

Oncogenomic investigation in the field of colon cancer carcinogenesis and melanoma-stroma interaction

Ph.D. thesis

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Budapest, 2010**

Introduction

The carcinogenesis is a complex, multifactorial process influenced by several factors, in which the altered expression of genes maintaining the homeostasis of the cell plays a leading role; in the clinical appearance the environmental effects are also necessary beside the genetic predisposing factors however. The exploration of the complex genetic mechanisms consisting in the background of the development of the illness is the object of intensive researches nowadays.

Due to the sequencing of the human genome, the expanding molecular genetic and informatics' knowledge, a new science was arisen some years ago, which created the genome scaled molecular genetics, the genomics. One of the most important trends of genomics is the structural genomics, main task of which is to identify disease related gene variants (mutations and polymorphisms) and haplotypes which may provide help in the exploration of the background of the tumors, and in the development of new diagnostic and therapeutic procedures. Functional genomics, the other important trend of genomics, allows us to monitor the expression of genes, which can help to identify disease related gene expression changes and patterns.

In our studies, we present the clinical significance of the colorectal cancer and melanoma, the molecular biology contexts recognized already and the microarray results. In our investigation, we applied functional genomic approaches to examine the genomic background of the carcinogenesis and the metastatic process of colorectal cancer, and we focused on the tumor-stroma interaction in melanoma as well. Our data gives new insights into the genetic mechanisms underlying neoplastic transformation of colorectal cells and into the interaction of elastin protein and melanoma cells.

Goals of our study

We designed our experiments along two capital directions. We have investigated the gene expression profiles of colon cancer samples in order to improve our understanding of the genetic mechanisms of carcinogenesis and to identify new potential tumor markers useful for clinical practice. We focused on the tumor-stroma interaction as well and we investigated the relation of elastin-derived peptides and melanoma cells. Goals of our study were the following:

1. We have investigated the gene expression profiles of lymph-node metastasis negative and distant metastasis positive colon cancer tissues compared to the adjacent non-cancerous mucosa from surgical resections by two-colour whole human genome oligonucleotide microarray. In these experiments, we focused on the followings:

- applying different bioinformatical techniques, which aimed us to identify new genes, gene groups involved in the pathogenesis and the progression of colorectal cancer,
- validation of the selected genes on multiple levels,
- identifying genes, gene groups and pathways that have not yet been investigated in the pathogenesis of colorectal cancer,
- identified new genes and pathways validation in cancer cell lines,
- functional examination of the new hypothesis.

2. We focused on the tumor-stroma interaction investigating the effects of elastin-derived peptides on the invasion potential of melanoma cells. To examine this, we focused on the following points:

- presenting the elastin protein, elastin fragments and elastin-derived peptides on the sections of patients with melanoma,
- investigating the receptors responsible for the effects of the elastin-derived peptides,
- examining the effects of elastin-derived peptides on several steps of the invasion process of melanoma cells.

Materials and methods

Patients and tissue collection

A total of 24-24 (lymph-node negative and distant metastasis positive) pairs of colorectal cancer and corresponding non-cancerous tissues were obtained from patients with sporadic colorectal adenocarcinoma who underwent surgical resection at the Uzsoki Teaching Hospital (Budapest, Hungary).

Cell lines and culture conditions

The HCT116, HT29 and WiDr human colon cancer cell lines were maintained in EMEM medium supplemented with 10% fetal calf serum and 0.6 g/l gentamicin at 37°C in 5% CO₂ atmosphere. Two human melanoma cell lines were used: the WM35 human melanoma cell line with a low invasive potential and the HT168-M1 human melanoma cell line with high invasive potential. Both cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 0.6 g/l gentamicin at 37°C in 5% CO₂ atmosphere. The cells were cultured until 80–90% confluent.

Peptide synthesis and treatment

Peptides were synthesized stepwise, with Fmoc (9-fluorenyl-methyloxycarbonyl) technique on special resin. VGVAPG and VAPG elastin-derived peptides (100 µg/ml), VPVGGA and PGVA scrambled peptides (100 µg/ml) and receptor blocking treatments (pretreated the cells with blocking antibodies and V14 peptide for 1 hr at 37°C, 5% CO₂ before the adding elastin-derived peptides) were performed for 12, 18 and 24 hrs.

RNA isolation, quality determination

RNA from the tissue and the cell lines was isolated and prepared by RNeasy columns, the quality and quantity of total RNA was determined with Agilent 2100 Bioanalyzer.

Microarray experiments

The Agilent Whole Human Genome Oligo Microarray 4x44K chips were used for microarray experiments. Samples from colorectal cancer tissues were identically labeled with Cy5 dye, while corresponding non-cancerous mucosa tissues were labeled with Cy3 dye, and served as a control. Cy3 and Cy5-labeled samples were mixed and hybridized to Whole Human Genome Oligo 4x44k microarrays, then the slides were washed with an ozone safe method and scanned by Agilent Microarray Scanner. All steps were carried out according to the manufacturer. Data were then normalized by the Feature Extraction software version 7.5 with default parameter settings for two-color oligonucleotide microarrays.

Statistical and bioinformatics analysis of the microarray data

Statistical and bioinformatics analysis of the microarray data was performed by the GeneSpring GX program. In GeneSpring the normalization and data transformation steps recommended by Agilent Technologies for two-color data were applied. Experiment interpretation was built by GeneSpring, and the expressed genes showing a >2.0-fold differential expression were further analyzed by statistical tests. The statistical comparisons were performed between the colorectal cancer and the corresponding non-cancerous tissues by one-way ANOVA and T-test, respectively. A multiple testing correction method by Benjamini-Hochberg was applied with $p < 0.05$ cutoff in our statistical tests.

Gene Ontology analysis

Based on the results of the microarray data analysis, the differentially expressed genes were studied for Gene Ontology (GO) terms, which provide information on the cellular component, biological process, and molecular function of the protein. The GeneSpring GX program calculates P values for each GO term using a standard hyper-geometric distribution to compare the frequencies of individual GO terms within the selected list of genes to the frequencies of those terms on the entire microarray (P values = 0.05 were considered to be significant). The p-value for individual GO terms, also known as the enrichment score, signifies the relative importance or significance of the GO term among the entities in the selected gene list compared to the entities in the whole dataset. False discovery rate (FDR) was controlled at 5% for all analyses. This strict correction ensures the enrichment is statistically significant.

Ingenuity pathways analysis (IPA)

Identification and analysis of canonical signaling pathways and functionally clustered gene networks was done with the Ingenuity Pathways Analysis system (Ingenuity Systems).

Reverse transcription and mRNA expression analysis by TaqMan real-time PCR

1 µg of total RNA was reverse transcribed to cDNA, and then the real-time PCR reactions were carried out for selected genes in an ABI Prism 7000 thermal cycler instrument according to the manufacturer. Hu18S-normalized signal levels were calculated using the comparative Ct method and expressed in percents of the respective marker level measured in placebo controls.

Histology

One part of each colon sample were taken into fixative for 24 h, and then dehydrated, embedded and 5 µm sections were stained with haematoxylin and eosin. Formalin-fixed and paraffin-embedded tissues of superficially spreading melanomas and their cutaneous metastases were cut and mounted onto SuperFrost slides. Specimens were deparaffinized and Weigert's resorcin-fuchsin staining was performed on the sections to demonstrate elastin protein and elastin fragments.

Flow cytometry

Cells (10^6 per sample) were fixed in a PBS solution containing 4% paraformaldehyde, washed with PBS containing 1% bovine serum albumin (BSA) twice, and stained with the primary antibody for 45 minutes at +4°C. For intracellular antigens the washing buffer was supplemented by 0.1% saponin. After two subsequent washes, cells were stained with secondary antibody for 45 minutes at +4°C in the dark. After two additional washes, cells were resuspended in PBS-BSA. 20000 cells were measured and analyzed on a FACSCalibur flow cytometer. Results were evaluated with the Cell Quest Pro software. Specific signals were identified by comparing all measured signals with nonspecific background staining given by the secondary antibody only. All experiments were performed in triplicate.

Protein isolation

For protein isolation from the tumors and the corresponding non-cancerous mucosa, samples were homogenized in a buffer containing 10 mmol/L Tris-HCl (pH 8.0), 10 mg/mL leupeptin, 0.5 mmol/L EGTA, 2% NaF, 1% Triton X-100, 25 mmol/L phenyl-methylsulfonyl-fluoride, and 2% Na-orthovanadate. Debris was removed by centrifugation, and protein yield was assessed by spectrophotometry.

Western blot analysis

10 µg aliquots of heat-denatured, β-mercaptoethanol-treated protein samples were loaded on precast Ready Gels. Gels were blotted onto polyvinylidene difluoride membranes, blocked, and blots were probed with primary antibodies, as stated. Blots were washed and secondary antibodies were applied, as appropriate. After subsequent washes, immunoreactive bands were visualized with the ECLPlus Western blotting Detection System. Image analysis was done using a Fluorchem 8000 image analysis platform and the ChemiImager 5500 image analysis software package. Specific band size was determined with the Full Range Rainbow Molecular Weight Marker.

Immunohistochemical analysis

Protein level validation of expressions was performed by immunohistochemistry using Biogenex i6000 automated staining system according to the manufacturer's protocol. Signal intensities were determined densitometrically with the NIH Image by measuring three randomly chosen areas per slide at 10x magnification.

Transmission electron microscopy

For transmission electron microscopy, a 1 mm³ specimen from the paraffin-embedded tissue of melanoma was taken, and postfixed with osmium tetroxide. After inclusion, sections of 40-50 nm were cut and stained with toluidine blue. Ultrathin sections were cut with an ultramicrotome and stained with uranylacetate and lead citrate. These sections were then examined under an electron microscope (Philips CM10, 80 kV).

MTT-assay

We washed cultured cells with the medium, MTT was added into wells being assayed, for 1 hr at 37°C. The converted dye is solubilized with DMSO, and the absorbance of the converted dye is measured at a wavelength of 570nm with background subtraction at 650nm by ELISA plate reader.

Quantitative cell migration assay.

The CHEMICON QCM Haptotaxis Cell Migration Assay - Fibronectin, Colorimetric (Chemicon) was carried out according to the manufacturer's protocol. This system utilizes Fibronectin-coated Boyden chambers and BSA-coated migration control Boyden chambers to provide a quantitative indication of the melanoma and colon cancer cells' migration. Cells migrating through 8 µm chamber pores were eluted with extraction solution, stained and counted. Optical density (OD) of the stained cells is then correlated with cell migration. Cell migration was measured by plotting the OD at a wavelength of 560 nm.

Cell adhesion assay

Twenty-four-well plates were coated for 18 hr at 4°C. Control wells were coated with TBS. Unoccupied sites were blocked with 1 mg/ml BSA at room temperature for 1 hr. Cells were pretreated before adhesion assay. Cells were released with 0.02% EDTA in PBS, washed twice in medium with 10% FCS, and then suspended in the medium with 10% FCS. Cells were added to the coated wells and allowed to attach for 4 hr at 37°C, 5% CO₂. After gentle washing twice with PBS (pH 7.4), the attached cells were fixed for 20 min with 4% paraformaldehyde in PBS and then stained with 0.25% crystal violet for 4 hrs. The cells were rinsed with deionized water and the stain released using 1% sodium dodecyl sulfate (SDS) and quantified by spectrophotometry (560 nm) using an ELISA plate reader.

Statistical analysis

Data were analyzed using Microsoft Excel or SigmaStat 3.5 programs. Depending on data type, unpaired t-test, one-way ANOVA and Tukey HSD post hoc test were applied to analyze the statistical significance of our results.

Results and discussion

In the course of literary research completed in PubMed and Google Scholar databases assessed, that on the platform of Agilent Whole Human Genome Oligo Microarray 4x44K no microarray examination was found yet in the topic of colorectal cancer. In our study, we have investigated the gene expression profiles of lymph-node metastasis negative and distant metastasis positive colon cancer tissues compared to the adjacent non-cancerous mucosa from surgical resections. Two-colour whole human genome oligonucleotide microarray was carried out and significantly deregulated genes were analyzed. We found considerable gene expression changes in spite of the fact that we applied strict statistical conditions: 2314 genes in the lymph-node metastasis negative group and 1496 genes in the distant metastasis positive group changed significantly compared to the non-cancerous mucosa. Based on the results of the microarray data analysis, the differentially expressed genes were studied for Gene Ontology (GO) terms, which provide information on the cellular component, biological process, and molecular function of the protein. The GeneSpring GX program

calculates p values for each GO term using a standard hypergeometric distribution to compare the frequencies of individual GO terms within the selected list of genes to the frequencies of those terms on the entire microarray (p values = 0.05 were considered to be significant). The GO analysis clearly revealed highly significant overrepresentation of gene set families related to chemokine, cytokine and ECM-degrading enzyme activity in the lymph-node metastasis negative group, while found overrepresentation of adhesion molecules, cytoskeletal compounds and transport proteins in the distant metastasis positive group compared to the controls.

I list the following significantly altered top genes in our experiment, which were found by others as well in their experiments:

1. upregulated genes:

- a. CXCL1,
- b. CXCL2,
- c. CDH3,
- d. MMP1,
- e. MMP3,
- f. MMP11.

2. downregulated genes:

- a. ADH1A,
- b. CXCL12,
- c. CA2.

In the course of Ingenuity pathway analysis we found that the decreased level of IGF-1 was associated with the decreased level of MDR1/P-gp and regulated via the MAPK-cascade. The members of the supposed signaling pathway were validated by TaqMan real-time PCR and immunohistochemistry methods on 24-24 paired samples of patients with colorectal cancer. According to the literary data the expression of IGFBP3 was increased, while the expression of IGF1 and MDR1 were decreased in tumor tissues compared to the adjacent non-cancerous mucosa. Lower level, but significant changes were

detected in the member of the signal transduction molecules: decreased expression of MAPK3, p90 RSK and c-FOS, while increased expression of MKP2 and PAC1 were found.

We validated our results *in vitro* as well. We have provided evidence that the exogenous IGF-1 resulted increased expression of P-glycoprotein in three human colon cancer cell lines (HCT-116, HT-29 és WiDr) applied TaqMan real-time PCR and flow cytometry techniques. We justified that the MAPK3 signaling pathway was concerned in the effect of IGF-1 applied U0126 and PD098059 inhibitor. Why is the reduced level of P-gp worth it for the cancer cells? We carried out functional investigations with anti-P-gp antibody which binds to the extracellular region of the molecule and inhibit the function. We found that the antibody treatment do not affect the tumor cell proliferation (by MTT-assay), but increased the migration and adhesion of the cells.

More of the details of this signaling pathway were announced already in the literature, we proved it in full whole one's after all.

Several studies have suggested that the progression and the metastatic potential of melanoma tumors are influenced by elastin, which is one of the most important components of the ECM in the skin, in the wall of blood vessels and in the lung. Melanomas containing more elastin are associated with higher stages of the disease: lymph node or distant metastases, higher Clark-level and greater tumor thickness. Several enzymes belonging to the matrix metalloproteinases (MMPs), serine and cysteine proteases superfamily mediate the degradation of elastin. The level of these enzymes is increased in various stages of the disease and leads to the generation of elastin-derived peptides (EDPs), which alter tissue homeostasis.

EDPs have several biological effects on a wide range of cells including normal and tumor cells, for example cell proliferation, migration and chemotaxis, tumor progression, endothelial cell migration and angiogenesis.

The aim of the present work was to investigate the interaction between EDPs and melanoma cells applying several methods.

The presence of elastin, tropoelastin protein and VGVAPG peptide at the invasion site of melanoma was demonstrated with histochemical and immunohistochemical reactions. Not only the VGVAPG elastin-derived peptide, which exhibits the XGXXPG consensus sequence in its primary structure, but also the shorter VAPG bind directly to 3 cell surface receptors: galectin-3, integrin $\alpha\beta3$ and elastin-binding protein.

Our results suggested that the interactions between elastin and melanoma cells were one of the most important pathophysiological factors in tumor progression and metastatic cascade. Our study provides evidence that not only the VGVAPG elastokine, elastin-derived matrikine, which exhibit the XGXXPG consensus sequence in its primary structure, but the shorter VAPG elastin-derived peptide also have significant biological effects on human melanoma cells leading to increased invasive potential. EDPs have complex effects on the ability of melanoma cells to migrate. Both VGVAPG and VAPG are chemotactic for melanoma cells. Moreover, migration of melanoma cells toward a fibronectin matrix was increased after the treatments with elastin-derived peptides. These results may suggest that after the degradation of elastin at the site of invasion, VGVAPG and VAPG peptides elicit the migration of the posterior melanoma cells. These cells migrate in all directions. In addition, both VGVAPG and VAPG peptides indirectly promote the migration of melanoma cells by increasing the expression of CXCR-4 and CXCL-12 through the binding of EDPs to galectin-3 and integrin $\alpha\beta3$ receptors. Both VGVAPG and VAPG peptides increase the invasive potential of melanoma cells and the generation of EDPs, enhancing the expression of elastin-degrading MMP-2 and MMP-3 by the binding of EDPs to galectin-3 and EBP receptors. The peptides also increase the motility of tumor cells through increasing the attachment and the expression of CD44, ICAM-1 and NCAM on melanoma cells through galectin-3 and integrin $\alpha\beta3$ receptors leading to enhanced adhesion capacity to ECM and to other cells. VGVAPG and VAPG

elastin-derived peptides are able to increase the angiogenic potential and nutrition of melanoma cells enhancing the expression of the lymphangiogenic VEGF-C by the binding of EDPs to the galectin-3 receptor.

Conclusions

1. In our study, we have investigated the gene expression profiles of lymph-node metastasis negative and distant metastasis positive colon cancer tissues compared to the adjacent non-cancerous mucosa from surgical resections. Two-colour whole human genome oligonucleotide microarray was carried out and significantly deregulated genes were analyzed:

- (i) we found considerable gene expression changes in spite of the fact that we applied strict statistical conditions: 2314 genes in the lymph-node metastasis negative group and 1496 genes in the distant metastasis positive group changed significantly compared to the non-cancerous mucosa,
- (ii) GO analysis clearly revealed highly significant overrepresentation of gene set families related to chemokine, cytokine and ECM-degrading enzyme activity in the lymph-node metastasis negative group, while found overrepresentation of adhesion molecules, cytoskeletal compounds and transport proteins in the distant metastasis positive group compared to the controls,

- (iii) after Ingenuity Pathway Analysis, the IGF1 – MAPK3 – MDR1 signaling pathway was hypothesized in colon cancer,
- (iv) the members of the supposed signaling pathway were validated by TaqMan real-time PCR and immunohistochemistry,
- (v) we have provided evidence that the exogenous IGF-1 resulted increased expression of P-glycoprotein in three human colon cancer cell lines (HCT-116, HT-29 és WiDr),
- (vi) we justified that the MAPK3 signaling pathway was concerned in the effect of IGF-1 applied U0126 and PD098059 inhibitor,
- (vii) after the functional inhibition of P-gp the migration and adhesion of colon cancer cells were increased.

2. Our results suggest that the interactions between elastin and melanoma cells are one of the most important pathophysiological factors in tumor progression and metastatic cascade. We concluded:

- (i) VGVAPG and VAPG elastin-derived peptides bind directly to three cell surface receptors: the elastin-binding protein (EBP), the galectin-3 and the integrin $\alpha\beta3$;
- (ii) VGVAPG and VAPG elastin-derived peptides are chemotactic for melanoma cells;
- (iii) they can increase the migration of melanoma cells and the expression of CXCR-4 and CXCL-12 chemokines;

- (iv) they enhance the expression of the elastin-degrading MMP-2 and MMP-3;
- (v) they increase the attachment of melanoma cells and the expression of different adhesion molecules;
- (vi) they increase the expression of the lymphangiogenic VEGF-C,
- (vii) the galectin-3 receptor can mediate all these effects.

Publications

Publications related to the subject of the thesis:

1. **Pocza P.**, Suli-Vargha H., Darvas Zs., Falus A.: Locally generated VGVAPG and VAPG elastin-derived peptides amplify melanoma invasion via the galectin-3 receptor. *Int J Cancer*. 2008 May 1; 122(9):1972-80. IF= 4,734
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Summary of Impact Factors: 16,203

Other publications:

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Cumulative Impact Factors: 42,188

Acknowledgement to:

- Prof. Dr. András Falus, who taught me, followed my work carefully, helped me in evaluating and publishing the results;
- Dr. Zsuzsanna Darvas, who provided a lot of scientific help;
- Prof. Dr. Ferenc Jakab, Dr. Zsolt Baranyai, Dr. Tamás Mersich, Dr. Kristóf Dede, Dr. István Besznyák, Dr. Bence Atkári and Dr. Attila Zaránd who helped me to get all surgical samples;
- Dr. Zoltán Wiener, Dr. Zoltán Pócs, Dr. Gergely Tölgyesi, Dr. Katalin Éder and Dr. Viktor Molnár, who provided a lot of scientific help and advice, and helped me to design my experiments;
- Viktor Molnár and Péter Pócsa PhD students for the scientific discussions and professional support in the molecular biological, and statistical methods, and histology;
- Farkasné Nagy Krisztina, Kovács Andrea és Orbán Andrea technicians for cell culturing and a lot of other helps;

- Melinda Rácz technician for her help in microarray experiments;
- All the colleagues of the Department of Genetics, Cell and Immunobiology, who supported my PhD work;
- Finally, last but not at least to my family for all the patience and support they gave me during my PhD work.