Phenotype/ genotype of malignant tumor sin bone metastasis

Dr. Gayane BadalianVery

Semmelweis University
School of clinical doctoral studies (Ph.D)

Head of the prgram: Dr. Szendrői Miklós, D.Sc.
Opponents : Dr. Simon Károly, Ph.D
Dr. Zalatnai Attila, D.Sc.

Head of the examining board: Dr. Szende Béla, D.Sc.
Members of examining board: Dr. Sápi Zoltán, Ph.D
Dr. Kulka Janina, Ph.D

Budapest
2007.

1. Introduction

There are three major models of the tumor progression/metastatization: clonal evolusion/selection , parallel development and the same-gene models 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. The clinical validity of these models can only be determined by direct comparison of metastatic and primary tumor tissues of various cancer types.

Phenotype/genotypes changes of cancers during dissemination of primary tumors to the distant sites may occur quite frequently serving as one of the major reasons of failure of targeted therapies. In clinics the candidates of targeted therapies are chosen mainly by genotyping of primary tumors, and basically genotyping of metastatic sites are not among the required criteria's before putting the patient on certain therapy.

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. 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. In addition secondary formation of bone occurs in response to bone destruction. 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.

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. 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. On the other hand, in

recent years EGFR emerged as a useful target in lung cancer 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.

Sporadic clear cell RCC is characterized by genetic aberration of the VHL gene, including inactivating mutations as well as inactivation caused by hypermethylation. 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, providing well-characterized molecular targets for novel anti-angiogenic therapies. VEGF level is increased in clear cell RCC patients and the cytokine activates endothelial cells through VEGFRs (mostly KDR). 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.

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. Upregulated EGFR could also result in overexpression of several genes involved in the angiogenic phenotype, including VEGF. 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.

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), Overexpression of the Her2/neu oncoprotein in breast cancers is associated with shortened survival, enhanced aggressiveness, resistance to hormone- and chemotherapy and eventually decreased sensitivity of targeted cells to therapy. The extracellular domain of her2/neu is the target of trastuzumab (Herceptin®), the humanized anti-her2/neu monoclonal antibody. It has

also been proven that the anticancer efficacy of trastuzumab is highly dependent on the Her2/neu amplification of breast cancer.

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. 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.

2 .Aims of thesis

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

3. Materials and methods

3.1 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. Paraffin embedded tumor samples of both the bone metastases as well as primaries were available for analysis. EGFR protein expression and K-RAS mutations were studied in these patients. The majority of the NSCLC patients were male (9/11) and adenocarcinomas dominated over squamous cell cancer (9/2). Except one case, bone metastases developed following surgery of the primary tumor.

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. 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. 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.

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). In total we investigated 48 metastatic cases. We were able to obtain paraffin embedded samples of the primary breast cancers in 23 patients. IHC studies of EGFR and FISH analysis were performed on these samples.

3.2. a. Immunohistochemistry

EGFR and VEGFR-2 protein expression in RCC and NSCLCs

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 a final IHC score was produced by multiplying % data with intensity values. Statistical analysis was performed using MS Excel program applying t test.

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

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. Furthermore, the C-terminal domain of the EGFR was also reliably detectable at both locations, while phosphorylated EGFR was practically absent (data not shown).

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. 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,).

3.1-b EGFR phenotype of RCC

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. 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.

3.2-b Detection of VEGF expression in RCC metastasis

The cytoplasm of RCC tumor cells was diffusely positive for VEGFR2, while in the majority of the cases scattered tumor cells contained VEGFR2 in their cytoplasm. 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 in to the category of scattered reaction pattern. VEGFR2 positivity is a rare feature in bone metastases of RCC (10%): out of the seven VEGFR2+ cases six lost this phenotype in bone metastases. On the other hand, appearance of VEGFR2 positive phenotype in bone metastasis was exceptionally rare (1/20).

3.3-a.HER-2/NEU status of BRC in bone metastasis

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. 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. 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%). Furthermore, we also detected lower her2/neu gene copy number in the bone metastasis of the two Her2 amplified BRC compared to the primary tumor.

4. Discussion

Bone is among the most frequent sites for metastasis of lung cancers. 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. Quite 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 downregulation was a rare event (<20%) and even upregulation was observed in a significant proportion of cases (>30%). This observation 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.

K-RAS is the hallmark of a relatively early genetic aberration during smoke-induced lung carcinogenesis. On the other hand, constitutively active (mutated) K-RAS in NSCLC may define a more aggressive and/or drug-resistant genotype It is also established that EGFR mutations do not occur together with K-RAS mutation. 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 mets 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.

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, and activated KDR (autophosphorylated forms) was detected on cancer cells as well. 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, 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, and clonal connection to the primary tumor can only be proven in half of the cases, while the development of metastases from a minor subpopulation in the primary RCC is also frequent. 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.

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. 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. Never the less, the main targets of any systemic therapy in metastatic breast cancer are metastatic foci. 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. 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. Recently some reports provided evidences that the Her2 genotype of BRC may change in metastasis: loss was reported in one case in liver metastases and loss of Her2 amplification was reported in 21 % but appearance of Her2 amplification was very frequent in visceral metastases (30%).

Bone is one of the most frequently involved sites in spreading of breast cancer metastasis too. Her2/neu genotype was analyzed in a very small number of patients (<10), 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. 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. 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 et al: 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

7.Acknowledgement

I am depply indepted to so many individuals who were invelved in various ways in complition of this work. First and for most I offer my gratitudes to Prof. Szendroi Miklos and Prof. Timar Joszef who undertook the challenge of accepting me in their programs and providing me with invaluable advises and guidenece.

Several colleagues have added to the luster of this work providing their expertees in hungerian translation and thechnical supports. I offer spetial thanks to Dr. Szendroi Atilla, Dr. Ladanye Andrea, Dr. Raso Erzsebet, Dr. Lorinc Tamas, Derecskei Katalin, and Barbai Tamas.

This work was supported by the Ministry of Education, NKFP1a-0024-05.