

Anatomic Site-Related Expression of Cancer-Associated Molecules in Ovarian Carcinoma

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Abstract: Ovarian cancer presents as disseminated disease in the majority of cases. Tumor metastasis to the peritoneal and/or pleural cavity is evident in two-thirds of cases at diagnosis and relapse is most often detected at this anatomic site. Despite the fact that the primary tumor is amenable to surgical removal in the majority of cases, ovarian cancer research, including the evaluation of therapeutic targets, has concentrated on primary disease. In recent years, we analyzed the site-dependent expression of cancer-associated and regulatory molecules in primary tumors, effusions and solid metastases. Our data show that some molecules (e.g., Ets transcription factors) are expressed at all anatomic sites in ovarian carcinoma and that their expression in primary and metastatic disease is associated with poor prognosis. However, the majority of molecules (e.g., cadherins, integrins, and nerve growth factor receptors) are differentially expressed along tumor progression and have different prognostic value depending on the organ sampled. Specifically, cancer-associated molecules with a well-characterized clinical significance in solid tumors (e.g., matrix metalloproteinases) have no such role in effusions. Finally, a growing number of molecules are differentially expressed in primary diagnosis (pre-chemotherapy) and disease recurrence (post-chemotherapy) specimens, reflecting the effect of disease progression and chemotherapy. This review will present the current knowledge in this area.

Keywords: Ovarian carcinoma, effusion, metastasis, tumor progression, chemotherapy, prognosis.

INTRODUCTION

Ovarian carcinoma is the most lethal gynecologic cancer and currently ranks fifth in causing cancer-related deaths among women [1]. For reasons that are not entirely understood, ovarian carcinoma spreads primarily to the serosal surface of the peritoneal cavity and its lining organs, with accumulation of effusion fluid (ascites) that contains numerous tumor cells. This process is clinically manifested as abdominal discomfort or pain, swelling or heaviness, which are the most common presenting symptoms of this disease [2]. Carcinoma cells in ascites often form extensive and multi-focal tumor deposits in the walls of the peritoneal cavity, with a variable degree of stromal invasion. Involvement of pelvic organs, such as the uterus and the fallopian tubes, probably occurs by means of direct extension, while the mechanism behind tumor spread to other abdominal structures is less clear. However, the co-existence of lymph node metastases and peritoneal spread in some patients suggests that lymphatic spread is an additional mechanism for tumor dissemination in ovarian carcinoma [3-4]. Distant lymphatic and/or hematogenous spread of ovarian carcinoma can involve any organ, including the brain [5] and the liver [6], but is distinctly less common. Finally, the pleural space may be involved as well, either at diagnosis or, more commonly, at a later stage, and the pleural cavity is the most common anatomic site for stage IV disease [7].

Ovarian carcinoma is treated by combined surgery and chemotherapy, the latter with platinum compounds and

paclitaxel as choice agents. Although the 5 year survival rate for ovarian cancer patients has improved in recent years, it is still disappointingly low, largely due to the fact that most patients present with metastatic disease, as well as the result of primary or acquired drug resistance [8-10].

Since the primary tumor site in the ovary is amenable to surgical removal in the majority of patients, and since recurrent ovarian carcinoma is in the overwhelming majority of cases diagnosed at metastatic sites, one would expect research on this tumor to focus on metastatic rather than primary disease. In fact, the effort directed at studying primary tumors far surpasses the investment in effusion or solid metastasis research. This discrepancy may be added to three general observations:

1. The expression and biological role of cancer-related molecules in cell lines or *in vivo* models may differ from those seen in clinical cancer.
2. Tumors at disease recurrence are more advanced in terms of tumor progression and are often obtained following radiotherapy or chemotherapy. They may therefore show different expression patterns compared to specimens obtained at primary diagnosis.
3. Observations and findings that apply to one tumor type do not necessarily hold true for other cancers.

These factors, added to differences in cohort size, tumor sub-types and the methods used in different studies, provide some explanation as to why it is almost impossible to achieve universal agreement regarding the validity of molecular markers for targeted therapy or prognosis evaluation in ovarian carcinoma.

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One issue that affects cancer cells in effusions is the unique microenvironment. In solid organs, the synthetic capacity of tumor cells is complemented by three cell populations-stromal myofibroblasts, endothelial cells and leukocytes, predominantly macrophages. In addition, tumor cells are able to induce leaky vessels and obtain nutrients and oxygen, in addition to gaining access to the circulation. In contrast, effusions are a hypoxic microenvironment with reduced access to nutrients, where stromal myofibroblasts and endothelial cells are no longer participating in paracrine tumor-promoting pathways. Mesothelial cells, the native resident cell population of the serosal cavities, together with leukocytes, are able to synthesize many of the proteins that positively regulate tumor growth at this site. However, the main 'responsibility' for survival is left to the diversity and flexibility of cancer cells, possibly explaining why so few cancer types are able to spread to this anatomic site.

This review will focus on data regarding the differential expression of cancer-related molecules in malignant effusions compared to solid primary and metastatic ovarian cancer, as well as differences between primary vs. recurrent tumor cells in effusions. Differences or similarities between ovarian carcinoma and other tumors that involve the serosal cavities will be discussed. Many of the molecules discussed in this review have been analyzed in a large number of excellent studies of solid tumors by other investigators. However, due to space limitations, this paper will focus on effusion biology. The data in the present review question the validity of several molecular targets identified in primary ovarian carcinoma, in other cancers or in *in vitro* models. In the opinion of this writer, these data call for re-evaluation of our research strategy in ovarian cancer.

ADHESION MOLECULES

Cadherins

Cadherins are Ca^{2+} -dependent integral membrane glycoproteins that mediate homophilic contacts with neighboring cells. The cadherin adhesion complex also includes p120catenin, β -catenin and γ -catenin, with intracellular binding to α -catenin and through it to actin molecules in the cellular cytoskeleton [11]. Cadherins have a central role in differentiation and tissue organization during embryonic development and in maintaining tissue structure in the mature organism. E-cadherin, the major cadherin molecule in epithelial cells, is a putative tumor suppressor molecule, and is downregulated in different cancer types epigenetically (through CpG promoter hypermethylation, transcriptional regulation, and post-translational modification) or through mutation along disease progression [12-13]. In different models, E-cadherin may be replaced by (neural) N-cadherin, a cellular event termed epithelial to mesenchymal transition (EMT) that is associated with altered intracellular signaling and enhanced migration and invasion [13]. Loss of β - and γ -catenin similarly affects signal transduction pathways and results in enhanced tumor aggressiveness [12-13].

While this model holds true for several cancer types, it does not apply to ovarian carcinoma, where mutations in the E-cadherin or β -catenin genes are rare [14-15]. At the protein level, E-cadherin and several catenins are only focally

expressed in primary ovarian carcinomas, but their expression is upregulated in effusions and solid metastases, suggesting that E-cadherin has a tumor promoting rather than tumor suppressor nature in this tumor [16]. An additional confounding factor is the fact that ovarian carcinoma cells in effusions show co-expression of E-, N- and P-cadherin, a finding that argues against the EMT model [17]. Finally, the prognostic role of E-cadherin in solid ovarian carcinoma is uncertain [18-20]. Lower E-cadherin mRNA levels in ovarian carcinoma effusions correlate with poor survival [21], but this is not the case for protein expression [unpublished observations].

We analyzed the expression and clinical role of three transcription factors that are negative regulators of E-cadherin - Snail and Slug, members of the Snail superfamily [22] and Smad interacting protein 1 (Sip1), member of the crystallin enhancer binding factor 1 family [23], in ovarian and breast carcinoma effusions. We found that a higher Sip1/E-cadherin ratio is associated with more advanced disease (stage IV vs. stage III disease) and correlates with poor overall survival in ovarian carcinoma, while Snail has a similar role in breast carcinoma [21]. In a recent study of primary and metastatic ovarian carcinomas we found higher Slug mRNA and protein expression in solid metastases and higher Snail protein expression in primary tumors and solid metastases compared with effusions, while SIP1 mRNA expression was highest in effusions [24]. The results of these two studies show that the clinical role and expression of E-cadherin transcriptional regulators varies according to tumor type and site.

Integrins

Integrins are a family of heterodimeric glycoproteins composed of α and β subunits that are involved in invasion, metastasis, angiogenesis, proliferation and apoptosis. Intracellular signaling *via* integrin receptors is initiated in response to cues originating from other cells (e.g., stromal myofibroblasts) or different ECM proteins, including laminin, fibronectin, collagen, vitronectin, entactin, tenascin and fibrinogen, and mediates synthesis of many cancer-associated molecules [25]. Altered expression of integrins (down- or up-regulation) has been detected in the majority of malignant tumors, but varies considerably according to the origin of the neoplasm [26].

In vitro studies have demonstrated that different integrins are involved in the interaction of ovarian carcinoma cells with ECM molecules in ovarian cancer, and that attachment to the peritoneal mesothelium involves the $\beta 1$ integrin subunit and CD44, an adhesion molecule of the immunoglobulin superfamily [27-29]. In clinical material, we detected frequent expression of the αv integrin subunit in ovarian carcinoma at all anatomic sites, but found higher expression of the $\beta 1$ subunit in effusions compared to solid lesions [30]. In a study of laminin receptors, we analyzed the expression of the $\alpha 6$ subunit and the non-integrin 67kDa laminin receptor in 88 effusions and 116 corresponding solid tumors [31]. We found higher expression of the $\alpha 6$ subunit mRNA in effusions compared to corresponding solid tumors, and confirmed the presence of this subunit in carcinoma cells in 17/27 effusions using flow cytometry [31]. These results differ from those reported in a limited

analysis that included 6 effusion specimens, where decreased expression of the $\alpha 6$ and $\beta 4$ subunits (components of the $\alpha 6\beta 4$ integrin laminin receptor) was found compared to solid lesions [32]. The 67kDa receptor was frequently expressed in both effusions and solid lesions, on both mRNA and protein level. Parenthetically, $\alpha 6$ integrin and the 67kDa laminin receptor are frequently expressed in breast carcinoma, but do not show differential expression with respect to anatomic site [33]. The sustained or upregulated expression of ECM receptors of both the integrin and non-integrin type in metastatic ovarian carcinoma suggests that these molecules may have a central role in tumor progression in this cancer.

PROTEASES

Matrix Metalloproteinases (MMP)

MMP, a family of more than 20 zinc- and calcium-dependent enzymes, degrade all major basement membrane and ECM components and are crucial mediators of all central events in tumor progression, including local invasion, angiogenesis and metastasis [34]. MMP have been previously divided into sub-families based on substrate specificity. However, due to the overlapping substrate range between different members, MMP are currently classified into 8 classes based on domain structure [35]. MMP-2 (Gelatinase A, 72kD type IV collagenase) and MMP-9 (Gelatinase B, 92kD type IV collagenase), the only enzymes with a gelatin-binding domain, are crucial for tumor metastasis due to their ability to degrade collagen type IV, a component of all basement membranes [34]. Additional MMP substrates include other MMP members, proteinases of different families (e.g., plasminogen), growth factors (transforming growth factor; TGF), tyrosine kinase receptors (Her2/neu, FGFR1), adhesion molecules (CD44, E-cadherin, αv integrin), and other molecules [34-35]. MMP activity is negatively regulated in a reversible manner by specific inhibitors, TIMP1-4, through the formation of a 1:1 stoichiometric binding, as well as by $\alpha 2$ macroglobulins, thrombospondins, and the membrane-bound RECK protein [34]. However, TIMP-2 also participates in cell surface-mediated activation of MMP-2 with membrane type 1-MMP (MT1-MMP, MMP-14) [35]. Different ECM proteins, growth factors and cytokines activate MMP synthesis (e.g., *via* integrin receptors), with transcriptional regulation mediated through binding of Ets family members, AP-1 and AP-2, and additional factors [34-35].

MMP and TIMP have been shown to be expressed in and synthesized by both carcinoma cells and stromal myofibroblasts in ovarian carcinoma, as shown by studies of protein and mRNA expression, respectively, although results from various groups differ [36-42].

In our two cohorts, MMP-2, MMP-9 and TIMP-2 mRNA was found in both tumor and stromal cells, while MT1-MMP was predominantly expressed in tumor cells, suggesting that ovarian cancer cells are able to produce MMP-2 and its co-activators TIMP-2 and MT1-MMP in an autonomous manner [40-41]. Similar results were reported in an additional study of MMP-9 and TIMP-1 [42]. Our comparative analysis of ovarian carcinoma at different anatomic sites showed upregulated expression of MMP-2 in effusions compared to primary tumors [41]. We additionally

showed that MT1-MMP and MT2-MMP, but not MT3-MMP, are expressed in ovarian carcinoma effusions [43].

The prognostic role of MMP and TIMP in ovarian carcinoma is not entirely decided, but several studies by others and we show correlation between tumor and/or stromal MMP and TIMP expression and survival in this cancer type [40, 44-45]. In our series of patients with a follow-up period of up to 20 years, TIMP-2 mRNA expression in stromal cells and MMP-9 and TIMP-2 mRNA expression in carcinoma cells of primary tumors correlated with poor outcome in univariate analysis. In metastatic lesions, the presence of TIMP-2 mRNA in stromal cells and of MMP-2 and MT1-MMP mRNA in tumor cells correlated with poor outcome. In a multivariate analysis, TIMP-2 mRNA expression in stromal cells and MMP-9 mRNA expression in tumor cells were independent predictors of poor survival [40]. In contrast, MMP-1, MMP-2, MMP-9 and TIMP-2 expression in effusions does not correlate with survival [40], suggesting that the clinical role of MMP and TIMP is limited to solid lesions. This would be supported by our findings regarding EMMPRIN (CD147), a membrane glycoprotein that mediates signaling events leading to MMP synthesis [48] and binds MMP-1 and integrins on the surface of tumor cells [46-48]. We have shown that EMMPRIN mRNA and protein are widely expressed on ovarian carcinoma cells in effusions and solid tumors, and that its presence is associated with MMP and integrin subunit expression and with activation of the mitogen-activated protein kinase (MAPK) signaling pathway [49-50]. However, only EMMPRIN expression on peritumoral stromal and endothelial cells in solid tumors correlated with poor survival [49].

Kallikrein 4

Kallikrein 4 is member of the tissue Kallikreins (KLKs), a family of serine proteases that currently consists of 15 different members, all encoded by a single gene cluster located at chromosome region 19q13.4 [51]. The roles of KLKs are poorly defined at present, but current data suggest that they are involved in proteolysis of various molecules in body fluids, such as semen, and activation of other proteases [51]. We recently found that Kallikrein 4 is upregulated in ovarian carcinoma effusions compared to solid lesions [52]. However, expression of this protease was significantly lower in breast carcinoma and malignant mesothelioma effusions, suggesting that this protease has different roles in tumor progression in these malignancies (Davidson *et al.* submitted).

ANGIOGENIC MOLECULES AND GROWTH FACTORS

Angiogenic Molecules

The ability of solid tumors to grow locally, and subsequently disseminate to distant organs, is dependent upon the formation of new blood vessels (angiogenesis), the presence of which increases nutrient supply and facilitates vascular invasion by tumor cells [53]. This process involves a large number of angiogenic factors, including vascular endothelial growth factor (VEGF), basic and acidic fibroblast growth factor (aFGF, bFGF), TGF α and TGF β , platelet-

derived growth factor (PDGF), interleukin-8 (IL-8) and heparanase [53-54].

The VEGF family currently consists of seven members, VEGF-A to VEGF-F and placental growth factor (PlGF), that mediate their effects through the tyrosine kinase receptors VEGFR1-3 [55]. VEGF-A has six isoforms consisting of 121-206 amino acid residues, as a result of alternative splicing. It induces proliferation, sprouting, migration and tube formation in endothelial cells and is a key molecule in tumor angiogenesis [55].

bFGF (FGF-2), a 146 amino-acid polypeptide, is part of a family that at present consists of 22 members in vertebrates, the majority of which are secreted [56]. FGF signaling involves various receptors, including FGF tyrosine kinase receptors, integrins and heparan sulfate proteoglycans, and induces proliferation, motility and angiogenesis [57-58].

IL-8 (CXCL8) is a member of the chemokine family, small molecules that regulate the immune response and mediate several cancer-related events, including angiogenesis (see below) [59-60].

As in essentially all solid tumors, angiogenesis plays an important biological role in local growth and metastasis in ovarian cancer. Angiogenic factor expression has been shown to be elevated in ovarian carcinoma compared to benign lesions and in metastatic compared to primary tumors in several studies (reviewed in [61]). We analyzed the expression of VEGF, bFGF and IL-8 in two cohorts of ovarian cancer patients, one in which the material consisted of solid primary and metastatic tumors, with patient follow-up of up to 20 years, the other with effusions and corresponding solid lesions. We found frequent expression of the three angiogenic molecules in tumor and stromal cells, but observed no correlation between VEGF, bFGF or IL-8 expression and survival [62-63]. In addition, although bFGF expression was consistent at all anatomic sites, VEGF and IL-8 were downregulated in carcinoma cells in effusions [63]. These data suggest that angiogenic molecules exert their most crucial effect in the primary tumor and therefore in the earlier stages of tumor progression. We hypothesize that carcinoma cells in effusions may reduce the synthesis of several of these molecules due to lack of need to induce angiogenesis in the effusion fluid. In support of this hypothesis, we recently observed reduced expression of IL-8 and bFGF in breast carcinoma effusions compared to corresponding solid tumors [64], and reduced expression of bFGF and heparanase in malignant mesothelioma effusions [65]. The anti-angiogenic drug bevacizumab (Avastin) is currently being tested in a randomized study as part of triple agent therapy with carboplatin and paclitaxel in suboptimally debulked ovarian cancer [66]. It remains to be seen whether this drug can be effective in treating patients with malignant effusions.

Nerve Growth Factor (NGF) Receptors

Neurotrophins are a family of growth factors, consisting at present of the prototype compound nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, NT-4 and NT-6 [67-69]. Neurotrophins bind in a specific manner to the tyrosine kinase receptors TrkA, TrkB and TrkC. NGF binds to TrkA, thereby activating TrkA

autophosphorylation at several sites such as tyrosine 490, promoting SHC binding and phosphorylation, coupling of GRB2-SOS complexes, activation of Ras and signaling *via* the mitogen activated protein kinase (MAPK) and phosphoinositol-3-kinase (PI3K)/AKT pathways, with resulting survival and proliferation [70-71]. p75, an additional neurotrophin receptor, belongs to the tumor necrosis receptor family, has a different structure, lacks intrinsic catalytic activity and is able to bind all neurotrophins [67]. p75 is able to activate both pro-survival and apoptotic signaling pathways [71].

Though originally discovered as a proto-oncogene of the nervous system, TrkA is expressed in tumors of both neural (pheochromocytoma and neuroblastoma) and non-neural origin, the latter consisting primarily of carcinomas [72-73]. In contrast, p75 expression was detected in some neural and soft tissue tumors, while most carcinomas were negative [74].

We analyzed the clinical role and anatomic site-related expression of activated phospho-TrkA (p-TrkA) and p75 in ovarian carcinoma, breast carcinoma and malignant mesothelioma (MM). We found p-TrkA in a large number of specimens in all three tumor types, with less frequent expression of p75 [75-78]. Total Trk and p-TrkA expression was higher in solid tumors compared to effusions in ovarian carcinoma, with an opposite finding for p75 [75-76]. However, in breast carcinoma and MM, p-TrkA was upregulated in effusions compared to solid tumors [77-78]. NGF was expressed in ovarian and breast carcinoma cells, consistent with the presence of an autocrine growth factor pathway for NGF-TrkA in these tumors [75, 77]. p-TrkA was additionally expressed on endothelial cells, predominantly in ovarian carcinoma and MM, supporting its role as an angiogenic factor, as has been shown in different experimental models [79-80]. p-TrkA and NGF expression correlated with poor survival in ovarian and breast carcinoma, respectively [76-77]. p-TrkA is additionally a marker of poor survival in malignant melanoma [81]. We recently showed that p-TrkA expression in ovarian carcinoma is a late event, occurring in FIGO stage III-IV, but only rarely in stage I carcinomas or in tumors of low malignant potential (LMP, borderline tumors) [82]. These data support a role for TrkA in tumor progression of three solid tumors involving the serosal cavities, and possibly in a subset of melanomas, and suggest that this molecule may be an attractive target for molecular therapy in these cancers.

Granulin-Epithelin Precursor (GEP)

GEP (progranulin/ PC-cell derived growth factor) is a 68 kDa secreted protein with multiple glycosylated variants, the most common of which is 88 kDa in size [83-84]. GEP also is cleaved into its component granulins (epithelins), small proteins of 6 kDa in size that have inhibitory function, opposing that of GEP [85]. GEP has been shown to have a role in both physiological processes, including embryogenesis and wound repair, and pathological processes, such as tumorigenesis [83, 86-87]. GEP was identified as an autocrine growth factor for ovarian carcinoma through its upregulation in tumor cells of invasive ovarian carcinomas compared to borderline tumors, and is additionally expressed in peritumoral myofibroblasts and endothelial cells [88].

Transfection of OVCAR-3 ovarian carcinoma cells with antisense GEP reduces cell growth and proliferation, and induces loss of density-independent growth [88]. GEP synthesis is regulated by endothelin-1 (ET-1) and lysophosphatidic acid (LPA), two additional growth factors with a central role in ovarian carcinoma, as well as by cyclic AMP (cAMP) in HEY-A8 and OVCAR-3 ovarian carcinoma cells. Activation of synthesis *via* cAMP is mediated through exchange protein activated by cAMP (EPAC) and the extracellular regulated kinase (ERK) MAPK family member [89]. Neutralizing anti-GEP antibody induced apoptosis in ovarian carcinoma cells [89].

We recently analyzed 190 effusions and 189 solid tumors for GEP expression. GEP was expressed in tumor cells in 90-95% of ovarian carcinoma effusions and solid lesions, but staining was seen in a higher percentage of cells in primary tumors and solid metastases compared to malignant effusions. Frequent GEP expression was seen in reactive mesothelial cells in effusions and in stromal and endothelial cells in solid tumors [90]. GEP expression was lower in stromal cells in solid metastases obtained post-chemotherapy compared to pre-chemotherapy specimens, and stromal expression in the latter group correlated with worse overall survival [90]. The data obtained in these *in vitro* and clinical studies suggest that GEP may be a new molecular therapeutic target in ovarian carcinoma.

SIGNALING MOLECULES

MAPK

The MAPK intracellular signaling pathway is a four-level cascade, in which each kinase activates the following kinase substrate through a complex network, enabling the cell to maintain diversity and specificity while responding to various extracellular cues [91-95]. MAPK tyrosine and threonine phosphorylation at the final level of the cascade occurs in an enzyme-specific manner by the MEK family of MAPK kinases. This double phosphorylation activates the 3 MAPK family members- ERK, c-jun amino-terminal kinase (JNK) and the high osmolarity glycerol response kinase (p38 $\alpha, \beta, \gamma, \delta$) [92-93]. This tightly regulated process is negatively regulated by dual specificity phosphatases (DUSP) that deactivate the enzymes [96-97].

JNK and p38 are activated by a large spectrum of stress-related stimuli [95], whereas ERK is largely activated by growth factor signals [92]. Activation of MAPK is followed by phosphorylation of a variety of cytosolic substrates, as well as their translocation to the nucleus, where they activate a large number of transcription factors, including AP-1 and Ets-1 [92]. It is now widely accepted that the simplified scheme, by which p38 and JNK mediate apoptotic signals, while ERK promotes growth, differentiation and proliferation, does not fully reflect the complex biology of cancer cells, especially in clinical material.

Our first study of MAPK in clinical material focused on ovarian carcinoma effusions. We analyzed the expression (total enzyme level, pan-MAPK) and activity (phosphorylated fraction, p-MAPK) of ERK, JNK and p38 in 64 specimens. Higher p38 and JNK level and activity were significantly associated with favorable clinicopathologic parameters, such as younger age and better tumor

differentiation. In univariate survival analysis, pan-ERK and pan-JNK levels and p-ERK activity correlated with longer overall survival. pan-ERK and pan-JNK were independent prognostic markers in Cox multivariate survival analysis [98]. These findings raise serious doubts regarding the validity of ERK signaling, a molecular target that is actively investigated by the industry in recent years [99], as a therapeutic target in ovarian carcinoma. This evidence is further supported by the finding that mRNA expression of PAC-1, member of the DUSP family that inactivates MAPK through dephosphorylation, correlates with poor survival in ovarian carcinoma patients with effusions [100]. In agreement with our findings, correlation between p-ERK expression in the primary tumor and improved survival was recently shown in a large cohort of ovarian carcinoma patients [101].

Following these rather unexpected results, we performed a comparative analysis of MAPK expression levels and activation in malignant mesothelioma and compared it to the benign counterpart of this tumor, reactive mesothelial cells. We found that malignant mesothelioma cells and reactive mesothelial cells have comparable MAPK expression and activation [102], a finding that argues against the importance of these enzymes in the malignant transformation of mesothelial cells.

Recently, we studied MAPK activation along tumor progression in breast carcinoma, by comparing primary tumors, lymph node metastases and effusions. We found that p-p38 and p-JNK, but not p-ERK are upregulated in breast carcinoma effusions, a finding that we attribute to a stress-related protective mechanism rather than apoptosis, in view of the minimal fraction of apoptotic cells at this anatomic site and the aggressive nature of the disease once tumor cells reach the pleural cavity [103]. Higher p38 activation ratio (p-p38/pan-p38 ratio) correlated with shorter survival. p-ERK expression in effusions had no clinical role, while its presence in primary tumors correlated with improved survival [103]. These findings are in agreement with two recent reports in which p-ERK expression in primary breast carcinoma has been shown to correlate with improved outcome [104-105]. Once again, these data strongly argue against the rationale behind targeting ERK signaling in cancer. Despite the undisputed role of ERK in the induction of proliferation, the cellular interactions and downstream pathways that this enzyme mediates in cancer cells are probably far too complex to be categorized as tumor-promoting, and are probably in part associated with less aggressive behavior.

Analysis of Proliferation, Survival and Apoptosis Pathways Using Proteomics

Another approach we recently used for studying signaling in ovarian carcinoma was lysate array proteomics. In collaboration with the laboratory of Dr. Elise Kohn at NCI/NIH, we analyzed the total expression and activation of 13 molecules, including tyrosine kinase receptors, MAPK, AKT, GSK3 β , CREB and caspases, in 61 malignant and 9 benign effusions [106]. Malignant effusions had higher expression of AKT, CREB, JNK, p-ERK, and p-CREB compared to benign specimens. Malignant pleural effusions could not be differentiated from ascites by signaling profiles,

a finding that we have repeatedly observed in other studies. Expression and activation levels were separately analyzed for clinical significance in patients with pre- and post-chemotherapy effusions. Using this approach, Cox proportional hazards model analysis showed that high p38 and pEGFR/EGFR ratio independently predict poor survival in pre-chemotherapy cases, while p-JNK level is associated with worse outcome in post-chemotherapy patients [106]. The data for MAPK differ from those in the above-mentioned study using Western blotting [98], a finding that is most likely related to the separate analysis of pre- and post-chemotherapy cases. Differences in the method applied and the antibodies used may have also contributed to the discrepancy. However, it is noteworthy that ERK had no relationship to aggressive clinical behavior in this study as well, despite its upregulation in malignant compared to benign effusions. We are currently analyzing the clinical role of the AKT pathway in a larger cohort.

TRANSCRIPTION FACTORS

The oncogene v-ets was discovered as part of the gag-myb-ets fusion protein of the avian retrovirus E26, and is able to induce leukemia *in vivo*. The Ets transcription factor family consists of 27 members to date, all containing an 85 amino acid DNA-binding domain (the Ets domain) that confers the ability to bind to DNA sequences having the core motif GGAA/T (Ets-binding site, EBS) [107-109]. Another conserved area that is present in 11 members is the pointed (PNT) domain, which mediates protein-protein interactions and oligomerization [107]. Ets factors have 200 known target genes, including proteases (MMP-1, -3 and -9, cathepsin) and their inhibitors (TIMP-1), cell cycle molecules (Cyclin D1, p21), apoptosis promoters and inhibitors (Fas, PARP, Bcl-2, Bcl-XL), adhesion molecules (E-cadherin, integrins), immune response mediators (interleukins, immunoglobulins), and angiogenesis mediators (the VEGF receptors Flt-1 and flk-1, Tie-1 and -2) [110]. In these multiple target genes, Ets factors can mediate transcriptional activation or repression according to the binding factor and the DNA sequence involved.

Analyses of our two cohorts of ovarian carcinoma patients have shown that Ets-1 and PEA3 mRNA expression is a biological marker of poor survival in both solid lesions and effusions, providing one of the rare examples of clinical relevance at both disease sites [111-114]. We were additionally able to show that Erg and Ets-2, two additional Ets family members, are expressed in ovarian carcinoma cells in effusions [114]. The co-expression of at least 4 Ets factors at an anatomic site where cellular economy dictates downregulation of redundant molecules, suggests that Ets transcription factors may be essential for the biology of ovarian carcinoma. This is also supported by the co-expression of Ets members with its target genes or regulators, including integrins, MMP and angiogenic molecules, an association we demonstrated in both cohorts [111-115].

THE IMMUNE RESPONSE

One of the complex areas that best exemplify the difficulty in extrapolating *in vitro* data into clinical practice

is the immune response to cancer. There is little doubt that cancer cells are able to counteract and even manipulate to their own benefit many of the defensive and offensive mechanisms that are mounted by the host immune system, since leukocytes are present in essentially all tumors while proving effective in only a minority. The nature of this phenomenon is, however, far from clear. Two areas in this field of tumor biology underscore this point.

HLA-G

One of the mechanisms by which tumor cells are able to escape the immune response *in vitro* or in animal models is through down-regulation of major histocompatibility complex (MHC) class I molecules, which are present on all normal cells, or by expressing non-classic human leukocyte antigens such as HLA-G and HLA-E [116-117].

HLA-G is present as a membrane-bound or a soluble form, and its expression in normal tissues is limited to trophoblastic cells, where it is postulated to mediate immune tolerance during pregnancy [117-118]. Although a variety of tumors, including melanomas, lymphomas and carcinomas of the lung, breast and kidney, express HLA-G, there are few studies that have dealt with the clinical role of this molecule and its importance in mediating immune response evasion in clinical cancer is largely unproven.

Measurement of HLA-G in the ascites fluid using ELISA shows significantly higher levels in malignant effusions compared to their benign counterparts [119]. In analysis of more than 300 ovarian carcinoma specimens, of which approximately 50% were effusions, we found that HLA-G is expressed in 33-50% of ovarian carcinomas depending on anatomic site. HLA-G expression in tumor cells was significantly lower in post-chemotherapy compared to pre-chemotherapy effusions. Surprisingly, the presence of HLA-G-positive tumor cells in effusions obtained prior to the institution of chemotherapy correlated with better overall survival, while its expression in primary tumors and solid metastases showed no correlation with clinical parameters or survival [120]. The reduced expression of HLA-G in post-chemotherapy effusions and its correlation with improved survival may be related to preferential susceptibility of HLA-G-expressing cells at this site, but argue against a role for this molecule in mediating more aggressive clinical behavior in ovarian carcinoma.

Recently, we studied the expression of HLA-G in breast carcinoma and malignant mesothelioma (MM) [121]. We found predominantly focal HLA-G expression in 26% of solid lesions in both tumor types, with higher expression in effusions (41% in breast carcinoma, 54% in MM). This difference was statistically significant for MM. MM cells in effusions showed higher HLA-G expression compared to both ovarian and breast carcinomas, but results were opposite in solid lesions. Flow cytometry analysis showed that expression of the normal HLA molecules (HLA-ABC) is conserved in cancer cells in effusions in both tumor types. Breast cancer patients with HLA-G-positive tumor cells had a trend for shorter disease-free survival, but no significant correlation with disease outcome was found [121]. The results of this study suggest that HLA-G is not universally expressed in MM and breast carcinoma, and that its

expression does not involve loss of HLA-ABC. Although the upregulated expression of HLA-G in MM effusions may suggest a role in immune response evasion in some cases, establishing the clinical relevance of this marker requires additional data.

Chemokine Receptors

The chemokine family consists of 41 members that are divided into four classes, C-C, C-X-C, C and C-X₃-C, depending on the location of the first two cysteines in their sequence, and exert their biologic role *via* specific chemokine receptors [59]. Chemokines produced by cancer and stromal cells attract lymphocytes and monocytes expressing their receptors to the tumor site. In addition, expression of chemokine receptors on tumor cells has been hypothesized to create an autocrine loop that mediates pro-growth signals (e.g., the above-described CXCL8/IL-8 and its receptors CXCR1/2), regulates angiogenesis (CXCL8, CXCL10, CXCL12) and promotes metastasis (CXCL12 and its receptor CXCR4) [59]. These effects have been largely shown *in vitro* and in animal models [59-60].

IL-8 is expressed in clinical ovarian carcinoma, although its expression is reduced in effusions compared to solid tumors [62-63]. Work by others investigators have shown expression of multiple chemokines and the receptors in leukocytes isolated from ovarian carcinoma effusions and secretion of chemokines into the effusion fluid [122-123]. However, these studies have not shown that chemokine

receptors are expressed in ovarian carcinoma cells and have not provided information regarding the clinical significance of these biological pathways. In order to investigate this issue, we analyzed the expression of 5 chemokine receptors (CXCR1, CXCR4, CCR2, CCR5 and CCR7) in 73 ovarian carcinoma effusions using flow cytometry. Since recent studies have shown correlation between the presence of CD3⁺ T lymphocytes and regulatory T cells and survival in primary ovarian carcinoma [124-125], we additionally studied the presence and clinical role of B and T lymphocytes, monocytes, neutrophils and natural killer (NK) cells in our cohort using leukocyte markers (CD3, CD4, CD8, CD4/CD8 ratio, CD16, CD19, CD14). CXCR4, CCR5 and CCR7 were abundantly expressed on leukocytes, but all receptors were rarely expressed on cancer cells. Surprisingly, the presence of NK cells and CD19-positive B lymphocytes predicted poor survival [126]. The presence of T lymphocytes (CD4-positive T helper or CD8-positive T suppressor/cytotoxic cells) in effusions showed no correlation with survival. The rare expression of chemokine receptors in ovarian carcinoma cells in effusions argues against an autocrine chemokine pathway in this malignancy. Our data additionally demonstrate that the presence of immune response effectors in effusions fails to improve the clinical outcome and that the host anti-tumor response at this metastatic site is therefore ineffective. Work in progress from our laboratory demonstrates rare expression of chemokine receptors also in MM [127] and breast carcinoma (in preparation).

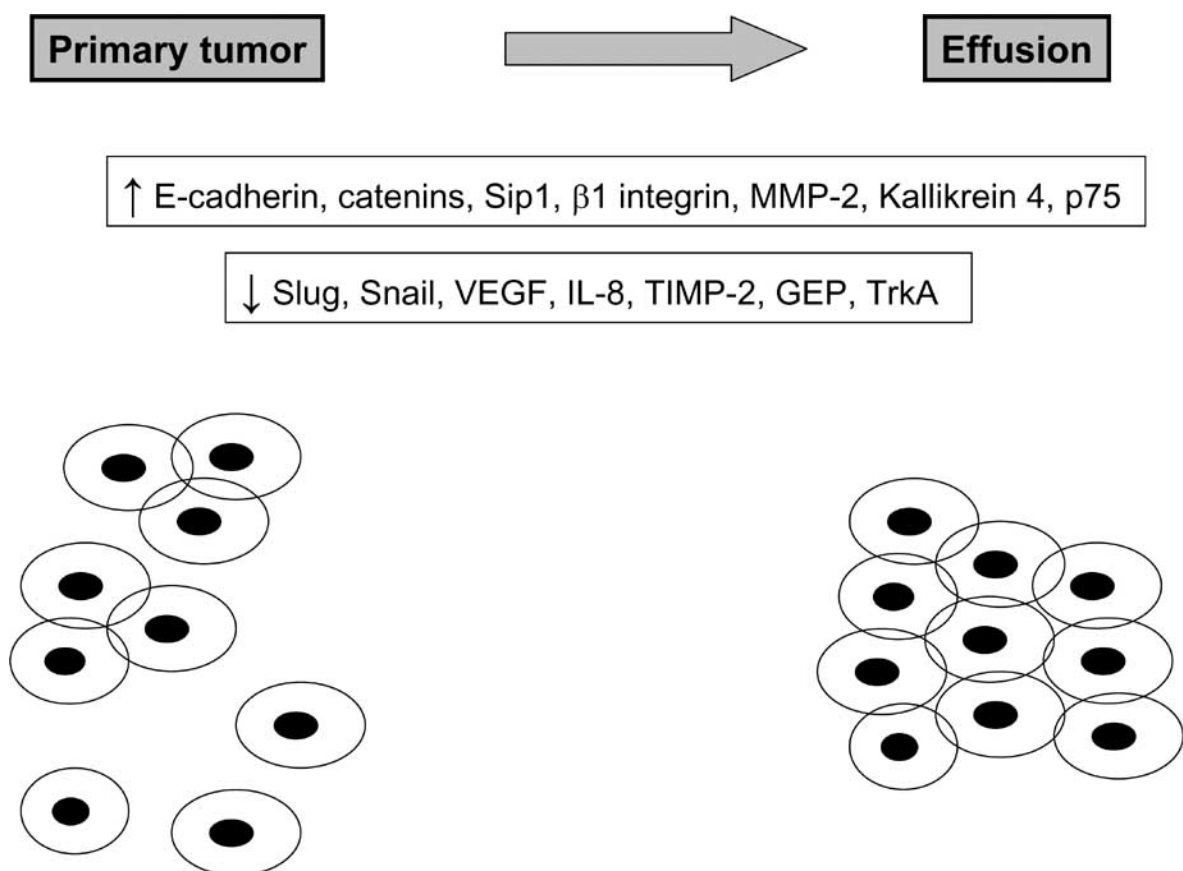


Fig. (1). Cancer cells in effusion show increased intercellular adhesion and altered expression of cancer-associated molecules, including adhesion molecules, proteases, angiogenic molecules and tyrosine kinase receptors.

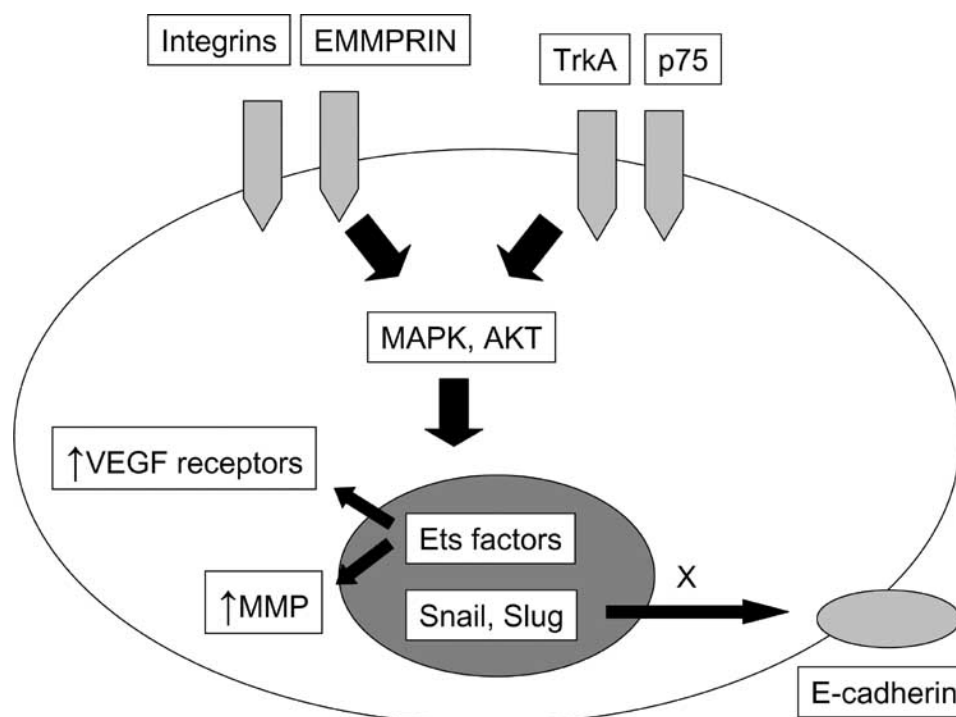


Fig. (2). Simplified view of some of the known interactions involving molecules analyzed in our cohort.

FUTURE DIRECTIONS

The data obtained from our studies clearly demonstrate that tumor heterogeneity is a complex issue that extends far beyond the presence of different cell populations in the primary tumor. Metastasis to solid sites, and especially to effusions, is associated with profound changes in tumor cells (Fig. 1) and may be regulated by cross talk with cells in the tumor microenvironment or by survival cues of the cancer cell itself (Fig. 2). There is therefore clear need to analyze the expression and clinical role of possible molecular targets in the cells that are designed to be targeted. In the case of ovarian carcinoma, these cells are primarily metastatic cells.

Our laboratory continues to investigate biological pathways that provide carcinoma cells in effusions with survival advantage. An aspect of our collaborative work continues to focus on metastasis-related pathways and their differential expression at different anatomic sites, most recently in analysis of the phospholipase A₂ autocrine pathway [128]. Other collaborative studies focus on the use of advanced molecular tools such as SAGE and digital karyotyping in order to discover novel molecules that are involved in ovarian carcinoma, with simultaneous expression analyses of primary and metastatic tumors [129-134]. One promising aspect of high throughput studies is the possibility to probe for tumor type-specific expression patterns. Using gene expression arrays, we recently found that MM and ovarian carcinoma cells in effusions differentially express more than 150 genes, including adhesion molecules (claudins), ECM components (vitronectin, laminin), growth factors (IGF-II), proteases (MMP-7), signaling molecules (Notch3) and cell cycle molecules (cyclin E1) [135]. Many of these molecules have an established biological and/or clinical role in solid ovarian carcinoma, but have not been studied in effusions. We are

currently expanding the analysis of these molecules to our entire cohort. It is our hope that more research by other groups will focus on metastatic cells and that this combined effort will enable us to design more effective future treatment to ovarian carcinoma and other tumors.

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