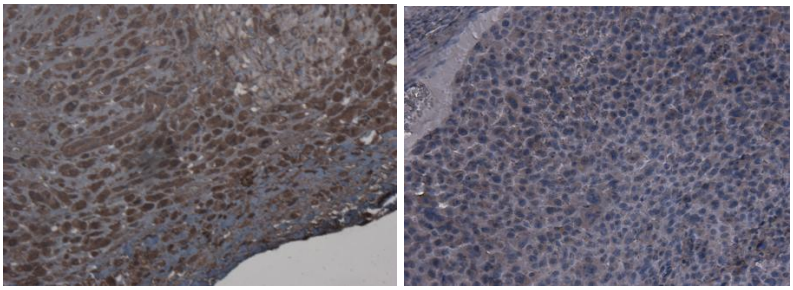
Strong CD147 immunoreactivity was found in all trophoblastic cell types of normal placenta (CT 100%, ST 100%, ET 100%). Choriocarcinoma had significantly stronger staining of CD147 than extravillous trophoblast cells in partial and complete mole ($p<0.01$, $p<0.01$). The stromal cells in partial mole showed significantly stronger expression of CD147 than in stromal cells in normal placenta and complete mole (p=0.025, p=0.014) (Table 8) (Figure 8).

IL-6 expression in extravillous trophoblast cells in both normal placenta and complete mole was significantly stronger than in choriocarcinoma (p=0.005, p=0.004). Placental site trophoblastic tumor (PSTT) exhibited significantly stronger staining for IL-6 than choriocarcinoma (p=0.03). The stromal cells in normal placenta had significantly stronger expression of IL-6 than the stromal cells in complete mole (p=0.001) (Table 8) (Figure 9).

Figure 7. Immunohistochemical analysis of expression of MMP-28 in normal placenta and gestational trophoblastic diseases (A,B,C: magnification, 10x, D,E: magnification, 100x)

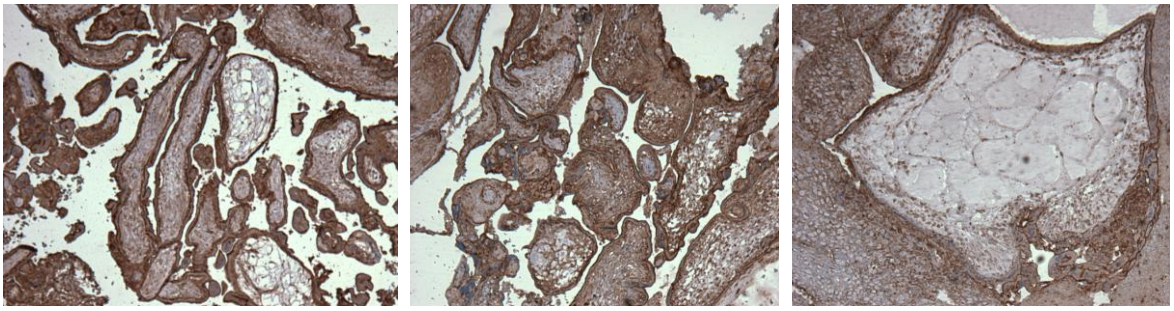


A**. Normal placenta** **B. Partial mole** **C. Complete mole**

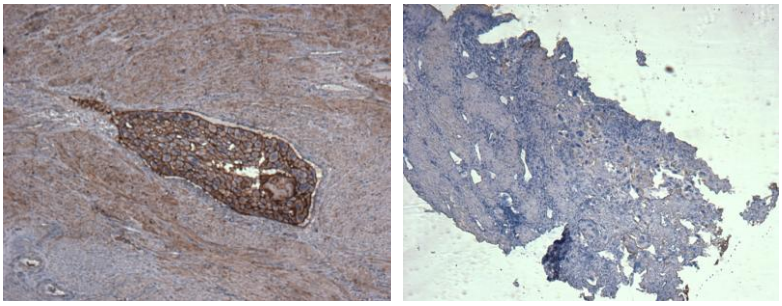


D. Choriocarcinoma **E. PSTT**

Figure 8. Immunohistochemical analysis of expression of CD147 in normal placenta and gestational trophoblastic diseases (A,B,C: magnification, 10x, D,E: magnification, 100x)

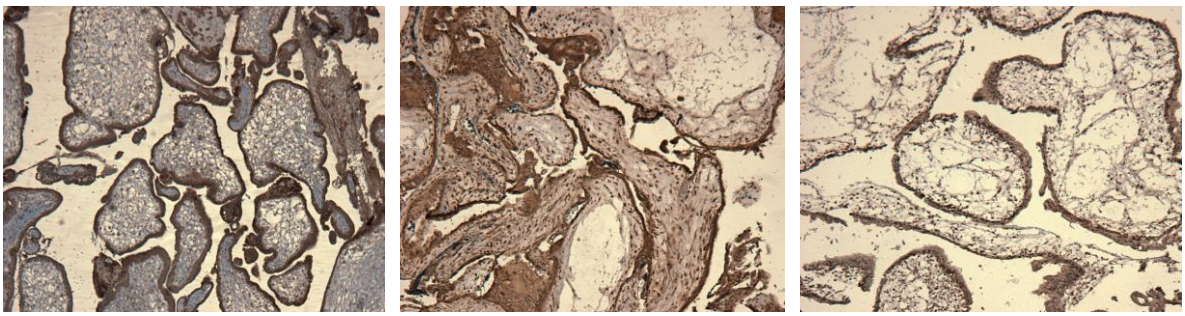


A. Normal placenta **B. Partial mole** **C. Complete mole**

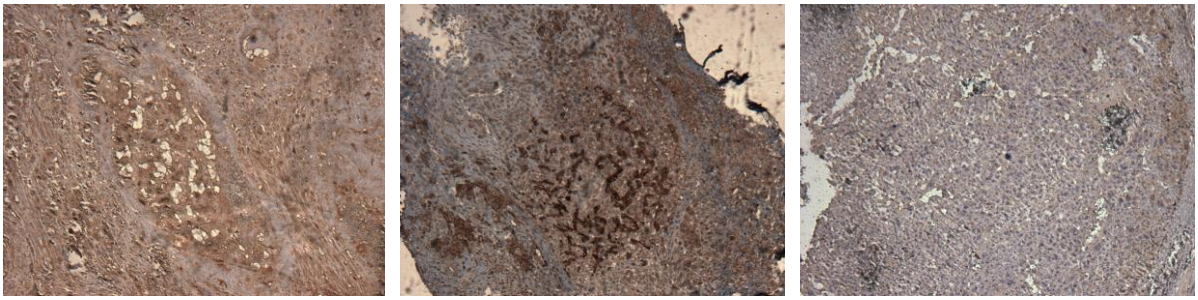


D. Choriocarcinoma **E. PSTT**

Figure 9. Immunohistochemical analysis of expression of IL-6 in normal placenta and gestational trophoblastic diseases (A,B,C: magnification, 10x, D,E,F: magnification, 100x)



A. Normal placenta **B. Partial mole** **C. Complete mole**



D. Choriocarcinoma **E. Metastatic choriocarcinoma** **F. PSTT**

There were no significant differences in the expression of MMP-1,-2,-3,-9,-13 and TIMP-1 between choriocarcinoma and PSTT but VEGF expression in PSTT were significantly higher than in choriocarcinoma (p=0.007). Choriocarcinoma had significantly stronger staining of MMP-14 and MMP-28 than PSTT (p=0.03, p=0.04). TIMP-3 and TIMP-4 expression in PSTT were significantly higher than in choriocarcinoma (p=0.03, p=0.01) (Table 9).

TABLE 9. Immunostaining of Choriocarcinomas and Placental site trophoblastic tumors for MMPs, TIMPs, VEGF and Angiopoietin-1,-2 (Mean level of Expression, Range 0-3)

Antibody	**Choriocarcinoma** (n=10)	**PSTT** (n=10)	**p value**
MMP-1	**2.6**	**1.4**	**NS**
MMP-2	**3.0**	**2.0**	**NS**
MMP-3	**0.8**	**1.0**	**NS**
MMP-7	**2.0**	**1.0**	**NS**
MMP-9	**1.4**	**1.8**	**NS**
MMP-13	**1.4**	**1.8**	**NS**
MMP-14	**2.6**	**1.4**	**0.03**
MMP-21	**3.0**	**3.0**	**NS**
MMP-28	**2.6**	**1.2**	**0.04**
TIMP-1	**1.4**	**1.2**	**NS**
TIMP-3	**1.0**	**2.0**	**0.03**
TIMP-4	**1.2**	**2.4**	**0.01**
CD147	**2.6**	**1.0**	**NS**
VEGF	**1.2**	**3.0**	**0.007**
Ang-1	**0**	**1.6**	**0.01**
Ang-2	**0.2**	**0.6**	**NS**

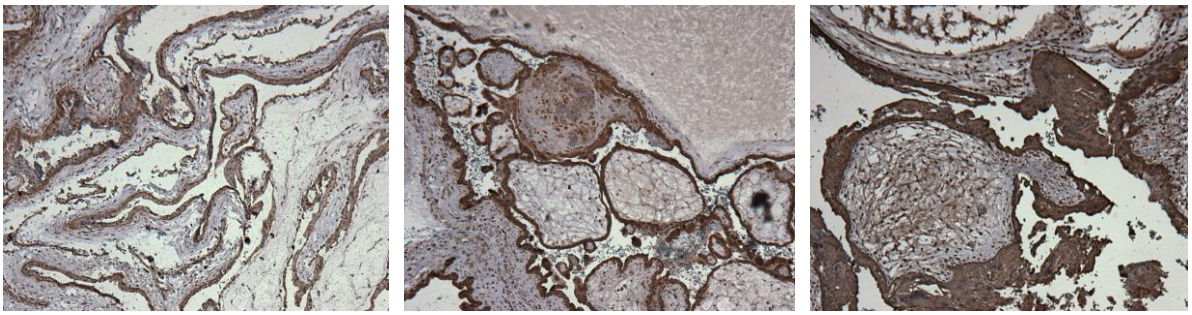
VEGFR-1 in cytotrophoblast cells in partial mole was significantly higher than in cytotrophoblast cells in normal placenta and complete mole (p=0.01, p=0.007). Choriocarcinoma had weaker staining for VEGFR-1 but not significantly different as compared to extravillous trophoblast cells in normal placenta, complete and partial mole. The syncytiotrophoblast cells in partial mole had significantly stronger expression of VEGFR-1 than the syncytiotrophoblast cells in normal placenta and in complete mole (p=0.011,

p=0.001). Expression of VEGFR-1 in PSTT was weak but not significantly different as compared to choriocarcinoma. VEGFR-1 expression in stromal cells in partial mole was significant lower than in stromal cells in normal placenta and in complete mole (p=0.001, p=0.001) (Table 10).

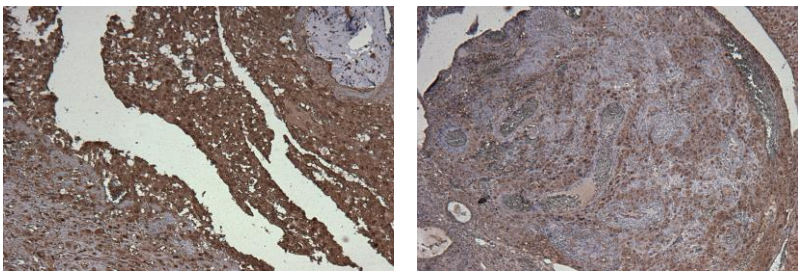
The syncytiotrophoblast cells in normal placenta had significantly stronger expression of VEGFR-2 than the syncytiotrophoblast cells in partial and complete mole (p=0.001, p=0.003). The cytotrophoblast cells in normal placenta showed stronger intensity of staining for VEGFR-2 than cytotrophoblast cells in partial mole (p=0.001). VEGFR-2 expression in cytotrophoblast cells in partial mole was significantly higher than in cytotrophoblast cells in complete mole (p=0.003) (Table 10).

Choriocarcinoma showed stronger intensity of staining for VEGFR-3 than the extravillous cells in normal placenta, partial and complete mole (p=0.036, p=0.038, p=0.05). VEGFR-3 in extravillous cells in normal placenta was significantly higher than in extravillous cells in partial and complete mole (p=0.039, p=0.012). The syncytiotrophoblast cells in normal placenta had significantly stronger expression of VEGFR-3 than the syncytiotrophoblast cells in partial and complete mole (p=0.039, p=0.012) (Table 10) (Figure 10).

Figure 10. Immunohistochemical analysis of expression of VEGFR-3 in normal placenta and gestational trophoblastic diseases (A,B,C: magnification, 10x, D,E: magnification, 100x)



A. Normal placenta **B. Partial mole** **C. Complete mole**



D. Choriocarcinoma **E. PSTT**

TABLE 10. Immunostaining of Placentas, Partial and Complete Moles, Choriocarcinomas and PSTTs for VEGFR-1,-2 and -3 (Mean level of Expression, Range 0-3)

Type of sample		VEGFR-1	VEGFR-2	VEGFR-3
Normal Placenta (n=10	CT	1.7	2.8	2.8
	ST	1.7	2.8	2.7
	ET	1.9	2.0	1.4
	SC	1.0	1.6	2.3
Partial Mole (n=10)	CT	2.4	1.9	2.6
	ST	2.4	1.9	1.6
	ET	1.7	1.5	2.2
	SC	1.7	1.7	2.2
Complete Mole (n=10)	CT	2.1	2.4	2.8
	ST	2.0	1.4	1.9
	ET	1.5	1.4	2.5
	SC	1.0	1.4	2.5
Choriocarcinoma (n=10)		1.2	1.2	3.0
PSTT (n=10)		0.8	1.4	2.0

Note: ST: syncytiotrophoblast, CT: cytotrophoblast, ET: extravillous trophoblast, SC: stroma cell, PSTT: Placental site trophoblastic tumor

Table 11 reviews the expression of VEGFR-1,-2 and -3 in vascular endothelial cells in normal placenta, partial mole, complete mole, choriocarcinoma and PSTT. The vascular endothelial cells in PSTT showed significantly lower staining for VEGFR-1 than the vascular endothelial cells in normal placenta and complete mole ($p=0.014$, $p=0.01$). There were no significant differences in VEGFR-2 and VEGFR-3 expression in vascular endothelial cells between normal placenta, partial mole, complete mole, choriocarcinoma and PSTT.

TABLE 11. Immunostaining of vascular endothelial cells in Choriocarcinomas and Placental site trophoblastic tumors for VEGFR-1,-2 and -3 (Mean level of expression, Range: 0-3)

Type of sample	VEGFR-1	VEGFR-2	VEGFR-3
Normal Placenta (n=10)	1.8	1.6	1.4
Partial Mole (n=10)	1.6	2.1	1.4
Complete Mole (n=10)	1.6	2.1	2.2
Choriocarcinoma (n=10)	0.6	2.4	2.0
PSTT (n=10)	0	2.0	1.4

2. MMP-1,-2,-3,-9, VEGF and VEGFR-2 in normal trophoblast and JEG-3 and Jar choriocarcinoma cell lines

Concentrations of MMP-1,-2,-3,-9 and VEGF and VEGFR-2 were measured in neat cell culture supernatants and cell lysates prepared from normal trophoblastic cell line and two choriocarcinoma cell lines JEG-3 and Jar, using an innovative electro-chemiluminescence assays (ECL). This assay system is highly sensitive and has been previously described and has been validated against the traditional ELISA method [17]. The protein levels were normalized by total protein concentrations produced by similar numbers of cells, as evidenced by similar readings in a MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay. Levels of MMP-1,-3,-9 in lysate samples support our earlier immunohistochemical data. [16] While the level of MMP-2 was higher in the supernatant than the lysate in normal trophoblast and choriocarcinoma cell lines, MMP-1 was higher in the lysate in both choriocarcinoma cell lines. (Table 12)

MMP-1 levels in supernatants in normal trophoblast cells were significantly higher than in supernatants in JEG-3 and Jar choriocarcinoma cells (p=0.0001, p=0.0001) and in the lysate in normal trophoblast cells (p=0.0003). Levels of MMP-1 in lysates were significantly higher than in supernatants in both Jar and JEG-3 (p=0.0001, p=0.0001).

Levels of MMP-2 in supernatants were significantly higher than in lysates in normal trophoblast cells and in JEG-3 and Jar choriocarcinoma cells (p=0.0002, p=0.0001, p=0.0008). MMP-2 levels in supernatants in Jar was significantly higher than in supernatants in normal trophoblast cells and JEG-3 (p=0.001, p=0.0001). The lysate of Jar had

significantly higher MMP-2 levels than the lysate in normal trophoblast cells and JEG-3 (p=0.0004, p=0.0001).

MMP-3 levels in the supernatant of normal trophoblast cells was significantly higher than the supernatant in Jar and JEG-3 (p=0.0001, p=0.0001) and in the lysate in normal trophoblast cells (p=0.0002). MMP-3 levels in the lysate of normal trophoblast cells was significantly higher than the lysate in Jar and JEG-3 (p=0.008, p=0.0014). The lysate in Jar had significantly higher levels of MMP-3 than the supernatant in Jar (p=0.01).

TABLE 12. Mean levels and Standard deviations of MMP-1, MMP-2, MMP-3, MMP-9 and VEGF and VEGFR-2 in Supernatant and Lysate of Normal Placenta, JEG-3 and Jar Choriocarcinoma Cell Lines

Type of cell		MMP-1	MMP-2	MMP-3	MMP-9	VEGF	VEGFR-2
Normal trophoblast cell	Supernatant (n=10)	243.9 (SD: 112.9)	23825.9 (SD: 233.5)	208.0 (SD: 87.6)	38.0 (SD:4.49)	7.8 (SD:172.7)	0.025 (SD:0.015)
	Lysate (n=10)	57.7 (SD: 46.2)	1615.5 (SD: 846.1)	3.78 (SD: 2.5)	22.7 (SD:32.7)	0.0 (SD:14.0)	0.013 (SD:0.005)
JEG-3 choriocc. cell	Supernatant (n=10)	4.36 (SD: 5.3)	384.7 (SD: 287.0)	0.4 (SD: 0.6)	3.4 (SD:3.3)	1196.5 (SD:401.1)	0.0036 (SD:0.001)
	Lysate (n=10)	192.0 (SD: 208.3	83.9 (SD: 29.0)	0.6 (SD: 0.8)	2.5 (SD:2.2)	482.0 (SD:460.1)	0.0011 (SD:0.001)
Jar choriocc. cell	Supernatant (n=10)	2.5 (SD: 3.7)	135459.8 (SD: 521.7)	0.2 (SD: 0.4)	1.0 (SD:1.5)	1713.9 (SD:702.9)	0.0039 (SD:0.001)
	Lysate (n=10)	151.0 (SD: 196.8)	8013.6 (SD:3092.1)	1.27 (SD: 0.9)	2.0 (SD:1.8)	191.1 (SD:61.1)	0.0021 (SD:0.001)

Note: Supernatants are pg/ml and Lysates are pg/mg/ml.

Levels of MMP-9 in the supernatant of normal trophoblast cells were significant higher than the supernatants in Jar and JEG-3 (p=0.0001, p=0.0001) and the lysate in normal trophoblast cells (p=0.04). MMP-9 levels in the lysate of normal trophoblast cells were significantly higher than in the lysate in Jar and JEG-3 (p=0.001, p=0.004).

VEGF levels in the supernatant of normal trophoblast cells were significantly lower than the supernatant in JEG-3 and Jar (p=0.0014, p=0.0013). Levels of VEGF in the supernatant were significantly higher than in the lysates of JEG-3 and Jar (p=0.0406, p=0.0047). VEGF levels

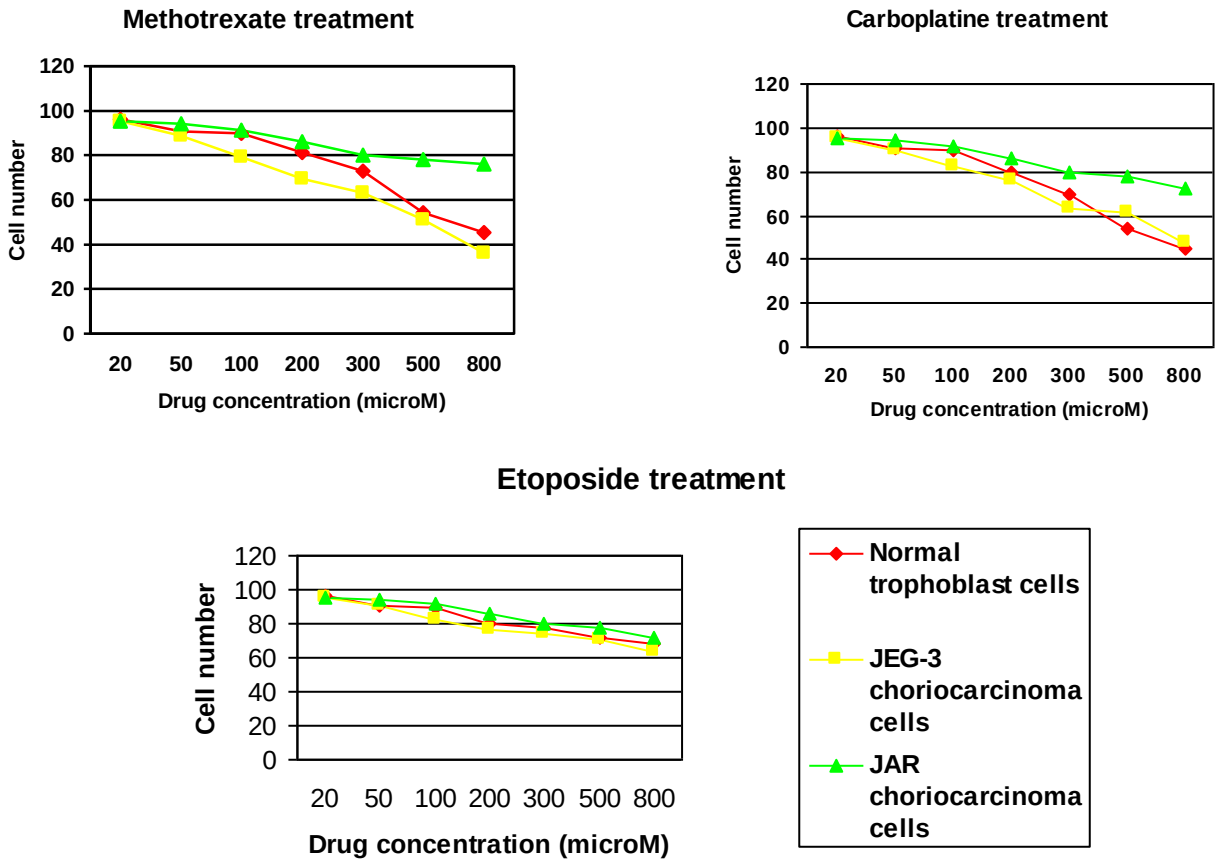
in lysate in normal trophoblast cells were significantly lower than in lysate in Jar and JEG-3 (p=0.0006, p=0.0014).

Levels of VEGFR-2 in the supernatant of normal trophoblast cells were significantly higher than the supernatants in JAR and JEG-3 (p=0.0002, p=0.0002) and the lysate in normal trophoblast cells (p=0.019). VEGFR-2 levels in the lysate of normal trophoblast cells were significantly higher than in the lysate in JAR and JEG-3 (p=0.0002, p=0.0002).

3. Cell proliferation assay

The number of Jar choriocarcinoma cells was significantly higher than the number of normal trophoblasts and JEG-3 choriocarcinoma cells ($p<0.05$, $p<0.05$) by the same concentration methotrexate and carboplatin treatment. The cell number was significantly decreased with increasing drug concentration by normal trophoblast cells and JEG-3 choriocarcinoma cells. The number of Jar choriocarcinoma cells was not changed significantly with increasing methotrexate and carboplatin treatment. There was no significant difference among the number of normal trophoblast cells, Jar and JEG-3 choriocarcinoma cells by etoposide treatment. (Figure 11)

Figure 11. Methotrexate, Carboplatine and Etoposide treatment of normal trophoblast cells and JEG-3 and Jar choricarcinoma cells



DISCUSSION

Gestational trophoblastic diseases are well recognized to have varying propensities for local invasion and metastasis. [2] Matrix metalloproteinases (MMP)-mediated degradation of the extracellular matrix (ECM) has a key role in tumor cell invasion and metastasis. [16,17] Angiogenesis is also required for these processes. MMPs, MMP inducer, their tissue inhibitors and regulators may play an important role in the pathogenesis of gestational trophoblastic diseases. Better knowledge of the expression of these factors may contribute to the understanding of the biology of normal placenta and gestational trophoblastic diseases.

I. Regulation of MMP activity

MMPs must be present in the right cell type and pericellular location, at the right time, in the right amount, to accomplish their normal or pathologic functions. They must be activated or inhibited appropriately. MMP gene expression is regulated by numerous stimulatory and suppressive factors that influence multiple signaling pathways. The expression of various MMPs can be up- or downregulated by several cytokines and growth factors, including interleukins, interferons, EGF, KGF, NGF, basic FGF, VEGF, PDGF, TNF-α, TGF-β, phorbol esters, integrin-derived signals, extracellular matrix proteins, cell stress, and changes in cell shape. [16] Many of these stimuli induce the expression and/or activation of c-fos and c-jun proto-oncogene products, which heterodimerize and bind activator protein-1 (AP-1) sites within several MMP gene promoters. [16,17]

1. Tissue inhibitors of matrix metalloproteinases

The tissue inhibitors of matrix metalooproteinases (TIMPs) represent a family of at least four 20–29-kDa secreted proteins (TIMPs 1–4) that reversibly inhibit the MMPs in a 1:1 stoichiometric fashion. [17] TIMP-3 has ability to regulate the invasion of trophoblast cells throught MMP-2 and MMP-9. It can inhibit the angiogenesis by blockage of VEGF binding to VEGF receptor-2. TIMP-4 is upregulated in cervical cancer, ovarian cancer, invasive endometrial cancer, and in ductal in situ breast cancer too. TIMP-4 inhibits the tumor cell's invasion in many human cancer.

Choriocarcinoma has significantly less expression of TIMP-3 and TIMP-4 than normal placenta, partial and complete mole what can contribute to its invasiveness. PSTT showed significantly stronger staining for TIMP-1,-3 and -4 compared to choriocarcinoma what can explain its less invasive behavior.

2. CD147

CD147 can induce MMP-1,-2,-3,-9 and also VEGF. [18,19] CD147 has been implicated in the tumorigenicity of breast and ovarian cancer. [40,41] CD147 expression is also closely correlated with tumor invasion and metastasis in breast cancer. [40] CD147 has role to mediate multidrug resistance in malignancies e.g. hepatocellular cancer. [18,19]

The strong expression of CD147 in cytotrophoblast cells of normal placenta confirm that the placenta undergoes a process of vasculogenic mimicry (pseudovasculogenesis). The cytotrophoblast cells of placenta with epithelial phenotype are able to convert to endothelial phenotype during normal pregnancy thereby forming and remodeling new vessels. [42] CD147 is marker of this process, what means the embryonic-like ability of aggressive, but not nonaggressive, tumor cells to form a pattern of vasculogenic-like networks, with concomitant expression of vascular-associated cell markers. [41]

Vasculogenic mimicry (VM) has been observed in several cancer including melanoma, breast, prostatic, ovarian and lung carcinoma. VM involves several signaling molecules e.g. vascular endothelial cadherin, MMP-2 and laminin 5 gamma 2 chain that are also involved in embryonic vasculogenesis. Matrix metalloproteinase-2 and membrane type 1-matrix metalloproteinase appear to play a key role in the development of vasculogenic-like networks and matrix remodeling by aggressive cancer cells. [41] Endostatin inhibits the endothelial-cell-driven angiogenesis but not the vasculogenic mimicry. [41] Endostatin has ability to inhibit the endothelial cell migration and induce endothelial cell apoptosis and cell cycle arrest. Endostatin overexpression of extravillous trophoblast cells was suggested in studies. [43] (Figure 12)

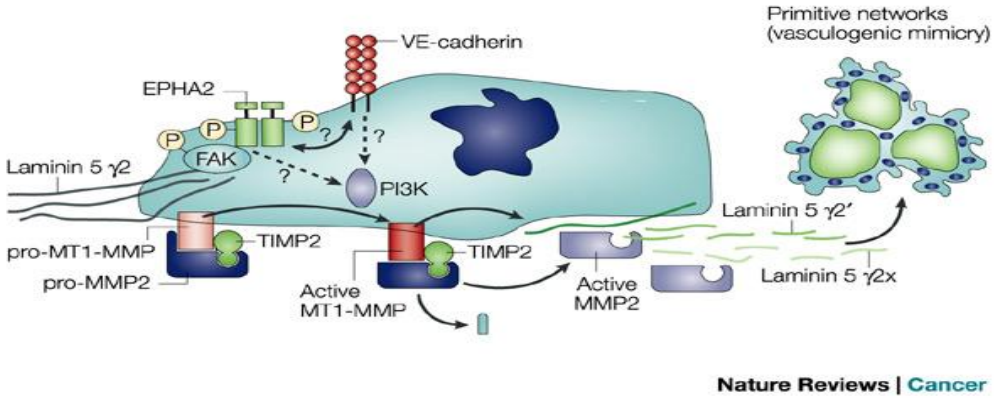


Figure 12. Molecular mechanism of vasculogenic mimicry [44]

Vasculogenic mimicry is one example of tumor cell plasticity. The mutations of rapidly dividing and growing cancer cells may be one reason to develop drug resistance. Endothelial cells have more genetically stability than the tumor cells thereby giving opportunity to targeting endothelial cells using antiangiogenic therapy. [44]

Increased CD147 expression may also cause higher MMP-1 and MMP-2 levels in choriocarcinoma and thereby increase its invasiveness.

Further study, what clarify the relationship between CD147, laminin (an epithelial anchoring filament component which after modulation by MMPs induces endothelial cell migration), endostatin and MMP-2 in GTD may provide important new insights into the drug resistance of gestational trophoblastic neoplasms.

3. Interleukin-6

IL-6 can be secreted by T cells and macrophages to stimulate the immune response to inflammation or trauma or implantation e.g. maximum IL-6 mRNA and protein expression was identified in the endometrium at the time of implantation. [45] Smooth muscle cells in the tunica media of blood vessels produce IL-6 too thereby increasing the activity of MMPs especially MMP-2 and -9 what are important in invasion. IL-6 is correlated with a higher metastatic potential of tumor cell. Serum level of IL-6 is associated with poor prognosis and resistance to chemotherapy in cancer patients. IL-6 production could contribute to peripheral T lymphocyte dysfunction thereby getting opportunity to tumor cells to escape the immune surveillance. The lack of the classical MHC expression of trophoblast cells inhibits the maternal immune response against the placenta. The lymphocytes can infiltrate the tumor

tissue, and have ability to product cytokines e.g. IL-1,-6,-8. The endothelial mitogen function of these factors is well known. [46] We didn't detect immune cell infiltration by choriocarcinoma.

Interestingly, choriocarcinoma has significantly less interleukin-6 (IL-6) expression than normal placenta, complete mole and PSTT. By contrast, several tumors (e.g. ovarian, breast cancer) are associated with overproduction of IL-6. [47-49] The explanation of this phenomenon may be due to the highly elevated hCG level in choriocarcinoma. HCG increases the levels of steroid hormones (estradiol and progesterone) and estrogen has the ability to suppress IL-6 expression. The suppressive effect of estrogen on IL-6 was first recognized in human endometrial stromal cells. [49] In contrast, IL-6 overexpression was detected in metastatic choriocarcinoma in lung and liver. The different immune response than in the uterus may be the reason of the elevated level of IL-6 in metastatic choriocarcinoma.

II. Angiogenesis

1. MMPs in angiogenesis

The formation of new blood vessels from existing vessels, the process of angiogenesis, is required for tissue development, repair, and for the growth of solid tumors. MMPs can generate both angiogenesis-inhibiting and –promoting signals. MMP-2,-9 and -14 are involved mainly in tumor angiogenesis and to a lesser extent MMP-1 and -7. Increased expression of MMP-1,-2 and MMP-3 is linked with lymphatic invasion and lymph node metastases. Inhibition of MMP-2,-9,-14 attenuates both angiogenesis and lymphangiogenesis and reduces lymph node metastasis. The lymphatic vasculature but not aortic vasculature is impaired by targeted deletion of MMP-2. [17]

The action of MMPs on ECM will not only affect the structure of the vessel wall but also cause the release of a number of biologically active molecules. MMPs can promote the angiogenesis by liberating sequestered angiogenic factors. [23]

The activity of MMP-2 can release transforming growth factor-β (TGF-β) bound to the ECM. TGF-β regulates trophoblast invasion and motility. [17]

MMP-9 has a key role in tumor angiogenesis by regulating the bioavailability of vascular endothelial growth factor. The modulation of VEGF bioavailability by MMP-9 may affect lympangiogenesis and promotes dissemination of metastases into the lymph. The direct cleavage of matrix-bound VEGF by MMP-3,-7,-9,-16 results in modified VEGF molecules with altered bioavailability, which changes the vascular patterning of tumors in vivo. [17]

2. Vascular endothelial growth factor and their receptors in angiogenesis

Vascular endothelial growth factors and their receptors are central regulators of angiogenesis. [23,24] Trophoblast cell's ability to produce vascular growth factors is described by several studies. [38,50,51] These factors have capacity to induce the proliferation, migration and invasion of trophoblast cells. [23,24]

Strong VEGF expression of trophoblast cells in both normal placenta and molar pregnancy is known from earlier studies. PSTT exhibits a high level of expression of VEGF and other studies have also described the overexpression of VEGF in PSTT. [52,53]

MMPs also can regulate vascular stability and permeability. [23] VEGF was described first as vascular permeability factor which promotes vascular leakage. VEGF has ability to induce the synthesis and releasing of nitrogen-monoxide and prostacyclins from the cells. The increased permeability of vessels results the increased interstitial pressure. The high interstitial pressure can compress the lumen of vessels thereby inhibiting the income of chemotherapy into the vessels.

Interestingly, we detected highly expressed VEGF in PSTT with immunohistochemistry and also in Jar choriocarcinoma cells with electro-chemiluminescens assay. The PSTT's less sensitivity of chemotherapy is well known. Our results showed chemotherapy resistance by Jar choriocarcinoma cells too. Further studies have to prove the role of VEGF in drug resistance.

VEGF is the only angiogenic factor what is continuously expressed and genetically stable during the tumor life. These angiogenic factors may be targets for novel therapies in gestational trophoblastic neoplasia. The direct and continuous VEGF inhibition has been validated as an effective clinical cancer therapy. Bevacizumab (Avastin) is an efficacious treatment for a wide variety of cancers including colorectal and breast cancer. [54,55] Bevacizumab, a humanized monoclonal antibody, has the ability to inhibit both endothelial

cell proliferation and angiogenesis. By binding to biologically active forms of VEGF, it prevents VEGF's interaction with VEGFR-1 and -2. Our findings suggest that Bevacizumab or other anti-VEGF agents could be potential useful drug in the treatment of PSTT and choriocarcinoma. Agents that inhibit VEGF receptors may also be a valuable therapy in the treatment of drug-resistant choriocarcinoma. (Figure 13)

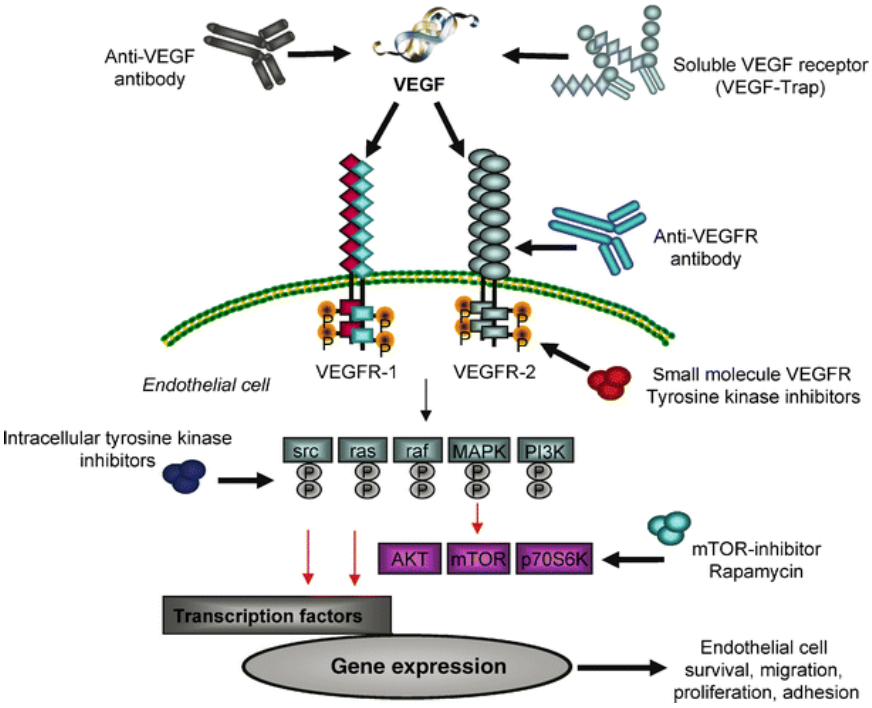


Figure 13. Schematic overview on different therapeutic strategies blocking VEGF-induced tumor angiogenesis. Prominent substances represent the VEGF antibody (Avastin), VEGF-Trap, and antibodies blocking VEGFR-2. Intracellular small-molecule tyrosine kinase inhibitors are capable to block VEGFR-1 and VEGFR-2 signaling. More downstream rapamycin effectively blocks mTOR-downregulating gene expression responsible for endothelial cell proliferation and survival [51]

MMPs can also negatively regulate angiogenesis by generating anti-angiogenic peptides. Collagen XVIII is a minor component of the spiral artery basement membrane produced by the extravillous trophoblast and vascular smooth muscle cells. The cleavage of collagen XVIII by MMP-2 or -9 releases endostatin, which inhibits EC proliferation and stimulates apoptosis. [17] Angiostatin is an N-terminal breakdown product of plasminogen that can be generated by MMP-2,-3,-7,-9, and -12; endostatin and tumstatin are anti-angiogenic protein fragments from the C-terminal portions of collagens XVIII and IV, respectively; and the free hemopexin domain of MMP-9 itself may act as an angiogenesis inhibitor. [17]

PSTT exhibits a high level of expression of VEGF and Angiopoietin-1 and other studies have also described the overexpression of VEGF in PSTT. [56,57] Inhibition of VEGF may

provide an useful treatment of PSTT which can be relatively resistant to cytotoxic chemotherapy.

It is important to recognize that our data concerning VEGF and choriocarcinoma is conflicting. While immunostaining for VEGF using a murine antibody was weak in choriocarcinoma tissues, VEGF levels in two choriocarcinoma cell lines were high in an electro-chemiluminescence assay using a human antibody to VEGF. Further studies should be done to clarify the relationship between VEGF and choriocarcinoma.

VEGFR-3 is up-regulated on vascular as well as nonvascular tumors and has been shown to be important for angiogenesis as well lymphangiogenesis by stimulating metastasis. [30,58] The increased expression of VEGFR-3 in choriocarcinoma compared to normal placenta, partial and complete mole may contribute to the invasiveness of this disease.

III. MMPs in cancer

Malignant cancers are distinguished from in situ cancers and benign neoplasms by their ability to cross tissue boundaries and spread to other organs of the body. Cancer cells must cross multiple ECM barriers as they traverse the epithelial basement membrane and interstitial stroma, enter blood vessels or lymphatics, and extravasate to form metastatic deposits at a distant site. Because of this requirement and because invasive behavior is associated with the upregulation of MMPs in virtually all human cancers, MMPs are thought to facilitate invasion and metastasis by degrading structural ECM components. [16,17]

MMPs are generally present in greater amounts and activated more often in and around malignant cancers than in normal, benign, or premalignant tissues, with the highest expression taking place in areas of active invasion at the tumor-stroma interface.

Significant positive correlations have been found between MMP expression and various indicators of a poor prognosis in virtually all types of cancer, and in some instances, increased MMP levels represent an independent predictor of shortened disease-free and overall survival.

Several MMPs have been implicated as key agonists in tumor invasion, metastasis, and angiogenesis, including MMP-1,-2,-3,-9, and -14. Without the aid of ECM-degrading MMPs, endothelial cells would probably be unable to penetrate the ECM, and cancer cells would be

unable to cross the matrix barriers that otherwise contain their spread. MMPs promote the invasiveness of tumor cells by degradation of different types of ECM's components and they have the ability to activate other MMPs e.g. MMP-7 activates MMP-2 and MMP-9 and MMP-14 activate MMP-2. [59,60] Furthermore CD147 can induce MMP-1,-2,-3 and -9. [18,19] The expression of MMPs has been investigated in several human malignancies but the expression of MMPs and TIMPs in the tumor microenvironment is quite diverse. MMP-7, MMP-14 and MMP-28 have been reported to be highly expressed in breast carcinoma. [59,60,61] MMP-14 has an important role in the migration and invasion of tumor cells. [62] MMP-21 has been detected in ovarian and colon carcinomas. [63] MMPs have therefore been implicated to contribute to the invasiveness and spread of several malignancies. data indicate that MMPs do far more to influence cancer than merely remove the physical barriers to invasion and metastasis.

Overexpression of MMP-28 in epithelial cells can contribute to irreversible epithelial to mesenchymal transition with loss of E-cadherin, a major cell adhesion molecule, from the cell surface. [64,65] A number of MMPs can promote early cancer development, and MMP-3 and -28 can also promote late epithelial-to-mesenchymal phenotypic changes that are associated with more aggressive malignant behavior. Downregulation of E-cadherin is causally involved in the malignant progression of some cancers, and MMP-generated E-cadherin breakdown products promote tumor cell invasion. In addition, MMPs can trigger epithelial-to-mesenchymal phenotypic conversions, a process that is associated with more aggressive malignant behavior and one that occurs naturally during development and wound repair when stationary and otherwise adherent epithelial cells become migratory and invasive. Thus MMPs can potentially contribute, both negatively and positively, to all stages of cancer evolution by numerous distinct pathways. [16,17,64]

The molecular communication between malignant epithelial cells and non-malignant stroma may be further understood through study of MMPs. [17] Studies indicate that stroma cells probably have ability to promote cancer development. In epithelial cancers, however, most of the upregulated MMPs are expressed by the supporting stroma cells rather than by the carcinoma cells themselves. Thus MMPs from adjacent stroma cells are often induced and commandeered by the malignant epithelial cells. In our study there were no correlations between the MMP or TIMP level of stroma cells and trophoblast cells.

MMP overexpression usually correlates with more aggressive malignant behavior and poor clinical outcome. Relatively benign cells acquire malignant properties when MMP activity is increased or TIMP activity diminished, and highly malignant cells are made less aggressive by reducing their MMP levels or suppressing their MMP activity.

MMP-14,-21 and -28 are overexpressed in choriocarcinoma compared to normal placenta, partial mole and complete mole. Choriocarcinoma has significantly less expression of TIMP-3 and TIMP-4. The increased expression of these MMPs and decreased expression of TIMPs in choriocarcinoma may contribute to the invasiveness of this disease. Earlier study has reported that choriocarcinoma also had higher expression of MMP-1 and -2 and lower expression of TIMP-1 than normal placenta, partial mole and complete mole. [15]

It was previously showed that the expression of MMP-1 and MMP-2 but not MMP-3 and MMP-9 was higher in choriocarcinoma than normal placenta. The current study of MMP measurement in choriocarcinoma and normal trophoblast cell lysate and supernatant is supportive of the prior data. Interestingly, the level of MMP-1 was higher in the lysate than supernatant in the choriocarcinoma cell lines. In contrast, the level of MMP-2,-3 and -9 were higher in the supernatant than in the lysate in choriocarcinoma cells and normal trophoblast cells. There may be a distinctly different processing mechanism to limit MMP-1 secretion by choriocarcinoma cells.

Placental site trophoblastic disease is a rare and unique form of GTD with unpredictable malignant potential and highly variable clinical course. It could also present as a fulminant metastatic disease, resistant to conventional treating modalities. The pathophysiology of PSTT is distinctly different from choriocarcinoma. PSTT produce low levels of human chorionic gonadotropin and are less sensitive to chemotherapy than choriocarcinoma. [4] Placental site trophoblastic tumors generally grow more slowly and metastasize later than choriocarcinomas. The lower capacity of PSTT to invade and metastasize may be partly explained by the expression of MMPs and their inhibitors in PSTT cells. MMP-21 is highly expressed in both PSTT and choriocarcinoma. However, as compared to choriocarcinoma, PSTT has less expression of MMP-14 and -28 and higher expression of TIMP-1,-3 and -4.

The known expression patterns of MMPs may provide an opportunity to develop novel therapies in gestational trophoblastic neoplasia. (Figure 14) Drug-resistant gestational trophoblastic tumors may be effectively treated with matrix metalloproteinase inhibitors

(MMPIs). Synthetic MMPIs (e.g. Marimastat, Prinomastat, Tanomastat) have been investigated in both breast and ovarian cancer and these agents may exhibit activity against gestational trophoblastic diseases. [66,67,68] Our findings provide a potential opportunity to develop novel therapy targeting MMPs for choriocarcinoma and PSTT.

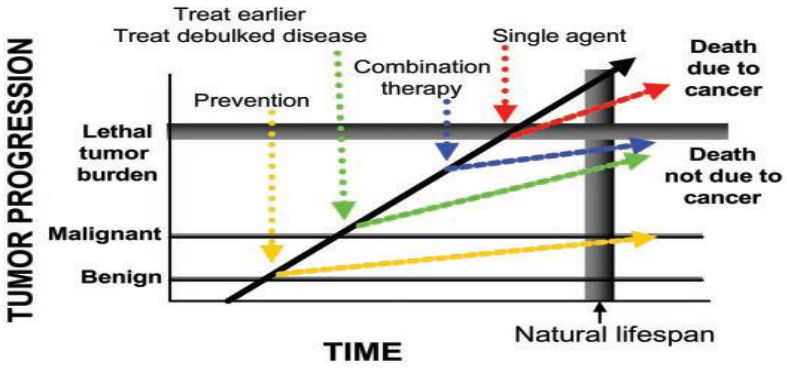


Figure 14. MMP inhibition in control tumor progression. Tumor progression is presumed to be a linear function (black arrow) progressing through stages of benign disease, malignant conversion, and a lethal tumor burden. Effective therapies would reduce the rate of tumor progression so that the arrow crosses the "natural life-span" line before the "death due to cancer" line. Effective cancer prevention would reduce the rate of tumor progression so that the "natural life-span" line is crossed before the "malignant disease" threshold is reached. Treatment of patients with an MPI as a single agent at advanced stages of disease is likely to have minimal impact on survival, resulting in death due to cancer (red arrow). Treatment with an MPI, in combination with other cytotoxic or cytostatic agents, is likely to cause a steeper reduction in the rate of tumor progression (blue arrow). Treatment at earlier disease stages, or treatment of debulked disease (i.e., treated by surgery, radiation, or cytotoxic therapy), should significantly affect tumor progression and prevent death due to cancer (green arrow). Treatment of premalignant disease is likely to prevent malignant conversion (gold arrow) and may be an effective strategy for cancer prevention. [69]

Taken together, these data indicate that we start to understand only the MMP's ability to degradate of ECM. Further studies have to clarify its roles on other fields including angiogenesis, cell growth and cell survival.

CONCLUSION

Matrix metalloproteinases are associated with multiple human cancers and were early considered as drug targets to treat cancer. The first drug development programs based on the notion of blocking MMP mediated metastasis were started about 25 years ago and led to a number of small-molecule metalloproteinase inhibitor drugs (MPIs) in phase III clinical trials. The understanding of MMPs expression pattern in cancer patients could facilitate a fully

rational decision about when and in what combination MPIs and anticancer drugs should be used in the future.

VEGF inhibition has been validated as an effective clinical cancer therapy. Bevacizumab (Avastin) is an efficacious treatment for a wide variety of cancers including colorectal and breast cancer. [55,70] Bevacizumab, a humanized monoclonal antibody, has the ability to inhibit both endothelial cell proliferation and angiogenesis. By binding to biologically active forms of VEGF, it prevents VEGF's interaction with VEGFR-1 and -2. Our findings suggest that Bevacizumab or other anti-VEGF agents could be potential useful drug in the treatment of PSTT and choriocarcinoma. Agents that inhibit VEGF receptors may also be a valuable therapy in the treatment of drug-resistant choriocarcinoma. [71]

The findings obtained from the present study provide a potential opportunity to develop novel therapy targeting MMPs, VEGF and VEGFRs for choriocarcinoma and placental site trophoblastic tumor. By advancing knowledge of the mechanisms regulating invasion and angiogenesis in normal placenta and gestational trophoblastic diseases this information may contribute to the understanding of the biology of gestational trophoblastic disease and foster development of new therapies.

SUMMARY

Gestational trophoblastic diseases are well recognized to have varying propensities for invasion and metastasis. During normal pregnancy, extravillous trophoblasts exhibit tumor cell like invasive capacity and locally invade maternal tissues. This tightly regulated process of trophoblast invasion involves enzymatic degradation of the extracellular matrix. MMPs along with other proteases have been reported to be involved in trophoblastic invasion of maternal tissues. Angiogenesis has also a key role in trophoblastic invasion during normal pregnancy and in tumor cell invasion and metastasis.

This study was undertaken to investigate the expression of matrix metalloproteinases, their inhibitors, regulators and vascular endothelial growth factor and their receptors in normal placenta and gestational trophoblastic diseases including partial mole, complete mole, choriocarcinoma and placental site trophoblastic tumor.

Paraffin sections of 10 normal first-trimester placentas (NP), 10 partial moles (PM), 10 complete moles (CM), 10 choriocarcinomas (CCA) and 10 placental site trophoblastic tumors (PSTT) were studied immunohistochemically for expression of MMP-7, MMP-14, MMP-21, MMP-28, TIMP-3, TIMP-4, CD147, IL-6, VEGFR-1,-2,-3. Immunolocalization of MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1, VEGF and Angiopoietin-1,-2 was performed on 10 choriocarcinomas and 10 PSTTs. The level of MMP-1,-2,-3,-9, VEGF and VEGFR-2 was determined in supernatants and lysates of normal trophoblast, JEG-3 and Jar choriocarcinoma cells with electro-chemiluminescence assays.

CCA showed stronger intensity for MMP-14 and MMP-28 than PSTT ($p<0.05$, $p<0.05$). CCA and PSTT had stronger expression of MMP-21 than NP, PM and CM ($p<0.05$, $p<0.05$, $p<0.01$). PSTT ($p<0.05$, $p< 0.05$), NP ($p< 0.01$, $p< 0.01$) and CM ($p<0.01$, $p< 0.05$) showed stronger staining for TIMP-3 and TIMP-4 than CCA. Higher levels of MMP-2,-3 and -9 but not MMP-1 were found in supernatants than in lysates in normal trophoblast and JEG-3 and Jar CCA cells. Choriocarcinoma had significantly stronger staining of CD147 than partial and complete mole ($p<0.01$, $p<0.01$). PSTT exhibited significantly stronger staining for IL-6 than choriocarcinoma ($p=0.03$). VEGF and Angiopoietin-1 expression in PSTT were significantly higher than in choriocarcinoma ($p=0.007$, $p=0.01$). Choriocarcinoma showed stronger intensity of staining for VEGFR-3 than normal placenta, partial and complete mole ($p=0.036$, $p=0.038$, $p=0.05$).

The high expression of MMPs and low expression of MMP inhibitors in choriocarcinoma might contribute to its invasiveness and metastatic potential. Similarly, the low expression of MMPs and high expression of MMP inhibitors in PSTT might partly explain its lower invasiveness. Placenta site trophoblastic tumors exhibited strong staining for VEGF and choriocarcinoma showed strong staining for VEGFR-3.

Gestational trophoblastic neoplasm is highly curable tumor but infrequently, patient with chemotherapy resistant choriocarcinoma and PSTT need new chemotherapeutic agents. Further understanding of the role of matrix metalloproteinases and angiogenetic factors in the biology of gestational trophoblastic diseases may lead to novel therapies. Agents that inhibit the activity of MMP, VEGF and VEGF receptors may prove to be useful in the therapy of gestational trophoblastic neoplasia.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor Attila Pál for his guidance, enthusiasm and inspiration to make this work possible.

I would also like to express my thanks to Dr.Gábor Németh and Dr.Vilmos Fülöp who were my supervisors and Professor János Szöllősi who was my tutor, they have always guided me with their advices.

I greatly acknowledgements to Professor Ross Berkowitz and Shu-Wing Ng, providing me the possibility to work in the Laboratory of Gynecologic Oncology, Brigham and Women's Hospital, Harvard Medical School.

I am indebted to Professor Yick Fu Wong, Dr. David Kindelberger and Dr. Raina Fichorova for their advice and help in managing laboratory measurements and analyzing all the data gathered for this work.

I additionally offer my thanks to Professor György Bártfai and my former and present colleagues.

Finally, I wish to thank my parents and friends for their help and patience during my work.

REFERENCES

[1] Horowitz NS, Goldstein DP, Berkowitz RS: Management of gestational trophoblastic neoplasia. Semin Oncol 2009;36(2):181-9.

[2] Berkowitz RS, Goldstein DP: Chorionic tumors. N Engl J Med 1996;335:1740-1748.

[3] Lurain JR: Gestational trophoblastic diseases I. Am J Obstet & Gynec 2010;

[4] Seckl MJ, Sebire NJ, Berkowitz RS: Gestational trophoblastic disease. Lancet 2010;376(9742):717-729.

[5] Berkowitz RS, Goldstein DP: Current management of gestational trophoblastic diseases. Gynecol Oncol 2009;654-662.

[6] Kohorn EI, Goldstein DP, Hancock BW, et al: Combining the staging system of the international Federation of Gynecology and Obstetrics eith the scoring system of the World Health Organization for trophoblastic neoplasia. Report of the Working committee of the International Society for the Study of Trophoblastic Disease and the International Gynecologic Cancer Society. Int J Gynecol Cancer 2000;10:84-88.

[7] Kim SJ, Bae SN, Kim JH, et al: Effects of multiagent chemotherapy and independent risk factors in the treatment of high-risk GTT—25 years experiences of KRI-TRD. Int J Gynaecol Obstet 1998;S85–S96.

[8] Bower M, Newlands ES, Holden L, et al: EMA/CO for high-risk gestational trophoblastic tumours: results from a cohort of 272 patients. J Clin Oncol 1997;2636–2643.

[9] Lurain JR, Singh DK, Schink JC: Management of metastatic high-risk gestational trophoblastic neoplasia: FIGO stages II-IV: risk factor score > or = 7. J Reprod Med 2010;55(5-6):199-207.

[10] Lewis SH, Perrin E: Pathology of the Placenta 2nd edition. 1999.

[11] Crescimanno C, Foidart JM, Noel A, et al: Cloning of choriocarcinoma cells shows that invasion correlates with expression and activation of gelatinase A. Exp Cell Res 1996;227:240-251.

[12] Lala PK, Graham CH: Mechanism of trophoblast invasiveness and their control: the role of proteases and protease inhibitors. Cancer Metast Rev 1994;9:369-379.

[13] Huppertz B, Kerschanska S, Demir AY, et al: Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. Cell Tissue Res 1998;291:133-148.

[14] Bishof P, Haenggli L, Campana A: Gelatinase and oncofetal fibronectin expression is dependent on integrin expression on human trophoblasts. Hum Reprod 1995;10:734-742.

[15] Vegh GL, Tuncer ZS, Fulop V, et al: Matrix metalloproteinases and their inhibitors in gestational trophoblastic diseases and normal placenta. Gynecol Oncol 1999;75:248-253.

[16] Sternlicht MD, Werb Z: How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001;17:463-516.

[17] Kessenbrock K, Plaks V, Werb Z: Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010;141(1):52-67.

[18] Iacono KT, Brown AL, Greene MI, et al: CD147 immunoglobulin superfamily receptor function and role in pathology. Exp Mol Pathol 2007;83(3):283-95.

[19] Kanekura T, Chen X: CD147/basigin promotes progression of malignant melanoma and other cancers. J Dermatol Sci 2010;57:149-54.

[20] Castellucci M, Theelen T, Pompili E, et al: Immunohistochemical localization of serine-protease inhibitors in the human placenta. Cell Tissue Res 1994;278:283-289.

[21] Folkman J, Cole P, Zimmerman S: Tumor behavior in isolated perfused organs: In vitro growth and metastases of biopsy material in rabbit thyroid and canine intestinal segment. Ann Surg 1966;164:491-502.

[22] Langer R: Control of tumor growth in animals by infusion of an angiogenesis inhibitor. Proc Natl Acad Sci USA 1980;77:4331-4335.

[23] Ferrara N: Vascular endothelial growth factor: Basic science and clinical progress. Endocrine Reviews 2004;25: 581-611.

[24] Hicklin DJ, Ellis LM: Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. Journal of Clinical Oncology 2005;1011-1027.

[25] Carmeliet P, Ferreira V, Breier G, et al: Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996;380:435–439

[26] Ferrara N, Carver Moore K, Chen H, et al: Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996;380:439–442

[27] Carpini JD, Karam AK, Montgomery L: Vascular endothelial growth factor and its relationship to the prognosis and treatment of breast, ovarian, and cervical cancer. Angiogenesis 2010;13:43-58

[28] Köhne CH, Lenz HJ: Chemotherapy with targeted agents for the treatment of metastatic colorectal cancer. Oncologist 2009;14:478-88

[29] Subramanian J, Morgensztern D, Govindan R: Vascular endothelial growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clin Lung Cancer 2010;11:311-9

[30] Stuttfeld E, Ballmer-Hofer K : Structure and function of VEGF receptors. IUBMB Life 2009; 61:915-22

[31] Cao Y: Positive and negative modulation of angiogenesis by VEGFR1 ligands. Sci Signal 2009;2(59):re1

[32] Achen MG, Stacker SA: Molecular control of lymphatic metastasis. Ann N Y Acad Sci 2008;1131:225-34

[33] Wei LH, Kuo ML, Chen CA, et al: Interleukin-6 in cervical cancer: the relationship with vascular endothelial growth factor. Gynecol Oncol 2001;82:49–56

[34] Salgado R, Vermeulen PB, Benoy I, et al: Platelet number and interleukin-6 correlate with VEGF but not bFGF serum levels of advanced cancer patients. British Journal of Cancer 1999;80:892–897

[35] Benoy I, Salgado R, Colpaert C, et al: Serum interleukin 6, plasma VEGF, serum VEGF, and VEGF platlet load in breast cancer patients. Clinical Breast Cancer 2002;2:311–315

[36] Kanekura T, Chen X: CD147/basigin promotes progression of malignant melanoma and other cancers. J Dermatol Sci 2010;57:149-54

[37] Bougatef F, Quemener C, Kellouche S, et al: EMMPRIN promotes angiogenesis through hypoxia-inducible factor-2alpha-mediated regulation of soluble VEGF isoforms and their receptor VEGFR-2. Blood 2009;114:5547-56

[38] Nagymanyoki Z, Growdon WB, Sarno J, et al: Vascularization and expression of angiogenic factors in partial and complete molar pregnancies. J Reprod Med 2008;53:589-94.

[39] Fichorova RN, Richardson-Harman N, Alfano M, et al: Biological and technical variables affecting immunoassay recovery of cytokines from human serum and simulated vaginal fluid: a multicenter study. Analytical Chemistry 2008;80(12):4741-51.

[40] Xue S, Li SX, Wu ZS, et al: Expression of CD147, matrix metalloproteinases and transforming growth factor beta1 in breast cancer. Zhonghua Bing Li Xue Za Zhi 2009; 38(8):524-8.

[41] Millimaggi D, Mari M, D' Ascenzo S: Vasculogenic mimicry of human ovarian cancer cells: role of CD147. Int J Oncol 2009;35(6):1423-8.

[42] Khankin EV, Royle C, Karumanchi SA: Placental vasculature in health and disease. Semin Thromb Hemost 2010;36(3):309-20.

[43] Pollheimer J, Husslein P, Knöfler M: Invasive trophoblasts generate regulatory collagen XVIII cleavage products. Placenta 2005;Suppl A:S42-5

[44] Hendrix MJ, Seftor EA, Hess AR et al: Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nat Rev Cancer 2003 Jun;3(6):411-21.

[45] Łukaszewicz M, Mroczko B, Szmitkowski M: Clinical significance of interleukin-6 (IL-6) as a prognostic factor of cancer disease. Pol Arch Med Wewn 2007;117(5-6):247-51.

[46] Wang X, Fu S, Freedman RS, et al: Immunbiology of gestational trophoblastic diseases. Int J Gynecol Cancer 2006;16,1500-1515

[47] Sotiriou C, Lacroix M, Lespagnard L, et al: Interleukins-6 and -11 expressions in primary breast cancer and subsequent development of bone metastases. Cancer Lett 2001;169(1):87-95.

[48] Duan Z, Ames RY, Ryan M, et al: CDDO-Me, a synthetic triterpenoid, inhibits expression of IL-6 and Stat3 phosphorylation in multi-drug resistant ovarian cancer cells. Cancer Chemother Pharmacol 2009;63(4):681-9.

[49] Tabibzadeh SS, Santhanam U, Sehgal PB, et al: Cytokine-induced production of IFN-beta 2/IL-6 by freshly explanted human endometrial stromal cells. Modulation by estradiol-17 beta. Journal of Immunology 1989;142(9):3134-3139.

[50] Clark DE, Smith SK, Licence D, et al: Comparison of expression patterns for placenta growth factor, vascular endothelial growth factor (VEGF), VEGF-B and VEGF-C in the human placenta throughout gestation. J Endocrinol 1998;159(3):459-67.

[51] Eichhorn ME, Kleespies A, Angele MK, et al: Angiogenesis in cancer: molecular mechanisms, clinical impact. Langenbecks Arch Surg. 2007;392:371-9.

[52] Lan C, Li Y, He J, et al: Placental site trophoblastic tumor: lymphatic spread and possible target markers. Gynecol Oncol 2010;116(3):430-7.

[53] Cole ME, Broaddus R, Thaker P, et al: Placental-site trophoblastic tumors: a case of resistant pulmonary metastasis. Nat Clin Pract Oncol 2008;5(3):171-5.

[54] Bischoff J, Ignatov A: The Role of Targeted Agents in the Treatment of Metastatic Breast Cancer. Breast Care (Basel) 2010;5(3):134-141.

[55] Murphy JE, Ryan DP: American Society of Clinical Oncology 2010 colorectal update. Expert Rev Anticancer Ther 2010;10(9):1371-3.

[56] Lan C, Li Y, He J, et al: Placental site trophoblastic tumor: lymphatic spread and possible target markers. Gynecol Oncol 2010;116(3):430-7

[57] Cole ME, Broaddus R, Thaker P, et al: Placental-site trophoblastic tumors: a case of resistant pulmonary metastasis. Nat Clin Pract Oncol 2008;5(3):171-5

[58] Smith NR, Baker D, James NH, et al: Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. Clin Cancer Res 2010;16(14):3548-61

[59] Masanori I, Hiroyuki Y, Yasushi A, et al: Role of matrix metalloproteinase-7 (Matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. Exp Biol Med 2006;231:20-27.

[60] Itoh Y. Metalloproteinase binding proteins: WO2009097397. Expert Opin Ther Pat 2010;20(8):1091-5.

[61] García MF, González-Reyes S, González LO, et al: Comparative study of the expression of metalloproteases and their inhibitors in different localizations within primary tumours and in metastatic lymph nodes of breast cancer. Int J Exp Pathol 2010;91(4):324-34.

[62] Andrea K, Ulrike K, Michaela K, et al: Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. BMC Cancer 2009;9:188.

[63] Ahokas K, Lohi J, Lohi H, et al: Matrix metalloproteinase-21, the human orthologue for XMMP, is expressed during fetal development and in cancer. Gene 2002;13:301(1-2):31-41.

[64] Sabeh F, Shimizu-Hirota R, Weiss SJ: Protease-dependent versus -independent cancer cell invasion programs: three-dimensional amoeboid movement revisited. J Cell Biol 2009;185:11-19.

[65] Rodgers UR, Kevorkian L, Surridge AK, et al: Expression and function of matrix metalloproteinase (MMP)-28. Matrix Biol 2009;28:263-72.

[66] Keller U, Bellac CL, Li Y, et al: Novel matrix metalloproteinase inhibitor [18F] Marimastat-Aryltrifluoroborate as a probe for in vivo positron emission tomography imaging in cancer. Cancer Res 2010;70(19):7562-9.

[67] Butler GS, Dean RA, Tam EM, et al: Pharmacoproteomics of a metalloproteinase hydroxamate inhibitor in breast cancer cells: dynamics of membrane type 1 matrix metalloproteinase-mediated membrane protein shedding. Mol Cell Biol 2008;28(15):4896-914.

[68] Hirte H, Vergote IB, Jeffrey JR, et al: A phase III randomized trial of BAY 12-9566 (tanomastat) as maintenance therapy in patients with advanced ovarian cancer responsive to primary surgery and paclitaxel/platinum containing chemotherapy: a National Cancer Institute of Canada Clinical Trials Group Study. Gynecol Oncol 2006;102(2):300-8

[69] Lisa M. Coussens LM, Fingleton B, Matrisian LM: Matrix Metalloproteinase Inhibitors and Cancer—Trials and Tribulation. Science 2002; 295: 2387-2392

[70] Bischoff J, Ignatov A: The Role of Targeted Agents in the Treatment of Metastatic Breast Cancer. Breast Care (Basel) 2010;5(3):134-141

[71] Moreno-Aspitia A: Clinical overview of sorafenib in breast cancer. Future Oncol 2010;6(5):655-63

ABBREVIATIONS

GTN: gestational trophoblastic neoplasm
GTD: gestational trophoblastic disease
PSTT: placental site trophoblastic tumor
CHM: complete hydatidiform mole
PHM: partial hydatidiform mole
CCA: choriocarcinoma
CT: cytotrophoblast cell
ST: syncytiotrophoblast cell
ET: extravillous trophoblast cell
FIGO: Federation of Gynecology and Obstetrics
WHO: World Health Organization
ECM: extracellular matrix
MMP: matrix metalloproteinase
TIMP: tissue inhibitor of matrix metalloproteinase
VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
Ang-1: Angiopoietin 1
Ang-2: Angiopoietin 2
CD147: extracellular matrix metalloproteinase inducer
IL-6: interleukin-6
VM: vasculogenic mimicry

TABLE OF CONTENTS