Phenotype/ Genotype of Malignant Tumors in Bone Metastasis

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Abbreviations

APC: Adenomatous polyposis coli gene

BCL2: B-cell lymphoma 2

BHD: Birt- Hogg-Dube syndrome

BMP: Bone Matrix Proteins

BRC: breast cancer

CA: 15-3: Carcinoma antigen 15-3

CEA: Carcino- embryonic antigen

CBFA-1: Core binding factor α–1

CD: Cluster Determining

CFP-15: Cystic fluid protein 15

CGMA: Comparative genomic micro-array analysis

CK: Cytokeratins

COX-2: Cyclooxygenase

CRC: Colon cancer

CSF: Colony Stimulating Factor

c-myc: oncogene

DAPK: Death associated protein kinase

DCC: Deleted in colorectal cancer

DKK: Dick-kopf

EGFR: Epithelial growth factor receptor

ER: Estrogen receptors

FGF: Fibroblast growth factor

FGFR: Fibroblast growth factor receptor

FH: Fumarate hydratase

FHIT: Fragile Histidine Triad

FISH: Fluorescence in situ hybridization

HER-2/NEU: erBb2 (Human EgfR/Neuroblastoma)

HGF: Hepatocyte growth factor

HIF-1: Hypoxia induced factor-1

IGF: Insulin like growth factor

IHC: Immune-histochemistry

IL: Interleukins

INF: Interferon

LTB4: Leukoterin B4

MIP-1: Macrophage inflammatory protein 1

MMP: Matrix metaloproteins

MUC-1: mucin-1

MUCI/EMA: Mucin 1/epithelial antigenic marker

NSCLC: Non small cell lung cancers

OPN: osteopontin

PAP: Prostate acid phosphatase

PDGF: platelet derived growth factor

PSA: Prostate Specific Antigen

PSMA: Prostate specific membrane anrigen

PTHrP: Parathyroid hormone related peptide

RAF: Ras associated factor

RANKLE: Receptor activation of nuclear factor kB

Ras: Rho-A superfamily (small GTP-ase)

Rb: Retinoblastoma

RCC: Renal cell Carcinoma

RT-PCR: Real time polymerase chain reaction

SCLC: Small cell lung cancers

SEMA3B:Semaphorin 3B

SFA: Surfactant

T_4: Thyroxin

uPA: Urokinase like plasminogen activator

TGF: Transforming Growth factor

WNT: (W: wingless, Int: integration) signaling pathway

TMN: tumor/nodal/distant metastasis

VEGF: Vascular endothelial growth factor

TTF-1: Thyroid transcription factor 1

VHL: Von Hipple Linda

XIAP: X-linked inhibitor of apoptotic protein

MAC: Medified , Astler Coller system

1 Introduction

1.1. Bone

Bone is specialized form of supporting tissue in which the extracellular components are mineralized, thus conferring the property of marked rigidity and strength whilst retaining some degree of elasticity. Bone also constitutes a store of calcium and other inorganic ions, and actively participates in the maintenance of calcium homeostasis in the body as a whole. The structure of individual bones provides for the maximum resistance to mechanical stresses whilst maintaining the least bony mass. To accommodate changing mechanical stresses and the demands of calcium homeostasis, all bones in the body are in a dynamic state of growth and resorption throughout life.

Like other supportive connective tissues, bone is composed of cells and organic extracellular matrix containing proteoglycan ground substance and collagen fibers. Inorganic salts, predominantly calcium hydroxyapatite crystals, form the mineral components of the bone. Ground substance constitutes only a small proportion of the organic extracellular matrix of bone and contains proteoglycans similar to those found in cartilage except that the proportion of sulphated glycoprotein is much less than in cartilage. The fibrous component of extracellular material is mainly type 1 collagen which exhibits a similar banding pattern to that of common collagenous supporting tissue.

The cells found in bone are of three types: osteoblasts, osteocytes and osteoclasts, in a short summery we describe the characteristics of these cells.

1.2. Osteoblasts

Osteoblasts are the bone forming cells. They arise from mesenchymal stem cells, which form osteoblasts, adipocytes and muscle cells. (1) A transcription factor that is critical for the differentiation of osteoblasts is Runx-2, and core binding factor $\alpha 1$ (CBFA1). CBFA1 drives the expression of most genes associated with osteoblast

differentiation (2). Bone does not develop in animals lacking CBFA1 gene. The differentiation of osteoblast is less well understood than the differentiation of osteoclast (3). It is clear that there is an early osteoblast precursor that produces alkaline phosphatase and a more differentiated precursor that produces increasing amounts of osteocalcin and classified matrix (4). Osteoblasts eventually become osteocytes. Bone morphometric proteins are critical factors that stimulate the growth and differentiation of osteoblasts. (5)

1.3. Osteoclasts

Osteoclast arise form the precursor cell in the monocyte-macrophage linage that differentiates into inactive osteoclasts (6). Activated osteoclasts resorb bone and eventually undergo apoptosis. Both locally produced cytokines and systemic hormones regulate the formation and activity of osteoclasts. The bone micro-environment plays a critical role in the formation of osteoclast through the production of macrophage colony stimulation factor and receptor activator of nuclear factor-kB (RANK) ligand (RANKL) by stromal cells and osteoblasts (7, 8).
RANKL a member of the family of tumor necrosis factors is expressed on the surface of osteoblasts and the stromal cells and is released by activated T cells (7). Most osteotropic factors, such as parathyroid hormone, 1, 25-dihydroxyvitamin D_3, and prostaglandins induce the formation of osteoclasts by increasing the expression of RANKL on the marrow stromal cells and osteoblasts rather than by acting directly on osteoclast precursors (9, 10). RANKL binds to RANK receptors on osteoclasts precursor and induces the formation of osteoclast by signaling through the nuclear factor-$_k$ B and Jun N-terminal kinase pathway. A soluble form of RANKL produced by activated T cells has been detected in the joint fluids of animals with adjuvant arthritis (11). The ratio of RANKL to osteoprotegerin regulates the formation and activity of osteoclasts. Overproduction of osteoprotegerin causes severe osteopetrosis, whereas the absence of osteoprotegerin results in marked osteopenia (12).

Osteoclast resorbs bone by secreting proteases that dissolve the matrix and producing acid that releases bone mineral into the extracellular space. The adherence of osteoclasts to the bone surface is critical for bone resorptive process, since agents that interfere with osteoclast attachment block bone resorption. Agents that affect the adherence of osteoclast to bone or inhibit protease production might be useful for treatment of bone metastasis (13). The importance of RANKL in bone destruction has led to development of recombinant osteoprotegerin and antibodies against RANKL as potential treatment for bone metastasis (12).

1.4. Control of normal bone remodeling

The adult skeleton continually turns over and remodels itself through the coordinated activity of the osteoclasts and osteoblasts on the trabecular surfaces and haversian system. In normal bone, there is a balanced remodeling sequence: first osteoclasts reabsorb bone, and then osteoblasts form bone at the same site.

Both systemic factors and locally acting factors induce the formation and activity of osteoclasts. Systemic hormones such as parathyroid hormone, 1, 25 Dihydroxy vitamin D_3, and thyroxin (T_4), stimulate the formation of osteoclasts by inducing the expression of receptor activator of the nuclear factor-*k*B (RANKL) on marrow Stromal cells and osteoblasts.(12) In addition, osteoclasts produce interleukin-6, interleukin-1, prostaglandins, and colony stimulating Factors (CSF), which induces the formation of osteoclasts. Accessory cells such as T cells can produce cytokines that can inhibit the formation of osteoclasts such as interleukin-4, interleukin-18 and interferonγ (10, 12).

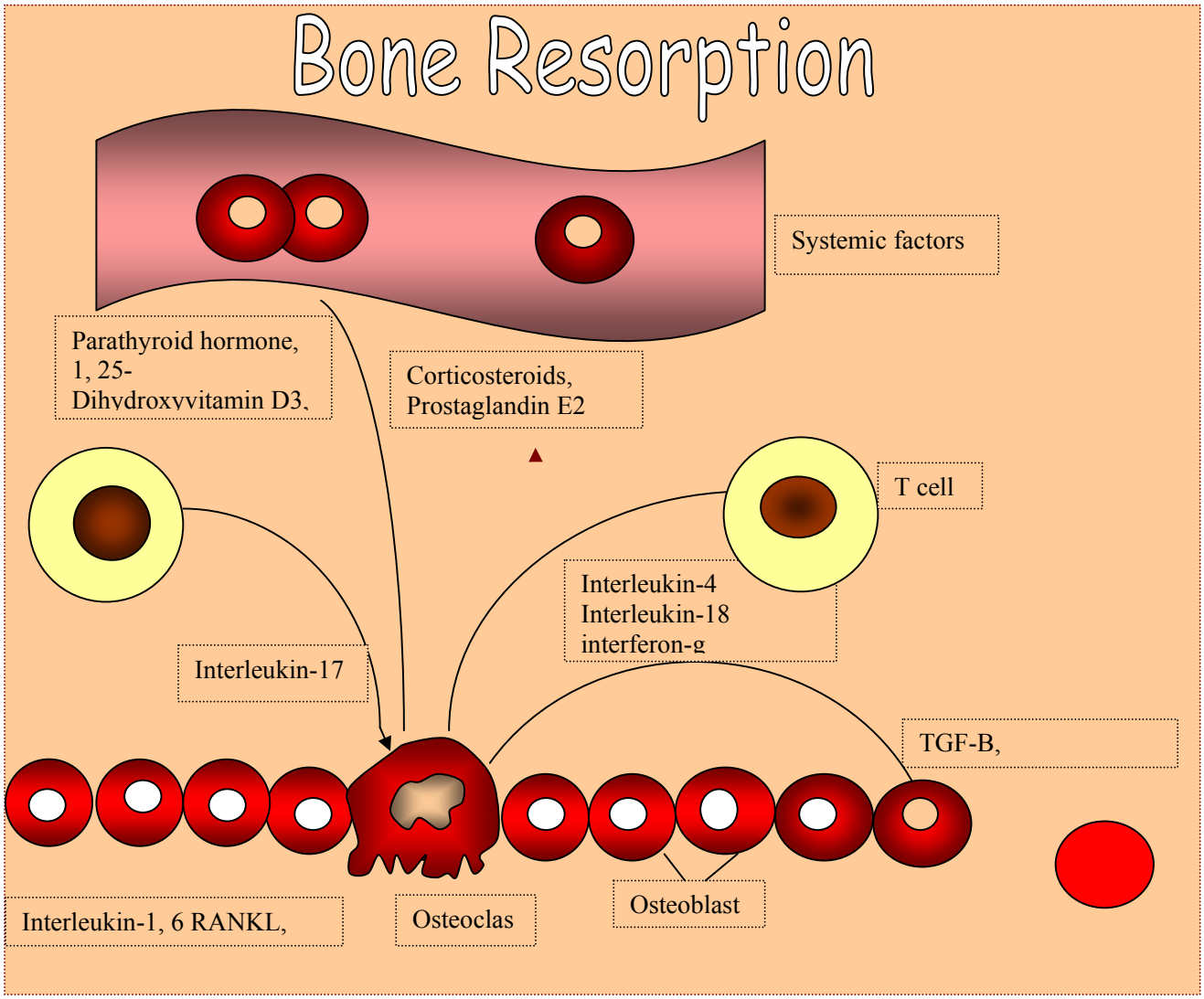


Figure 1: Regulation of bone resorption: both systemic factors and locally acting factors induce the formation and activity of Osteoclasts. Systemic hormones such as parathyroid hormone, 1, 25 Dihydroxy vitamin D_3, and thyroxin (T_4), stimulate the formation of osteoclasts by inducing the expression of receptor activator of the nuclear factor-*k*B (RANKL) on marrow Stromal cells and osteoblasts. In addition, osteoclasts produce interleukin-6, interleukin-1, prostaglandins, and colony stimulating Factors (CSF), which induces the formation of osteoclasts. Accessory cells such as T cells can produce cytokines that can inhibit the formation of osteoclasts such as interleukin-4, interleukin-18 and interferonγ.

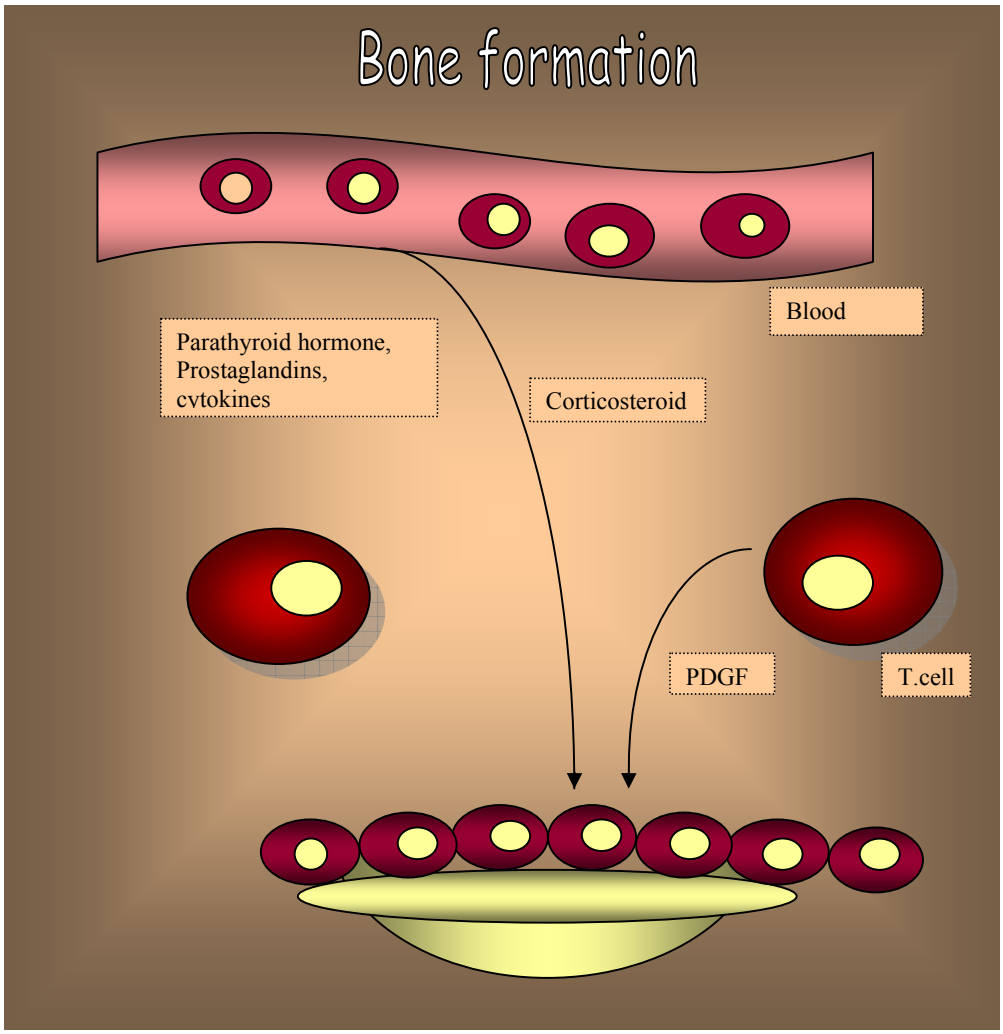


Figure 2: Both systemic factors and locally acting factors can enhance the proliferation and differentiation of osteoblasts. These include Parathyroid hormone, prostaglandins, cytokines as well as growth factors, such as platelet derived growth factor (PDGF) derived from lymphocytes. In addition bone matrix is a major source of growth factors, which can enhance the proliferation and differentiation of osteoblasts. These include the bone morphogenic protein (BMP), TGF-β, insulin like growth factors, and fibroblast growth factors. Corticosteroids can induce apoptosis of osteoblasts and block bone formation.

1.5. Bone metastasis

Although the genetic basis of tumor genesis may vary greatly between different cancer types, the cellular and molecular steps required for metastasis are similar for all cancer cells. In carcinomas the influence of microenvironment are mediated in large part, by bidirectional interactions (adhesion, survival, proteolysis, migration, immune escape mechanisms, angiogenesis and homing in target organs) between epithelial tumor cells and neighboring stromal cells, such as fibroblast as well as endothelial and immune cells. In this work I summarize recent advances in understanding the molecular mechanisms that govern this frequently lethal metastatic progression along and axis from primary tumor to distant organ sites, namely bones. Eventually, we discuss new therapeutic approaches targeting the microenvironment

Majority of the bone tumors have metastatic origin. Bone is the most frequently affected site in human malignancies. In case of certain tumors such as breast and prostate, in advanced stages of the disease approximately 70% of patients develop bone metastasis (14). The prevalence of metastasis declines to approximately 30% in other tumors originating from lungs, thyroid or kidneys (12). Among tumors with the least metastatic potential such as colon and bladder carcinoma up to 20% of advanced disease spread into bones. Detection of bone metastasis in the majority of malignancies is equivalent to incurable stage of the disease. Small group of patients may show long term survival even in the presence of bone spreading. Survival of patients with bone metastasis still may vary from one or two months to several years, the longest survival is seen in 20% of the tumors originating from breast (15).

1.5. Types of bone metastasis

Metastases have been characterized as osteolytic and osteoblastic. This classification also represents the two extremes of a continuum in which deregulation of normal bone remodeling occurs (12). Patients can have both osteolytic and osteoblastic metastasis or mixed lesions containing both elements. Most patients with breast cancer have predominantly osteolytic lesions, although at least 15 to 20 percent of

them have predominantly osteoblastic lesion (10). In addition secondary formation of bone occurs in response to bone destruction (16). The reactive process enables us to detect osteolytic lesions by means of bone scanning, which identifies site of active bone formation. Only in multiple myeloma do purely lytic bone lesions develop. In contrast the lesions in prostate are predominantly osteoblastic (12).

1.6. Pathomechanism of bone metastasis

The basis of bone metastasis is hematogenous spreading. Dissemination of solid tumors occurs through the lymphatic and blood vessels leading to regional lymph nodes and organ metastasis respectively. Hematogenous spreading of solid cancers represents the biggest clinical challenge in oncology and has a fundamental influence in outcome of the disease.

The vascular structure of primary tumor has a significant importance in initiation of spreading. Density of vasculature in tumor tissue is important determining factor for the chances (presence) of metastasis. Tumor cell in primary tumor enter circulation via the walls of the vessels in the tumor mass. These cells (1) adhere to the endothelium of post capillary venulles, (2) lysing the connective tissue underlying the endothelial cells and getting into the circulation (16).

Lung cancer is one of the most frequent human malignancies categorized as highly aggressive malignancy, showing relatively high metastatic potential. Tumors originating from lungs enter pulmonary vein and get directly into the circulatory system, in and other word their dissemination is strictly hematogenous.

Prostate cancers belong to the groups of tumors in which prognosis and consequently progression is associated to the extent of vascularization. Prostate cancers are the most frequent source of bone metastasis, these cells penetrate the pre-capillary venous plexus and via the circulation they find their way to lumbar and lumbo-sacral vertebrae, where they eventually penetrate the bone matrix (12, 17).

The majority of tumor cells entering the circulation get destroyed due to mechanical damages which are resulted from the turbulence of blood flow in large arteries. This so called natural defense is less effective on the tumor cells entering the venous system (17). Another effective defense mechanism in circulation is the presence of white blood cells, monocytes and natural killer cells express direct killing effect on tumor cells either by lyses of the cytoplasmic membrane or phagocytosis of detected cells. The majority of tumor cells die due to this effect, leaving only 0.1% to 1% of the circulating tumor cells as the survivals (18).

On the contrary to white blood cells platelets play a defending role, these cells aggregate around the tumor cells and practically build a barrier against the immune cells and mechanical damage. In another word interaction of tumor cells with platelets plays an important role in hematogenous spreading and perhaps bone metastasis (17). 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. These include β3 integrins, selectins, and CD44H (19)

Extravasation of tumor cell occurs in capillary bed, as the aggregates of tumor mass and platelets get stacked in the capillaries the tumor cells adhere to the endothelial lining of these vessels destroying the basement membrane and eventually getting into the various tissues.

There is organ selectivity in metastasis, supporting Seed and Soil theory which implicates that both the unique phenotype of tumor cells and the unique phenotype of the host organ together are responsible for organ selectivity (17).
Bone specificity of the metastatic process may depend on the ectopic expression of Bone Matrix Proteins (BMP). Tumors which are characterized by osteopontins, osteocalcin and or bone sialoprotein expression are breast, prostate, and thyroid cancers, all of which are characterized by a strong metastatic preference for bone

(20, 21). Bone is an abundant source of inactive growth factors, which are activated during the bone resorption process, (26) which can then stimulate the growth of tumor cells. Parathyroid hormone related peptide is probably the factor produced by the cancer cells and most solid tumors that stimulate the formation of osteoclasts. Both parathyroid hormone related peptide and parathyroid hormone bind the same receptor (PTHR1) and induce the expression of RANKLE on marrow stromal cells. Parathyroid hormone in the main regulator of calcium homeostasis, at the same time parathyroid hormone related peptides hove similar biological effects on the bones. In the amino acid sequences of parathyroid hormone and parathyroid hormone related peptides, 8 out 13 amino acids are identical, and both peptides have similar three-dimensional structure (42).

Several factors account for the frequency of the bone metastasis. Blood flow is high in areas of red marrow, accounting for the predilection of metastasis for those sites (12). Production of adhesive molecules by the tumor cells enables them to attach to marrow Stromal cells and the bone matrix. These adhesive interactions induce the tumor cells to increase the production of angiogenesis factors and the bone-resorbing factors that further enhances tumor growth in the bone. Bone is also a large repository of immobilized growth factors; these growth factors are released and activated during resorption providing fertile ground in which tumor cells can grow.

Dissemination to the bones mainly happens through the arteries, cancers originating from the prostate are exceptions since they reach the bones through the venous system (22). Prostate cancer cells secrete endothelin-1, uPA, and PSA (prostate specific antigen), which in turn induce bone Stromal cells to produce bone morphogenic growth factors such as bFGF, TGFβ, HGF, IGF and BMPs, leading to the formation of new bone around the metastatic foci (17).

From the arterial system tumor cells get to the sinusoid of the bones, these sinusoid systems are simple and quite similar to the hepatic and lymphatic sinusoids. All of

them present similar microscopic structure with a thin virtual basement membrane bordering non fenestrated endothelial cells. Basically all of the chemical factors of local organ get easily through these sinusoids, eventually all of the information's of critical importance for the survival of the tumor cells reach them without requiring theses cells penetrating the vascular structures (19).

Bone matrix is very unique tissue for tumor cells. Bone tissue is mineralized and therefore it makes a good mechanical defense for the tumor cells due to mineralization, the dynamic reconstruction and remodeling of the bone enables the tumor cells penetrating and surviving in bone matrix. Osteoclast and osteoblasts are the major cells involved in remodeling of the bone. The molecular mechanism of the dynamism is later in detailed (12).
Bone metabolism is governed by a delicate, through highly regulated, balance between bone formation (by osteoblasts) and bone resorption (by osteoclasts). This results in an ongoing process of bone remodeling which confers strength and decreased fraction risk. Biphosphonates are the most efficacious anti-resoptive agents available in the management of metabolic bone disease and bone metastasis. These agents inhibit osteoclast function and interfere with recruitment and differentiation of osteoclast precursors (91)

It is well known for a while that metastasis of malignant tumors to the bone is achieved in either of these ways, the disseminating cells solve the bone tissue and the surroundings by lytic enzymes and consequently destroying the arachnoids structure of the bone leading to pathologic fracture. These groups of patients are presented with severe pain succeeding pathological fracture as weigh bearing bones are unable to fulfill their function. Breast cancers usually demonstrate pathological fractures, it was taught previously that tumor cells are capable for production of lytic enzymes solving the bone tissue, but nowadays this theory is not accepted anymore. For the development of lytic metastasis tumor cells activate osteoclasts, so the spreading is called osteoclastic. Tumor cells stimulate physiological resorption of bone by production of certain chemical mediators (18).

The other form of dissemination is osteoblastic, this is characterized by continues bone formation; osteogenesis goes on around the tumor tissue by induction factors derived, either from tumor or bone tissue. The mediators secreted activate the osteoblasts to build the bone tissue around the tumor. These groups of the patients do not suffer form the pathological fracture of their weight bearing bone. It will be worth to talk about the molecular mechanism of the two subtypes of metastasis. Knowing the exact mechanism of metastasis will enable the physicians to target several components involved in metastasis and consequently interfere with dissemination process.

As mentioned previously physiologically construction of the bones is mediated by osteoblasts. Activity of these cells is mediated by certain proteins generally called cytokines. Namely growth factors are involved in this process, transforming growth factor (TGF), insulin like growth factor (IGF) and fibroblast like growth factor (FGF) and BMP protein are the most important ones to be mentioned (!7).

The hormonal status of the case may influence the osteoblastic activity, elevated PTH level (parathyroid hormone) has a positive effect whereas elevated corticosteroid levels demonstrates a negative effect on osteoblastic activity.

The interferon's (IFN) are natural glycoproteins with antiviral, anti-proliferative, and immunomodulatory properties. The major classes are (1) IFN-α, derived from leukocytes, (2) IFN-β, derived from fibroblasts, (3) IFN-γ, derived from activated lymphocytes. Both, natural and recombinant interferons possess activity in renal cell carcinoma. Overall response rates are 12% to 14%. There are no apparent differences in response rates using different interferons. Likewise, there is no well-established dose response relationship for IFNs, although the toxicity is dose related (23).

In the case of the breast cancer causing osteolysis, the main mediator is parathyroid-hormone-related-peptide (PTHrP), whereas in osteoblastic lesions, known mediators include endothelin-1 and platelet-derived growth factor (21).

In osteolytic metastasis, there is a vicious cycle in bone microenvironment, whereby bidirectional interactions between tumor cells and osteoclast leads to both osteolysis and tumor growth (74).
Bisphosphonate interrupts the vicious cycle and causes not only a reduction in osteolytic bone lesion, but also decrease the tumor burden in bone. (74)

Both systemic factors and locally acting factors induce the formation and activity of osteoclasts (12). Systemic hormones such as parathyroid hormone, 1, 25-dihydroxyvitamin D3, and thyroxine (T4) stimulate the formation of osteoclasts by inducing the expression of receptor activator of nuclear factor-kB ligand (RANKEL) on marrow Stromal cells and osteoblasts. In addition osteoblasts produce interleukin-6, interleukin-1, prostaglandins and colony stimulating factor (CSF), which induces the formation of osteoclasts. Accessory cells such as T cells can produce cytokines that can inhibit the formation of osteoclasts, such as interleukin-4, interleukin-18 and interferon gamma. TGF-β denotes (transforming growth factor β) (9).
Several factors accounts for frequency of bone metastasis. Blood flow is high in areas of red marrow (24), accounting for predilection of metastasis for those sites. Furthermore tumor cells produce adhesive molecules that bind them to the marrow stromal cells and bone matrix. These adhesive interactions cause the tumor cells to increase the production of angiogenetic factors and bone-resorbing factors that further enhances tumor growth in bone (25).

1.8. Osteolytic metastasis

In osteolytic metastasis, the destruction of the bone is mediated osteoclasts rather than the tumor cells. However the factors responsible for the activation of the osteoclasts vary depending on the tumor. In multiple myeloma, osteoclasts accumulate only at bone resorbing surfaces adjacent to myeloma cells; their levels are not increased in areas uninvolved with tumor. In addition to increase in bone resorption, bone formation is suppressed so that, bone lesion in patients with myeloma becomes purely lytic (27).

Several osteoclastogentic factors have been implicated in the increased activity of osteoclasts in myeloma (28). The leading candidates are interleukin-1, interleukin-6, macrophage inflammatory protein1α,and RANKL. Interleukin-1 is a potent stimulant of osteoclast formation, but levels of interleukin-1produced by myeloma cells are extremely low (29). They believe that interleukin-1 is not a major mediator of myeloma bone disease.

Interleukin-6 is a growth factor or at least blocks apoptosis of myeloma cells (30). It is present in marrow plasma samples from patients with myeloma. Interleukin-6 is a potent stimulant of osteoclasts formation and can enhance the effects of the parathyroid hormone-related peptide on the formation of osteoclasts in vivo. Interleukin-6 levels have net consistently been correlated with the presence of bone lesions, however (31). When a myeloma cells adheres to marrow stromal cells, the production of interleukin-6 by marrow stromal cells increases. Thus interleukin-6 appears to have an important role in enhancing the growth or prolonging the survival of myeloma cells, though its role in myeloma bone disease remains to be determined.

RANKL is a major mediator of myeloma bone disease. It is suggested that myeloma cells produce RANKL, (32, 33) it is still unclear that the amount of RANKL produced by myeloma cells is sufficient to induce the formation of the osteoclasts. Instead it may simply prevent apoptosis of osteoclasts. RANKL is produced by marrow sromal cells in myeloma. In the microenvironment of bone of myeloma patients, RANKL production is increased and osteoprotegerin is markedly decreased (34). Blocking the binding of RANKL to RANK receptors or osteoprotegerin inhibits bone destruction (35).

Macrophage inflammatory protein 1α also appears to be a key regulator of bone destruction in myeloma (36). Macrophage inflammatory protein 1α is a potent inducer of osteoclast formation in vivo, independently of RANKL, and enhances both RANKL stimulated and interleukin-6 stimulated osteoclast formation (37). Macrophage inflammatory protein 1α levels is correlated strongly with the presence

of osteolytic lesions, moreover, macrophage inflammatory protein 1α also enhances adhesive interactions between myeloma cells and stromal cells by up regulating the expression of β1 integrin on myeloma cells. Adhesive interactions between marrow stromal cells and myeloma cells increases the production of interleukin-6, RANKL, and macrophage inflammatory protein 1α further increasing bone destruction (38).

Bone lesions in myeloma are purely lytic, that is there is no osteoblastic response. This phenomenon explains the clinical observation that in about half of the cases of myeloma, bone scans are normal in presence of severe osteolytic bone destruction. The basis of decreased osteoblastic response in myeloma is unknown. Myeloma cells can produce tumor necrosis factor α, which inhibits osteoblastic growth and differentiation (39). However, tumor necrosis factor α has not been implicated in the suppression of bone formation in myeloma. On the other hand myeloma cells express dick-kopf 1 (DKK1), a Wnt-signaling antagonist and the presence of high levels is correlated with focal bone lesions in patients with myeloma. A high level of DKK1 interferes with differentiation of osteoblasts in myeloma patients (40).

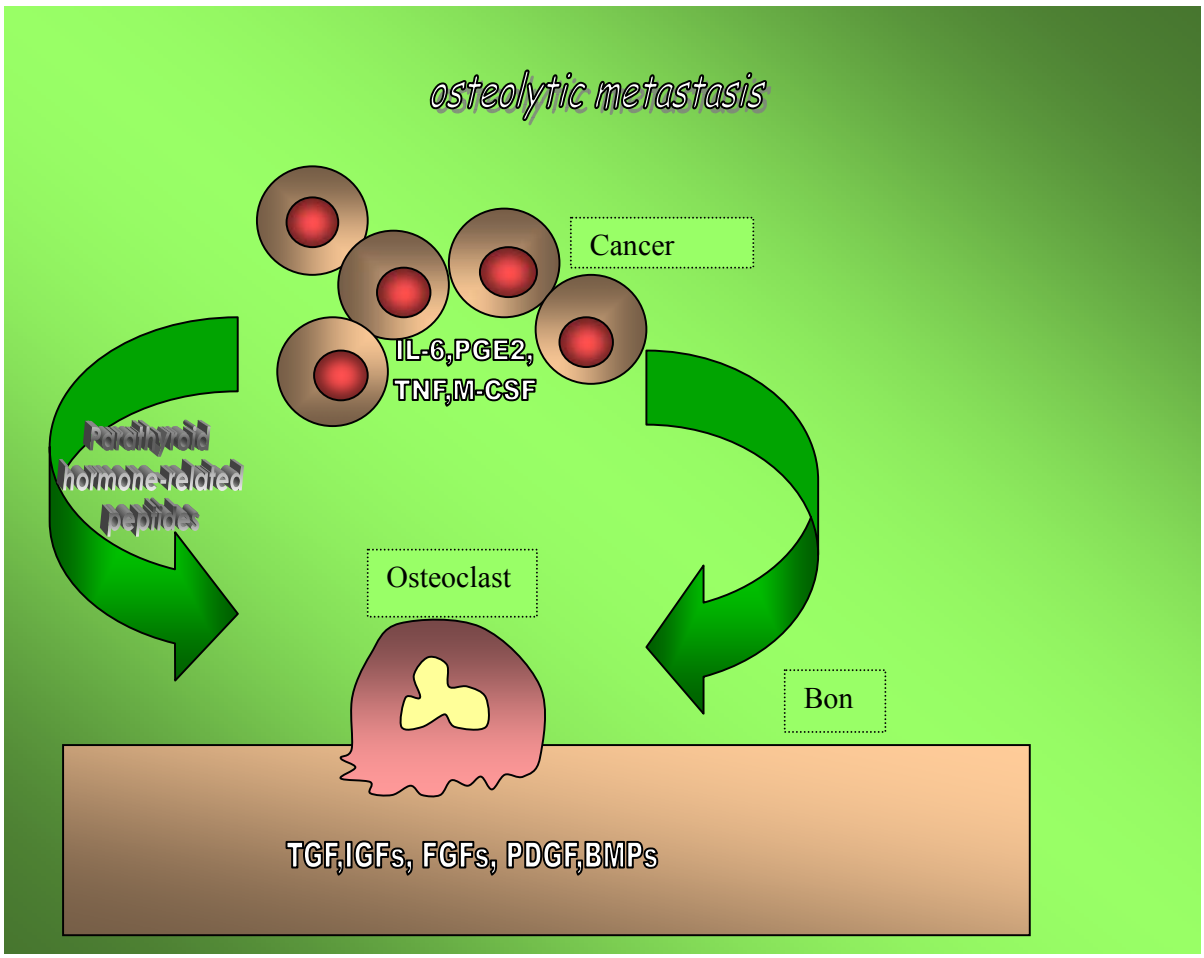


Figure 3: The vicious cycle of osteolytic metastasis

Tumor cells, in particular breast-cancer cells, secrete parathyroid hormone related peptide as the primary stimulator of osteoclastogenesis. In addition tumor cells produce other factors that increase the formation of osteoclasts, including interleukin-6, prostaglandin-E_2, tumor factor (PDGF), and bone morphogenetic protein (BMP), which increases the formation of parathyroid–hormone related peptide by tumor cells as well as growth factors that increase tumor necrosis factor, and macrophage colony stimulating factor (MCSF). These factors increase the expression of receptor activator of nuclear factor-$_k$B ligand, and (RANKL), which directly acts on osteoclasts precursors to induce the formation of osteoclasts and bone resorption. The process of bone resorption releases factors such as transforming growth factor β (TGF-β), insulin-like growth factors (IGF), fibroblast growth factor (FGF), and platelet-derived growth factor. This symbolic relationship between bone destruction and tumor growth increases further bone resorption and proliferation of tumor cells.

1.9. - HER-2 and Breast cancer

The Her2/neu oncoprotein is a transmembrane receptor, belonging to the epidermal growth factor receptor (EGFR) family, with tyrosine kinase activity. Her2/neu has been shown to be over-expressed, most commonly by gene amplification, in a number of human malignancies, including breast (BRC). (1) Overexpression of the Her2/neu oncoprotein in breast cancers is associated with shortened survival, enhanced aggressiveness, resistance to hormone- and chemotherapy (2-4)) and eventually decreased sensitivity of targeted cells to therapy (5). The extracellular domain of her2/neu is the target of trastuzumab (Herceptin®), the humanized anti-her2/neu monoclonal antibody. (6) It has also been proven that the anticancer efficacy of trastuzumab is highly dependent on the Her2/neu amplification of breast cancer. (7)

A careful selection of patients is crucial for raising the clinical benefit of trastuzumab, and avoiding unnecessary exposure of patients who most likely will not benefit from it. (6) Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are standard methods to determine her2/neu protein expression or gene amplification, respectively. Clinical data indicate that patients with IHC 3+ and/or FISH-positive BRC gain the greatest clinical benefit from Herceptin® treatment. (8)

It is still a matter of debate whether Her2/neu status should be evaluated by early testing, i.e. systematically performed examinations on the primary tumor at the time of diagnosis of early breast cancer, or by pretreatment testing, i.e. performed on either the primary tumor or on a metastatic site at the time of tumor relapse. (9) To date, the Her2/neu status in the majority of the cases is evaluated in the primary tumor sample, since routine biopsy of metastatic sites is not a standard procedure (10) Never the less, the main targets of any systemic therapy in metastatic breast cancer are metastatic foci. (10) Primarily one expects to find great similarity between primary site and metastatic foci on protein expression. Whereas, experimental and some clinical data suggest that, 1. The primary tumor is genetically heterogeneous, 2. The clones responsible for organ dissemination may not be even present in the primary tumor but may develop through sequential genetic alterations, or 3. They compose very small percentage of the primary. (11) Earlier studies exclusively used Herceptest to assess the maintenance of Her2 genotype in metastatic

lesions and found it concordant in case of lymph node, liver and lung metastases (12-16). Recently some reports provided evidences that the Her2 genotype of BRC may change in metastasis: loss was reported in one case in liver metastases (17) and loss of Her2 amplification was reported in 21 % but appearance of Her2 amplification was very frequent in visceral metastases (30%) (18).

1.9.-a Osteolytic metastasis from breast cancer

Dissemination of BRC cells occurs through lymphatic and hematogenous channels. Nodal metastases are present in about two thirds of cases at the time of diagnosis, axillary's nodes, lymph nodes along the internal mammary arteries, and the supraclavicular are usually the primary site of spread. More distant dissemination eventually ensues, with metastatic involvement of eventually any organ or tissue in the body. Favored locations are the lungs, skeleton (namely bones), liver and adrenals (14).

Tumor cells in breast cancer produce factors that directly or indirectly induce the formation of osteoclasts. In turn, bone resorption by osteoclasts releases growth factors from the bone matrix that stimulate tumor growth and bone destruction (41). This reciprocal interaction between breast cancer cells and the bone microenvironment results in a vicious cycle that increases both bone destruction and the tumor burden.

Bone is an abundant source of inactive growth factors, which are activated during the bone resorption process, (26) which can then stimulate the growth of breast cancer cells. Parathyroid hormone related peptide is probably the factor produced by breast cancer cells and most solid tumors that stimulate the formation of osteoclasts. Both parathyroid hormone related peptide and parathyroid hormone bind the same receptor (PTHR1) and induce the expression of rankle on marrow stromal cells. Parathyroid hormone in the main regulator of calcium homeostasis, at the same time parathyroid hormone related peptides hove similar biological effects on the bones. In the amino acid sequences of parathyroid hormone and parathyroid hormone related

peptides, 8 out 13 amino acids are identical, and both peptides have similar three-dimensional structure (42).

The production of parathyroid hormone related peptide is increased in metastasis of breast cancer to bone. This peptide induces the formation of osteoclasts and bone resorption, which in turn releases transforming growth factor β. Transforming growth factor β further increases production of peptide by breast cancer cells (43). In the vicious cycle of breast cancer metastasis, bone destruction increases local calcium levels, which promotes tumor growth and production of parathyroid hormone related peptide. Breast cancer cells also produce, or induce, interleukin-6, prostaglandin E_2, macrophage colony stimulating factor, interleukin-1 and tumor necrosis factor α , (44) which may also play an important role in the induction of osteoclast formation by breast cancer metastasis. Prostaglandin E_2 can increase the expression of RANKL and directly enhances the effect of RANKL on the formation of osteoclasts (12, 43).

Osteopontin is also widely expressed in breast cancer and has been assessed as a potential prognostic factor in metastatic breast cancer. Osteopontin is a secreted phosphoprotein that is synthesized in a variety of tissues, high level of OPN is detected in the bones, where it is produced by both osteoclasts and osteoblasts (33). Osteoclastogenesis is reduced in the absence of OPN, and the osteoclasts which are produced are less motile and have impaired ability to resorb bone. OPN is widely expressed in variety of human tumors and has been assessed as potential prognostic factor in metastatic breast cancer.

1.9.-b. Metastatic genes of BRC

Although the metastatic potential of tumors does not necessarily depend on their proliferation potential, but in certain tumors such as breast cancers, the proliferation rate of the tumor is frequently associated with poor prognosis. Therefore, markers of proliferation potential have been studied extensively.

NM23 was the first metastatic suppressor gene to be described. Decreased expression of NM23 gene and low levels of NM23H1 protein, in breast cancers is associated with poor survival. The metastasis suppressor, E-cadherin, is usually

down-regulated in invasive breast cancer and it's proved to be an independent negative prognostic factor for node status (17).

CD44 and its variants are differentially expressed in breast cancers, it was demonstrated that expression of v6 variant correlates to favorable prognosis in node negative patients, while the expression of CD44v3-4 indicates the potential for local lymphatic spread (17, 45).

Down regulation of β1 integrins (laminin collagen receptors) is detected mainly in invasive breast cancers. On the other hand, highly invasive breast cancers were shown to express αvβ3 integrins, these proteins show high affinity to osteopontins and osteonectins, (found extensively in the bones), suggesting that these adhesion molecules may mediate the specific recognition of the bone by circulating metastatic cells.

Up regulation of MMP genes, namely MMP-2 overexpression, is and strong independent prognostic marker for unfavorable outcome and metastasis (46).

1.9.-c. Detection of micrometastasis in breast cancer

Despite apparent curative surgery in the treatment of breast carcinoma 25% of node negative patients still develop lymph node and distant metastatic disease. These groups of patients usually have occult micrometastatic disease at the time of surgery and they are under-staged. Up to 38% of patient with stage 1 and 2 breast cancers have demonstrated micrometastasis and reduced survival (14, 47).

The formal TMN staging is based almost exclusively on the anatomical stage of the disease, which is assessed using a combination of tumor size or depth (T), lymph node spread (L), and presence or absence of metastasis (M). The TNM system has provided a standardized, anatomical basis for staging and several important functions (47). It provide basis for prediction of survival, choice of initial treatment, stratification of patients in clinical trials, accurate communication between healthcare providers, and uniform reporting of outcome

The anatomically based TNM staging system remains useful for the system listed above, but new factors are both complicating the situation and providing new opportunities when it comes to predicting survival and/or selection of therapy. Development of biomarkers has opened a new chapter in cancer therapy. First, individual molecular markers and patterns of markers are successfully subdividing traditional tumor classes into subsets that behave differently from each other. Second, chemotherapeutic and biological agents are more effective and more widely used than when TNM staging was introduced, especially in the adjuvant staging. Third, many new targeted agents such as tamoxifen, are effective only if their receptive molecular markers are mutated or expressed in sufficient levels (12, 14, 47).

Specifically in the case of bone metastasis, biomarkers could be used for detection of the origin of metastasis and subsequently choice of initial treatment.

Several biomarkers could be used for early detection of metastasis from breast cancers (17, 47).

1. CA15-3 is a glycoprotein found in serum of patients of breast cancer is used for monitoring
2. CA27-29 is a glycoprotein found in serum of patients with breast cancer used for monitoring
3. Cytokeratins, belonging to the membrane proteins could be detected by immunohistochemistry; they are used as prognostic factors
4. Estrogen receptor and progesterone receptor, belonging to the membrane proteins, detected on primary tumors of breast cancers, used for the selection of hormonal therapy,
5. HER2/NEU proteins detected in breast tumors, by immunohistochemistry, used for prognosis and selection of therapy
6. HER2/NEU protein detected in serum, could be used for monitoring
7. c-erbB2 protein usually a predictor of a poor outcome
8. VEGF protein overexpression, correlated with high rate of metastasis and consequently poor outcome,

1.10. Dissemination of lung cancers

Bronchial carcinomas are without doubt the number one cause of cancer related deaths in industrialized countries. The pick incidence of lung cancer occurs between ages 40 and 70 years; currently the male to female ratio is 2 to 1. Male cigarette smokers are about 10 times more likely to die of bronchogenic carcinoma than are nonsmokers (48).

The four major histological types of bronchogenic carcinomas are squamous cell carcinoma, adenocarcinoma, large cell undifferentiated carcinoma, and small cell carcinoma. It has become apparent that for most therapeutic decisions the first three can be lumped into a category termed non-small cell lung carcinoma (NSCLC) to distinguish them from small cell lung cancer (SCLC) (49).

Like all cancers, lung cancers result from genetic changes that affect oncogene and tumor suppressor genes. SCLCs are characterized by changes in several oncogens, including amplification of myc family. L-myc amplification is associated with particularly aggressive behavior (17). Overexpression or increased function of EGFR, cyclin-D1, and BCL2 genes are frequently detected in lung cancers. Mutational inactivation of P53, p16, FHIT, RASSFIA, SEMA3B, and *Rb* are also common in SCLC. In addition all SCLCs have a deletion of the short arm of chromosome 3 (3p14-25), where at least three tumor suppressor genes are suspected to reside (49).

Genomic profiling of lung cancer revealed that squamous cell cancer is characterized by overexpression of several members of the arachidonic acid metabolizing system (LTB4 dehidrogenase and COX-2) which produce bone regulatory prostanoid metabolites 3. It is worth to mention that PTHrP or bone matrix protein expressions characterizing other bone metastatic cancer types are not expressed by squamous cell lung cancer.

The genetic alterations in NSCLC are somewhat different. Alteration in myc, p53, and *Rb* are present, but less frequently than in SCLC. On the other hand, mutations of K-ras, not seen in SCLC, are found in about 30% of adenocarcinomas, and the activation of these oncogenes is associated with poor prognosis (49).

A proportion of Non Small Cell Lung Cancers (NSCLC) are characterized by EGFR protein over expressions partly due to gene amplification and in a subset of adenocarcinomas by tyrosine kinase domain mutations (49, 50). On the other hand, smoking induces K-RAS mutation which occurs in 20-30% of NSCLC being more frequent in adenocarcinomas than in squamous cell cancers (51). It is worth to note that the K-RAS and the EGFR-TK mutations are mutually exclusive (50, 52). EGFR overexpression in NSCLC has no prognostic importance whereas, EGFR amplification and especially TK mutations predict favourable response to EGFR targeted therapies. (53) On the other hand, K-RAS mutation was reported to be a negative prognostic and predictive factor, although other studies challenged this view (51) It is important to note that almost all of the aforementioned studies on EGFR and K-RAS were performed on primary tumors and much less information is available concerning the metastatic tissue.

Tumor induced angiogenesis is and independent prognosticator for disease progression in lung cancers. Over-expression of VEGF, bFFGR, and FGRFR1 add correlate with unfavorable outcome (17, 50).

Lung cancers are characterized by high metastatic potential; therefore identification of metastatic potential specific markers would be highly desirable to clinical oncologists.

1.10 a. Metastasis genes in NSCLC

Expression of CD44 is associated with poor survival but on the contrary to breast cancers no splice variants have been detected in lung cancers. NM23 is not expressed by bronchial epithelium but it can be expressed by NCSLC, a feature that strongly predicts poor survival. Besides the NM23H1 expression is associated with c-myc expression and proliferative potential of the tumor (17).

1.10. b. Biomarkers in lung cancers

On the contrary to plenty of tumors, there are no biomarkers in value for early detection of the disease.

There are controversial data for carcino-emberionic antigen (CEA), used in diagnosis of NSCLC and neurospecific enolase, used for detection of SCLC. Though detection of disease in early stages is quite difficult, there are several markers for prediction of the outcome of the disease. Methylation of DAPK and IL-10 gene leading to reduced expression, in combination with BCL2 and Ki-67 overexpression is powerful indicators of poor survival (48).

Aggressive subtypes of adenocarcinomas are found to express p16/INK4, arachidonic acid metabolizing enzymes, COX and LTB4 dehydrogenase, as well as proteases such as cathapsin-L and uPA. P63 and caspase4 involved in the regulation of apoptotic potential, HER2 and cytochrome p450, as well as matrix proteins namely laminin and bone morphogenic protein-2 are also highly expressed in aggressive subtypes of adenocarcinomas (17 45, 48). On the other hand, the good prognosis signature contained surfactant protein A, TTF1, and hespin protease (17).

In adenocarcinomas, loss or reduced expression of laminin/collagen receptor $\alpha 3$ ($\beta 1$) is associated with poor survival. Loss of expression of αv ($\beta 3$) from NSCLC cells predicts the recurrence of the disease in N0 graded patients. Appearance of MUC-1 in adenocarcinoma is a bad prognostic factor (17, 48).

1.10. d. Differential diagnosis of the primary or metastatic tumors in the lungs

A significant portion of primary tumors of the lungs are adenocarcinomas, on the other hand adenocarcinomas of different origin are frequently spreading to the lungs (49). In order to achieve a proper therapeutic approach one has to distinguish between tumors of pulmonary origin and metastatic ones (17). Adenocarcinomas of the lung originate either from type 2 pneumocytes of Clara cells, which do maintain distinct and specific ultra-structural characteristics in their tumors. Pneumocytes contain cytoplasmic lamellar bodies due to the production of surfactant, a unique intranuclear inclusion (apoprotein) and microvilli without glycocalix. Clara cells also have lamellar bodies and occasionally dense core granules. These ultra-

structural features are maintained in the well and moderately differentiated adenocarcinomas of the lungs (49, 50). Certain proteins could be used as markers too, cytokeratin 7 (CK-7), villin, and thyroid transcription factor-1 (TTF-1) could be used to differentiate the adenocarcinomas with pulmonary origin. Metastatic adenocarcinomas of colon show enhanced expression of CK-20 (17). Presence of glycocalix positive microvilli on the tumor cells supports the diagnosis of colonic adenocarcinoma. Adenocarcinomas of breast origin express cystic fluid protein-15 (CFP15) which is almost never expressed in primary tumors of the lung (54). Surfactant protein A or B could be occasionally expressed by adenocarcinomas of the breast, on the other hand prostate, colonic and renal adenocarcinomas, never express surfactant protein, so this protein could be used for differential diagnosis (53). Electron microscopy could be used for identification of adenocarcinomas of breast origin, since these cells usually contain intra-cytoplasmic lumens decorated with microvilli (53, 54)

Table 1: Differential diagnostic markers of primary and metastatic adenocarcinomas of the lung (17)

	SFA/B	**TTF-1**	**CK-7**	**CK-20**	**CEA**	**villin**	**CFR-15**	**ER** **S100**
Lung primary	+	+	+	-	+	+	-	+/- +
Breast cancer	+/-	-	+	-	+	+	+	+ +/-
Colon cancer	-	-	-	+	+	+	-	- -

SFA: Surfactant

TTF: Thyroid transcription factor

CK: Cytokeratin

ER: Estrogen receptors

1.11. Cancer of prostate

Carcinoma of prostate is the most common visceral cancer in males in US, ranking as the second most common cause of cancer related deaths in men older than 50 years of age, after carcinoma of the lungs. It is predominantly the disease of older males with a pick incidence between ages of 65 and 75 years. Latent cancers of the prostate are even more common than those, which are clinically apparent, with and overall frequency of more than 50% in men older than 80 years of age (55).

Histologically, most prostate carcinomas are adenocarcinomas exhibiting variable degree of differentiation. The better-differentiated lesions are composed of smaller glands that infiltrate the adjacent stroma in an irregular, haphazard fashion.

Prostate cancer spreads to the bones relatively in early stages of the disease, causing multifocal metastasis. Bone metastasis particularly to the axial skeleton is common and may cause either osteolytic or more commonly osteoblastic lesions (12, 17). Accordingly staging is more complex and grading system applied is more complicated. Gleason score seems to fit these requirements. Capsular invasion is a very strong prognostic factor, since the encapsulated lesions seems to have a much better prognostic outcome (55). Presence of androgen receptors on the prostate cancer could be considered as positive sign and a good prognostic outcome. Prostate cancers show different sensitivity to androgen and based on this they are divided to several groups: Androgen dependent, androgen sensitive, and androgen independent (17). Emergence of androgen independent phenotype is mediated by profound genetic changes in cancer cells, including expression of growth factor receptors such as EGFR, c-erb-B2, loss of suppressor genes mainly, p53, DCC and APC, and amplification of oncogens, including c-myc and bcl-2 genes.

Extent of vascularization may affect the prognostic outcome in prostate cancers. Determination of vascular density of tumor is obligatory to establish prognosis (56, 57).

It is a repeated finding that in prostate cancers with poor prognosis the cell adhesion molecule and strong suppressor of metastasis E-cadherin is down regulated. Poor prognostic cancers are also characterized by loss of laminin/collagen receptors β4

integrin and ectopic expression of $\alpha 2b\beta 3$. In advanced cancers both uPA and MMP-2 are over-expressed.

1.11 a. Metastatic genes in prostate cancer

Expression of metastatic associated genes is highly unusual in prostate cancer. Loss of CD44 (tumor suppressor gene) and expression of NM23H1 (metastatic gene) occurs in cancer characterized by poor prognosis and systemic dissemination. Meanwhile epithelium of prostate expresses a unique metastatic suppressor gene, KIA-1, coding for a motility related membrane glycoprotein CD82 (17). It has been demonstrated that the expression of KIA-1 is down-regulated in more aggressive tumors (56, 57)

1.11. b. Biomarkers of carcinoma of prostate

1. PSA (prostate specific antigen) total in serum used for screening and monitoring.
2. PSA % in serum for differential diagnosis between benign prostatic hyperplasia and carcinoma
3. Alkaline phosphatase in serum for differential diagnosis of benign condition and malignant tumor
4. RT-PCR for PSA m-RNA is recently being used for detection of micro metastasis and selection of therapeutic approach (12, 17).
5. PAP (Prostatic Acid Phosphatase) is found in high titers in serum of patients with carcinoma
6. PSMA (Prostatic Specific Membrane Antigen) could be detected in prostatic cancer patients with rather advanced disease

1.11. c. Osteoblastic metastasis in prostate cancer

The exact mechanism of osteoblastic metastasis and the factors involved are unknown. Endothelin-1 has been implicated in osteoblastic metastasis from breast cancer (58). It stimulates the formation of bone and the proliferation of osteoblasts

in bone organ cultures (59), and serum endothelin 1 levels are increased in patients with osteoblastic metastasis from prostate cancer (12). Treatment with a selective endethelin-1A-receptor antagonist decreases both osteoblastic metastasis and tumor burden (58). Treatment with antagonist has no effect on the growth of tumor at orthotropic sites. This suggests that blocking osteoblast-inducing activity by tumors may decrease tumor growth and osteoblastic activity and suggests that a vicious cycle may also be involved in osteoblastic metastasis in which tumors induces osteoblasts activity (58,17) and subsequent release from the osteoblasts of growth factors that increases tumor growth. In addition to endothelin-1, platelet-derived growth factor, a polypeptide produced by osteoclasts in bone microenvironment, urokinase, and PSA may be involved (60).

Overproduction of urokinase like plasminogen activator (u-PA) by prostate cancer cells increases bone metastasis (61, 12). These data are confirmed by significantly reduced metastasis potential by cells transfected with anti-sense DNA to u-PA. PSA (a kallikrein serine protease) released by human prostate cancer cells, cleaves parathyroid-hormone related peptide in their N-terminal, which may block tumor induced bone resorption. It may also activate osteoblastic growth factors released in the bone microenvironment, during the development of bone metastasis, such as insulin like growth factor 1&2, or transforming growth factor β (60). These data suggest that a vicious cycle may also be responsible for osteoblastic metastasis.

Bone metastases in prostate cancer are predominantly osteoblastic, with increased numbers of irregular bone trabeculae (62). However markers of bone resorption are also increased in prostate cancer, there is usually no histological evidence of increased numbers of osteoclasts. In prostate cancer, levels of bone resorption markers are higher in patients with bone metastasis than in patients without bone metastasis and reflect the extent of bone metastasis more accurately than does the PSA level (12).

1.12. Renal cell carcinoma

Renal cell carcinomas are adenocarcinomas arising from tubular epithelial cells, and represent 2% of all cancers in adults. These cancers are usually large by the time they are discovered measuring 3 to 15 cm in diameter. They may arise anywhere in the kidney (63). Depending on the amount of lipid and glycogen present, the tumor cells may appear almost totally vacuolated (lipid-laden, clear cell) or may be solid. Renal cell carcinoma can be classified into several subtypes according to histology. Clear cell carcinoma is the most common form, and accounts for majority of cases of renal tumors. Other histological types are papillary, chromophobe and collecting duct carcinomas showing gradual reduction in prevalence from papillary toward collecting duct cancers (63, 70).

Our knowledge about the molecular genetics of RCC has grown significantly in the past decade, and as a result, we have now a much more appropriate picture on the carcinogenesis of RCC. Sporadic clear cell RCC is characterized by inactivating mutation or methylation of VHL, defining this suppressor gene as primary regulator of the carcinogenesis of RCC (63,64) The consequence of the loss of VHL function in clear cell RCC is the overexpression and activation of HIF1α, a master regulator of the hypoxia-responses of cells. Overexpression of HIF1α in RCC results in the up-regulation of several growth factors including VEGF, responsible for the up-regulated angiogenic phenotype of RCC, and overexpression of various receptors including VEGFR2 (63,64). On the other hand, clear cell RCC is characterized by frequent up-regulation of EGFR protein as well, which is not due to mutation or amplification of the gene (65, 66, 67). However, the prognostic significance of the overexpression of EGFR in RCC is controversial (68, 69, 70).

Almost half of the patients with RCC experience distant metastasis during the course of their disease. Metastatic RCC is a disease historically resistant to chemotherapy. A limited subset of patients will experience clinically meaningful benefit from interleukin-2 and /or interferonα therapy, but there was no effective therapy for patients who did not respond or relapsed after cytokine-based treatment. However, this trend was rapidly shifted recently with the advent of molecular targeted therapy of RCC (63, 71). Both anti-

VEGF antibody therapy (72), as well as multi-targeting the signaling pathways in metastatic RCC by VEGFR2 (73) or RAF-inhibitors (74) resulted in significant inhibition of disease progression, identifying these treatments as novel standard of care. However, the molecular marker(s) of drug sensitivity of RCC is not yet defined. On the other hand, trials also indicated that EGFR in RCC could also be potential target for molecular therapy, since several EGFR inhibitors shown clinical activity in metastatic RCC (75, 76). Meanwhile there are limited data available on the pheno-genotype of metastatic (bone) RCC although these new therapies are introduced in advanced stage. It would be worth to compare the EGFR and VEGFR expression profile on primary and metastatic tumors of renal origin, to be able to predict the tumor response to chemotherapy.

1.12. a. Metastatic genes in RCC

VHL tumor suppressor gene, MET proto-oncogene encoding a receptor tyrosine kinase, FH, encoding fumarate hydratase, can be involved in tumorgenesis (17).

Familiar RCC occurs due to an inactivating mutation of VHL gene while papillary RCC is due to mutation in c-met oncogene. VHL gene allele deletion (loss of heterozygosity) has been demonstrated in 84% to 98% of sporadic renal tumors (63, 64). VHL gene inactivation also occurs through methylation of CpG rich DNA (17, 63), rendering the gene transcriprionally inactive, practically VHL suppressor gene could be defined as the main regulator of carcinogenesis of RCC. In consequence to the loss of VHL gene function, HIF1α gene-a master regulator of hypoxia of the cells-gets activated and over expressed. Up-regulated HIF1α in RCC results in up-regulation of several growth factors including EGF and VEGF (72, 73), responsible for epithelial proliferation and angiogenesis respectively. On the other hand, clear cell RCC is frequently characterized by EGFR overexpression (64), irresponsive to gene mutation. EGFR overexpression is correlated with an aggressive clinical course in variety of cancers including renal cell carcinomas (17, 65). High EGFR titer is a poor prognostic factor, usually associated with shortened survival.

In the table below you may view the familiar syndromes associated with RCC (17, 49).

Renal tumor	**Disease**	**gene**
Clear cell RCC bilateral .multiple	Von Hipple-Lindau	VHL
Clear cell RCC unilateral solitary		Translocation on 3q or VHL
Clear cell RCC	Hereditary paraganglioma	SDHB
Clear cell RCC angiomyolipoma	Tuberous sclerosis	TSC1-2(9q-16p)
Papillary RCC bilateral multiple	Hereditary papillary RCC	MET (7q)
Papillary RCC unilateral solitary	Hereditary leiomyomatosis RCC	FH (1-q)
Papillary RCC, Wilm's tumor	hyperthyroidism, jaw tumors	HRPT (1q)

MET is a proto-oncogen encoding a receptor tyrosine kinase that is normally activated by hepatocyte growth factor (HGF). The MET-HGF pathway is responsible for many regulatory cell functions. Most of the germ line mutations occur in the MET activation loop or in the ATP-binding pocket leading to ligand independent MET activation.

Birt-Hogg-Dube (BHD) syndrome is a genodermatosis, and BHD gene encodes folliculin, without a known function. Almost half of the mutation occurs in the mononucleotide tract of cyanosis due to slipping in the DNA polymerase during replication.

(CA9) plays a role in the regulation of intra-and extracellular PH during period of hypoxia in tumor cells. CA9 is one of the genes under the control of HIF-1 and seems to play a very important role in RCC. Down regulation of CA9 in RCC patients is a predictor of metastasis.

Comparative genomic micro-array analysis (CGMA) identified consistent down-regulation of gene expression on chromosome 3 and frequent up-regulation of a short segment on chromosome 5 (5q) in clear cell RCC samples, as well as gain in expression at chromosome 7, 16p, and 17 in papillary RCC.

1.12. b. Prognostic biomarkers of RCC

Classical negative prognostic factors of RCC are aneuploidy and high S-phase fraction (12, 73). P53 positive cases are evaluated as poor prognostic ones. Among the adhesion molecules, cadherin-6 and MUCI/EMA are also the markers of poor prognosis (49). On the contrary to prostate cancers where expression of androgen receptors was usually demonstrating a favorable course of disease, up-regulation of androgen receptors in RCC is a predictive factor for a poor outcome (17). Certain cytokines such as interleukin-6 are elevated in patients with RCC; they usually indicate the advanced stage of the disease. Renal cancers are one of the most vascularized types of cancers in human. VEGFR are constitutively present in serum of RCC patients, but its level does not have prognostic significance (70, 73). VEGFR can also be detected in RCC cells, and the intensity of staining usually correlates with grade and stage of tumor (49).

Inactivation of VHL and overexpression of HIF-1, induces production of several growth factors such as EGFR, VEGF, IGF, which in turn they increase the metabolism of the cell, ending up to overexpression of glucose transporters, glycolytic enzymes, which are categorized as poor prognostic factors (49).

The expression of apoptotic inhibitor XIAP is decreased with increasing grade of RCC, reflecting a poor prognosis (77). Adhesion molecules cadherin-6 has also been proved to be the marker of poor prognosis. RCC do not express estrogen or progesterone receptors, but in small proportion of cases androgen receptors could be detected, which usually correlate with poor prognosis (49, 78)

On the other hand BCL2 expression associated with low Ki-67 labeling index and rare p53expression, are favorable factors for prognosis.

Numerous investigations reported the prognostic significance of CD44 and v6 isoform overexpression, in clear cell type of RCC. On the other hand up-regulation of osteopontins expression is associated with increased metastatic potential and consequently poor prognosis of the disease.

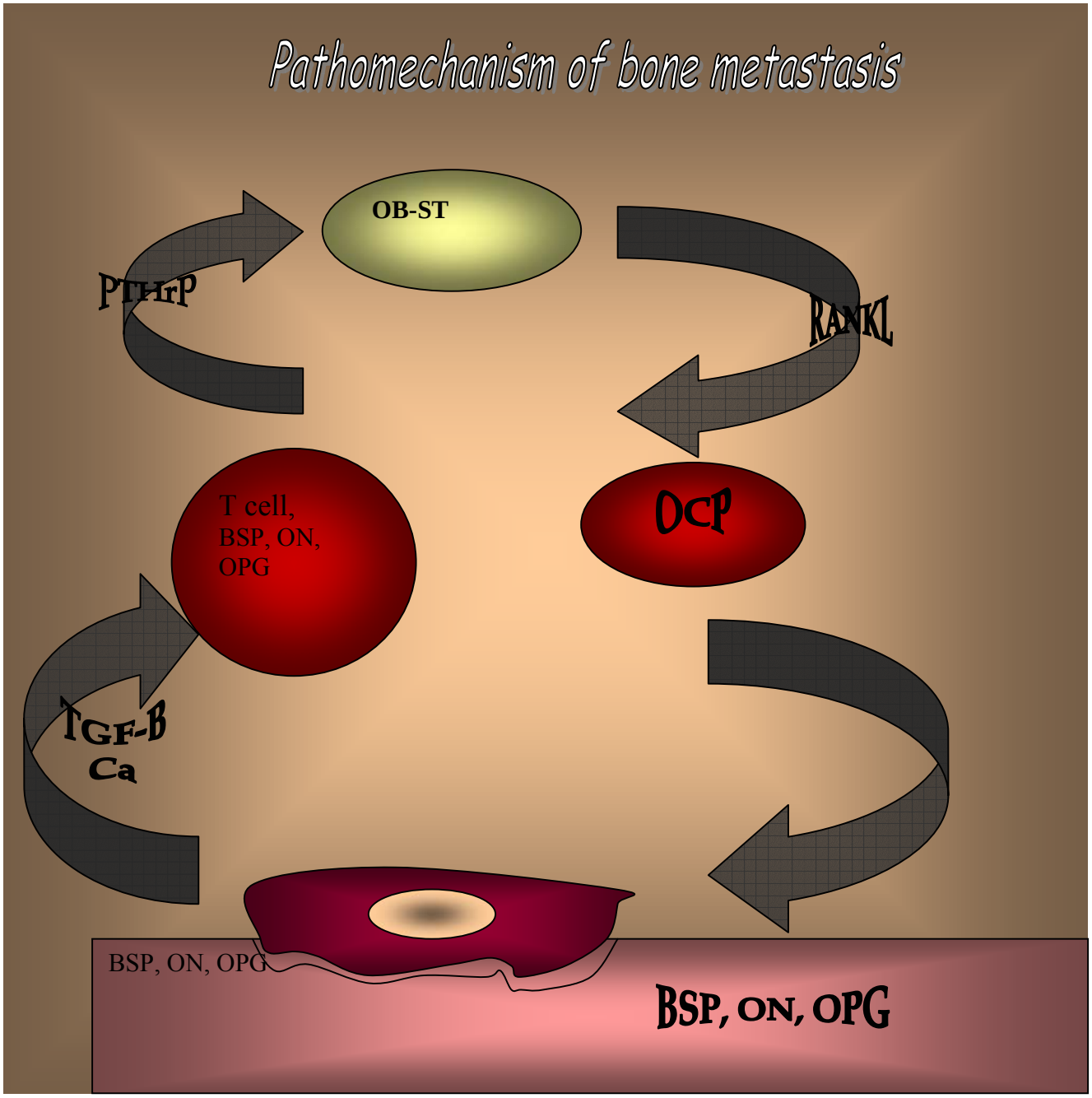


Figure 4: Pathomechanism of bone metastasis

1.13. Colon cancers (CRC)

The major histological type of large bowel cancers is adenocarcinoma which accounts, for 90% to 95% of large bowel tumors. Colloid or mucinous adenocarcinoma represents about 17% of large bowel tumors.

There are several kind of grading for tumor of large bowel, TNM, Dukes, MAC (modified, Astler-Coller system correlated with TNM) are the accepted ones used in clinics for diagnosis and monitoring (17).

Prognostic pathology of colorectal cancer involves primarily macro-and microscopic features of the primary tumor. One of the oldest staging systems of the tumors, Ducks staging has survived into this century

1.13. a. Metastasis genes in CRC:

Expression of CD44 and its splice variant, v6, has been extensively studied in colorectal cancer. Up regulation of CD44 is a strong negative prognosticator, both for development of metastasis and poor survival (79, 80). Enhanced VEGF expression is found in patients with advanced disease and it's usually correlated with poor outcome of the disease (81). U-PA emerged as a strong independent prognosticator for colorectal cancer, where also the ratio of tumor versus Stromal cells has predictive value for metastasis and disease progression. Catapsin B was found to be increased in invading tumors (49). In MMP family of proteases, several members are expressed by colorectal cancer, and the expression of MMP-2 seems to be a constitutional event suggesting that this is necessary but not sufficient for metastatic potential (82). K-RAS oncogene overexpression was also found as negative prognostic factor.

1.13. b. Prognosis of CRC

Invasion of lymphatic vessels by tumor tissue is usually correlated with later occurrence of metastasis and poor survival (49). In local recurrences, perineural invasion must be examined carefully, since its presence predicts disease progression and distant metastasis.

1.15. EGFR family

Is a member of ErbB family receptors, a superfamily of four closely related receptor tyrosine kinases.

1. ErbB1 or HER1 fund almost on all epithelial cells
2. ErbB2 or HER2 found predominantly on breast cancer cells
3. ErbB3 or HER3
4. ErbB4 or HER4

EGFR exist on the cell surface and is activated by binding of its specific ligands, including epithelial growth factor and transforming growth factor α (TGFα).

Binding of ligand to EGFR induces dimmerization of receptor. EGFR dimerization stimulates its instinct intracellular protein kinase activity. As a result auto-phosphorylation of five tyrosine residues in C-terminal domain of EGFR occurs. The autophosphorilation elicits downstream activation and signaling by several other proteins that associate with the phosphorylated tyrosine through their own phosphotyrosine-binding SH2 domains. These downstream signaling proteins initiate several signal transduction cascades principally the MAPK, AKt and JNK pathways', leading to DNA synthesis and cell proliferation (68).

Mutations that lead to EGFR overexpression (known as up-regulation) or over-activity have been associated with a number of cancers, including breast and lung cancers.

The identification of EGFR as an oncogene has lead to the development of anticancer therapeutics directed against EGFR, including gefitinib and erlotinib for lung cancers, cetruximab for colon cancer, and transtuzumab for breast cancer (53, 65, 74).

These therapies are based in the use of monoclonal antibodies against EGFR or in protein kinase inhibitors. The monoclonal antibodies block the ligand binding domain with the use of anti EGFR antibodies. With the binding site blocked, signal molecule can no longer attach there and activate the tyrosine kinases. In this way tumor growth could be down-regulated.

1.16. VEGFR family

Vascular endothelial growth factor (VEGF) is a highly specific mitogen for vascular endothelial cells. Several VEGF isoforms are generated as a result of alternative splicing from a single VEGF gene (57). VEGF induces endothelial cell proliferation, promotes cell migration and inhibits apoptosis. In vivo VEGF induces angiogenesis so it plays a role in regulation of vascularization (73). Deregulated VEGF expression contributes to development of solid tumors by promoting tumor angiogenesis. VEGF also contributes to the developments of tumors because of its ability to induce permeablization of blood vessels. VEGF induces the formation of fenestrations in blood vessels and the formation of vesiculo-vascular organelles that form channels through which blood born proteins can extravasate. This leads to the formation of an extra-vascular fibrin gel, which provides a matrix that supports the growth of endothelial cells and tumor cells and allows invasion of Stromal cells into the developing tumor (147).

These are the receptors for vascular endothelial growth factors on the surface of endothelial cells, and they constitute three main subgroups.

1. VEGFR-1 (Flt-1)
2. VEGFR-2 (Flk-1/KDR)
3. VEGFR-3 (Flt-4)

VEGFR-1&2 are expressed predominantly on the endothelial cells, but a few additional types of cells express these receptors. The VEGFR-1 is expressed on trophoblast cells, monocytes and renal mesangial cells. VEGFR-2 on the other hand is also expressed on hematopoietic stem cells and retinal progenitor cells. VEGFR-3 receptor, which is expressed on lymph vessels, binds VEGF-C and VEGF-D (81, 147).

1.17. From primary tumors to metastasis

There are several developmental models for metastasis. These are categorized in three main subgroups.

1. Clonal evolution/selection theory: in this model metastasis develops rather late in the course of the disease and the metastatic cells usually demonstrate the clonal characteristics of subgroups of cells in primary tumor.

2. Parallel development theory: In this model metastasis develops in early stages of the disease, meaning that micro metastasis is initiated with the growth and development of primary tumor and consequently these groups of cells can produce totally new clones at the metastatic site.
3. Same gene model theory: this is rather a borderline between two extreme subtypes mentioned previously

1.18. Therapeutic approach for managements of patients with bone metastasis

Facing a patient with distant metastasis one may raise several questions:
What could possibly be the best therapeutic approach for patient?
How can we enhance the effectiveness of therapy?
Which elements make the cancers resistant to chosen therapy?
And eventually how can we avoid the recurrence of cancers?
Of course there are several therapeutic approaches such as surgery, radiation therapy, chemotherapy, and recently introduced targeted therapies. Every each mode has its advantages and disadvantages of course.
Targeted therapy seems to be quite promising field, on the contrary to chemotherapy these agents act on more specific groups of cells reducing the unwanted side effects of chemotherapy.
Several models exist for tumor metastasis to the bones; consequently one should be pretty conscious while putting the patient with distant metastasis on targeted therapy about the mode of dissemination, as it can dramatically affect the outcome.
Based on the theories mentioned previously, the genotype of tumor cells on metastatic site can either be identical, borderline or totally different from the primary tumor. In our work we compared the genotype/phenotype alterations between primary and bone metastatic sites in RCC, NSCLC, and breast cancers.
It is always worth to genotype the metastatic samples since in this way we can avoid exposing the patients on unnecessary medications.

1.19. Aim of our studies

It is generally accepted, spatially by clinicians that the phenol/genotype of a given cancer metastasis is similar to the primary tumor. Meanwhile there are rare clinical date in the literature on this subject, further more very few pathological studies were aimed on characterization of bone metastasis of certain cancers , even though this is a rather frequent site of distant metastasis. Therefore we have categorized four aims in our studies.

1. Comparison of the EGFR phenotype of RCC and NSCLC in bone metastasis.
2. Comparison of VEGFR phenotype of primary tumors and bone metastasis in RCC
3. Comparison of K-RAS mutational status of primary tumors and bone metastasis in NSCLC
4. Comparison of HER-2 /neu status of primary tumors and bone metastasis in BRC

2. Materials and methods

2.1 Patients

We have selected 11 NSCLC cases for this retrospective histopathological analysis from the archive of the Department of Orthopaedics with the approval of the local ethical committee. NSCLC patients presented with bone metastases, which were at least partially removed by surgery (patient's data are on **Table 3**). Paraffin embedded tumor samples of both the bone metastases as well as primaries were available for analysis. The majority of the NSCLC patients were male (9/11) and adenocarcinomas dominated over squamous cell cancer (9/2). (**Table 3**) The age distribution was 47 and 72 years. Except one case, bone metastases developed following surgery of the primary tumor. Besides bones, liver kidneys and lymphatic system were the most frequent sites affected by tumor dissemination. Majority of cases were put on various chemotherapeutic protocols beside surgical intervention. The survival of the patients varied between 21 and 75 months.

We applied our studies on primary and bone metastatic samples of 20 patients diagnosed by RCC. The samples were paralelly analized for EGFR, and VEGFR by immune histochemistry (**Table 4**).

Bone metastases tumor samples of breast cancer were retrospectively collected from the pathology departments of following institutes (Departments of Orthopedics and Traumatology of Semmelweis University and National Institute of Traumatology). Samples were derived from open biopsies of bone metastases obtained during transfocal stabilization of impending or comleted pathological fractures, or resection of bone metastases. We excluded 9 cases because of over-decalcination or insufficient amount of tumor tissue in the section. In total we investigated 48 metastatic cases. We were able to obtain paraffin embedded samples of the primary breast cancers in 23 patients. The characteristics of the patients are shown in **Table 5**. We have assessed the histological grade of the metastases inn 44 of the 48 cases by the Bloom-Richardson method as previously described. (141)

Table 3. NSCLC patient's characteristics.

Case No.	sex	Age (years)	Histology of primary	Adjuvant therapy	Primary survival (month)	Bone metastasis	Metastasis therapy	Overal survival (month)
1	F	53	AC	NA	NA	Fe,l	TEP	28
2	M	76	AC	S	18	Fe,l	TEP	36
3	M	67	SQCC	S+CY	35	T,r	PR	42
4	M	57	SQCC	S+CPD, DOXO	31	Fe,r	PR	47
5	F	47	AC	S+CPD	51	Fe,l	TEP	75
6	M	49	AC	S+DOXO	41	Fe,r	PR	59
7	M	72	AC	S+DOXO	46	Fe,r	TEP	55
8	M	66	AC	S+chemo	19	U,r	PR	21
9	M	56	AC	S	25	Fe,r	PR	38
10	M	53	AC	S+DOXO	49	Fe,l	TEP	42
11	F	60	AC	S+CPD	11	Fe,l	TEP	24

AC= adenocarcinoma, Chemo= undefined chem,otherapy, CPD= cis-platinum, DOXO= doxocylin, F= female, Fe=femoral, M=male, PR= partial resection, S= surgery, SQCC= squamous cell carcinoma, TEP= total epiphyseal prothesis, T=tibial, U=ulnar metastasis, r=right, l=left,

Forty paraffin-embedded surgical samples of clear cell RCC were used throughout this study where material was available from both primary and bone metastatic lesions (Table 7). The male to female ratio was 15:5 and the age distribution was 48-78 years. All the cases were diagnosed as clear cell renal cell cancer (CC-RCC) with 3 and 4 cases containing sarcomatoid and chromophobic regions, respectively. Fuhrman grading was used to stratify the cases. The bone metastases have been operated with either palliative of curative total endoprothesis. Four cases were treated with hormone, four patients with

bisphosphonates and one patient with interferon-α. The survival of the patients was in the range of 6-240 months.

Table 4. Clinical data of the renal cell cancer patients

No.	Sex	Age (years)	Histology	Grade	Localization of bone met.	Therapy	Survival (months)
1	M	78	CC	3	F		7
2	M	68	S-CC	4	F		33
3	F	72	S-CC	4	F		21
4	M	61	CH-CC	2	Hu	H	30
5	M	69	S-CC	4	Hu		14
6	F	64	CC	3	Hu		25
7	F	70	CH-CC	3	F		18
8	M	71	CC	1	T		42a
9	F	78	CC	2	F	H,B	11
10	M	69	CC	2	I		14
11	M	67	CH-CC	2	F		180
12	M	54	CC	1	F		48a
13	M	48	CC	2	SC		12a
14	M	68	CC	2	Hu	H	65
15	M	59	CC	2	I		6
16	M	64	CC	1	Hu		39a
17	M	64	CC	1	R		18
18	F	61	CH-CC	2	Hu	B	72a
19	M	57	CC	2	Hu	IFN, B	84
20	M	52	CC	2	F	H,B	240

a= alive, B= bisphosphonate, CC= clear cell, CH-chromophobe, F=female, F=femure, H= hormone, Hu= humerus, I= os ilei, IFN= interferon alpha, M= male, S= sarcomatoid, SC= sculp, T= tibia

Table 5: Characteristics of patients with breast cancer

n	48
age (median)	59
sex (female/male)	47/1
histology (n=48)	invasive ductal carcinoma n=35 invasive lobular carcinoma n=8 other/not classifiable n=5
grade of primary tumors (n=23)	I.: 1/23 II.: 10/23 III.: 12/23
grade of bone metastases n=44*	I.: 3/44 II.: 29/44 III.: 12/44

*4 out of 48 bone metastases the sample size was not enough for grading

Ninety six paraffin embedded samples were available for IHC studies, the median age of the patients was determined to be 59, and histological grading was performed both on primary and metastatic samples. 35 patients were diagnosed with invasive ductal carcinoma, 8 patients with invasive lobular carcinoma whereas 5 patients did not fall into these categories. With the exception of one case all the patients were females.

2.2. a. Immunohistochemistry

EGFR and VEGFR expression in NSCLC and RCC

Tissue samples were routinely fixed in 10% (v/v) neutral buffered formalin, dehydrated on a graded series of alcohol and xylene and embedded into paraffin at temperature not exceeding 60^{o}C. Three to four micron sections were mounted on Superfrost slides (Shandon) and manually deparaffinized. We have used the antigen retrieval technique suggested by the EGFR pharmDx^{TM} kit (Dako, Glostrup, Denmark): 100 μl 0.1% Proteinase K diluted in TRIS-HCl buffer containing 0.015 mol/L sodium azide was exposed to the sections for 5 min at room temperature, followed by HQ water washings (3+2 min). Alternatively, slides were immersed in 0.05 mM citrate buffer (pH=6), and exposed to 750 W microwave for 3x5 min (MFX-800-3 automatic microwave, Meditest, Budapest, Hungary). Three % H_2O_2 for 5 min at room temperature was used to block endogenous peroxidase activity.

The extracellular domain of EGFR protein was detected by the EGFR pharmDx^{tm} kit (Dako) using mouse monoclonal anti-human EGFR (EGFR-EC, clone 2-18C9) [6], dextran polymer conjugated with HRP and goat anti-mouse IgG and DAB substrate-chromogen applied rigorously following the manufacturer's instructions. As positive control, slides provided by the manufacturer (formalin-fixed and paraffin-embedded pellet of HT29 human colorectal carcinoma cell line) as well as human head and neck carcinoma tissue sample previously diagnosed 3+ in 100% of cells for membrane EGFR by using EGFR pharmDx^{TM} and CONFIRM anti-EGFR (Ventana) were used.

To detect the cytoplasmic domain of EGFR (EGFR-CY), we have used rabbit polyclonal antibody PU335-UP (Biogenex,) with no dilution. In case of negative control, instead of the primary antibody, slides were exposed to the diluents but were processed in the same way as other slides.

To detect VEGFR2/KDR, a mouse monoclonal antibody from R&D Systems (Abingdon, UK) diluted 1:20 was used. Samples were incubated overnight with the primary antibodies at a temperature of 4^{o}C. As positive control, a human Kaposi sarcoma tissue was used, while in negative controls the primary antibody was omitted.

On the successive day LSAB Kit (Dako) was used as developer reagent. Immunoreaction was visualized by using diaminobenzidine (DAB for EGFR) or AEC (for KDR) as chromogen. Nuclei were visualized by hematoxyline.

DAKO HercepTest for Immunoenzymatic Staining to Detect HER-2/*neu* Protein expression in BRC

The HercepTest (DAKO, Corp) is a subjectively scored immunohistochemical assay used to determine HER-2/*neu* protein overexpression in histological sections of breast cancer specimens. The HercepTest is approved by the FDA (September, 1998) for selection of women with breast cancer to receive trastuzumab humanized monoclonal antibody therapy. In this study, the HercepTest was performed according to the approved protocol as described by the manufacturer. Tissue sections were cut, mounted on plus slides, heat-treated for antigen retrieval, and immunostained. Antigen retrieval involved boiling the tissue sections at 95°C to 99°C in 10 mmol/L citrate buffers for 40 minutes. The sections were cooled and treated with peroxidase-blocking reagent for 5 minutes, rinsed, and treated with sufficient primary rabbit HER-2/*neu* antibody to cover the entire tissue section for 30 minutes. The sections were rinsed again and treated for 30 minutes with visualization reagent, a solution containing both secondary goat anti rabbit antibody and horseradish peroxidase linked to a common dextran polymer backbone. After rinsing away excess visualization reagent, the sections were incubated in diaminobenzidine for 10 minutes to identify the location of immunoprecipitates. The tissue sections were processed with the DAKO Autostainer Universal Staining System according to the instructions of the manufacturer (DAKO, Corp). The sections were counterstained with hematoxylin and mounted in Permount. Immunostaining was interpreted with a bright-field Olympus microscope according to the scoring system of the manufacturer as 0, 1+, 2+, and 3+ (DAKO, Corp); 2+ and 3+ immunostaining was considered to be overexpression and 0/1+ immunostaining was considered to be low expression.

Morphometry

Reactions were evaluated by 2 experts and the % of positive cells as well as the intensity of the reaction (0, 1+, 2+, 3+) was determined at least in 3 areas of the tumor. Mean levels were determined for each tumor and multiplying % data with intensity values produced a final IHC score. Statistical analysis was performed using MS Excel program applying t test.

2.2. b. Molecular techniques

. PCR-RFLP analysis for K-ras gene point mutations in codon-12

DNA was extracted from formalin fixed and paraffin-embedded tissue using the MasterPure™ DNA Purification Kit according to the instructions of the manufacturer. We have used two primer pairs (nested PCR). DNA amplifications were performed using DyNAzymeTM and Mastercycler gradient thermal cycler supplied by Eppendorf. The reaction mixture of reagents for samples was prepared, containing 2.5 µl 10X PCR puffer+Mg^{2+} (DyNazyme^{TM}), 200 µM/each dNTP, 1.00 pM/reaction of each primer, 0.8 U of DyNAzyme^{TM} polymerase /reaction in the first step and 0.25 U DyNAzyme^{TM} polymerase/reaction in the nested step. The Inner sense primer was mismatch primer, and the product of PCR contained in the wild type of K-ras gene the recognition site of BstNI restriction endonucleas. Outer primer pair 5'-GCCTGCTGAAAATGACTGAAT-3' and 5'- GGTCCTGCACCAGTAATATG -3'; 35 cycles of denaturation at 95 ^{o}C for 1 min, primer annealing at 55 ^{o}C for 1 min, chain elongation at 72 ^{o}C for 2 min. Inner primer pair 5'-GAATATAAACTTGTGGTAGTTGGACCT-3'and 5'-GGTCCTGCACCAGTAATATG -3'; 35 cycles of denaturation at 95 ^{o}C for 1 min, primer annealing at 55 ^{o}C for 1 min, chain elongation at 72 ^{o}C for 2 min. The amplified products were digested with BstNI (New England BioLabs) restriction endonucleas. Ensimatic digestions were at 60 ^{o}C and 3 h in a total volume of 30µL. Digested PCR product were separated on 4% agarose gel in TAE buffer and visualized under UV light following ethidium bromide staining.

. Fluorescence in situ hybridization (FISH):

FISH was perfomed in the cases where samples had 2+ or 3+ her2/neu IHC status in the bone metastases and/or in the primary tumors, or if discodance was found in her2/neu status detected by IHC between primary tumors and their corresponding bone metastases. FISH was performed by using the Oncor INFORM system (Ventana) as previosuly described (142) and by using the protocol No.2 of the Benchmark automata stainer. Briefly, slides were depraffinised. After denaturation at 90 ^{0}C for 10 minutes, they were incubated with protease 3 for ten minutes. After digestion, slides were incubated with one drop INFORM HER-2/neu probe, following by incubation with FITC/anti-biotin and anti mouse/FITC. In the next step slides were counterstained with 4, 6-diamino-2-phenylindole (DAPI). Slides were assessed for her2/neu gene copy number as previously described: for each specimen, gene copy level was assessed in two areas of at least 20 nonoverlapping tumor cell nuclei by using an epifluorescence microscop (Nikon Eclipse-600). A tumor was considered to be amplified if there were more than four copies of her2/neu per cell. (142)

3. Results

3.1. Phenotype/genotype of NSCLC

3.1-a. Comparison of EGFR phenotype of bone metastasis to primary tumors of NSCLC

We have selected 11 NSCLC cases for this retrospective histopathological analysis from the archive of the Department of Orthopaedics with the approval of the local ethical committee. NSCLC patients presented with bone metastases, which were at least partially removed by surgery. Paraffin embedded tumor samples of both the bone metastases as well as primaries were available for analysis.

The majority of the NSCLC patients were male (9/11) and adenocarcinomas dominated over squamous cell cancer (9/2). (**Table 3**) The age distribution was 47 and 72 years. Except one case, bone metastases developed following surgery of the primary tumor. Primary tumors of the patients happened to come to attention when the diameters of lesions were ranging between 4 and 11 cm. Besides bones, liver kidneys and lymphatic system were the most frequent sites affected by tumor dissemination. Majority of cases were put on various chemotherapeutic protocols beside surgical intervention. The survival of the patients varied between 21 and 75 months.

Immunohistochemical studies of EGFR expression in primary and bone metastatic NSCLC indicated that similarly to the primary tumors, in bone metastatic (decalcified) cancer tissues EGFR protein could be detected by protocol modifications of the EGFR PharmDX kit such as microwave antigen retrieval and extended incubation with the primary antibody (**Fig.5a, c.**). Furthermore, the C-terminal domain of the EGFR was also reliably detectable at both locations (**Fig.5b, d**), while phosphorylated EGFR was practically absent (data not shown).

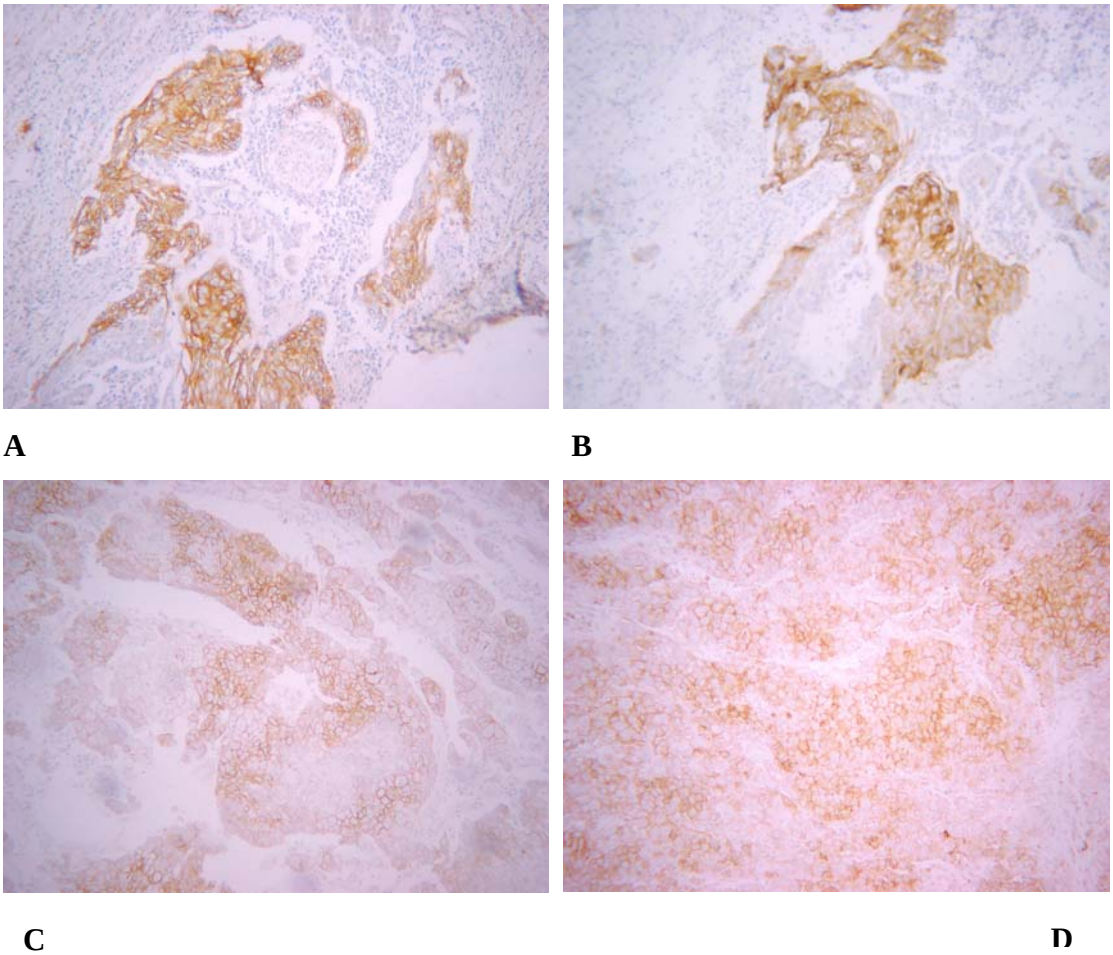


Fig.5. Detection of EGFR protein in NSCLC samples by immunohistochemistry (Case No.5). A, C: Immunoreaction of EGFR-EC domain using EGFRPharmDX-kit. B, D: Immunoreaction of EGFR-CY domain using polyclonal anti-C-terminal antibody. A, B: primary tumor, C, D bone metastasis

Morphometric analysis of the EGFR protein expression of the primary tumors compared to the bone metastases demonstrated no significant difference in expression level as detected, by both anti-EGFR antibodies (**Fig. 6**). Comparison of the EGFR expression levels of primary and metastatic NSCLC in the individual cases further supported this observation, since five out of eleven cases maintained EGFR expression levels (**Table 6**) and four out of eleven cases demonstrated increased expression level at least by 50% (**Table 6**).

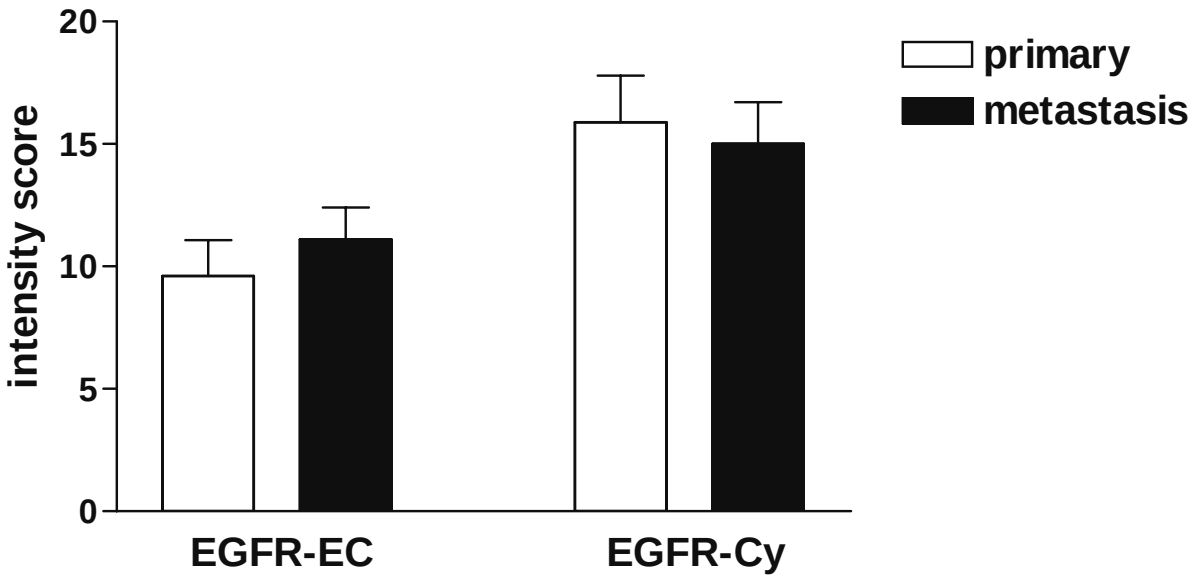


Fig.6. Comparison of EGFR protein expression of NSCLC in primary and bone metastatic cases (n=11). Data are EGFR intensity scores, expressed as mean±S.E and derived from Table 2. EGFR-EC= extracellular domain, EGFR-CY= cytoplasmic domain.

In 45.5% of the cases the EGFR expression was maintained, only in 18.2% of cases down-regulation of protein expression could be detected by morphemotric analysis, whereas in 36.4% of the cases up-regulation of EGFR was observed.

Table 6: EGFR protein expression in bone metastases of NSCLC

	EGFR-	EC	EGFR-	Cy	EGFR
	primary	metastasis	primary	metastasis	Status in metastasis
1	10.0	17.0	19.25	23.88	maintained
2	19.76	17.0	23.88	18.0	maintained
3	17.0	10.0	24.88	6.25	downregulated
4	6.88	14.0	12.5	18.0	upregulated
5	13.0	4.32	16.88	9.0	downregulated
6	7.25	12.75	8.8	13.75	upregulated
7	5.88	13.0	12.0	18.8	upregulated
8	7.0	5.0	8.0	17.5	maintained
9	8.0	11.25	24.88	14.88	maintained
10	6.0	9.25	14.0	18.5	upregulated
11	4.88	8.63	11.5	7.25	maintained
				EGFR maintained	5/11 (45.5%)
				EGFR upregulated	4/11 (36.4%)
				EGFR downregulated	2/11 (18.2%)

Data are expressed as mean of IHC score. Maintained= no change in IHC score in metastasis, downregulated= decrease of IHC score >50, upregulated= increase in IHC score >50%

3.1-b K-RAS mutation in bone metastasis of NSSCLC

RFLP-PCR analysis of the paraffin embedded tumor tissues for K-RAS codon-12 mutation demonstrated that 5 out of 11 NSCLC cases (45.5%) were mutant (**Table 7**). However, primary and metastatic tumors showed a great variability in respect of K-RAS mutation status which was rarely maintained (1/5 cases), but was rather altered. (4/5). It is of importance that the frequency of loss of K-RAS mutation or acquisition of this genotype in bone metastases occurred with equal frequency (40%, respectively, **Table 7**).

Table 7: K-RAS mutation status of NSCLC

	K-Ras status	N(%)	
Case No.	primary	metastasis	NSCLC case
1	wt	M	mutant
2	wt	wt	wt
3	wt	wt	wt
4	wt	wt	wt
5	wt	M	mutant
6	M	M	mutant
7	wt	wt	wt
8	wt	wt	wt
9	M	wt	mutant
10	wt	wt	wt
11	M	wt	mutant
K-Ras mutation	3/11 (27.3%)	3/11 (27.3)	5/11 (45.5%)

Wt= wild-type, M= mutated at codon 12

When the EGFR protein expression changes were compared to the alteration of K-RAS mutation status of the metastatic lesion it was found that in case of tumors carrying wt-K-RAS EGFR expression was upregulated in metastases in 3 out of 6 cases (50%), while only 1 out of 6 was downregulated (**Table 8**.) In the mutant K-RAS cases on the other

hand, in 3 out of 5 cases the EGFR status was maintained in metastases (60%), while up- or downregulation were rare (**Table 8**).

Table 8: Comparison of EGFR protein expression and K-RAS mutation changes in bone metastases of lung cancers

Case N.	KRAS change	EGFR change
2	Wt maintained	maintained
3	Wt maintained	down
4	Wt maintained	up
7	Wt maintained	up
8	Wt maintained	maintained
10	Wt maintained	up
1	Mutated in metastasis	maintained
5	Mutated in metastasis	down
6	Mutated in primary and metastasis	up
9	Mutated in primary	maintained
11	Mutated in primary	maintained

3.2-Phenotype of RCC in bone metastasis

3.2.a .EGFR phenotype of RCC

Forty paraffin-embedded surgical samples of clear cell RCC were used throughout this study where material was available from both primary and bone metastatic lesions (**Table 9**). The male to female ratio was 15:5 and the age distribution was 48-78 years. All the cases were diagnosed as clear cell renal cell cancer (CC-RCC) with 3 and 4 cases containing sarcomatoid and chromophobic regions, respectively. Fuhrman grading was used to stratify the cases. In majority of the cases primary tumors were diagnosed first, but still there were two cases in which the primary tumors came to attention when the patients seek medical care for the pathological fracture in their weigh baring bones. Majority of tumors were invading the long bones such as humerus and femurs. Both solitary and multiplex foci could be detected in affected bones. Invasion of soft tissue and elsewhere metastasis, mostly to contra-lateral kidney, liver and lungs were found in couple of patients. The bone metastases have been operated for either palliative purposes or curative intentions such as replacement of the affected segments with total endoprothesis.

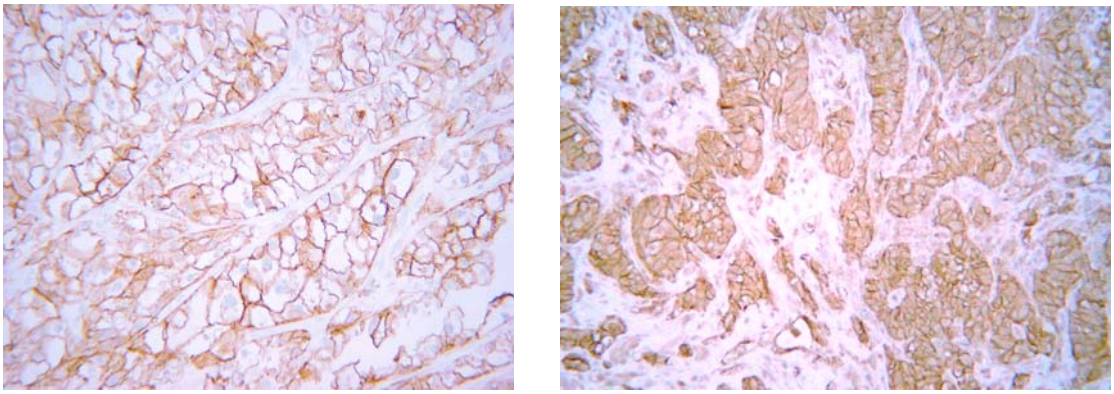
Patients in our cohort studies received different treatments during the course of their disease. Four cases were treated with bisphosphonate, four patients received hormonal therapy, and one patient was put on interferon-α. Some patients received just the surgical therapy and there were no data on chemotherapeutic protocols. The survival of the patients varied greatly ranging from 6-240 months.

Table 9. Clinical data of the renal cell cancer patients

No.	Sex	Age (years)	Histology	Grade	Localization of bone met.	Therapy	Survival (months)
1	M	78	CC	3	F		7
2	M	68	S-CC	4	F		33
3	F	72	S-CC	4	F		21
4	M	61	CH-CC	2	Hu	H	30
5	M	69	S-CC	4	Hu		14
6	F	64	CC	3	Hu		25
7	F	70	CH-CC	3	F		18
8	M	71	CC	1	T		42a
9	F	78	CC	2	F	H,B	11
10	M	69	CC	2	I		14
11	M	67	CH-CC	2	F		180
12	M	54	CC	1	F		48a
13	M	48	CC	2	SC		12a
14	M	68	CC	2	Hu	H	65
15	M	59	CC	2	I		6
16	M	64	CC	1	Hu		39a
17	M	64	CC	1	R		18
18	F	61	CH-CC	2	Hu	B	72a
19	M	57	CC	2	Hu	IFN, B	84
20	M	52	CC	2	F	H,B	240

a= alive, B= bisphosphonate, CC= clear cell, CH-chromophobe, F=female, F=femure, H= hormone, Hu= humerus, I= os ilei, IFN= interferon alpha, M= male, S= sarcomatoid, SC= sculp, T= tibia

We have demonstrated EGFR protein expression in 20 cases of clear cell RCC using two antibodies specific for the extracellular and the cytoplasmic domain of EGFR (***Fig.7a, b***). In the majority of cases, EGFR protein could be detected at various levels in primary RCC, similarly to previous reports. None of the primary RCC cases were negative for EGFR protein, even though the scores differed significantly (maximum of 5-fold). Analysis of the EGFR protein expression in the corresponding bone metastases of the 20 cases demonstrated that RCC maintained a certain level of EGFR expression. Statistical analysis indicated that the EGFR scores for both anti-EGFR antibodies decreased about 30% in bone metastases of RCC (p=0.006, EGFR-EC and p=0.048, EGFR-CY, respectively, **Fig.8**).



A **B**

Fig.7: Expression of EGFR protein in RCC detected by immunohistochemistry.
A. Detection of the extracellular domain of EGFR. Note the strong membrane reaction on the majority of the cancer cells (EGFR PharmDx^{TM} kit). B. Detection of the cytoplasmic domain of EGFR. Note the intense membrane labeling on cancer cells and a diffuse cytoplasmic label as well. Bar: 100 μ

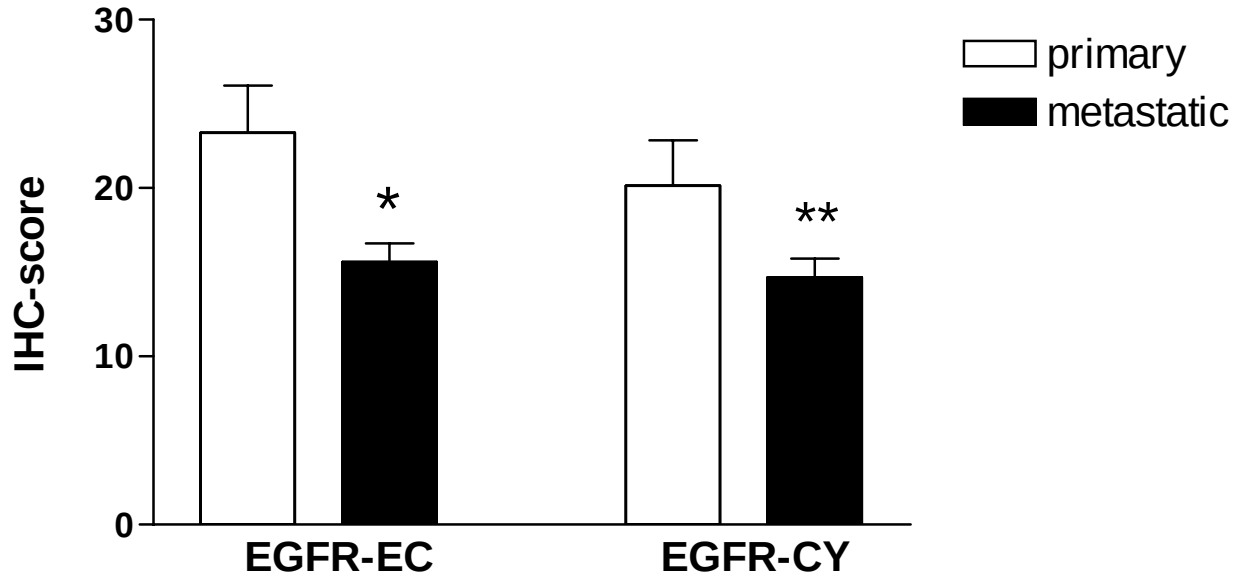
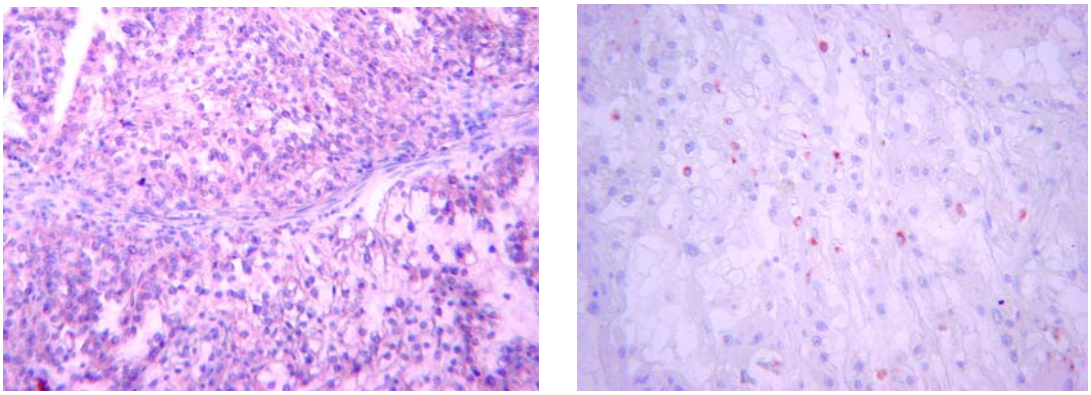


Fig.8. Comparison of EGFR protein expression of RCC in primary and metastatic cases (n=20). EGFR-EC= extracellular domain, EGFR-CY= cytoplasmic domain. *p=0.006, **p=0.048

We have analyzed the cause for this alteration; therefore we have compared samples from primary and metastatic sites individually (**Table 10**). In the majority of the cases (50%) inconsistent (unconfirmed) alterations were found in EGFR expression in metastases including both up- or downregulation detected by one of the antibodies. On the other hand, in 35% of the cases a reduced expression of EGFR protein was found by both antibodies and in 15% of cases the reduction was greater than 50% (**Table 10**), while confirmed upregulation occured only occasionally (1/20).

3.2-b Detection of VEGF expression in RCC metastasis

Next we have tested VEGFR2 protein expression in primary and metastic RCC using IHC. Only a small fraction of primary RCC contained positive tumor cells (**Fig. 9**)



A **B**

Fig.9: Expression of VEGFR2/KDR protein in RCC detected by immunohistochemistry. Strong cytoplasmic reaction for KDR in cancer cells of a case with diffuse positivity. B. Cytoplasmic labeling for KDR in scattered isolated cancer cells of a case with low level of positivity. Bar: 100 μ

RCC expressed two VEGFR2 patterns: rarely the cytoplasm of tumor cells was diffusely positive for VEGFR2 (**Fig.9a**), while in the majority of the cases scattered tumor cells contained VEGFR2 in their cytoplasm **(Fig.9b**). In both cases the majority of tumor cells remained negative for VEGFR2. Analysis of the primary tumors indicated that 35% of the cases were positive for VEGFR2, and the overwhelming majority fell into the category of scattered reaction pattern (**Table 11**). VEGFR2 positivity is a rare feature in bone metastases of RCC (10%, **Table 11**): out of the seven VEGFR2+ cases six lost this phenotype in bone metastases. On the other hand, appearence of VEGFR2 positive phenotype in bone metastasis was exceptionally rare (1/20).

Table 10. IHC scores of EGFR protein in primary and bone metastatic RCC cases

	EGFR- EC		EGFR- CY		
Case No	primary	metastatic	primary	metastic	Confirmed changes
1	9.38	8.75	8.75	12.0	maintained
2	33.0	25.0	14.5	23.0	inconsistent
3	29.25	18.25	9.5	16.0	inconsistent
4	7.5	8.5	10.0	7.5	inconsistent
5	13.0	25.0	15.0	18.75	upregulated
6	25.0	17.5	16.75	12.5	reduced
7	25.0	25.0	18.32	16.5	maintained
8	15.0	14.7	16.5	7.8	inconsistent
9	44.0	7.75	31.0	13.75	reduced
10	12.5	12.5	11.5	8.4	inconsistent
11	11.83	15.6	12.5	12.0	inconsistent
12	12.25	12.8	9.32	12.5	inconsistent
13	45.0	17.0	26.25	36.0	inconsisten
14	18.3	18.75	12.65	16.1	inconsistent
15	14.0	11.0	11.38	8.36	reduced
17	30.0	6.72	12.5	8.75	reduced
18	48.0	12.5	45.0	12.5	reduced
20	32.0	27.0	21.75	12.0	reduced
21	28.0	12.5	40.0	14.5	reduced
22	13.75	13.7	28.75	18.0	inconsistent
maintained		8/20		2/20	2/20
reduced		10/20		11/20	7/20
upregulated		2/20		7/20	1/20

Confirmed changes= EGFR-EC and EGFR-CY gave similar results. IHC= immunohistochemistry

Table 11: VEGFR2/KDR protein expression in clear cell RCC

	Primary N (%)	Bone metastasis N (%)
negative	13/20 (65%)	18/20 (90%)
positive	7/20 (35%)	2/20 (10%)
diffuse cytoplasmic	1/20	0/20
scattered focal	6/20	2/20

3.3. Breast cell cancer

3.3-a. HER-2/neu genotype of BRC in bone metastasis

Bone metastases tumor samples of breast cancer were retrospectively collected from the pathology departments of following institutes (Departments of Orthopedics and Traumatology of Semmelweis University and National Institute of Traumatology). Samples were derived from open biopsies of bone metastases obtained during transfocal stabilization of impending or comleted pathological fractures, or resection of bone metastases. We excluded 9 cases because of overdecalcination or insufficient amount of tumor tissue in the section. In total we investigated 48 metastatic cases. We were able to obtain paraffin embedded samples of the primary breast cancers in 23 patients. The characteristics of the patients are shown in Table 1. We have assessed the histological grade of the metastases inn 44 of the 48 cases by the Bloom-Richardson method as previously described. (19)

Table 12: Her 2/neu status in the primary tumors and their corrsponding bone metastases of the 23 paired cases

Case No.	Histology	Grade of the primary tumor	Grade of the bone metastases	IHC* in the primary tumors	IHC* in the bone metastases	FISH** in the primary tumors	FISH** in the bone metastases
1	IDC	3	3	-	+	NT	not-tested
2	IDC	2	2	+++	-	A	non-amplified
3	IDC	2	2	-	+++	A	amplified
4	IDC	3	2	-	-	NT	not-tested
5	IDC	1	2	-	-	NT	not-tested
6	IDC	3	2	-	-	NT	not-tested
7	IDC	3	3	-	-	NT	not-tested
8	IDC	2	1	-	-	NT	not-tested
9	IDC	3	3	+++	+++	A	Amplified
10	IDC	3	2	-	++	non-amplified	non-amplified
11	IDC	2	2	-	-	NT	not-tested
12	IDC	3	2	-	-	NT	not-tested
13	IDC	2	2	+	-	NT	not-tested
14	IDC	2	3	-	-	NT	not-tested
15	IDC	2	2	++	-	A	non-amplified
16	ILC	2	2	-	-	NT	not-tested
17	IDC	3	3	-	-	NT	not-tested
18	IDC	3	***	-	-	NT	not-tested
19	IDC	3	3	+	-	NT	not-tested
20	IDC	3	3	+	-	NT	not-tested
21	IDC	2	2	-	+	NT	not-tested
22	IDC	3	3	-	-	NT	not-tested
23	IDC	2	2	-	-	NT	not-tested

* Her2/neu status detected by immunohistochemistry
** Her2/neu status detected by florescence in situ hybridization
*** not graded because of the litle amount of tumor
IDC: invasive ductal carcinoma, A: amplified, NT: not tested, ILC: invasive lobular carcinoma

In the 48 metastatic lesions 9/48 (18.75%) of the cases showed her2/neu overexpression by IHC (3+: 5/48, 2+: 4/48). We found her2/neu gene amplification in 1 out of the 4 IHC 2+ scored cases, and in 4 out of the 5 IHC 3+ scored cases. (Table 12,) In one of the IHC 3+ scored cases we were not able to get any signals, when repeatedly analyzed by FISH. Taken together, five of the 48 bone metastatic breast cancer patients (10.5%) had her2/neu gene amplification.

23 paired cases of primary and metastatic tissue samples were available for analysis. Using IHC and FISH we found that 4 out of the 23 primary tumors (17.3%) had amplified Her2/neu gene (Table 12.) In bone metastases of the 23 primary tumors we found 2 cases only where the HER2/neu status was maintained (9.5%) while in two other cases the initial amplified status was changed to a Her2/neu negative one (9.5%).

Table 13: Comparison of HER-2/neu status of BRC in bone metastases compared to the primary tumor.

	primary tumor			bone metastasis		
Case	IHC	FISH	Status	IHC	FISH	Status
1	3+	A	+	3+	A	+
2	3+	A	+	0	N	-
3	0	A	+	3+	A	+
4	2+	A	+	0	N	-
5-23	0/1+	n.t.	-	0/1+	n.t.	-

A= amplified, IHC= immunohistochemistry, FISH= fluorescence in situ hybridization, N= non-amplified, n.t.= not tested

4. Discussion

There are three major models of the tumor progression/metastatization: clonal evolusion/selection (104), parallel development (105) and the same-gene models (106) providing sharply different explanations for tumor progression. The two extremes are parallel development and the same gene models, where the previous predicts very early generation of disseminated cancer cells to distant organs with highly diverse genetic profiles of the primary and metastasis whereas the later suggesting metastasis as a relatively late event of tumor progression, therefore genetic diversity of the metastases suggested to be minimal (105,106). The clinical validity of these models can only be determined by direct comparison of metastatic and primary tumor tissues of various cancer types. Concerning the progression of lung cancer it seems that all the three metastasis models are applied by the nature, supported by LOH (107), CGH (108) and SNP analyses (109) of metastatic and primary lung tumor tissues.

Bronchial carcinomas are without doubt the number one cause of cancer related deaths in industrialized countries. The pick incidence of lung cancer occurs between ages 40 and 70 years; currently the male to female ratio is 2 to 1. Male cigarette smokers are about 10 times more likely to die of bronchogenic carcinoma than are nonsmokers (48).

Like all cancers, lung cancers result from genetic changes that affect oncogene and tumor suppressor genes. SCLCs are characterized by changes in several oncogens, including amplification of myc family. L-myc amplification is associated with particularly aggressive behavior (17). Overexpression or increased function of EGFR, cyclin-D1, and BCL2 genes are frequently detected in lung cancers. Mutational inactivation of P53, p16, FHIT, RASSFIA, SEMA3B, and *Rb* are also common in SCLC. In addition all SCLCs have a deletion of the short arm of chromosome 3 (3p14-25), where at least three tumor suppressor genes are suspected to reside (49).

Non small cell lung cancers (NSCLC) are characterized by EGFR protein over expressions partly due to gene amplification and in a subset of adenocarcinomas by tyrosine kinase domain mutations (49, 50). On the other hand, smoking induces K-RAS mutation which occurs in 20-30% of NSCLC being more frequent in adenocarcinomas than in squamous cell cancers (51). It is worth to note that the K-RAS and the EGFR-TK

mutations are mutually exclusive (50, 52). EGFR overexpression in NSCLC has no prognostic importance whereas, EGFR amplification and especially TK mutations predict favourable response to EGFR targeted therapies. (53) On the other hand, K-RAS mutation was reported to be a negative prognostic and predictive factor, although other studies challenged this view (51) It is important to note that almost all of the aforementioned studies on EGFR and K-RAS were performed on primary tumors and much less information is available concerning the metastatic tissue.

Tumor induced angiogenesis is and independent prognosticator for disease progression in lung cancers. Over-expression of VEGF, bFFGR, and FGRFR1 add correlate with unfavorable outcome (17, 50).

Lung cancer was long thought to be relatively resistant to chemotherapy; therefore it was treated mainly by surgery and radiotherapy. However, this trend changed in the past decade with the development of clinically effective chemotherapeutic protocols and introduction of molecular targeted therapy (95,101). While the pharmacogenomics of chemosensitivity of lung cancer is still unable to provide highly efficient predictive markers, identification of certain genes might change this trend (12). On the other hand, in recent years EGFR emerged as a useful target in lung cancer (95, 96) and EGFR-targeting agents (mostly small molecular TK inhibitors) presented unprecedented success treating NSCLC and adenocarcinomas in particular where the role of predictive pathology is ill-defined yet (103).

Bone is among the most frequent sites for metastasis of lung cancers but studies on bone metastatic disease or on the metastatic tissues is surprisingly rare in the literature. That is why we have collected a small cohort of bone metastatic NSCLC cases where both the primary and the metastatic tissues were available for analysis. Since EGFR targeted therapy of NSCLC is applied at advanced organ metastatic stage of the disease we were interesting whether the EGFR expression profile of the primary tumor is maintained in bone metastases. IHC determination of EGFR protein expression is frequently criticized in the literature due to its highly inconsistent results. We have used several commercial anti-EGFR antibodies for immunodetection of EGFR in bone metastases of NSCLC. Since this tissue has to be decalcified before processing the sensitivity of the EGFR protein detection was in question. We have fine-tuned the EGFR PharmDX protocol

(100) to detect the extracellular domain of the protein but we also used a control antibody which detects the C-terminal portion of the receptor. Both in primary and bone metastatic tissues the two antibodies provided highly comparable results on EGFR protein expressions. More interestingly, we found that the expression level of EGFR protein is highly similar in bone metastases compared to the primary tumors. This conclusion is further supported by individual comparison of corresponding primary and metastatic tissues where down-regulation was a rare event (<20%) and even up-regulation was observed in a significant proportion of cases (>30%). These observations suggest that EGFR expression status may not change during metastatic progression of NSCLC, therefore the profile determined in the primary tumor is predictive for the metastatic tissue as well. Our data are supported recently by studies on brain metastases of lung cancer where the mutational status of EGFR was also found to be preserved (110).

K-RAS is the hallmark of a relatively early genetic aberration during smoke-induced lung carcinogenesis (107) On the other hand, constitutively active (mutated) K-RAS in NSCLC may define a more aggressive and/or drug-resistant genotype (107) It is also established that EGFR mutations do not occur together with K-RAS mutation (106, 108) However there were no data on the possible changes in this status during the progression of lung cancer. Here we have shown data that the K-RAS mutational status of the primary tumor does not predict the status of the metastatic tissue of NSCLC, since we have observed both emergence of mutant clones in metastatic samples from wt-primary and loss of mutant clones in metastases in addition to the maintained mutant status. Our data support that at least two progression models occur in NSCLC, the same-gene as well as the clonal selection. It is noteworthy that loss or emergence of K-RAS mutant clones in NSCLC metastasis did not affect the EGFR protein expression pattern, which could be important for fine tuning the molecular targeted therapies.

Lung cancers are characterized by high metastatic potential; therefore identification of metastatic potential specific markers would be highly desirable to clinical oncologists.

Renal cell carcinomas are adenocarcinomas arising from tubular epithelial cells, and represent 2% of all cancers in adults. These cancers are usually large by the time they are discovered measuring 3 to 15 cm in diameter. They may arise anywhere in the kidney

(63). Depending on the amount of lipid and glycogen present, the tumor cells may appear almost totally vacuolated (lipid-laden, clear cell) or may be solid. Renal cell carcinoma can be classified into several subtypes according to histology. Clear cell carcinoma is the most common form, and accounts for majority of cases of renal tumors. Other histological types are papillary, chromophobe and collecting duct carcinomas showing gradual reduction in prevalence from papillary toward collecting duct cancers (63, 70).

Our knowledge about the molecular genetics of RCC has grown significantly in the past decade, and as a result, we have now a much more appropriate picture on the carcinogenesis of RCC. Sporadic clear cell RCC is characterized by inactivating mutation or methylation of VHL, defining this suppressor gene as primary regulator of the carcinogenesis of RCC (63,64) The consequence of the loss of VHL function in clear cell RCC is the overexpression and activation of HIF1α, a master regulator of the hypoxia-responses of cells. Overexpression of HIF1α in RCC results in the up-regulation of several growth factors including VEGF, responsible for the up-regulated angiogenic phenotype of RCC, and overexpression of various receptors including VEGFR2 (63,64). On the other hand, clear cell RCC is characterized by frequent up-regulation of EGFR protein as well, which is not due to mutation or amplification of the gene (65, 66, 67). However, the prognostic significance of the overexpression of EGFR in RCC is controversial (68, 69, 70).

. Prognostic biomarkers of RCC

Classical negative prognostic factors of RCC are aneuploidy and high S-phase fraction (12, 73). P53 positive cases are evaluated as poor prognostic ones. Among the adhesion molecules, cadherin-6 and MUCI/EMA are also the markers of poor prognosis (49). On the contrary to prostate cancers where expression of androgen receptors was usually demonstrating a favorable course of disease, up-regulation of androgen receptors in RCC is a predictive factor for a poor outcome (17). Certain cytokines such as interleukin-6 are elevated in patients with RCC; they usually indicate the advanced stage of the disease. Renal cancers are one of the most vascularized types of cancers in human. VEGFR are constitutively present in serum of RCC patients, but its level does not have prognostic significance (70, 73). VEGFR can also be detected in RCC cells, and the intensity of staining usually correlates with grade and stage of tumor (49).

Inactivation of VHL and overexpression of HIF-1, induces production growth factors, IGF, which in turn they increase the metabolism of the cell, ending up to overexpression of glucose transporters, glycolytic enzymes, which are categorized as poor prognostic factors (49).

Sporadic clear cell RCC is characterized by genetic aberration of the VHL gene, including inactivating mutations as well as inactivation caused by hypermethylation (62, 63). As a consequence, the HIF pathway becomes constitutively activated in this tumor with the consequences of upregulation of the HIF-regulated genes such as VEGF, PDGF, bFGF, EPO, TGF-α, GLUT1 and CA-IX. This results in a consitutive angiogenic phenotype of this cancer. That is why clear cell RCC is one of the most vascularized human cancers (76), providing well-characterized molecular targets for novel anti-angiogenic therapies (62, 63). VEGF level is increased in clear cell RCC patients and the cytokine activates endothelial cells through VEGFRs (mostly KDR) (63). However, it was also reported that in clear cell RCC not only angiogenic cytokines, but their receptors are also upregulated including VEGFR1 and VEGFR2/KDR, suggesting that an autocrine activation loop may also exist (83, 86).

It is known for decades that clear cell RCC is characterized by overexpression of EGFR which is not due to amplification and/or activating mutation of the gene (64). Upregulated EGFR could also result in overexpression of several genes involved in the angiogenic phenotype, including VEGF (87). Therefore, it seems that the angiogenic phenotype of clear cell RCC is regulated by both the inactivated VHL and the upregulated EGFR-driven signaling pathways. Accordingly, both pathways provide attractive molecular targets for therapy (71, 75).

The expression of apoptotic inhibitor XIAP is decreased with increasing grade of RCC, reflecting a poor prognosis (77). Adhesion molecules cadherin-6 has also been proved to be the marker of poor prognosis. RCC do not express estrogen or progesterone receptors, but in small proportion of cases androgen receptors could be detected, which usually correlate with poor prognosis (49, 78)

On the other hand BCL2 expression associated with low Ki-67 labeling index and rare p53expression, are favorable factors for prognosis.

Numerous investigations reported the prognostic significance of CD44 and v6 isoform overexpression, in clear cell type of RCC. On the other hand up-regulation of osteopontins expression is associated with increased metastatic potential and consequently poor prognosis of the disease.

Almost half of the patients with RCC experience distant metastasis during the course of their disease. Metastatic RCC is a disease historically resistant to chemotherapy. A limited subset of patients will experience clinically meaningful benefit from interleukin-2 and /or interferonα therapy, but there was no effective therapy for patients who did not respond or relapsed after cytokine based treatment. However, this trend was rapidly shifted recently with the advent of molecular targeted therapy of RCC (63, 71). Both anti-VEGF antibody therapy (72), as well as multi-targeting the signaling pathways in metastatic RCC by VEGFR2 (73) or RAF-inhibitors (74) resulted in significant inhibition of disease progression, identifying these treatments as novel standard of care. However, the molecular marker(s) of drug sensitivity of RCC is not yet defined. On the other hand, trials also indicated that EGFR in RCC could also be potential target for molecular therapy, since several EGFR inhibitors shown clinical activity in metastatic RCC (75, 76). Meanwhile there are limited data available on the pheno-genotype of metastatic (bone) RCC although these new therapies are introduced in advanced stage. It would be worth to compare the EGFR and VEGFR expression profile on primary and metastatic tumors of renal origin, to be able to predict the tumor response to chemotherapy.

Unfortunately, biomarkers are mostly lacking to define the effectiveness of these novel therapies in RCC. One of the problem is that the vast majority of the studies have been performed on the primary tumors while these novel therapies are administered in disseminated disease. That is why our study seems to be unique in the literature concerning both the EGFR phenotype as well as KDR protein expression in progressing RCC. Our data indicated that although KDR protein expression is relatively frequent in primary clear cell RCC (35%, as determined on paraffin-embedded sections), this phenotype is almost completely lost in bone metastases. By molecular analysis, KDR and other VEGF receptor expressions were found to be elevated in primary RCC [83,84], and activated KDR (autophosphorylated forms) was detected on cancer cells as well (88) . On the other hand, similarly to previous reports, primary RCC was found to be

constitutively expressing EGFR protein at a high level as detected by two antibodies targeting extracellular and intracellular domains, but this phenotype changed in bone metastases: the average level of protein expression decreased significantly and the decrease characterized 35% of the cases [7/20] where in a subpopulation of patients (15%) this decrease was substantial, exceeding 50%. On the other hand, upregulation of EGFR protein expression was a rare event (1/20). In a previous analysis we also compared the microvascular density of bone metastatic RCC to the primary tumor and discovered that in a significant proportion of the cases (45%) a lower density can be detected, (76) suggesting the development of a less angiogenic phenotype in bone metastases. In our genetic analysis of bone metastases of RCC as compared to the primary tumors we were able to show that the genetic aberrations are more numerous in metastases (89), and clonal connection to the primary tumor can only be proven in half of the cases (89), while the development of metastases from a minor subpopulation in the primary RCC is also frequent (89, 90).

Our study supports previous reports that in a significant proportion of RCC cases the geno/phenotype of the progressing tumor may change which can affect the strategy of the selection of the standard-, and especially the novel targeted therapies. Therefore we suggest to reanalyze the therapeutic targets in clear cell RCC in metastatic tissues if they are available.

No cancer is more feared by women than carcinoma of the breast and for good reason. In United States 185,000 new breast cancers are discovered yearly causing 45000 deaths scoring the second leading cancer death in US. For unknown reasons (possibly related in some part to better case finding), there has been an increased in the incidence of breast cancer throughout the world.

Although the metastatic potential of tumors does not necessarily depend on their proliferation potential, but in certain tumors such as breast cancers, the proliferation rate of the tumor is frequently associated with poor prognosis. Therefore, markers of proliferation potential have been studied extensively in breast cancer.

NM23 was the first metastatic suppressor gene to be described. Decreased expression of NM23 gene and low levels of NM23H1 protein, in breast cancers is associated with poor

survival. The metastasis suppressor, E-cadherin, is usually down-regulated in invasive breast cancer and it's proved to be an independent negative prognostic factor for node status (17).

CD44 and its variants are differentially expressed in breast cancers, it was demonstrated that expression of v6 variant correlates to favorable prognosis in node negative patients, while the expression of CD44v3-4 indicates the potential for local lymphatic spread (17, 45).

Down regulation of β1 integrins (laminin collagen receptors) is detected mainly in invasive breast cancers. On the other hand, highly invasive breast cancers were shown to express αvβ3 integrins, these proteins show high affinity to osteopontins and osteonectins, (found extensively in the bones), suggesting that these adhesion molecules may mediate the specific recognition of the bone by circulating metastatic cells.

Up regulation of MMP genes, namely MMP-2 overexpression, is and strong independent prognostic marker for unfavorable outcome and metastasis (46).

Spread occurs through lymphatic and hematogenous channels. Nodal metastases are present in about two thirds of cases at the time of diagnosis, axillary's nodes, lymph nodes along the internal mammary arteries, and the supraclavicular are usually the primary site of spread. More distant dissemination eventually ensues, with metastatic involvement of eventually any organ or tissue in the body. Favored locations are the lungs, skeleton (namely bones), liver and adrenals (14).

Tumor cells in breast cancer produce factors that directly or indirectly induce the formation of osteoclasts. In turn, bone resorption by osteoclasts releases growth factors from the bone matrix that stimulate tumor growth and bone destruction (41). This reciprocal interaction between breast cancer cells and the bone microenvironment results in a vicious cycle that increases both bone destruction and the tumor burden.

Bone is an abundant source of inactive growth factors, which are activated during the bone resorption process, (26) which can then stimulate the growth of breast cancer cells. Parathyroid hormone related peptide is probably the factor produced by breast cancer cells and most solid tumors that stimulate the formation of osteoclasts. Both parathyroid hormone related peptide and parathyroid hormone bind the same receptor (PTHR1) and induce the expression of rankle on marrow stromal cells. Parathyroid hormone in the

main regulator of calcium homeostasis, at the same time parathyroid hormone related peptides hove similar biological effects on the bones. (42).

The production of parathyroid hormone related peptide is increased in metastasis of breast cancer to bone. This peptide induces the formation of osteoclasts and bone resorption, which in turn releases transforming growth factor β. Transforming growth factor β further increases production of peptide by breast cancer cells (43). In the vicious cycle of breast cancer metastasis, bone destruction increases local calcium levels, which promotes tumor growth and production of parathyroid hormone related peptide. Breast cancer cells also produce, or induce, interleukin-6, prostaglandin E_2, macrophage colony stimulating factor, interleukin-1 and tumor necrosis factor α , (44) which may also play an important role in the induction of osteoclast formation by breast cancer metastasis. Prostaglandin E_2 can increase the expression of RANKL and directly enhances the effect of RANKL on the formation of osteoclasts (12, 43).

Osteopontin is also widely expressed in breast cancer and has been assessed as a potential prognostic factor in metastatic breast cancer. Osteopontin is a secreted phosphoprotein that is synthesized in a variety of tissues, high level of OPN is detected in the bones, where it is produced by both osteoclasts and osteoblasts (33).

Osteoclastogenesis is reduced in the absence of OPN, and the osteoclasts which are produced are less motile and have impaired ability to reabsorbe bone. OPN is widely expressed in variety of human tumors and has been assessed as potential prognostic factor in metastatic breast cancer.

The Her2/neu oncoprotein is a transmembrane receptor, belonging to the epidermal growth factor receptor (EGFR) family, with tyrosine kinase activity. Her2/neu has been shown to be over-expressed, most commonly by gene amplification, in a number of human malignancies, including breast (BRC). (1) Overexpression of the Her2/neu oncoprotein in breast cancers is associated with shortened survival, enhanced aggressiveness, resistance to hormone- and chemotherapy (2-4)) and eventually decreased sensitivity of targeted cells to therapy (5). The extracellular domain of her2/neu is the target of trastuzumab (Herceptin®), the humanized anti-her2/neu monoclonal antibody. (6) It has also been proven that the anticancer efficacy of trastuzumab is highly dependent on the Her2/neu amplification of breast cancer. (7)

A careful selection of patients is crucial for raising the clinical benefit of trastuzumab, and avoiding unnecessary exposure of patients who most likely will not benefit from it. (6) Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are standard methods to determine her2/neu protein expression or gene amplification, respectively. Clinical data indicate that patients with IHC 3+ and/or FISH-positive BRC gain the greatest clinical benefit from Herceptin® treatment. (8)

It is still a matter of debate whether Her2/neu status should be evaluated by early testing, i.e. systematically performed examinations on the primary tumor at the time of diagnosis of early breast cancer, or by pretreatment testing, i.e. performed on either the primary tumor or on a metastatic site at the time of tumor relapse. (9) To date, the Her2/neu status in the majority of the cases is evaluated in the primary tumor sample, since routine biopsy of metastatic sites is not a standard procedure (10) Never the less, the main targets of any systemic therapy in metastatic breast cancer are metastatic foci. (10) Primarily one expects to find great similarity between primary site and metastatic foci on protein expression. Whereas, experimental and some clinical data suggest that 1. The primary tumor is genetically heterogeneous, 2. The clones responsible for organ dissemination may not be even present in the primary tumor but may develop through sequential genetic alterations, or 3. They compose very small percentage of the primary. (11) Earlier studies exclusively used Herceptest to assess the maintenance of Her2 genotype in metastatic lesions and found it concordant in case of lymph node, liver and lung metastases (12-16). Recently some reports provided evidences that the Her2 genotype of BRC may change in metastasis: loss was reported in one case in liver metastases (17) and loss of Her2 amplification was reported in 21 % but appearance of Her2 amplification was very frequent in visceral metastases (30%) (18).

Despite apparent curative surgery in the treatment of breast carcinoma 25% of node negative patients still develop lymph node and distant metastatic disease. These groups of patients usually have occult micrometastatic disease at the time of surgery and they are under-staged. Up to 38% of patient with stage 1 and 2 breast cancers have demonstrated micrometastasis and reduced survival (14, 47).

The formal TMN staging is based almost exclusively on the anatomical stage of the disease, which is assessed using a combination of tumor size or depth (T), lymph node

spread (L), and presence or absence of metastasis (M). Since its inception the TNM system has provided a standardized, anatomical basis for staging and several important functions (47). It provide basis for prediction of survival, choice of initial treatment, stratification of patients in clinical trials, accurate communication between healthcare providers, and uniform reporting of outcome

The anatomically based TNM staging system remains useful for the system listed above, but new factors are both complicating the situation and providing new opportunities when it comes to predicting survival and/or selection of therapy. Development of biomarkers has opened a new chapter in cancer therapy. First, individual molecular markers and patterns of markers are successfully subdividing traditional tumor classes into subsets that behave differently from each other. Second, chemotherapeutic and biological agents are more effective and more widely used than when TNM staging was introduced, especially in the adjuvant staging. Third, many new targeted agents such as tamoxifen, are effective only if their receptive molecular markers are mutated or expressed in sufficient levels (12, 14, 47).

Specifically in the case of bone metastasis, biomarkers could be used for detection of the origin of metastasis and subsequently choice of initial treatment.

Several biomarkers could be used for early detection of metastasis from breast cancers (17, 47).

1. CA15-3 is a glycoprotein found in serum of patients of breast cancer is used for monitoring
2. CA27-29 is a glycoprotein found in serum of patients with breast cancer used for monitoring
3. Cytokeratins, belonging to the membrane proteins could be detected by immunohistochemistry; they are used as prognostic factors
4. Estrogen receptor and progesterone receptor, belonging to the membrane proteins, detected on primary tumors of breast cancers, used for the selection of hormonal therapy,
5. HER2/NEU proteins detected in breast tumors, by immunohistochemistry, used for prognosis and selection of therapy
6. HER2/NEU protein detected in serum, could be used for monitoring

7. c-erbB2 protein usually a predictor of a poor outcome

8. VEGF protein overexpression, correlated with high rate of metastasis and consequently poor outcome,

Bone is one of the most frequently involved sites in spreading of breast cancer metastasis too. (146) Her2/neu genotype was analyzed in a very small number of patients (<10), (140, 22, 143) only one study analyzed a larger population (132). These case reports documented a relatively frequent change in her2/neu genotype in distant metastases in up to 20% of the case.

There are reports that the estrogen receptor (ER) status of BRC is different in bone metastases: usually the initial ER positive phenotype reverts to an ER negative one. (146). It is also reported that careful genetic analysis revealed a different cytogenetic profile on bone metastasis of BRC compared to the primary tumor. Furthermore, expression profiling also detected a unique gene signature in the bone metastases compared to other metastatic sites (145). These data together all suggest that the common dogma that BRC maintains the geno/phenotype in metastasis may not be valid anymore. Our recent data further support these notions, revealing that in a small proportion of cases (around 10%) the initial genotype may change detected by her2/neu genotype. This may not seem a significant proportion of BRC cases, but it must be considered that in our cohort studies 50% of the her2/neu amplified cases lost their genotype in bone metastasis. Unfortunately we were not able to perform a larger study on bone metastases because in approximately half of our cases, we were not able collect the primary samples for comparison. We suggest that in the subset of her2/neu-amplified BRC the conversion of the genotype may occur more frequently compared to the her2/neu-negative BRC which maintained its genotype in our studies. Since the therapy of the her2/neu amplified and non-amplified metastatic BRC is totally different, based on our data and on the literature, we suggest performing her2/neu testing both on primary tumor and samples obtained from BRC metastases, at least in case of her2/neu amplified primary tumors.

5. Conclusions

5.1. Comparison of corresponding primary and metastatic NSCLC tissues indicated that downregulation of EGFR was a rare event (<20%) compared to upregulation (>30%) in bone metastases. On the other hand, our data indicated that the K-RAS mutation status of the primary tumor does not predict the status of the bone metastatic tissue of NSCLC. Our data support that at least two progression models occur in NSCLC, the same-gene as well as the clonal selection one.

5.2. EGFR protein scores were reduced significantly in bone metastases of RCC due to the reduction of EGFR protein expression in about one third of the cases. VEGFR2 protein positive phenotype of clear cell RCC was relatively frequent (7/20, 35%) which was also lost in bone metastases. These data suggest a phenotypic/genotypic change of clear cell RCC during the progression to bones.

5.3. Our studies on BRC revealed that the HER-2 negative status was maintained during the progression to bones. However, the Her-2 positive BRCs frequently lost this genotype in bone metastases. These data suggest that in BRC both the same-gene- as well as the clonal selection models characterized the metastatic progression.

5.4. Collectively, our studies on the metastatic progression of major human cancer types suggest that determination of the pheno/genotype of the individual cancer must be repeated if metastatic tissues are available since the expression of the common molecular targets of therapies may differ from the one found in the primary tumor.

6. Publications

I. Gayane Badalian, Katalin Derecsei, Atilla szendroi, Miklos Szendroi,Jozsef Timar: EGFR and VEGF protein expression in bone metastases of clear cell renal cancer. 27, xxx-xxx 2007 Anticancer research (under press)

II. Gayane Badalina, Tamas Barbai, Erzsebet Raso, Katalin Derecskei, Miklos Szendroi, Jozsef Timar et al: Phenotype of bone metastases of non small cell lung cancer: Epidermal growth factor receptor expression and K-RAS mutation status. Pathology Oncology Research (accepted)

III. Tamas Lorincz, Jozsef Toth, Gayane Badalian, Jozsef Timar, Miklos Szendroi et al: HER-2/neu genotype of breast cancer may change in bone metastasis. Pathology Oncology Research Vol.12, No 3,149-152, 2006

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References

1. Aubin JE. (1998) Bone stem cells. J Cell Biochem Suppl. 30-31: 73-82

2. Yang X, Karsenty G. (2002): Transcription factors in bone: developmental and pathological aspects. Trends Mol Med 8:340-345,

3. Komori T., Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. (1997): Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell; 89:755-764,

4. Stein GS, Lian JB. (1993): Molecular mechanisms mediating proliferation/differentiations interrelationships during progressive developments of the osteoblast phenotype. Endocr Rev. 14: 424-442,

5. Wozney JM. (2002). Overview of bone morphogenic proteins. Spine Supple 1:S2-S8

6. Roodman GD. (1999) Cell biology of osteoclast. Exp Hematol 27: 1229-1241

7. Kodama H, Nose M, Niida S, Yamasaki A. (1991) Essential role of macrophage colony stimulating factor in osteoclast differentiation supported by Stromal cells. J Exp Med; 173: 1291-1294

8. Lacy DL, Timms E, Tan HL, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan G, Howkins N, Davy E, Caparreli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delanay J, Boyle WG (1998) Osteooprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell; 93: 165-176

9. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tesuda E, Morinaga T, Higashio K, Udagava N, Takahashi N, Suda T(1998): Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA. 95; 3597-3602

10. Hofbauer LC, Heufelder AE. (1998) Osteoprotegerin and its cognate ligand: a new paradigm of osteoclastogenesis. Eur J Endocrinol, 139: 152-4

11. Kong YY, Fiege U, Sarosi I. (1999) Activated T-cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature, 402: 304-9

12. Roodman GD. (2004) Mechanism of bone metastasis. N Engl J Med 350: 1655-64

13. Horton MA, Dorey EL, Nesbitt SA, Samanen J, Ali FE, Stadel JM, Nichols A, Grieg R, Helfrich MH, (1993) Modulation of vitronectin receptor-mediated osteoclast adhesion by Arg-Gly-Asp peptide analogues: A structure function analysis. J Bone Miner Res, 8: 239-247

14. Coleman RE, Rubens RD. (1987) The clinical course of bone metastasis from breast cancer. Br J Cancer, 55:61-66, 1987

15. Coleman RE. Metastatic bone disease (2001) Clinical features, pathophysiology and treatment strategies. Cancer Treat Rev, 27:165-76

16. Coleman RE, Seaman JJ. (2001) The role of the zoledronic acid in cancer: Clinical studies in treatment and prevention of bone metastases. Semin Oncol 28: Suppl 6:11-6

17. Timar J, Csuka O, Ororsz Z, Jeney A, Kopper L. (2001) Molecular pathology of tumor metastases. Pathol Oncol Res. Vol 7

18. Fidler IJ, (1997) Molecular biology of cancer: invasion and metastasis. Cancer In: (DeVita VT, Hellman S, Rosenberg SA (eds)): principal and practice of oncology, Fifth edition, Lippincott-Raven Publ. Philadelphia, 1:135-152

19. Tanigawa S, Hirokazu A, Mastumura M. (1997) Tumor angiogenesis and mode of metastasis in patients with colorectal cancer. Cancer Res 57:1043-1046

20. Bellanchene A, Albert V, Pollina L, Basolo F, Fisher LW, Castronovo V. (1998) Ectopic expression of bone sialoprotein in human thyroid cancer. Thyroid 8:637-641

21. Gillespie MT, Thomas RJ, Pu ZY, Zhou H, Martin TJ, Findlay DM. (1997) Calcitonin receptors, bone sialoprotein and osteopontins are expressed in primary breast cancer. Int J Cancer 73: 812-815

22. Koeneman KS, Yeung F, Chung LW. (1999) Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. Prostate 39:246-261

23. Yoneda T. (2005) Hiraga T.Crosstalk between cancer cells bone microenvironment in bone metastasis. BBRC 327:679-687

24. Kahn D, Weiner GJ, Ben-Haim S, Ponto LL, Madsen MT, Bushnell DL, Watkins GL, Argenyi EA, Hichwa RD. (1994) Position emission tomographic measurement of bone marrow blood flow to the pelvis and lumbar vertebrae in young normal adults. Blood, 83:958-963

25. Van der Pluijm G, Sijmons B, Voled-graven H, Deckers M, Papapolous S, Lowik C. (2001) Monitoring metastatic behavior of human tumor cells in mice with species-specific polymerase chain reaction: elevated expression of angiogenesis and bone resorption stimulators by breast cancer in bone metastasis. J Bone Miner Res 16:1077-1091

26. Pfeilschifter J, Mundy GR. (1987) Modulation of type β transforming growth factor activity in bone cultures by osteotropic hormones. Proc Natl Acad Sci USA, 84: 2024-8

27. Taube T, Beneton MN, McCloskey EV, Rogers S, Greaves M, Kanis JA. (1992) Abnormal bone remodeling in patients with myelo-metastasis and normal biochemical indices of bone resorption. Eur J Hematol, 49:192-8

28. Roodman GD. (2001) Biology of osteoclast activation in cancer. J Clin Oncol, 19:3562-71

29. Lust JA, Donovan KA (1999) The role of interleukin-1 beta in the pathogenesis of multiple myeloma. Hematol Oncol Clin North Am, 13:1117-25

30. Cheung WC, Van Ness B. (2002) Distinct IL-6 signal transduction leads to growth arrest and death in B cells or growth promotion and cell survival in myeloma cells. Leukemia 16: 1182-8

31. Iwasaki T, Hamano T, Ogata A, Hashimoto N, Kitano M, Kakishita E. (1999) Clinical significance of IL-6 gene expression in the bone marrow of patients with multiple myeloma. Int Hematol, 70: 163-8

32. Sezer O, Heider U, Jakob C, Eucker J, Possinger K. (2002) Human bone marrow myeloma cells express RANKL. J Clin Oncol, 20:353-4

33. Sezer O, Heider U, Jakob C, Zavarski I, Eucker J, Possinger K, Sers C, Krenn V. (2002) Immunecytochemistry reveals RANKL expression of myeloma cells. Blood, 99: 4646-7

34. Pearse RN, Sordillo EM, Yaccoby S, Wong BR, Liau DF, Colman N, Michaeli J, Epstein J, Choi Y. (2001) Multiple myeloma disrupts the trans osteoprotegerin cytokine

axis to stimulate bone destruction and promote tumor progression. Proc Natul Acad Sci USA, 98:11581-6

35. Oyajobi BO, Mundy GR. (2003) Receptor activator of NF-KappaB ligand, macrophage inflammatory protein-1alpha, and the proteosome: novel therapeutic targets in myeloma. Cancer 97: Suppl: 813-7

36. Uneda S, Hata H, Matsuno F, Harada N, Mitsuya Y, Kawano F, Mitsuya H. (2003) Macrophage inflammatory protein-1alpha is produced by human multiple myeloma (MM) cells and its expression correlates with bone lesion in patients with MM. Br J Hematol, 120: 53-5

37. Han JH, Choi SJ, Kurihara N, Koide M, Oba Y, Roodman GD. (2001) Macrophage inflammatory protein-1α is an osteoclastogenic factor in myeloma that is independent of receptor activator of nuclear factor kappaB ligand. Blood, 97:3349-53

38. Oyajobi BO, Franchin G, Williams PJ, Pulkrabek D, Gupta A, Munos S, Grubbs B, Zhao M, Chen D, Sherry B, Mundy GR. (2003) Dual effect of macrophage inflammatory protein-1alpha on osteolysis and tumor burden in murine 5TGM1 model of myeloma bone disease. Blood, 102:311-9

39. Pavalkov FM, Gerald RL, Ponic SM, Gallagher PJ, Jin Y, Norvel SM. (2003) Fluid shear stress inhibits TNF-alpha induced apoptosis in osteoblasts: a role for fluid shear stress-induced activation of P13-kinase and inhibition of caspase-3. J Cell Physiol, 194:194-25

40. Tian E, Zhan F, Walker R. (2003) The role of the WNT/β catenin signaling antagonist DKK-1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med, 349:2483-94

41. Chirgwin JM, Guise TA. (2000) Molecular mechanisms of tumor-bone interactions in osteolytic metastasis. Crit Rev Eukaryot Gene Expr, 10:159-78

42. Karaplis AC, Goltzman D. (2000) PTH and PTHrP effects on the skeleton. Rev Endothel Metabol Disord, 1:331-41

43. Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubs BG, Wieser R, Masaque J, Mundy GR, Guise TA. (1999) TGF-β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastasis development. J Clin Invest, 103:197-206

44. Wani MR, Fuller K, Kim NS, Choi Y, Chambers T. (1999) Prostaglandin E2 cooperates with TRANCE in osteoclast induction from hemopoetic precursors: Synergistic activation differentiation, cell spreading and fusion. Endocrinology, 140:1927-35

45. Jalkanen S, Reichert RA, Gallatin WM, Weisseman IL, Butcher EC. (1986) Homing receptors and the control of lymphocyte migration. Immunol Rev, 91:39-60

46. Gohji K, Fujimoto N, Hara I, Fujii A, Gotoh A, Okada H, Arakawa S, Kitazawa S, Miyake H, Kamidono S, Nakajima M. (1998) Serum matrix metalloproteinase -2 and its density in men with prostate cancer as a new predictor of disease extension. Int J Cancer, 79:96-101

47. Joseph A. Ludwing, Jhon N Weinstein (2005) Biomarkers in cancer staging, prognosis and treatment selection. Nat Rew Cancer, Vol 5,

48. Singhal S, Vachani A, Antin-Ozerkis D, Keiser LR, Albenda SM. (2005) Prognostic implication of cell cycle, apoptosis and angiogenesis. Biomarkers of non small cell lung cancer: a review, Clin Cancer Res, 2005

49. Kopper L, Tímár J. (2005) Genomics of lung cancer may change diagnosis, prognosis and therapy. Pathol Oncol Res 11:5-10

50. Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Complese PP, Iarussi T, Mucilli F, Mezzetti A, Cuccurullo F, Sacco R, Buttitta F. (2005) EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 23:857-865

51. Aviel-Ronen S, Blackhall FH, Shepherd FA, Tsao MS. (2006) K-ras mutations in non-small cell lung carcinoma: a review. Clin Lung Cancer 8:30-38

52. Aviel-Ronen S, Blackhall FH, Shepherd FA, Tsao MS. (2006) K-ras mutations in non-small cell lung carcinoma: a review. Clin Lung Cancer 8:30-38

53. Riely GJ, Politi KA, Miller VA, Pao W. (2006) Update on epidermal growth factor receptor mutations in non-small cell lung cancer. Clin Cancer Res 12:7232-7241

54. Hammer PS. (1998) Metastatic adenocarcinoma of unknown primary origin. Hum Path 29: 1319-1402

55. Humphry PA, Walther PJ (1993) Adenocarcinoma of prostate, part 2: Tissue prognosticators, Am J Clin Pathol, 100: 256

56. Honn KV, Aref A, Chen Q. (1996) Prostate cancer old problems and new approaches. Pathology Oncology research 2: 191-211

57. Osterling J, Fuks ZVI, Lee CT, Scher HI. 9 In (DeVita VT, Hellman S, Rosenberg SA, (eds)): Principal and practice of oncology, Fifth edition, Lippincott-Raven Publ. Philadelphia, 1997: 1222-1385

58. Guise TA, Yin JJ, Mohammad KS (2003) Role of endothelin-1 in osteoblastic bone metastasis. Cancer, 97 Suppl: 779-84

59. Kasperk CH, Borcsok I, Schairer HU, Schneider U, Nawroth PP, Niethard FU, Ziegler R (1997) Endothelin-1 is a potent regulator of human bone cell metabolism in vitro. Calcif Tissue Int, 60:368-74

60. Carmer SD, Chen Z, Peehl DM, (1996) Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH like domain: Inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts. J Urol, 156: 526-31

61. Achbarou A, Kaiser S, Tremblay G, Ste-Marie LG, Brodt P, Goltzman D, Rabbani SA (1994) Urokinase overproduction results in increased skeletal metastasis by prostate cancer cells in vivo. Cancer Res; 54: 2372-7

62. Rabbani SA, Desjardins J, Bell AW (1990) An amino-terminal fragment of urokinase isolated from a prostate cancer cell line (PC-3) is mitogenic for osteoclast like cells. Biochem Biophys Res Commun 173:1058-64

63. Pantuck AJ, Zeng G, Belldegrun SD, Figlin RA. (2003) Pathobiology, Prognosis, and targeted therapy for renal cell carcinoma: Exploiting the hypoxia-induced pathway. Clin Cancer Res, 9:4641-52

64. Rini BI, Small EJ. (2005) Biology and clinical development of vascular endothelial growth factor-targeted therapy in renal cell carcinoma. J Clin Oncol 23:1028-43

65. Dancey JE. (2004) Epidermal growth factor receptor and epidermal growth factor receptor therapies in renal cell carcinoma: J Clin Oncol 15:2975-7

66. Sakaeda T, Okamura N, Gotoh A, Shirakawa T, Terao S, Morioka M, Tokui K, Tanaka H, Nakamura T, Yagi M, Nishimura Y, Yokoyama M, Okumura K. (2005) EGFR

mRNA is upregulated, but somatic mutations of the genes are hardly found in renal cell carcinoma in Japanese patients. Pharm Res 22:1757-61

67. Moch H, Sauter G, Gasser TC, Bubendorf L, Richter J, Presti JC Jr, Waldman FM, Mihatsch MS. (1998) EGF-r gene copy number changes in renal cell carcinoma detected by fluorescence in situ hybridization. J Pathol 184:424-9

68. Hofmockel G, Riess S, Bassukas ID, Dammrich J. (1997) Epidermal growth factor family and renal cell carcinoma: expression and prognostic impact. Eur Urol 31:478-84

69. Kallio JP, Hirvikoski P, Helin H. (2003) Membranous location of EGFR immunostaining is associated with good prognosis in renal cell carcinoma. Br J Cancer 89:1266-9

70. Merseburger AS, Hennenlotter J, Simon P, Kruck S, Horstman M, Keuhs U, Kufer R, Stenzel A, Kuczyk MA. (2005) Membranous expression and prognostic implications of epidermal growth factor receptor protein in human renal cell cancer. Anticancer Res 25:1901-7

71. Patel PH, Chaganti RSK, Morzer RJ. (2006) Targeted therapya for metastatic renal cell carcinoma Br J Cancer 94:614-9

72. Yang JC. (2004) Bevacizumab for patients with metastatic renal cancer: an update. Clin Cancer Res 10:6367S-70S

73. Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI. (2006) Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol **24**:16-24

74. Ratain MJ, Eisen T, Stadler WM. (2006) Phase II placebo-controlled randomized discontinuation trial of sorafenib in patients with metastatic renal cell carcinoma. J Clin Oncol 24:2505-12

75. Hainsworth JD, Sosman JA, Spigel DR, Edwards DL, Baughman C, Greco A. (2005) Treatment of metastatic renal cell carcinoma with a combination of bevacizumab and erlotinib. J Clin Oncol 23:7889-96

76. Jarmann M, Stahel RA, Salzberg M. (2006) A phase II, open-label study of gefitinib (IRESSA) in patients with locally advanced, metastatic, or relapsed renal cell carcinoma. Cancer Chemother Pharmacol 57:533-9

77. Rump U, Krieg T, Caliskan E, Mahotka C, Ebert T, Willers R, Gabbert HE, Gerharz CD. (2004) XIAP expression is an independent prognostic marker in clear-cell renal carcinomas. Hum Pathol 35:1022-1088

78. Langer C, Rastschek M, Rehak P. (2004) Steroid hormone receptor expression in renal cell carcinoma: an immunohistochemical analysis of 182 tumors. J Urol 171: 611-614

79. Hofman M, Rudy W, Zoller M. (1991) CD44 splice variants confer metastatic behavior in rats: Homologous sequences are expressed in human tumor cell lines. Cancer Res 51:5292-5297

80. Nihei Z, Ichikawa W, Kojima K, Tago S, Miyanaga T, Hirayama R, Mishima Y. (1996) The positive relationship between the expression of CD44 variant 6 and prognosis in colorectal cancers. Surg Today 26: 760-761

81. Ishigami SI, Arii S, Furatani M, Nivano M, Harada T, Mizumoto M, Mori A, Onodera H, Imamura M. (1998) Predictive value of vascular endothelial growth factor

(VEGF) in metastasis and prognosis of human colorectal cancer. Br J Cancer 78:1379-1384

82. Parsons SL, Watson SA, Collins HM, Griffin NR, Clarke Pa, Steele RJ. (2001) Gelatinase (MMP-2 and 9) expression in gasterointestinal malignancy. Br J Cancer 78: 1495-502

83. Lorincz T, Timar J, Szendrői M. (2004) Alternation of micro-vascular density in bone metastases of adenocarcinomas. Pathol Oncol Res 10:149-53

84. Sasaki R. (1996) Microvessel count and vascular endothelial growth factor in renal cell carcinoma. Nippon Hinyokika Gakkai Zasshi 87:1032-40

85. Gunningham SP, Currie MJ, Han C, et al. (2001) Vascular endothelial growth factor-B and vascular endothelial growth factor-C expression in renal cell carcinomas: regulation by the von Hippel-Lindau gene and hypoxia. Cancer Res 61:3206-11

86. Tsuchiya N, Sato K, Akao T, Kakinuma H, Sasaki R, Shimoda N, Satoh S, Habuchi T, Ogawa O, Kato T. (2001) Quantitiative analysis of gene expression of vascular endothelial growth factor-related factors and their receptors in renal cell carcinoma. Tohoku J Exp Med 195:101-13

87. Ljungberg BJ, Jacobsen J, Rudolfsson SH, Lindh G, Grankvist K, Rasmuson T. (2006) Different vascular endothelial growth factor (VEGF), VEGF-receptor 1 and -2 mRNA expression profiles between clear cell and papillary renal cell carcinoma. BJU Int 98:661-7

88. Le QT, Denko NC, Giaccia AJ. (2004) Hypoxic gene expression and metastasis. Cancer Metast Rev 23:293-310

89. Fox SB, Turley H, Cheale M, Blazguez C, Roberts S, James N, Cook N, Harris A, Gatter K. (2004): Phosphorylated KDR is expressed in neoplastic and stromal elements of human renal tumors and shuttles from cell membrane to nucleus. J Pathol 202:313-20

90. Junker K, Romics I, Szendroi A, Riesz P , Moravek P, Hindermann W, Winter R, Schubert J. (2004) Genetic profile of bone metastasis in renal cell carcinoma. Eur Urol 45:320-4

91. Mundy GR. (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. Nature Rev Cancer 2:584-593

92. Roodman GD (2004) Mechanisms of bone metastasis. N Engl J Med 350:1655-1664

93. Garber ME, Troyanskaya OG, Schluens K, Petersen S, Thaesler Z, Pacina-Gengelbach m, van di Rijn M, Rosen GD, Perou CM, Whyte RI, Altman RB, Brown PO, Botstein D, Petersen I. (2001) Diversity of the gene expression of adenocarcinoma of the lung. Proc Natl Acad Sci USA 98:13784-13789

94. Beer DG, Kardia SL, Huang CC, Giordano TJ, Levin AM, Misek DE, Lin L, Chen G, Gharib TG, Tomas DG, Lizyness MA, Kuick R, Hayazaka S, Taylor JM, Iannettoni MD, Orringer MB, Hanash S. (2002) Gene expression profiles predict survival of patients with lung adenocarcinoma. Nat Med 8:816-824

95. Kopper L, Tímár J. (2005) Genomics of lung cancer may change diagnosis, prognosis and therapy. Pathol Oncol Res 11:5-10

96. Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Complesse PP, Iarussi T, Mucilli F, Mazetti A, Cuccurullo F, Sacco R, Buttitta F. (2005) EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 23:857-865

97. Aviel-Ronen S, Blackhall FH, Shepherd FA. (2006) K-ras mutations in non-small cell lung carcinoma: a review. Clin Lung Cancer 8:30-38

98. Soung YH, Lee JW, Kim SY, Nam SW, Park WS, Lee JY, Yoo NJ, Lee SH. (2005) Mutational analysis of EGFR and K-RAS genes in lung adenocarcinomas. Virchows Arch 446:483-488

99. Riely GJ, Politi KA, Miller VA, Pao W. (2006) Update on epidermal growth factor receptor mutations in non-small cell lung cancer. Clin Cancer Res 12:7232-7241

100. Derecskei K, Moldvay J, Bodos K, Tímár J. (2006) Protocol modifications influence the result of EGF receptor immunodetection by EGFRpharmdx^{tm} in paraffin embedded cancer tissues. Pathol Oncol Res 12: 243-246

101. Molina JR, Adjei AA, Jett JR. (2006) Advances in chemotherapy of non-small cell lung cancer. Chest 130:1211-1219

102. Bepler G, Li X, Shrma A. Clinical value of tumoral RRM1 and ERCC1 expressions for treatment response and therapeutic decisions in NSCLC. In: ASCO2006 Education Book, Ed.: M.C. Perry, ASCO, Alexandria, 2006:431-435

103. Patel JD. (2006) EGFR pathway targeted therapy in patients with aerodigestive malignancies. Curr Opin Oncol 18:609-614

104. Fearon ER, Vogelstein B. (1990) A genetic model for colorectal tumorigenesis. Cell 61:759-767

105. Gray JW. (2003) Evidence emerges for early metastasis and parallel evolution of primary and metastatic tumors. Cancer Cell 4:4-6

106. Bernards R, Weinberg RA. (2002) A progression puzzle. Nature 418:823

107. Shiseki M, Kohno T., Nishikawa R, Sameshima Y, Mizoguchi H, Yokota J. (1994) Frequent allelic losses on chromosomes 2q, 18q, and 22q in advanced non small cell lung carcinoma. Cancer Res. 54: 5643-5648

108. Petersen S., Aninat-Mayer M., Schlus Kl. (2000) Chromosomal alteration in the clonal evolution of the metastatic stage of squamous cell carcinoma of the lung. British journal of cancer 82:65-73

109. Takahashi K, Kohno T, and Matsumoto S, Nakanishi Y, Arai Y, Yamamoto S, Fujiwara T, Tanaka N, Yokota J. (2007) Clonal and parallel evolution of primary lung cancers and their metastases revealed by molecular dissection of cancer cells. Clin Cancer Res 13:111-120

110. Matsumoto S., Takahasi K., and Iwakawa R (2006) Frequent EGFR mutation in brain metastases of lung adenocarcinoma. Int J Cancer. 15:1491-1494

111. Mundy GR. (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. Nature Rev Cancer 2:584-593

112. Roodman GD. (2004) Mechanism of bone metastasis. N Eng1 J Med 350:1655-1664

113. Yoneda T, Hiraga T. (2005) Crosstalk between cancer cells bone microenvironment in bone metastasis. BBRC 327:679-687

114. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J. (2003) A multigenic program mediating breast cancer metastasis to bone. Cancer cell 3: 537-549

115. Minn AJ, Knag Y, Serganova I, Gupta GP, Giri DD, Doubrovin M, Ponomarev V, Gerald WL, Blasberg R, Massague J. (2005) Distinct organ specific metastatic potential of the individual breast cancer cells and primary tumors. J Clin Incest 115: 44-55

116. Jung K, Lein M, Stephan C, von Hossln K, Semjonow A, Sinha P, oening SA, Schnorr D. (2004) Comparison of 10 serum bone turnover markers in prostate carcinoma patients with bone metastatic spread: diagnostic and prognostic implications. Int J Cancer 111: 783-791

117. Stewart DA, Cooper CR, Sikes RA. (2004) Changes in extracellular matrix (ECM) and ECM associated proteins in the metastatic progression of prostate cancer. Reproduct Biol Endocrinol 2:1-14

118. Lehrer S, Diamond EJ, Mamkine B, Stone NN, Stock RG. (2004) Serum interleukin-8 is elevated in men with prostate cancer bone metastasis. Technol Cancer es Teat 3:411

119. Klezovitch O, Chevillett J , Mirosevitch J. Robert RL, Matusik RJ, Vasioukhin v. (2004) Hepsin promotes prostate cancer progression and metastasis. Cancer cell.6: 186-195

120. Mastro AM, Gay CV, Welch DR. (2003) The skeleton as a unique environment for breast cancer cells. Clin Exp Metast 20:257-284

121. Lorincz T, Toth J, Szendroi M, Timar J. (2005) Microvascular density of breast cancer in bone metastasis: influence of therapy. Anticancer Res25: 3075-3082

122. Lorincz T, Timar J, Szendroi M. (2004) Alternation of microvascular density in bone metastasis in adenocarcinomas. Pathol Oncol Res 10:149-153

123. Mendelsohn J, Baselga J. (2000) The EGF receptor family as targets for cancer therapy. Oncogene 19: 6550-6565

124. Slamon DJ, Clark GM, Wong SG. (1987) Human breast cancer: Correlation of relapse and survival with amplification of he Her2/neu oncogene. Science 235:177-182

125. Yu D, Hung MC. (2000) Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. Oncogene 19: 6115-6121

126. Tsuda H, Hirohashi S, Shimotaso Y. (1989) Correlation between long term survival in breast cancer patients and amplification of two putative oncogene co-amplification units: hst-1/int-2 and c-erbB-2/ear-1. Cancer Res 49:3104-3108

127. de Bono JS, Rowinsky EK. (2002) The ErbB receptor family: a therapeutic target for cancer. Trends Mol Med 8 (4 suppl): S19-S26

128. Ross JS, Fletcher JA. (1999) HER-2/neu (c-erb-B2) gene and protein in breast cancer. Am J Clin Pathol 112: 53-67

129. Cobleigh MA, Vogel CL, Triphaty D. (1999) Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in woman who has HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. JClin Oncol 17: 2639-2648

130. Smith IE. (2001) Efficacy and safety of Herceptin® in women with metastatic breast cancer: Result from pivotal clinical studies. Anticancer Drugs 12 (suppl 4): S3-S10

131. Di Leo A, Dowsett M, Horten B, Penault-Liorca F. (2002) Current status of Her2 testing. Oncology 63 (suppl 1): 25-32

132. Gancberg M, Di Leo A, Rouas G. (2002) Comparison of topoisomerase IIα (topoIIα) and HER-2 status between primary (P) breast cancer (BC) and corresponding metastatic (M) sites. Ann Oncol 13: 1036-1043

133. Fidler IJ, Review. (1987) Biologic heterogeneity of cancer metastasis. Breast Cancer Res Treart 9:17-26

134. Masood S, Bui MM. (2000) Assessment of Her-2/neu overexpression in primary breast cancers and their metastatic lesions: an immunohistochemical study. Ann Clin Lab Sci 30: 259-265

135. Shimizu C, Fukutomi T, Tsuda H, Akashi-Tanaka S, Watanabe T, Nanasawa T, Sugihara K. (2000) C-erbB-2 protein overexpression and p53 immunoreaction in primary and recurrent breast cancer tissues. J Surg Oncol 73: 17-20

136. Carlsson J, Nordgren H, Sjöström J, Waster K, Villman K, Bengtsson NO, Ostenstad B, Lundgvist H, Blomgvist C. (2004) HER2 expression in breast cancer primary tumours and corresponding metastases. Original data and literature review. Br J Cancer 90: 2344-2348

137. Vincent-Salomon A, Jouve M, Genin P, Freneaux P, Sigal-Zafrani B, Caly M, Beuzeboc P, Pouillart P, Sastre-Garau X. (2002) HER2 status in patients with breast carcinoma is not modified selectively by preoperative chemotherapy and is stable during the metastatic process. Cancer 94: 2169-2173

138. Tanner M, Järvinen P, Isola J. (2001) Amplification of Her-2/neu and topoisomerase IIα in primary and metastatic breast cancer. Cancer Res 61:5345-5348

139. Kunimoto K, Inoue S, Ichihara F. (2004) A case of metastatic breast cancer with outgrowth of HER2-negative cells after eradication of HER2-positive cels by humanized

anti-HER2 monoclonal antibody (trastuzumab) combined with docetaxel. Human Pathol 35: 379-381

140. Zidan J, Dashkovsky I, Steyerman C, Basher W, Cosacov C, Hadary A. (2005) Comparison of HER-2 overexpresion in primary breast cancer and metastatc sites and its effect on biological targeting therapy of metastatic disease. Br J Cancer 93: 552-556

141. Latinovic L, Heinze G, Birner P, Samonigg H, Hausmaninger H, Kubista E, Kwasny W, Gnant M, Jakesz R, Oberhuber G. (2001) Prognostic relevance of three histological grading mehods in breast cancer. Int J Oncol 19: 1271-1277

142. Press MF, Slamon DJ, Flom KJ, Park J, Zhou JY, Bernstein L. (2002) Evaluation of HER-2/neu gene amplification and overexpression: comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimens. J Clin Oncol 20: 3095-3105

143. Yoneda T. (1998) Cellular and molecular mechanism of breast and prostate cancer metastasis to bone. Eur J Cancer 34: 240-245

144. Gong Y, Booser DJ, Sneige N. (2005) Comparison of HER-2 status determined by fluorescence in situ hybridization in primary and metastatic breast carcinoma. Cancer 103: 1763-1769

145. Regitnig P, Schippinger W, Lindbauer M, Samonigg H, Lax SF. (2004) Change of HER-2/neu status in a subset of distant metastases from breast carcinomas. J Pathol 203: 918-926

146. Bruun Rasmussen B and Kamby C. (1989) Immunohistochemical detection of oestrogen receptor sin paraffin sections from primary and metastatic breast cancer. Pathol Res Pract 185:856-859

147. Prenzel N, Fischer O M, Streit S, Hart S, Ulrich A. (2001) The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocrine-Related Cancer, 8:11-31