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SEMINAR

Molecular Pathology of Tumor Metastasis

I. Predictive Pathology

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Millenium reviews of oncology agreed that the last century produced major developments mainly in the management of the primary tumor, but despite all of these results, cancer still remains among the leading causes of death due to the failure of clinical management of disseminated disease. This failure is primarily due to the lack of detailed information on the molecular mechanisms of tumor metastasis. Therefore, one of the hottest fields in experimental oncology is metastasis research, which provides more and more information about the molecular mechanisms. However, this information is fragmented and is not yet exploited in clinical practice. A new field of diagnostic pathology recently emerged, which translates basic research data to diagnostic practice to provide clinically relevant information on the biological potential (in this case metastatic potential) of the malignant tumors. Since tumor cell-extracellular matrix interactions are key features of tumor dissemination, expression of genes responsible for them can define the metastatic potential of malignant tumors. This review summarizes our recent knowledge on the metastatic geno- and phenotype of major human solid tumors: lung, colon, breast, prostate cancers and malignant melanoma. (Pathology Oncology Research Vol 7, No 3, 217–230, 2001)

Keywords: metastatic cascade, extracellular matrix interaction, metastatic phenotype, lung cancer, breast cancer, colon cancer, prostate cancer, malignant melanoma

Introduction

Millenium reviews of oncology agreed that the last century produced major developments in oncology, primarily in the management of the primary tumor, but despite all of these results, cancer still remains among the leading causes of death due to the failure of clinical management of the disseminated disease. This failure is primarily due to the lack of detailed information on the molecular mechanisms of tumor metastasis. Therefore, one of the hottest fields in experimental oncology is metastasis research, which provides more and more information about the molecular mechanisms. However, this information is fragmented and is not yet exploited in clinical practice. This series intends to summarize our knowledge on the molecular mechanisms of tumor dissemination and to show their clinical relevance in two related fields: predictive pathology (this issue), molecular diagnostics (upcoming issue). The series will be finished by a summary of the new pathomechanism-based therapeutic approaches (in a later issue).

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Molecular mechanism of metastasis

Uncontrolled tissue proliferation can result in benign or malignant tumors. There are several macroscopic and microscopic features which may help to distinguish between the two, but the ultimate feature which has 100% specificity and 100% selectivity is the tumor cell invasion. However, tumor progression may not stop at this point but develop into metastatic disease. Tumor (or malignant) progression can be considered both as a cascade of events as well as a continuous selection process *(Table 1).*[21] There are considerable similarities between various levels of this cascade. One key event is tumor-induced neoangiogenesis, without which tumor tissue cannot growth beyond the size of 1-2 mm^3 at the primary nor at the

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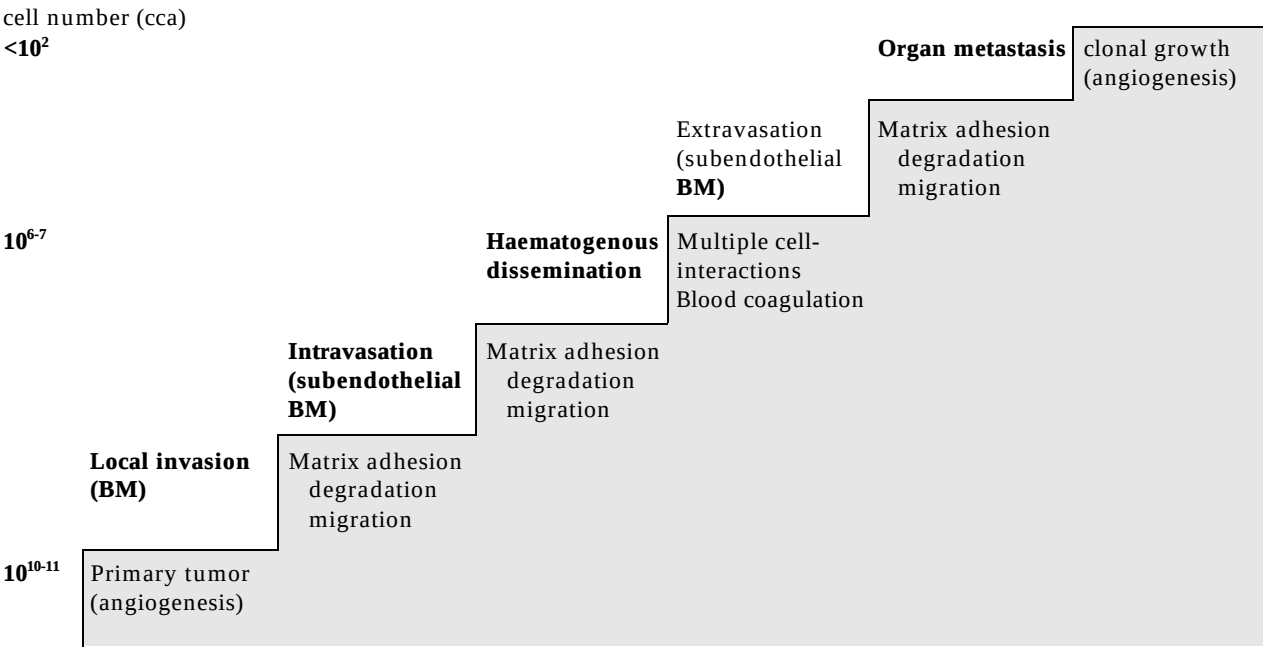
Table 1. **The metastatic "staircase"**



metastatic site.[25] The other continuous feature of this cascade is the surveillance of the immune system which must be permissive for the propagation of the process (as described in other excellent reviews). Thirdly, and possibly the most important event of this cascade, is the repeated interaction of the tumor cells with the surrounding (actual) extracellular matrix of the primary site, of the blood vessels, and of the metastatic site *(Table 1).*[54] Since both benign as well as malignant tumors can grow beyond the critical size, induction of angiogenesis is not specific for malignant tumors. It is accepted accordingly, that the malignant cell-ECM interactions must be considered as the key feature of malignancy therefore the details of the molecular mechanism have great importance.

ECM recognition

Tumor cell-ECM interactions are combinations of highly inter-related events of matrix adhesion, specific degradation of their components and migration in the partially degraded milieu. Meanwhile, even malignant tumor cells are socialized in the tumor tissue mediated by cell-cell adhesion molecules (CAMs). The best known cell-cell adhesion molecule of epithelial cells is E-cadherin, the loss of which is characterizes the majority of carcinomas and is now considered a natural metastasis suppressor. Its importance has recently been demonstrated by the discovery of the inactivating germline mutation in the CDH1 (E-cadherin-1) gene resulting in early onset of diffuse gastric cancer.[37] Similar mutations can be found in sporadic gastric and breast tumors as well. On the other hand several paracrine and autocrine growth factors induce tyrosine phosphorylation of the E-cadherin/catenin complex resulting in down-regulation of E-cadherin, and cell-cell adhesion. In other tumor types, similar cell-cell adhesion molecules could be involved in this process, for example, the N-CAM family, which is expressed in a wide variety of neural crest-related tumors.[71] The above described events produce a rich source of malignant cells with loosened cell-cell contact. Only these tumor cells would have the chance to leave the primary site, though this event is only a prerequisite for invasion and not metastasis.

Invasive tumor cells have to be able to identify the surrounding matrix (in case of carcinomas this is the basement membrane) and while they also have to be able to release anchoring matrix adhesions *(Figure 1.).* That usually requires down-regulation of the original matrix receptors, mainly the integrins such as $\alpha5\beta1$ FN-receptor and/or various laminin/collagenIV receptors ($\alpha6\beta1$, $\alpha6\beta4$)[48] and expression of new ones or upregulation of pre-existing ones, the function of which would be more permissive for rapid changes in adhesion/detachment cycles. Sometimes the new integrin is ectopic or even mutated[100,101] resulting in oncogenic signaling.[73] Though the close connection between matrix adhesion and degradation was realized earlier, we have just started to reveal those molecular mechanisms which actually control them. It turns out that some integrin receptors such as v3 vitronectin receptor can bind and even activate proteolytic enzymes such as uPA or MMP providing a local anchor for the otherwise soluble enzymes.[9]

Matrix degradation

It has also become clear in the past few years that invasiveness requires either significant matrix-degrading enzyme-binding potential of tumor cells provided by the expression of specific or non-specific enzyme receptors (see above) or increased activity of one or more enzymes[18] due to either up-regulation of the enzyme or down-modulation of their respective inhibitor. Matrix degradation is not a unique phenomenon, since normal cells are able to perform it in a strictly controlled way. The same enzymes are also involved in invasion by tumor cells. These enzymes include the serine-protease family (uPA, elastase, plasmin and cathepsin G), the matrix metalloproteinases (gelatinases, stromelysins, matrilysins) and the cysteine proteinases (cathepsin B, L). Physiologically all of these enzymes have their own specific inhibitors (PAI-1-2, TIMP-1-3, and steffin cystatins) responsible for immediate inactivation of the enzyme. Several enzymes are physiologically stored in lysosomes. However, in tumor cells due to inappropriate trafficking and control such enzymes may appear at the cell surface, as has been observed for cathepsins.[85] Another possibility for uncontrolled activity of matrix-degrading enzymes is down-regulation of their inhibitors.[57] The result of the concerted action of the matrix degrading enzymes is fragmentation of the matrix proteins, which not only provide an easy transit for tumor cells, but in the meantime release several matrix-bound factors including growth factors and cytokines, as well as chemotactic peptides.

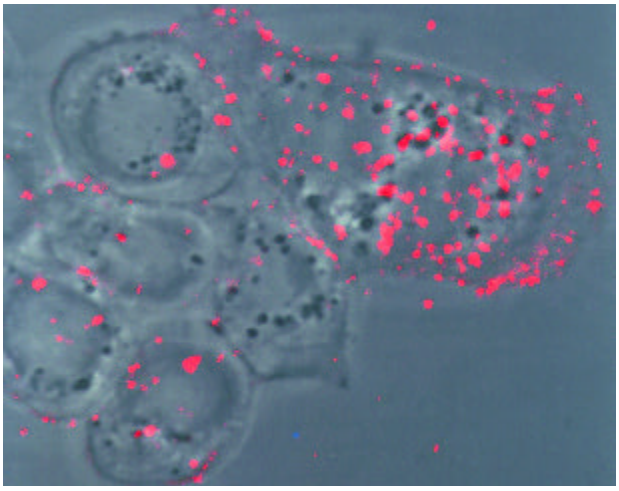


Figure 1. Demonstration of focal adhesion plaques in fibronectin-adherent human colon carcinoma cells (HT25). Cultured tumor cells were fixed, permeabilized and labelled immunocytochemically for phosphotyrosine using monoclonal antibody and Texas-red-conjugated streptavidin. Tumor cells were studied by confocal microcopy with optical section at the substrate attached membrane area. Note the dot-like appearance of accumulated phosphotyrosine-containing adhesion plaques.

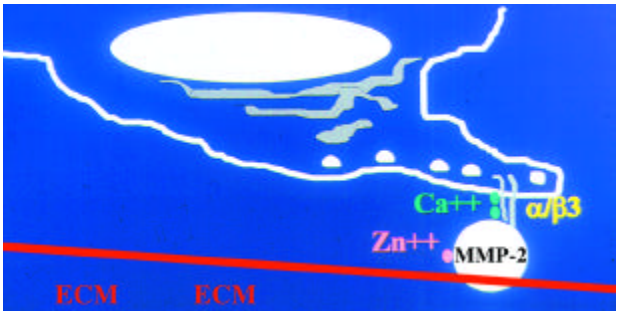


Figure 2. Schematic representation of the invadopodia in cancer cell. Explanations are in the text.

It is now recognized that the microinvasion of tumor cells is generated by a specialized membrane organelle, called *invadopodia (Figure 2.).* This organelle is generated from the plasma membrane by matrix receptors anchored to unique areas of the surrounding matrix resulting in a polarization of the entire cytoskeleton of the cell. The degradative enzymes stored intracellularly can only be released into the areas around that polarization, and soluble enzymes in the micromilieu, released by other cells will also be concentrated to these sites by the adhesion receptors. This will provide the critical concentration of degradative enzymes that now can produce spaces for cell migration. On the other hand, since matrix degradation is generated by several different enzymes that are activated along a cascade of interconnected activation processes, these sites would serve as activation centers at the surface membranes. Since these membrane areas are in the close vicinity of the focal matrix adhesion sites, degradation could start right on the spot.[57]

Migration

The third event, which is actually the integrating element of microinvasion, is the migration of tumor cells *(Figure 3).* Cell migration is a tightly regulated process of detachment and re-attachment of cells as well as continuous rearrangement of the cytoskeleton. The process, at least in tumor cells, utilizes Rho/Rac G-proteins, IP-cycle and PKCα as effector kinase acting on motor proteins.[85] It is now widely accepted that malignant tumor cells are characterized by loosely controlled migratory potential, regulated both by powerful autocrine as well as paracrine mechanisms. The motile activity of malignant cells is generated by cytokines produced by the tumor cells themselves. Interestingly, both of these cytokines are ectoenzymes, localized to the cell surface. The best characterized such cytokine to date is the autocrine motility factor (AMF), previously known as neuroleukin or phosphohexose isomerase.[104] Its receptor is identified, cloned and the signaling pathway is described. Non-metastatic cells also

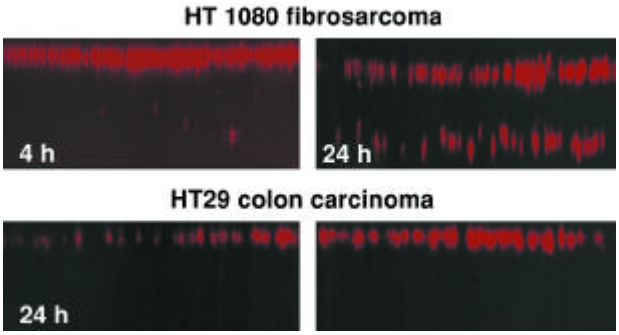
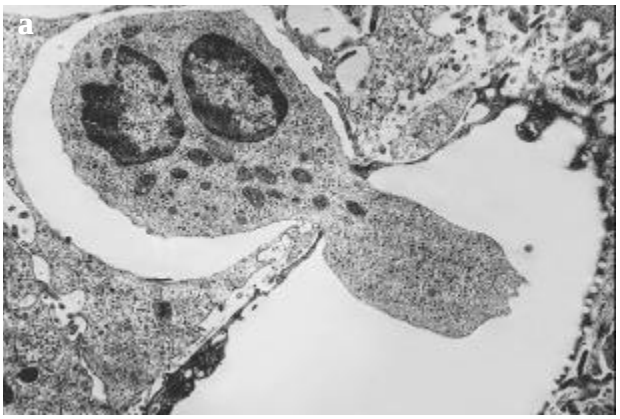


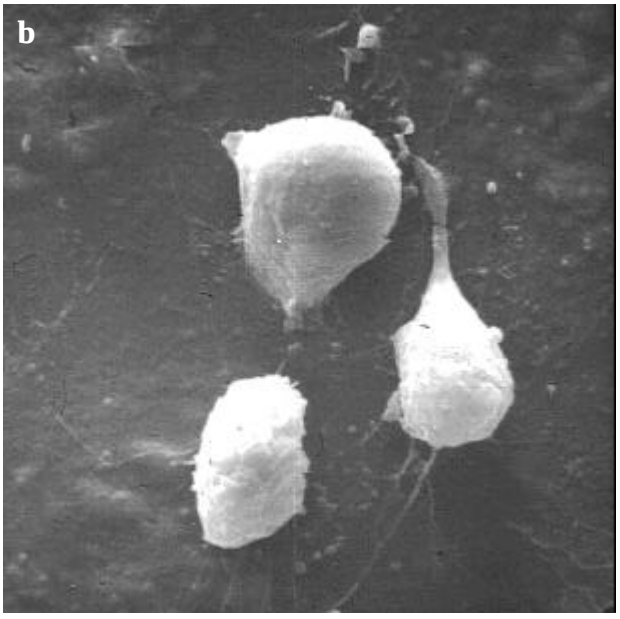
Figure 3. Migration of of human colon carcinoma cells into an artificial basement membrane (Matrigel). Tumor cells were seeded on the top of Matrigel and were incubated at 37ºC for 4-12 hrs. At the end of the incubation, plates containing Matrigel and tumor cells were fixed and nuclei were stained with propidium iodine. Samples were studied by confocal microscopy with 3D reconstruction. Note that the non-metastatic tumor cells stayed on the top of the Matrigel (HT29) while metastatic tumor cells migrated deeply into the gel during the incubation period.

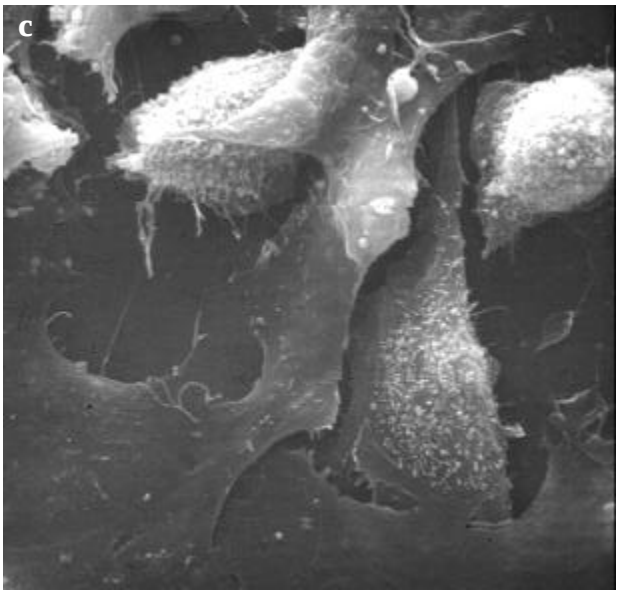
express the receptor but either the ligand is not provided or the signaling pathway is shut down, therefore the receptor seems to be more or less non-functional. In motile tumor cells, AMF is produced and the receptor associated signaling pathway is active. The significance of the expression of the AMFR in tumor progression is well documented in several human cancer types.[85] The other identified autocrine motility cytokine is autotaxin (ATX), which is the phosphodiesterase-I. enzyme, but its receptor is still not known.[81] On the other hand paracrine cytokines may have equal contribution to initiate tumor cell migration. It is now recognized that almost all cytokines could generate a motile response if its receptor can turn on the so-called motility signaling pathway.[96,97] The most ubiquitous among the paracrine motility cytokines is HGF/scatter factor. This is frequently produced by activated mesenchymal cells, and tumour cells often express its receptor, the c-met oncoprotein.[76]

Hematogenous dissemination

Tumor progression continues further over this point (local invasion) in most of the malignant tumors. The key issue here is the interaction of tumor cells with the local microvessels (post-capillary veins and lymphatic vessels) called intravasation *(Figure 4a.)*. However, the process is highly similar to the one operational at the interaction with the local matrix or the basement membrane, surrounding the primary tumor. Adhesion to, degradation of and migration through the subendothelial basement membrane take place in an identical fashion, and the regulation of the process is also similar. However, the difference in this case is in the unique interaction of tumor cells with the endothelial cells and the chemotactic response to the increasing concentration of nutrients that peaks in the vessel itself. It is notable that the intravasating tumor cells enter the vessel lumen by a non-destructive interaction with the endothelial cells.[32] At this point one of the strongest selection pressures is applied to tumor cells reaching this phase of dissemination. One such selection mechanism is mechanical, due to the effects of intravascular pressure and the mechanical forces caused by contacts with both the vessel wall and the circulating blood cells. The majority of the tumor cells will die due to these effects, estimated to be 99.0-99.9% of the circulating tumor cell population.[21] Another selection factor is the recognition of tumor cells by immune effector elements in the circulation, also resulting in a significant loss of viable tumor cells. It seems plausible that tumor cells characterized by exceptional survival potential or resistance to apoptosis are more prone to reach distant areas of the vasculature than others which have maintained their apoptotic machinery.[33] These cells would also be more resistant to immune attacks. On the other hand, intravascular tumor cells could build a mechanical defense with the help of aggregating platelets and fibrin clots. The prerequisite for this process is the ability of tumor cells to aggregate platelets and to initiate clotting. Adhesion molecules involved in both platelet aggregation as well as fibrinogen binding are those molecules whose expression is necessary for tumor cells to survive.[93] These include β3 integrins (especially the ectopically expressed αIIbβ3), and the thrombin receptors.[93] The platelet/fibrin clot around the circulating tumor cells does not simply provide a defense for them, but the activated platelets release cytokines and growth factors (such as PDGF) and bioactive lipids (such as 12-HETE) which could also initiate tumor cell activation, resulting in activation of either mitogenic, motogenic, or both signaling pathways.[93] Therefore the arrival of such "activated" tumor cells at the periphery of the vasculature and the mechanical entrapment of tumor cells in the narrowed lumen of the distant organ would provide a milieu which is superior for survival and increases the chance of tumor cells building up a successful interaction with the organ vasculature.[93] Besides the mechanical filtering, some circulating tumor cells may also recognize specific terminal organ vessels by expressing cell-cell adhesion molecules (addressins, otherwise used either by platelets (P-selectin, P-CAM), leukocytes (L-selectin, I-CAM) or lymphocytes (L-selectin, I-CAM, CD44H) during their transition through vasculature.[32] The consequence of specific tumor cell adhesion to endothelium would be transient retraction of the endothelial cells, thereby exposing the attractive subendothelial matrix (similar to basement membrane) to tumor cells.[93] Those tumor cells which are expressing







appropriate matrix receptors at their cell surface and are able to utilize them would then become adherent to the exposed subendothelial matrix. At each step of this process, further selection occurs based on the expression of the appropriate (degradative and/or motogenic) phenotype of tumor cells. This entire process is called extravasation *(Figure 4b,c.)* which can be considered an organ-specific event, since endothelial cells have organ specific phenotype, and their subendothelial matrix is organ specific, so that tumor cells have to be able to accommodate differently to these environments for successful exit from the circulation.[32]

Organ selective metastasis development

The problem of organ selectivity of tumor metastasis was recognized early in the 19th century by Paget[67], who suggested the "seed and soil" theory implicating that both the unique phenotype of tumor cells and the unique phenotype of the host organ together are responsible for the organ selective metastasis. This was contradicted by Ewing[19] some 40 years, who suggested that mechanical filtering was of greater importance. Most would now accept that both hypoetheses have merit, though Paget's seed and soil hypothesis is likely to be the greater influence of the distribution of metastases. Unfortunately, more than a century after Paget's work, we still know little about the detailed molecular mechanisms of the organ selectivity of tumor metastasis.

We have learned that the microvessels within individual organs, the primary filters for tumor cells, have organ-specific phenotypes based on the expression of various cell-adhesion, as well as MHC molecules, and that therefore this phenotype could serve as a primary recognition mechanism.[32] As a subsequent step, the retracted endothelium exposes the subendothelial matrix which both structurally different in each organ as well as containing different organ-specific cytokines, providing another recognition mechanism.[32] It is important to quote experimental studies

Figure 4. Forms of tumor cell – endothelial cell interactions.
(a) Early phase of intravasation of 3LL-HH tumor cell in vivo in the liver sinusoid. 3LL-HH tumor cells were implanted into the spleen as primary site and the liver was studied ultrastructurally on the 7th day using organ-perfusion fixation. Transmission electron microscopy.
(b) Coculture of low metastatic tumor cells with endothelial cells in vitro (overnight). Note the inactive round tumor cells at the apical surface of the endothelial monolayer. Scanning electron microscopy.
(c) Coculture of high metastatic tumor cells with endothelial cell monolayer in vitro (overnight). Note the elongated tumor cells invading the endothelial monolayer positioned subendothelially. Scanning electron microscopy.

in this respect where it has been shown that subendothelial basement membranes are differentially involved in the organ colonization of a highly metastatic tumor. In these experiments, whereas liver metastases were dependent on the sinusoidal heparan sulphate component of the subendothelial matrix, lung metastases were dependent on the laminin and fibronectin components of that matrix.[99] Ultimately, and this area is the only one which has produced some results in the past decades, tumor cells have to be able to respond to the local mitogens which are likely to be the rate limiting step of organ metastasis. Experimental metastasis models suggested that the selective liver and lung metastatic potential of various tumor types may depend on the responsiveness of tumor cells to lung- or brain-derived transferrin (Tf).[62] However, the tumor cell receptor to which Tf would bind is not identified yet. This could be the transferrin receptor (TfR) itself or other Tf-binding high affinity cell surface receptor(s). Unfortunately, studies on human lung- or brain-metastatic tumors did not support these observations. Liver-derived mitogens identified to promote liver metastasis development of human colon carcinoma cells were shown to be local growth factors such as TGFα, EGF and HGF. On the other hand, expression of EGFR and HGFR by colon carcinoma cells proved to be associated to their liver metastatic potential, suggesting at least a working model for the liver-selectivity of GI-tract tumour dissemination.[20,72]

Bone-specificity of the metastatic process may depend on the ectopic expression of bone matrix proteins. Tumors which are chataracterized by osteonectin, osteocalcin and/or bone sialoprotein expression are breast-, prostate- and thyroid cancers, all of which are characterized by a strong metastatic preference for bone.[5,30,50] It was also suggested that prostate cancer cell-secreted endothelin-1, uPA and PSA may initiate bone stromal cell-production of bone-morphogenic growth factors (bFGF, TGFβ, HGF, IGF) and bone morphogenic proteins (BMPs) leading to the formation of new bone around the metastatic foci[50] Osteolytic metastases are formed when tumor cells secrete osteoclast-activating cytokines, PTH-related peptides, TGFα, TNFα, TNFβ or M-CSF, IL-1,6 or prostanoids.[23]

"DORMANCY". Consequences of tumor cell extravasation would be several. Some tumor cells would not be able to survive in the new matrix environment and would die in the perivascular zone.[39] Some tumor cells would temporarily be unable to respond to local growth factors, but those having a superior survival potential due to the impaired apoptotic machinery would stay in a "dormant state" in the perivascular zone. Thirdly, tumor cells which either have a strong autocrine proliferation stimulatory potential or express growth factor receptors for those present in that organ's micromilieu would be able to initiate colony formation. At this point, tumor growth in the new organ would follow the process described for the primary site. Recent data suggest that one obvious factor which can induce dormancy of tumor cells in a new environment is their unability to induce angiogenesis to provide the ideal milieu for clonal expansion.[39] Unfortunately, extravasated tumor cells may regain such potential due to the background slow mitotic activity and genetic instability, leading at a later point to the acquisition of angiogenic potential with the late development of clinically apparent metastases.

Metastasis genes

Certain genes have emerged as possible key players in the process, and these are now known as metastasis genes. Interestingly, the first of such a gene identified and tested clinically was NM23/NME1.[88] This gene codes for the NDP kinase enzyme in humans and belongs to the small G-protein family. The physiological function of NM23 is not completely known, and more importantly the function of NM23 in tumor cells is still obscure.[27] It was found in several experimental tumor systems that loss of the NM23 expression follows the emergence of the metastatic potential. These geno- and phenotypic characteristics can be found in human breast cancer where loss of heterozygosity for NM23 gene (LOH) is described.[27] In colon, kidney and certain lung tumors loss of NM23 was identified as a prognosticator of poor survival.[22] Unfortunately, in several other human tumor types expression of NM23 does not change or even neoexpression takes place during progression,[82] questioning the universal metastasis suppressor role of this gene.

The first metastasis-promoting gene identified was a splice variant of CD44, which was first identified as the homing receptor of lymphocytes.[46] It turned out that activation of the expression of v6 splice variant of the CD44 can turn non-metastatic cells to metastatic ones.[41] Unfortunately, scientists and clinicians were not aware of the fact that the physiological function of CD44 is lymphocyte homing and that the experimental model where CD44v6 was found to be metastasis gene was a model where the tumor cells disseminated through lymphatics. Therefore, the numerous studies performed in various human malignancies again produced controversial results. The most consistent finding is that GI tract tumors which express CD44v6 are rendered metastatic.[12] Since one of the main dissemination mechanisms for these tumors is the lymphatics, this can be considered as a rational finding. However, it is not known if CD44 variants could have other roles than this in facilitating the organ metastatic process in such an indirect way. A newly described metastasis suppressor gene is the KAI-1 gene located on chromosome 11.[17] This was identified in prostate epithelium and non-invasive cancer cells but is lost in distant metastases. The gene codes for a transmembrane glycoportein, CD82, the homologue of CD9 which is involved in the regulation of

lymphocyte motility.[22] Another newly described putative metastasis suppressor gene is KISS-1 located on chromosome 1, which is lost in metastatic human melanoma cells.[52] The gene codes for a protein which affects cell-matrix interaction probably acting on the cytoskeletal organization.[22]

In conclusion, it can be said that the complexity of the metastatic cascade is unlikely to be controlled by just one or a few genes. The identified genes (NM23 or CD44v6) should rather be regarded as metastasis-associated genes which control certain more or less important (though unknown) steps of this complex process.

It has to be emphasized that the whole cascade of events described above is very inefficient and at each major step, the tumor cell population is diminished by at least one log due to failure to proceed further.[21] However, on the other hand, the entire process is based on selection, so that the tumor cells entering a subsequent stage would harbor more and more potential to proceed further. This process is facilitated by continuous genetic instability that could also lead to the appearance of new geno- and phenotypes compatible with the changing environment in which the tumour cells find themselves.[21] Therefore, one can envision the emergence of a genetic super-cell as a result of passage through the "stairs"of the metastatic cascade, where the resulting tumor cell population repeats the tumor tissue development process to produce more uncontrolled cells than those were present in the primary site. This entire process is critically influenced also by therapeutic interventions, which not only select drug-resistant clones from the original population, but also could facilitate the process of genetic instability.

Prognostic pathology of the metastatic potential

Lung cancer

Lung cancer is among the most frequent human malignancies and one of the most aggressive, characterized by high metastatic potential. Therefore identification of metastatic potential-specific markers would be highly desirable to clinical oncologists. However, only a few such markers have even been tested in clinical situations and only a few have been proved to have clinical significance. Lung cancer consists of three types, non-small cell lung cancer (NSCLC), adenocarcinoma (AC) and small-cell lung cancer (SCLC). For prognostic factors NSCLC has been studied in more detail than any other subtype. Tumor size and lymph node status are two independent prognosticators of poor outcome in early stage disease. However, in advanced disease, staging loses its significance. It is found that DNA aneuploidy can be used to identify a group of patients with poorer prognosis versus diploid tumors.[31] Accumulation of p53 in NSCLC is interpreted differently in the literature, but its coexpression with bcl-2 may be considered as marker of poorer prognosis.[14,49,68] Similar to breast cancer, expression of the c-erb-B2 receptor has been identified as marker of bad prognosis[49], as well as the expression of Cyclin D-1.[7] Furthermore, mutations in the K-ras oncogenes have also been found to be associated to a poorer survival.[58] Such studies have rarely been performed in case of the other type of lung cancers, and therefore invite the activity of both cancer biologists and clinicians.

METASTASIS GENES. Expression of CD44 is associated to a poor survival of NSCLC,[14] but no splice variants have been found in lung cancer. Interestingly, NM23 is not expressed by bronchial epithelium but it can be expressed by NSCLC, a feature which strongly predicts poor survival.[103] However, it turned out that NM23H1 expression is actually associated with c-myc expression and the proliferative potential of the tumor.

VASCULARIZATION. Tumor-induced angiogenesis is an independent prognosticator for disease progression in lung cancer.[14] Furthermore, expression of VEGF, bFGF, FGFRR1 all correlate with an unfavorable outcome.[66]

MATRIX ADHESION. In lung adenocarcinomas, loss of expression of the laminin/collagen receptor $\alpha 3(\beta 1)$ has been shown to be a negative prognosticator for survival,[1] which is not the case for NSCLC. Recently is was shown that the loss of the expression of $\alpha v(\beta 3)$ integrin from NSCLC cells predicts the recurrence of disease in node-(N0) tumors.[87] On the other hand, the appearance of a different adhesion molecule, MUC-1 mucin, in adenocarcinoma was shown to be associated with shortened survival.[91]

MATRIX DEGRADATION. The uPA/PAI-1 system plays also a role in the progression of the NSCLC similar to breast cancer,[68] but other proteinases have not been studied sufficiently and their prognostic significance has not been established.

MIGRATION. Though lung cancer is one of the most metastatic tumor types, motility factors have been extremely rarely studied in this tumor. It has recently been found that the expression of HGF/scatter factor is an independent negative prognosticator for survival in NSCLC patients,[84] suggesting that this paracrine motility cytokine plays a fundamental role in shaping the metastatic phenotype of this tumor.

Breast cancer

Breast cancer prognosis is one of the hottest area of basic and clinical cancer research, and many prognostic indicators (biomarkers) have been analyzed in the past decade.

However, the most reliable markers are still conventional pathologic data including number of involved axillary lymph nodes, the size of the tumor (1 cm cut off in case of infiltrating ductal and lobular cancers and 3 cm cut-off in case of infiltrating special histological type of cancers). Poor differentiation (histological and nuclear) is also associated with poor prognosis.[38] DNA ploidy measurements are helpful to select aneuploid tumors with unfavorable outcome, but the significance of ploidy as an independent prognosticator is not yet established. However, the biggest problem of DNA ploidy measurement is standardization, reproducibility and quality control. For this reason, the NIH have not recommended the use of DNA ploidy measurements for decision making in clinical practice.[38] Lastly, the detection of vascular or lymphatic invasion in the primary tumor evidently has high prognostic significance. Interestingly, the sex hormone receptor status is only a very weak prognosticator of disease outcome (metastasis) but a strong prognosticator of therapeutic response.[38]

The metastatic potential of tumors may or may not depend on their proliferation potential. Breast cancer is among the tumors where the proliferation rate of the tumor was frequently associated to poor prognosis,[47,74,77] and therefore markers of the proliferation potential have been studied extensively. Detection of S-phase fraction by flow cytometry has been suggested to be a good tool to predict proliferation rate of breast cancer,[38] but again the lack of standardization, reproducibility and quality control limits its use for clinical decision making. Several other proliferation markers have been studied in this respect and it seems that immunohistochemical detection of a protein S2 which labels the S-G2-M fractions would be a more reliable marker of the proliferating fraction than the Ki-67 (MIB-1), or PCNA.[77] The cell cycle regulator protein, Cyclin-D1, and the CDK inhibitor, p27/Kip1, have been studied with some success as prognosticators. It is now considered that a high Cyclin-D1 expression is a sign of good prognosis while the loss of p27 predicts a bad clinical outcome.[4,92] Analysis of the accumulation of p53 in breast cancer produced highly controversial results and can not be used as a prognosticator.[38] Meanwhile, a new marker has emerged from the large family of growth factor receptors; the c-erbB2 receptor. Overexpression of c-erbB2 is an independent marker of bad prognosis, and also predicts a poor response to chemotherapy, though it does allow the use of antibody treatment (Herceptin) directed against this molecule.[44,70]

METASTASIS GENES. NM23 was the first metastasis suppressor gene to be described, and its role in progression was substantiated by the extensive studies in breast cancer. It is now accepted that decreased expression of the NM23 gene and a low level of expression of the NM23H1 protein in breast cancer have an independent prognostic value for lymph node metastasis and poor survival.[13,105] The metastasis suppressor, E-cadherin, was shown to be down-regulated in invasive breast cancer and proved to be an independent negative prognosticator for node status.[3] On the other hand, CD44 and its splice variants are differentially expressed in breast cancer: it was demonstrated that the expression of v6 variant correlates to favorable prognosis in node-negative patients,[24] while the neoexpression of CD44v3-4 indicated the potential for local lymphatic spread.[3]

VASCULARIZATION. Breast cancer is among the malignant tumors where the role of tumor-induced angiogenesis has been recognized and has frequently been shown to have prognostic significance for metastatic potential.[28] It has to be emphasized here that it is evident that the low intratumoral vascular density which has an independent prognostic value for good prognosis, rather than the high vascularity which is more common in this tumor type.[28] It is also important that it is not enough simply to label vessels in and around the tumor, the measurement of proliferating vessels has a much stronger prognostic value.[28,51]

MATRIX ADHESION. A significant independent prognostic role for 67kD laminin receptor expression has been repeatedly demonstrated in breast cancer (similar to lung and prostate cancers), showing correlation with both tumor growth and metastasis.[56] On the other hand, the down-regulation of the majority of the β1 integrins (most of them laminin/collagen receptors) has been shown to be another aspect of the invasive phenotype of breast cancer.[36] More interestingly, bone-metastatic breast cancer was shown to express αvβ3 integrin which has several high affinity ligands in bone (osteopontin, osteonectin), suggesting that this adhesion molecule may mediate the specific recognition of the bone by circulating metastatic cells.[53]

MATRIX DEGRADATION. All the major family members of the proteinases have been studied extensively in breast cancer. From the serine proteinase family uPA and its receptor, uPAR, emerged as independent prognosticators for bad prognosis (i.e. metastasis).[18,55] Cathepsins were also studied in details but the role for cathepsin D still remains highly controversial as a prognosticator, similar to cathepsin B, which proved not to be an independent factor.[18,55] Interestingly, MMPs were rarely studied from this respect in breast cancer and there is only one significant clinical study which has indicated MMP-2 expression as a strong independent prognostic marker for unfavorable outcome and metastasis.[95]

MIGRATION. This area is the least frequently studied field of human breast cancer biology. Among paracrine factors, the receptor for HGF/scatter factor, the c-met oncoprotein, was studied recently and the expression of it in breast can-

cer was shown to be an independent prognosticator for poor outcome.[29] Evidently, major new developments can be expected from studies on such factors and the expression of their receptors in breast cancer.

Colon cancer

Prognostic pathology of colorectal cancer involves again primarily macro- and microscopic features of the primary tumor, with staging and grading of the tumor. One of the oldest staging system of tumors, Duke's staging has survived into this century with modifications. It is based on the local extension of the tumor (depth of invasion) and the degree of mesenteric lymph node involvement. Its value in predicting the course of colorectal cancer is not yet seriously challenged by any novel markers or features.[11] Rather, it has been augmented by a few new features such as lateral margin involvement, and histological grading of the tumor, both of which turned out to be independent prognosticators for colorectal cancer. One other new features of staging is the detection of vascular invasion. There has to be a specific discrimination between the blood vessels and lymphatics by the use of elastin staining. Interestingly, the detection of blood vessel invasion at intra- or extramural areas did not have independent prognostic role in the cancers of the colon or rectum. In contrast, lymphatic vessel invasion is an independent prognosticator for later occurrence of metastases and poor survival. From the point of view of local recurrence, perineural invasion must be considered the most important factor, which indirectly also predicts disease progression.[11]

Looking at the phenotype of these tumors, DNA ploidy is an independent prognosticator for colorectal cancer, but with the same provisos regarding reproducibility and standardization of techniques as previously discussed for breast cancer. Aneuploid tumors tend to proliferate faster, and accordingly, PCNA expression is a strong independent negative prognosticator in this tumor type.[11] Interestingly, growth factor receptors were not studied extensively on clinical materials. Among oncogenes, K-ras overexpression was found to be a strong independent negative prognosticator.[90] Among the oncosuppressor genes, loss of DCC gene and protein are independent prognosticators in colorectal cancer for poor outcome[78] but p53 accumulation is less significant from the point of view of progression. Cyclin D1 expression is an independent negative prognosticator, while p27 expression is an independent positive prognosticator for metastasis.[26] It seems that the loss of apoptotic potential may be an important new negative prognosticator in colorectal cancer.[40]

METASTASIS GENES. Expression of CD44 and its splice variant, v6, has been extensively studied in colorectal cancer in this decade and these studies ultimately identified CD44v6 expression (both at mRNA and protein level) as a strong independent negative prognosticator for both the development of liver metastasis and for poor survival.[41,63,75] On the other hand the significance of the expression of NM23 is not substantiated yet in colorectal cancer.

VASCULARIZATION. In colorectal cancer too, tumor induced angiogenesis has independent negative prognostic value to predict the outcome of the disease.[94] The main growth factor responsible for angiogenesis was identified as VEGF, expression of which is also a strong independent negative prognosticator for disease progression .[45]

MATRIX ADHESION. Among the matrix receptors, integrins have been extensively studied in colorectal cancer, but none of them emerged as a significant contributor to the metastatic phenotype. On the other hand, ectopic expression of MUC-1 mucin was shown to be an independent negative prognosticator for short survival and the development of metastases.[60]

MATRIX DEGRADATION. uPA emerged as strong independent negative prognosticator for colorectal cancer, where even the ratio of the tumor versus stroma has predicitve value for metastasis and disease progression.[18] It is important to mention that serum uPAR levels in colorectal cancer patients also have prognostic value, suggesting a critical role for uPA/uPAR system in the progression of colon cancer.[89] Cathepsin B was found to be increased in invading tumors,[80] but these observations were not followed by larger studies. Turning to the MMP family of proteases, several members are expressed by colorectal cancer, and the overexpression of MMP-2 seems to be a constitutional event suggesting that this is necessary but not sufficient for metastatic potential.[69]

MIGRATION. Studies of motility factors and their receptors are rare in the clinical literature, with the exception that the expression of AMFR was found to be an independent negative prognosticator both for the development of liver metastasis and shortened survival.[60] More recently, it was shown that human colon cancer cells expressing amplified c-met, respond to liver-derived growth factor, HGF by migration at a dose which is not mitogenic.[20] Furthermore, c-met is over-expressed in Duke's C but not in Duke's B tumors suggesting a specific role for the c-met/HGF system in the dissemination of colon cancer.

Prostate cancer

The development of prostate cancer is typically multifocal, and accordingly the staging and grading system applied to this cancer type is more complex than some

other organs. A special pathologic term was introduced, the Gleason score, which implies and evaluates these multifocal lesions. The TNM system was also extensively analyzed in prostate cancer and was adapted to this organ with several variations, often challenged in the literature. The most important points from the point of view of prognosis are capsular invasion, the presence of tumor at the surgical margin, the invasion of adjacent structures, as well as the lymphatic, vascular and perineural invasion.[65] Hormonal dependence of the tumor is one of the most significant positive prognosticator in prostate cancer, however determination of the expression of androgen receptor is not yet a routine feature of primary diagnosis. Prostate cancer can be divided into androgen-dependent, androgen-sensitive and androgen-independent forms which tend also to correlate with a worsening prognosis. Emergence of the androgen-independent phenotype is mediated by profound genetic changes in prostate cancers involving the expression of growth factor receptors such as EGFR, c-erb-B2, and mutations in a series of suppressor genes such as p53, DCC and APC, as well as amplification of c-myc and bcl-2.[42,43]

METASTASIS GENES. Expression of metastasis-associated genes is highly unusual in prostate cancer. Several independent studies demonstrated a unique so-called reciprocal expression of those genes: loss of CD44 and neoexpression of NM23H1 occurs in cancers characterized by a poor prognosis and systemic dissemination.[64,82] Accordingly, CD44 serves as a metastasis suppressor gene and NM23H1 as a metastasis gene in prostate cancer, unlike several other cancer types. Furthermore, prostatic epithelium expresses a unique metastasis suppressor gene, KAI-1,[17] which codes for a motility-related membrane glycoprotein CD82, a relative to CD9. It has been repeatedly shown that the expression of KAI-1 is down-regulated in the more aggressive tumors.[42,43,65]

VASCULARIZATION. Prostate cancer belongs to those tumors in which prognosis (and therefore its progression) is associated to the extent of vascularization. Accordingly the determination of the intratumoral vascular density is obligatory to establish prognosis.[42,65]

MATRIX ADHESION. It is a repeated finding that in prostate cancers with a poor prognosis that the cell adhesion molecule and metastasis suppressor, E-cadherin, is down-regulated, due to either the mutation in their APC-like cytoskeletal linker α-catenin or hypermethylation of the 5'-region of the gene.[42] Turning to the matrix receptors, prostate cancer is characterized by the loss of some laminin/collagene IV receptors such as β4 integrins[42] and by the ectopic expression of αIIbβ3.[100] However, the clinical significance of these features remains to be established.

MATRIX DEGRADATION. Progression of prostate cancer is clearly accompanied by the overexpression of the uPA/uPAR system in the tumor resulting in the appearance of these molecules in the systemic circulation as well making it possible to use as serum marker of progression.[58] However, uPA is not the only protease expressed by prostate cancer since MMP-2 was also shown to be expressed by advanced tumors and has been demonstrated to be associated with bone-metastasis.[34]

MIGRATION. Though prostate cancer is an aggressive disease, which frequently disseminates to distant organs, motility factors and their receptors have not been studied extensively enough to use them as prognosticators. The exception is the HGF/c-met paracrine motility cytokine-receptor system, where the overexpression of c-met in prostate cancer is established as a negative prognosticator in this tumor similar to breast cancer.[42]

Malignant melanoma

The life-threatening phase of this skin tumor is the stage when organ-metastases start to develop primarily in the lung, liver and brain, since regional block dissections or the more accurate sentinel lymph node dissection technique, applied by the help of radioscintigraphy, aid efficient removal of the potentially affected regional lymph nodes.[83] This technique however, is less efficient to control the development of organ metastasis. Accordingly, it would be highly desirable to identify the selective prognosticators to predict the organ-metastatic potential of melanoma.

The classical pathological categories based on the depth of invasion in the dermis and quantification of it (Clark and Breslow respectively) are still the best prognosticators for such purposes,[10] though the application of TNM categories may help to further improve it.[2] Today the use of the system allows prediction of the development of metastases and disease outcome with a relatively high accuracy compared to other tumors, but the success rate for individuals is still poor due to the unique and individual behavior of the tumors. For this reason, a great deal of clinical and experimental work is focused to find better pathological and clinical prognosticators, as summarized below. It is now evident that ulceration of the primary tumor and the anatomical location are both independent strong prognosticators for both disease progression and shortened survival.[2] The advent of elective lymph node dissection contributed significantly to the process of clinical management of melanoma and indicated both the therapeutic and prognostic significance of the resection of the appropriate lymph node.[83] Regional block-dissection and determination of the number of involved lymph nodes has independent prognostic significance.[2] Only those factors will be summarized below which could now be considered as bio-

logical prognosticators, the functions of which were shown to be directly related to the invasive/metastatic process in cases of malignant melanoma.

TUMOR PROLIFERATION. The mitotic index or determination of the proliferative fraction by markers of cell cycle in the primary tumor are not significant independent prognosticators for progression.[2,6] Interestingly, but not necessarily connected to this, EGFR appears to be over-expressed by invading melanoma cells suggesting some as yet unidentified role in the progression process. On the other hand the expression of oncosuppressor genes has been extensively studied, but p53 mutation is rare and accumulation of it does not have clear prognostic value.[2,6] It seems that the gene involved in the carcinogenesis of familiar melanoma (p16/CDKN2/Ink4) is not involved in the progression of sporadic melanoma.[6] Similarly, expression of bcl-2 in melanoma does not have independent prognostic impact.[8]

METASTASIS GENES. CD44 splice variants are expressed by skin melanoma. However, v6 expression does not correlate to progression, and instead, down-modulation of the v5 and up-regulation of the v10 variant was shown to have some negative prognostic impact.[15] Recently we have shown that the expression of CD44v3, the only HSPG form of this family of molecule, is closely associated to the organ-metastatic phenotype of the disease.[16] Expression of the NM23H1 gene in cutaneous melanoma and its prognostic impact is highly controversial, since down-regulation of gene expression has been demonstrated repeatedly in metastatic tumors, but expression at protein level did not follow that trend.NM23 cannot therefore be used as prognosticator incutaneous melanoma.

VASCULARIZATION. Published results from studies of the clinical and prognostic importance of vascularization in skin melanoma show considerable variation[2,10,15] which is at least in part due to the different techniques used for measurement. However, skin melanoma is a highly vascularized tumor with minor individual variations, which may suggest that this is the reason for the lack of prognostic significance.

MATRIX ADHESION. Identification of an adhesion molecule as prognosticator was first recognized in skin melanoma. It is now well accepted that the overexpression of the β3 integrin is a strong prognosticator for the development of metastasis.[15] However, identification of the actual integrin receptor heterodimer containing the β3 chain is still missing.

MATRIX DEGRADATION. Skin melanoma is another malignant tumor where the expression of uPA and uPAR has a strong independent negative prognostic value to predict

Table 2. **Summary of the metastatic phenotype of selected human malignancies**

	Lung cancer	*Breast cancer*	*Colon cancer*	*Prostate cancer*	*Malignant melanoma*
Metastasis genes					
CD44 splice variants	no change	v3-4 upregulation	v6 upregulation	H down regulation	V3 upregulation
NM23	upregulation	downregulation	no change	upregulation	?
Vascularization-angiogenesis	correlation	correlation	correlation	correlation	correlation (only intratumoral vessels)
Adhesion molecules	α6β4 downregulation αvβ3 downregulation	α/β1 downregulation αvβ3 upregulation 67-LNR upregulation E-cadherin-downregul.	?	?	α5β1 downregulation α/β3 upregulation ectopic αIIbβ3
ECM degrading enzymes	uPA upregulation	uPA upregulation uPAR upregulation MMP2 upregulation	uPA upregulation uPAR upregulation MMP2 upregulation	uPA upregulation uPAR upregulation MMP2 upregulation	uPA upregulation uPAR upregulation MMP2 upregulation
Motility					
Autocrine	?	?	AMFR upregulation	?	AMFR upregulation
Paracrine	?	c-met upregulation	c-met upregulation	c-met upregulation	?
Other		c-erbB2 upregulation	EGFR upregulation		

disease progression.[15] Meanwhile it seems that other proteases such as MMP-2 might contribute to malignant progression, since neoexpression of it has recently been shown to have strong independent prognostic role.[102] However, a third class of protease, the cathepsins (B&D) have also been shown to be expressed in cases with poor prognosis,[35] suggesting that all the three major protease classes are involved in the progression of this tumor.

MIGRATION. Although the AMF receptor was isolated and cloned in melanoma, the expression of AMF receptor in human skin melanoma has only been studied in a limited number of cases, though this suggests that its expression is upregulated with tumor thickness i.e. increased potential invasiveness.[98] The receptor for the paracrine motility regulator, HGF/scatter factor, c-met is constitutively expressed in melanoma but a prognostic role has not been asssociated with it.[79]

Conclusion

Modern surgical pathology has started to incorporate more and more laboratory techniques to answer an increasing number of clinical questions concerning not only the nature of the tumor, but also the individual biological potential, including its metastatic capability. Such questions cannot be answered without the knowledge of the details of the molecular mechanisms that are active during the dissemination of various tumor types. Although there are clear common patterns (altered matrix adhesion, degradation and increased motility, see the summary on *Table 2.*) such features are individual to the various tumor types. Increasing amounts of data are now available from experimental systems which have to be tested in clinical settings before we can build a rationale to predict with higher probability the individual behavior of various cancer types. New molecular techniques, such asDNA and protein microarrays may help to solve these problems.

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