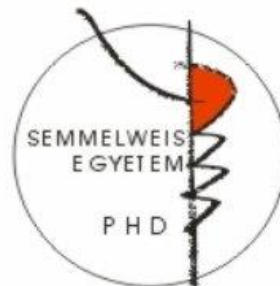


Host and tumoral factors in the progression of human melanoma

PhD thesis

Tamás Barbai

Doctoral School of Pathological Sciences
Semmelweis University



Consultant: Erzsébet Rásó, Ph.D.

Official reviewers: Gábor Méhes, Ph.D.
András Lászik, Ph.D.

Head of the Final Examination Committee: Péter Lakatos, D.Sc.
Members of the Final Examination Committee: Zoltán Szentirmay, PhD
Attila Patócs, Ph.D.

Budapest
2016

1. Introduction

The substance of metastasis research is the step by step cascade process of tumor progression. Stages in this process are separated into two aspects: tumor and host, with the border between the two not being sharp. Interaction between tumor cells and their microenvironment does not only provide opportunity for tumor growth, but can finally lead to the selection of a metastatic population of tumor cells. This relation can form the metastatic niche, involving angiogen phenotype, extracellular matrix rearrangement via production and storage of chemokines and growth factors, changes in migration potential, and due to stromal cells activation, leading to tumor formation by selected cells. Primarily, the stromal components are the fibroblasts, reprogrammed into tumor associated fibroblasts by the active participation of tumor cells, and making progress together to promote the molecular environment for the primary tumor. Surprisingly, it is this environment that can arrange this premetastatic and metastatic niche at the location of distant metastases and which supports colonization of the selected tumor cell clones. Published data have reported on several genes and their interactions participating in the metastatic cascade activation, but the complete spectrum of genes has yet to be revealed.

Chemokines:

Chemokines are small molecular weight proteins which can bind specific G protein coupled cell surface receptors. They play key role in many areas, such as migration, inflammation, immune response and tumor growth. The classification of chemokines is based on the pattern of the cysteine residue containing protein C terminus which can be divided into four major groups: CXC, CX3C, CC and C, where C is cysteine and X denotes any other amino acid. Functionally there are homeostatic and inflammatory cytokines. The nomenclature of cytokines is not uniform, special attention should therefore be paid to make sure that a certain chemokine molecule is the same in mice and humans. Several members of the tumor-host system have been proven to play role in tumor progression, for example tumor growth, migration, angiogenesis, EMT. The metastatic cascade involves the route by which tumor cells can travel from the primary tumor through the blood and lymph systems. The appearance of metastases follows a certain regularity indicating

that it might not be a random set of events. Physiologically, chemokines are involved in the management of leukocyte migration and by a similar method they promote organ specific tumor metastasis formation.

Collagen XVII

Interaction between epithelial cells and extracellular matrix has critical role in maintaining the integrity of cellular environment. One of its essential function is to anchor the epithelial cells to the basal membrane. The connection between the matrix and cells could alter due to several reasons, modifying the function of the tissue in question. Collagen XVII is a member of the integrin protein family, which provides at least five other members to the group of molecules responsible for forming cell-matrix connections. Collagen XVII is one of the transmembrane proteins adhere to basal membrane like an anchor. Its globular N-terminal head is attached to a plaque formed by hemidesmosomes, while its C-terminal tail is fixed to the basal membrane. In order to form the hemidesmosomal matrix-cell connection it is also linked to laminin-332, earlier called laminin-5, and to collagen-4. In the literature, autoantibodies have been described in bullous pemphigoid. The 120 kDa molecule, which functions as a matrix anchor, is cleaved by ADAM9 and 10 metalloprotease, resulting is a 60 kDa endodomain. Expression of collagen XVII is physiologically linked to differentiation of keratinocytes, however its expression have been detected in cells originating from neural crest, in hyperplasia of melanocytes, as well as in squamous cell carcinoma and in ductal carcinoma of the pancreas. Proliferating melanocytes express both ecto- and endodomain of collagen XVII protein. Accumulation of 60 kDa endodomain have been observed in melanomas.

CD44

The cell surface glycoproteine CD44 was first described on T-lymphocytes. The molecule is evolutionally preserved as it is 80% identical to its homologue in mice. The gene contains 20 exons, of which 10 are variable and are straddled by 5 non-variable exons on each side. Versatility at the level of the variable exons of CD44 is a great example of alternative splicing, considering the wide spectrum of variants

arising from one gene. Standard CD44 (CD44S) can predominantly be found in epithelial cells and hematopoietic tissue, and it either does not contain any of the variable exons or variable exon 1 only. On the contrary, diverse populations of these variants can be found in tumor cells. Post-translation, the variable region is extracellular within the CD44 protein and is situated close to the cell membrane. CD44 therefore can bind extracellularly to heparan sulfate, hyaluronic acid and other polysaccharides. The CD44 splice variants detected in malignant neoplasia show weaker hyaluronic acid (one of the structural glycosaminoglycans of cell-cell and cell-matrix interactions) binding propensities. Tissue structure is preserved throughout epidermal differentiation, however it is disrupted during the progression of undifferentiated tumour tissue (EMT phenomenon). One of the key steps of tumor progression is the appearance of new splicing variants alongside the standard CD44 molecule.

2. Objectives

Our goal was to identify novel host associated markers and study their behavior during the course of the melanoma progression.

- To identify the changes in gene expression related to stromal components (host derived) during tumor progression in human malignant melanomas implanted into mice with severe combined immunodeficiency using an expression chip representing 20.000 genes.
- To validate molecules showing significant changes, therefore potentially playing a part in progression, on animal samples and human clinical melanoma samples,
- to study their effect on migration and proliferation activity of tumor cells and
- to model the mechanism of tumor-host interaction *in vitro* in a system simulating cooperation.
- To determine the quantitative changes characteristic for tumor progression in the profile of 800 miRNA in the above described model, and to verify the probable prognostic importance of molecules showing the greatest change in expression on human clinical samples.
 - To examine the changes in the expression of collagen XVII (a molecule hypothetically associated with metastasis formation) in our experimental setup modeling the progression of xenograft melanoma.
- To observe the characteristics of alternative splicing, a phenomenon associated with tumor progression, with the aid of CD44, as this single gene can give rise to a great variety of protein isoforms.

3. Methods

Human xenograft model

Xenotransplantation was carried out by subcutaneous inoculation of freshly prepared cell suspension of human melanoma cell lines (HT199, HT168M1, WM983B, 5×10^6 cells/50 μ l/animal) in the left hind leg of adult and newborn animals. On the 30th day, the animals were sacrificed by bleeding under anesthesia. Primary *in vitro* cell cultures were formed from the primary tumor, circulating tumor cells and lung metastases of the same animal implanted as a newborn. Further, the primary tumor and circulating tumor cells from the adult animals were used to create cell cultures in a similar manner. This way the 'noise' of communication with the microenvironment, such as skin, blood, lung, was excluded. Total RNA was isolated from the cultures.

This study was carried out in strict accordance with the recommendations and was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (TUKÉB permit number: 83/2009).

Tumor samples

Frozen samples of melanoma were stored in RNA later. Random sampling was applied throughout the experiments. Stratification of melanomas was based on the percentage of melanoma cells containing melanin. Melanomas were classified as amelanotic, moderately pigmented and strongly pigmented according to the literature [33]. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice, and with the approval of a Semmelweis University Regional and Institutional Committee of Science and Research Ethics (114/2012).

RNA samples

Total RNA was extracted from the original cell suspension and from the tissue samples (subcutaneous primary tumors and metastases) at the time of evaluation, using NucleoSpin RNAII total RNA isolation kit (MACHEREY-NAGEL GmbH, Düren) according to the manufacturer's protocol.

The quality and integrity of total RNA were evaluated on 2100 Bio analyzer (Agilent Technologies, Palo Alto, CA) and the same samples were divided into individual aliquots for gene expression analysis on two different microarray platforms and for the TaqMan Gene Expression Assay based real-time PCR analysis.

Agilent Mouse Oligo Microarray analysis

The Agilent Mouse Oligo Microarray is comprised of 22,575 (60-mer) oligonucleotide probes representing 20,000 mouse genes and transcripts in the mouse genome. Probe labeling and hybridization were carried out following the manufacturer's specified protocols. Briefly, amplification and labeling of 5 µg of total RNA were performed using Cy5 for healthy control skin sample RNA and Cy3 for newborn subcutaneous tumor sample RNA. Hybridization was performed for 16 hrs at 60°C and arrays were scanned on an Agilent DNA microarray scanner. Following the manufacturer's protocol, Agilent's Stabilization and Drying Solution (#5185–5979) was used to protect against the ozone-induced degradation of cyanine dyes on microarray slides during hybridization and processing steps. Images were analyzed and data were extracted, background was subtracted and normalized using the standard procedures of Agilent Feature Extraction Software A.7.5.1. Arrays were analyzed. Linear & LOWESS, the default normalization method in the Agilent Feature Extraction Software A.7.5.1, were applied for normalizing Agilent microarrays. The method performs a linear normalization across the entire range of data, followed by a non-linear normalization (LOWESS) to the linearized data set.

cDNA synthesis

One µg of total RNA was reverse transcribed using oligo(dT)/random primer (2.5 µM) and MMLV reverse transcriptase (200 unit/µl, Finnzyme® Espoo, Finland). The reaction mixture was incubated at 37°C for 50 min, heated at 85°C for 10 min.

PCR

The primers were designed by ArrayDesigner software. DNA amplifications were performed using *AmpliTaq Gold® 360 PCR Master Mix (Thermo Fisher Scientific Inc.)* and Mastercycler gradient thermal cycler supplied by Eppendorf at 35 cycles

of denaturation at 95 °C for 1 min, primer annealing at 59 °C for 1 min, chain elongation at 72 °C for 2 min. After amplification 10 µl of PCR products were separated on 3% agarose gel and stained with ethidium bromide.

Quantitative PCR analysis

For quantitative measurement of the expressed genes (Fam 187b, Lass5, DEAD box 60 polypeptide, Gm4262, CCL11, Pex2, CathepsinL, mouse CCL12, Ninein, human CCL8, CCR1) q-PCR reactions were used. Each 25 µl reaction mixture contained 12.5 µl of 2X iQ SYBR® Green Supermix (Bio-Rad), 0.5 µl of each primer for final concentration of 200 nM and 11.5 µl of the diluted cDNA. The used primers are presented in a separate table (Table 2). Cycling conditions comprised 3 min of iTaq™ DNA polymerase activation at 95°C, 40 cycles at 95°C for 30 sec, at 55°C for 30 sec and at 72°C for 1 min. Starting quantities were defined on the basis of standard fivefold dilution series (1X-625X) carried out with control cDNA of mouse B16 and human K562 cell lines. Relative expression of the examined genes was determined by normalizing starting quantities to those of the housekeeping genes beta-2 microglobulin or beta actin from the same cDNA sample.

Immunohistochemistry

Formalin fixed paraffin embedded sections of tumors were cut at a thickness of 2 µm. Sections were deparaffinated and to remove the melanin pigments we used potassium permanganate (KMnO₄) with oxalic acid depigmentation protocol. Citrate buffer (pH 6) was used for microwave assisted antigen retrieval. After endogenous peroxidase quenching the sections were incubated overnight with mouse monoclonal CCL8 primary antibody in a dilution of 1:50 (Santa Cruz). Following extensive washings with PBS, sections were incubated for 1 h in the secondary biotinylated antibody, then the slides were developed by avidin-biotin peroxidase followed by incubation with diaminobenzidine or NovaRED™ Substrate (Vector Burlingame, CA). The negative control was prepared by omitting the primary antibody.

Cell lines

The A2058 melanoma cell line was provided by LA Liotta (NCI, Bethesda, MD). HT168 and HT168M1 lines are derivatives of A2058. HT199 was developed in the 1st Institute of Pathology and Experimental Cancer Research (Semmelweis University, Budapest, Hungary). WM983B and WM983A were gifts from M. Herlyn (Wistar Institute, Philadelphia, PA). Normal human melanocytes (C-12403), keratinocytes (C-12003) and dermal fibroblasts (C-12360) were derived from Promo Cell. Cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum (Sigma, St. Louis, MD) at 37°C in 5% CO₂ atmosphere.

Cell viability test

Cells (HT199, HT168, WM983A, fibroblast and melanocyte) were placed in 96-well tissue culture plates (6×10^3 /well) in RPMI 1640 medium containing 5% FCS. After overnight incubation at 37°C, adherent cells were treated with CCL8 (R&D Systems, Minneapolis, MN, USA) (at final concentration of 500 pg/ml, 1 ng/ml) for 12 hrs. At the end of the incubation period, a colorimetric assay (MTT test) was performed. Absorbance at 570 nm was measured with Bio-Tek Microplate Reader (Merck).

Transmembrane migration assay

Human melanoma (HT168, WM983A), fibroblast and melanocyte cell migration was measured using a real time system (Roche xCELLigence). Cells were seeded at 2×10^5 /ml in 100 µl of RPMI 1640 medium. The first round of cells were treated with 1 and 10 ng/ml of exogenous CCL8 (R&D Systems) in the upper chamber with 8 µm pore size and the second round in the lower chamber as a chemoattractant. The control cells were treated with equal volume of PBS. Cell migration was monitored every 5 min by CIM plate 16 for a period of 0-23 hrs.

ELISA:

The human CCL8 ELISA kit (R&D Systems, according to the manufacturer's protocol) was used to detect CCL8 in the serum of melanoma patients. Duplicated samples were analyzed using Bio-Tek Microplate Reader (Merck). Positive control wells were treated with recombinant CCL8 peptide.

miRNA assay

Digital quantification of microRNA was calculated using the nCounter System from nanoString Technologies.

Statistics

Results of quantitative PCR were analyzed with Stat Soft Statistica 11 software using unpaired t test. Cell viability and migration assays were analyzed using ANOVA and Scheffe post hoc test. Values of $P \leq 0.05$ were considered significant.

4. Results

Single cell suspension of the human melanoma cell line, HT168M1 was orthotopically implanted into adult and newborn SCID mice simultaneously. There was primary tumor growth in both hosts, but metastases only formed in the newborn host. The experiment was terminated after 30 days and RNA isolated from the metastatic primary tumor (newborn host). The RNA expression pattern of the metastatic host was investigated by Agilent Mouse Oligo Microarray Platform containing 20,000 genes. The stromal expression changes were compared with tissue derived from corresponding area of tumor implantation of healthy, age-matched controls. The nineteen genes showing the largest expression changes were then chosen and their expression further examined on samples from three genetically different human melanoma cell lines (HT168M1, WM983B, HT199) implanted into the same metastatic / non-metastatic model. The species specificity of the primers for every examined gene was checked by using them on human and mouse tumor cell lines. This was necessary to ensure that the expression changes were strictly mouse-derived stroma related, therefore only those primers giving clear products in the target species (mouse), but not in human, were validated. PCR products were identified by direct sequencing. Nine out of 19 genes were suitable to be used as host specific markers: Fam 187b, Lass5, DEAD box 60 polypeptide, Gm4262, CCL11, Pex2, Cathepsin L, CCL12 and Ninein. Total RNA was then isolated from the implanted *in vitro* cultures and *in vivo* subcutaneous primary tumors of all three melanoma cell lines, and the relative expression of target genes was determined by real time PCR. To assess the role of these genes in providing a

microenvironment prone to select a more metastatically potent subset of cells, we compared the relative expression pattern of these genes in the non-metastatic and metastatic version of each of the three melanoma cell lines growing in adult and newborn hosts respectively.

The expression threshold used was 1.5 fold or higher in at least two cell lines. Five of the nine genes fulfilled these requirements, namely Fam 187b, Lass5, DEAD box 60 polypeptide, Cathepsin L and CCL12. Unfortunately, any PCR-based technique would struggle to differentiate between host and tumor-derived expression of genes due to their genetic identity. Therefore, human homologues of the five genes were analyzed in our human melanoma cell lines. There was only one gene, CCL12 and its human homologue CCL8, which was expressed by only one human melanoma cell line (WM983B, which was derived from a melanoma metastasis) and therefore was proved to be suitable for further analysis. An almost two-fold difference was observed in the relative expression level of CCL12 between metastatic and non-metastatic model in all tested cell lines. This result 'nominated' this gene to operate in the selection of metastatic tumor cells.

We then examined the expression of CCL8 in five, genetically different human melanoma cell lines (HT199, HT168M1, WM983A, A2058, WM983B) and found that it was expressed by only one of them (WM983B). Additionally, we demonstrated CCL8 expression in *in vitro* cell cultures of human dermal fibroblasts and melanocytes. In order to find out which cell types are the potential targets of CCL8, the presence of CCL8 sensitive chemokine receptors was analyzed in the tumorous and several normal constituents of the tumor-host system. Specifically, we examined the expressions of CCR1, CCR2 and CCR5. Expression of CCR1 was detected in HT199, HT168 and WM983A tumor cell lines. Only dermal fibroblasts expressed CCR1 from the host side.

After identification of the chemokine/receptor pattern of both sides of the tumor-host system, our next step was to map its functional effects. First of all, the effect of CCL8 on dermal fibroblasts, melanocytes and CCR1-expressing tumor cell lines was examined, using MTT-test. The effect of CCL8 on viability was compared to untreated control, using two different concentrations of CCL8, after 12 hours of treatment. To determine the optimal concentration range of the treatment we tested

the viability of HT168M1 human melanoma cells. Based on the viability test the applied concentration was 0.5-10 ng/ml.

Significant difference was observed in HT199 of the tumor cells lines and in dermal fibroblasts of host cells in both cases at the concentration of 500 pg/ml, which reduced viability.

The effect of CCL8 on migration, one of the key steps of the metastatic cascade, was measured with xCELLigence-system on CIM (Cell Invasion Migration) plate, semiquantitatively. Based on the detection of impedance by microelectrodes, the migration activity of the untreated control culture was compared with that of the treated cells. CCL8 was applied in two different ways on cell cultures: either added directly to the culture or as a chemoattractant. In both cases the administered concentrations were 1 and 10 ng/ml. Analyses started 5 hours after treatment following which data were collected every 5 minutes for a duration of 18 hours. A summary of the results is presented in the table inserted in. According to our observations, the migration of tumor cells was inhibited by CCL8 when applied directly and it was increased when added as chemoattractant. On the other hand, CCL8 as a chemoattractant either did not alter the migration of non-tumor cells or inhibited them (fibroblasts in low concentration). When applied directly, CCL8 inhibited migration of both melanocytes and fibroblasts, while in lower concentration it significantly increased migration activity of fibroblasts.

Since the expression pattern of CCL8 was identified in the melanoma cell lines and non-tumoral cells (fibroblasts, melanocyte, keratinocyte) as well as human metastatic and non-metastatic melanoma samples in our metastatic animal model, we raised the question whether there was any correlation between chemokine expression and metastasis formation of human primary melanomas.

Human surgical primary melanoma samples were divided into two groups: non-metastatic (NM) primary tumor – patients with no metastasis detected during a follow-up period of five years and metastatic (M) primary tumor – patients developing metastasis within five years. Data were retrospectively collected to include sex, age, tumor phenotype (pigmentation), tumor site, histological type, Clark level and Breslow thickness. Patients of the metastatic group had distant organ metastases (single or multiple) in the lung, liver, brain, bones, skin and lymph nodes. Total RNA was then isolated and the CCL8 expression determined in each

sample, qualitatively in the first instance. Subsequently, the relative quantitative expression of CCL8 was also measured by real time PCR, comparing the CCL8 expression level with human beta actin expression (relative CCL8 expression). A total of 57 clinical primary melanoma samples were analyzed and no statistical difference was found between the metastatic and non-metastatic group. Metastatic primary tumor samples were then further divided into 6 subgroups (patients with lung, liver, skin, bone, brain and lymph node metastases) based on the target organ of the metastases. Because of patients with multiple metastases in different locations the number of samples proved to be sufficient for our study only when we examined primary tumors forming lung metastases. When re-analyzing the data considering this subgroup, we found a significant difference between non-metastatic primary human melanoma and primary melanoma with lung metastases, the latter group expressing lower levels of CCL8.

The expression of the CCL8 receptor, CCR1, was analyzed on the same samples, but no difference was observable between the analyzed non-metastatic primary human melanoma and primary melanoma with lung metastases groups.

It was not possible to separately examine CCL8 mRNA expression of the stromal components in our surgical samples, therefore we used immunohistochemistry to reveal the localization and expression of CCL8. Heterogeneous expression of this protein was detected primarily in the stromal cells, and in the tumor as well, though to a lesser extent. Systemic appearance of CCL8 in the serum of patients with melanoma was analyzed by ELISA kit, with recombinant CCL8 used to set the standard curve. During measurement, because of the detection limit, CCL8 concentrations lower than 60 pg/ml were considered to be zero. Altogether, 36 serum (17 metastatic and 19 non-metastatic) samples of patients with melanoma were analyzed and CCL8 protein concentration was higher than 60 pg/ml in 15(7 metastatic and 8 non-metastatic) of the analyzed serum samples. The level of CCL8 protein was found to be greater in non-metastatic group of patients, however because of low sample number and broad range of standard deviation it did not prove to be statistically significant.

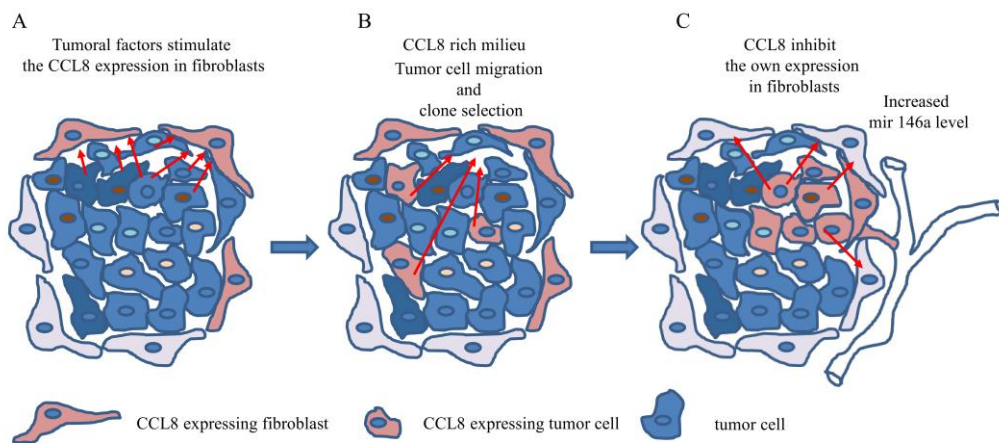
Our experimental animal model offered good possibility to examine tumor cell populations derived from different stages of the metastatic cascade, free of stromal components. Namely, cell cultures were created from metastatic and non-metastatic

primary tumors, circulating tumor cells as well as lung metastases, and CCL8 expression was measured qualitatively. CCL8 mRNA expression was detected in the lung metastases of the metastatic system (newborn model) and in circulating tumor cells of the non-metastatic system (adult model). We could not prove the presence of CCL8 mRNA in cell culture derived from the primary tumor, although by analyzing the whole subcutaneous tumor tissue CCL8 was detectable by immunohistochemistry in a small group of tumor cells. To further investigate the molecular cooperation between host and tumor, we treated human dermal fibroblasts with the supernatant of HT199 melanoma and K562 leukemia cell cultures as well as 'pure' CCL8. Untreated cells and glucose solution were used as control. Dramatic increase of CCL8 expression was observed after melanoma and leukemia supernatant treatment, but in contrast 'pure' CCL8 treatment terminated the continuous expression of CCL8 .

The rapid effect of CCL8 on the gene expression of dermal fibroblasts suggests that microRNAs might be involved. In the two groups (CCL8 treated and control) the quantitative change of 800 human microRNAs was measured. We created a CCL8 specific microRNA pattern by including microRNAs showing fivefold or higher differences between the two groups. One of the microRNAs, miR146a, was also found in the middle range of our list, therefore playing role in the regulation of CCL8 in accordance with the literature. miR146a level was measured in randomly selected five samples each of the metastatic and non-metastatic primary tumors of human melanoma patients, using nCounter assay. A statistically significant, higher miR146a expression level was found in the metastatic group (independent of metastasis localization) as compared with the non-metastatic group.

The apparent contradictions between our data from the animal model and clinical samples can be explained by a few facts. In the animal model we were only able to detect the relative chemokine expression of host cells with host (mouse) specific methods in contrast to clinical samples in which the whole cumulated (tumor and stroma) expression level was measured in order to validate the prognostic value. In conclusion we created a hypothetic process: factors of tumoral origin stimulate the chemokine expression of cells in their surroundings, mostly in fibroblasts and this chemokine functions as a chemoattractant molecule. As a consequence of the tumor-host interaction, some of the tumor cells might be able to express CCL8. The local

CCL8-rich environment could promote the selection of tumor cells with metastatic ability. The high CCL8 concentration inhibits the migration of tumor cells. *In vitro* studies showed that the exogenous CCL8 blocks the production of this protein in fibroblasts, thus if a CCL8 expressing population grows in the primary tumor, these cells could regulate the chemokine expression of tumor associated fibroblasts. Our data suggests that miR146a expression and CCL8 level are related. Therefore, miR146a could contribute to restoring the shifted balance, meaning that it is overexpressed in metastatic tumors. By contrast, in non-metastatic tumors low miR146a expression level is accompanied by relatively high CCL8 expression. However, this hypothesis should be supported by further analysis.



The communication between tumor cells and their environment is mediated by cell surface molecules. One of them is collagen XVII, the expression of which we detected (beside its physiological appearance) in dividing melanomas and xenotransplanted human primary melanomas. We were able to prove that metastatic cells seceding from primary tumor kept this feature when forming metastasis in distant organs. We studied the role of the molecule following proteolytic cleavage, in cell function by altering viability and adhesion, and by induction of apoptosis during *in vitro* experiments. Our results show that targeted treatment of melanoma cells expressing collagen XVII endodomain with collagen XVII reduces their viability and increases their apoptotic ability.

CD44 cell surface molecules transmit signals to tumor cells from their environment, however their role in metastasis formation of many tumors including melanomas remain controversial. The reason for this might be that in tumors the molecule called

'CD44' includes a group of molecules formed via alternative splicing and differ from each other in function and structure and these variants can be found on the surface of the tumor cells simultaneously.

During RNA editing a definite number and structure of messenger RNA is assembled from the variable exons of the molecule, and their protein products are differ in function. Data in the literature does not take into account the fact that these proteins are nominally similar but their function may be different. However, very few result can be found describing the generated molecular pattern and its characteristics in different tumor types. Studies performed by our team proved that CD44 pattern was preserved during progression in primary tumors and their metastases. We identified the types of CD44 molecules forming this pattern with new generation sequencing, establishing the chance to find CD44 molecule variants related to metastasis formation by correct identification of variants with different function.

5. Conclusions/ Novel findings:

- With the aid of our human melanoma xenograft animal model, we identified the changes of the gene expression profile in the stromal components of the primary tumor during tumor progression.
- From many aspects, we found CCL12 chemokine ligand to be the most prominent progression associated host derived factor after validating 19 gene, that showed the greatest change in expression of the studied 20.000 genes.
- Characterizing the role of CCL12 in tumor progression during our *in vitro* and *in vivo* studies, our findings are the following:
 - we detected the expression of CCL8 (nucleic acid and protein, as well), the human homologue of CCL12, and its receptor CCR1 on human primary melanomas
 - CCL8 did not alter the viability of tumor cells significantly, however its effect as a chemoattractant is remarkable, as CCL8 increased the migration of tumor cells

- in human clinical samples primary melanomas with lung metastasis were separable from non-metastatic samples on the level of CCL8 expression
 - examining the different steps of progression, we observed the presence of CCL8 (nucleic acid and protein) in circulating and metastatising tumor cells, as well.
 - in vitro CCL8 decreased its own expression in fibroblasts.
- Examining a panel of 800 miRNA we defined which miRNA showed significant change after CCL8 treatment in human dermal fibroblasts. We proved on human clinical samples that one of the miRNA-s in focus, miR146a is presented in different degree in metastatic and non-metastatic primary melanomas.
- We demonstrated that expression of collagen XVII did not alter in primary tumors and in distant organ metastases in our melanoma xenograft animal model, also we proved that targeted collagen XVII treatment of melanoma cells bearing collagen XVII endodomain decreased their viability and increased their apoptotic activity.
 - We defined the alternative splice pattern of CD44 characteristic for human melanomas and we verified that this pattern remained qualitatively stable during tumor progression, while quantitatively it showed a definite alteration.

6. List of the author's publications

Bibliography of the candidate's publications:

1. **Barbai T**, Fejős Z, Puskas LG, Tímár J, Rásó E. (2015) The importance of microenvironment: the role of CCL8 in metastasis formation of melanoma *Oncotarget*, 6(30):29111-28.
2. Krenacs T, Kiszner G, Stelkovics E, Balla P, Teleki I, Nemeth I, Varga E, Korom I, **Barbai T**, Plotar V, Timar J, Raso E. (2012) Collagen XVII is expressed in malignant but not in benign melanocytic tumors and it can mediate antibody induced melanoma apoptosis. *Histochem Cell Biol.*, 138(4):653-67.
3. Raso-Barnett L, Banky B, **Barbai T**, Becsagh P, Timar J, Raso E. (2013) Demonstration of a Melanoma-Specific CD44 Alternative Splicing Pattern That Remains Qualitatively Stable, but Shows Quantitative Changes during Tumour Progression. *PLoS One.*,8(1):e53883.

Publications without close relation to the dissertation:

4. Garay T, Molnár E, Juhász É, László V, **Barbai T**, Dobos J, Schelch K, Pirker C, Grusch M, Berger W, Tímár J, Hegedűs B. (2015) Sensitivity of Melanoma Cells to EGFR and FGFR Activation but Not Inhibition is Influenced by Oncogenic BRAF and NRAS Mutations. *Pathol Oncol Res.*, 21(4):957-68.
5. Lohinai Z, Hoda MA, Fabian K, Ostoros G, Raso E, **Barbai T**, Timar J, Kovalszky I, Cserepes M, Rozsas A, Laszlo V, Grusch M, Berger W, Klepetko W, Moldvay J, Dome B, Hegedus B. (2015) Distinct Epidemiology and Clinical Consequence of Classic Versus Rare EGFR Mutations in Lung Adenocarcinoma. *J Thorac Oncol.*, 10(5):738-46.

6. Cserepes M, Ostoros G, Lohinai Z, Raso E, **Barbai T**, Timar J, Rozsas A, Moldvay J, Kovalszky I, Fabian K, Gyulai M, Ghanim B, Laszlo V, Klikovits T, Hoda MA, Grusch M, Berger W, Klepetko W, Hegedus B, Dome B. (2014) Subtype-specific KRAS mutations in advanced lung adenocarcinoma: a retrospective study of patients treated with platinum-based chemotherapy. *Eur J Cancer*, 50(10):1819-28.
7. Moldvay J, **Barbai T**, Bogos K, Piurko V, Fillinger J, Popper HH, Tímár (2013) EGFR autophosphorylation but not protein score correlates with histologic and molecular subtypes in lung adenocarcinoma *J. Diagn Mol Pathol.*, 22(4):204-9.
8. Rásó E, **Barbai T**, Györffy B, Tímár J. (2013) Prognostic and predictive markers of malignant melanoma. *Magy Onkol.*, 57(2):79-83
9. Madaras L, Baranyák Z, Kulka J, Szász AM, Kovács A, Lan PH, Székely B, Dank M, Nagy T, Kiss O, Harsányi L, **Barbai T**, Kenessey I, Tőkés AM. (2013) Retrospective analysis of clinicopathological characteristics and family history data of early-onset breast cancer: a single-institutional study of Hungarian patients. *Pathol Oncol Res.*, 19(4):723-9.
10. Bánky B, Rásó-Barnett L, **Barbai T**, Tímár J, Becságh P, Rásó E. (2012) Characteristics of CD44 alternative splice pattern in the course of human colorectal adenocarcinoma progression. *Mol Cancer*, 14;11:83.
11. Patonai A, Erdélyi-Belle B, Korompay A, Somorác A, Törzsök P, Kovalszky I, **Barbai T**, Rásó E, Lotz G, Schaff Z, Kiss A. (2013) Molecular characteristics of fibrolamellar hepatocellular carcinoma. *Pathol Oncol Res*, 19(1):63-70.
12. Hegedûs Z, **Barbai T**, Tátrai P, Hegedûs B, Kiss A, Rásó E, Bodoky G. (2011) Effect of KRAS mutation status on the efficiency of Avastin therapy of colorectal cancer. *Magy Onkol.*, 55(2):99-100.

- 13.** Badalian G, **Barbai T**, Rásó E, Derecskei K, Szendrői M, Tímár J. (2007)
Phenotype of bone metastases of non-small cell lung cancer: epidermal growth factor receptor expression and K-RAS mutational status. *Pathol Oncol Res.*, 13(2):99-104.