

**Ph.D. Thesis**

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**Genetic and gene expression alterations  
during the melanoma progression**

**Zsuzsa Rákósy**

**Supervisor**

**Dr. Margit Balázs**

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**University of Debrecen  
Medical and Health Science Center  
Faculty of Public Health Department of Preventive Medicine  
Public Health Research Group of the Hungarian Academy of Sciences**

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## **Introduction**

Malignant melanoma is an aggressive, therapy-resistant malignancy of melanocytes. The incidence of the disease has been steadily increasing worldwide, resulting in an increasing public health problem. Exposure to solar UV radiation, fair skin, dysplastic nevi syndrome, and a family history of melanoma are major risk factors for melanoma development. The interactions between genetic and environmental risk factors that promote melanomagenesis are currently the subject of ongoing research.

At present, there are no systemic agents available that significantly extend the lifespan of patients with advanced disease, and the key to improved survival in all affected individuals remains early diagnosis and treatment. While early stage disease may result in occasional deaths, there are no available tests to predict which early stage tumors have a high likelihood of progression and therefore a worse prognosis. Thus, an urgent need exists for the identification of molecular signatures of melanoma progression which can be used to develop accurate prognostic markers and effective targeted therapies.

High-throughput gene expression profiling technologies offer an opportunity to uncover critical molecular events in the development and progression of human melanoma and can be used to design improved prognostic testing and effective treatment strategies. Previous transcriptome analyses in other malignancies have provided valuable information for the assessment of patient group classifications such as subgroups of patients that are likely to respond to a particular therapy. Expression profiling of metastatic melanomas was able to identify previously unrecognized subtypes of disease and predict phenotypic characteristics which may be of importance to melanoma progression. Such studies have been limited in utility due to the lack of concordance from one study to the next suggesting tumor heterogeneity.

Our aim was to study of gene expression signatures associated with melanoma progression using high density oligonucleotide microarrays for gene expression profiling of primary and metastases melanomas. Our also aim was to perform a detailed FISH analysis to define copy number alterations of 9p21 tumorsuppressor locus and EGFR oncogene on a large number of fresh and frozen primary melanomas, correlating the genetic alterations with the histopathological parameters of melanoma patients, and to compare the EGFR gene copy changes, mRNA level and protein expression status in a subset of lesions.

## Specific aims

Melanoma is an aggressive metastatic disease and beside the intensive research, the underlying molecular events that explain the development and aggressive clinical behavior of the lesion have been only partially characterized. Here we focus on the identification of the genetic alteration and gene expression signatures that are associated with melanoma progression. These particular progression associated genes may reflect the underlying molecular mechanisms of the various phases in the known tumor progression pathways of melanoma. As such, the pathways and molecules identified in this study have the potential to be utilized as therapeutic targets for melanoma as well as novel molecular markers for melanoma progression.

Performing array analyses and FISH studies our specific aims were the followings:

1. Examine the global gene expression profiling of primary and melanoma metastases.
2. Compare the array comparative hybridization (aCGH) and gene expression results. Identify the copy number changes that are behind the gene expression alterations.
3. Perform a detailed FISH analysis to define EGFR (7p12) gene and 9p21 locus copy number alterations in a large number of primary melanomas at single cell level.
4. Correlation of the EGFR and 9p21 FISH data to the clinical and histopathological parameters of melanoma patients.
5. Investigation of the influence of the gene copy changes to mRNA level and protein expression using PCR and immunohistochemistry techniques.

## **Materials and methods**

### **Tumor samples**

Tumor tissues were obtained from the Department of Dermatology, University of Debrecen, Hungary. Patients had not been given chemotherapy before surgery. The study was approved by the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center and conducted according to regulations. A written informed consent was always obtained from the patients.

Fresh tissues after surgical excision were immediately placed into RNA later solution (Applied Biosystems, Foster City, USA). Diagnoses of tumors were carried out on formalin fixed paraffin embedded tissue sections using hematoxylin and eosin staining. Melanoma tumor staging was determined according to the new melanoma TNM staging system. Tumor imprint preparations for interphase FISH analysis were made from fresh tissue using 3-aminopropyltrimethoxy-silane treated slides as described previously. Diagnoses of the tumors were carried out on formalin fixed paraffin embedded tissue sections using hematoxylin and eosin staining. Melanoma tumor staging was determined according to the new TNM staging system.

### **Fluorescence in situ hybridization**

Fluorescence in situ hybridization (FISH) was performed on tumor imprint preparations using chromosome 7 centomere- (cep7, SpectrumGreen) and locus specific 7p12 (EGFR locus, SpectrumOrange) and 9 centomere- (CEP 9, SpectrumGreen) and locus specific 9p21 (SpectrumOrange) probes from Vysis/Abbott (Vysis Inc., Downers Grove, IL, USA, Abbott Ltd. Budapest, Hungary). FISH was carried out according to the protocol of the supplier with slight modifications. Before microscopic evaluation nuclei were counterstained with DAPI (40,6-diamidino- 2-phenylindole) in antifade solution (Vector Laboratories, USA).

Approximately 100–300 nonoverlapping tumor cells were counted for each sample using a fluorescent microscope (OPTON, Oberkochen, Germany) equipped with selective filters for the detection of SpectrumGreen, SpectrumOrange and DAPI.30 Three color images were captured using a digital imaging analysis system (ISIS Metasystem GmbH, Altusheim, Germany).

## **Categories of gene alterations**

Cutoff for 9p21 loss relative to CEP 9 was defined according to published data; if the copy number of 9p21 was less than the copy number of CEP 9 in > 20 % of the cells, the alteration was defined as 9p21 loss. Homozygous deletion of 9p21 required the presence of only CEP 9 signals in > 20 % of the cells. Gain of the 9p21 region was defined if the copy number of 9p21 was higher than the copy number of CEP 9 in more than 20% of the cells.

*Categories for EGFR alteration* (i) EGFR deletion: the copy number of the EGFR gene was less than the copy number of chromosome 7 centromere (cep7) in more than 10% of cells; (ii) apparent amplification: the copy number of the gene was equal to cep7, but the cep7 signals were >2/ cells in more than 15% of cells; (iii) low level EGFR amplification was defined if the copy number of EGFR gene was higher than the copy number of cep7, but the ratio was less than 5 in more than 15% of cells; (iv) high level EGFR gene amplification was considered if the gene copy number was at least five times higher than the copy number of cep7 in more than 15% of cells.

## **Statistical Analysis**

Fisher exact test was applied to examine the relationship between EGFR gene alterations -cep7 ploidy and chromosome 9 and 9p21 deletions to compare the copy number alterations with the different clinical parameters including age and gender of patients, tumor thickness, histological subgroups and metastasis formation of the primary lesion. A p value less than 0.05 was considered statistically significant. Two sample Wilcoxon rank-sum (Mann-Whitney) test was performed to correlate the EGFR gene index to the clinical parameters of patients.

## **RNA isolation, quality control of RNA, microarray hybridization**

High quality total RNA was prepared from primary melanoma tissues using RNeasy Mini kit according to the protocol of the supplier (Qiagen, GmbH, Germany). RNA concentrations were measured using NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDropTechnologies, Wilmington, Delaware USA). The integrity of RNA samples were determined by the Agilent 2100 Bioanalyser using RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). All RNA samples had a 28S/18S ribosomal RNA ratio of > 1.5. In order to define that the RNA samples are free from biological inhibitors which can

suppress the reverse transcription step, P16 gene specific QRT-PCR were performed on all samples.

Gene expression analysis was carried out using Affymetrix GeneChip Human Genome U133 Plus 2.0 expression arrays, which contain 46,700 transcripts and variants representing 39,000 of the best characterized human genes (Affymetrix Inc., Santa Clara, CA). Hybridization, washing and scanning steps of gene chips were performed in the GeneCore Genomics Core Facility, Heidelberg Germany.

### **Statistical analysis of microarray data**

Gene expression analyses were carried out using the GeneSpring 7.3 software (Agilent Technologies). Raw intensity values from each chip were normalized to the 50th percentile of the measurements taken from that chip to reduce chip-wide variations in intensity. Each gene was normalized to the average measurement of that gene to enable comparison of relative changes in gene expression levels between different conditions. Cross-gene error model was applied with replicates. Only genes with a detection flag present at least in 18 samples were used for further analysis (25886 probe sets). Tumors were classified according to expression pattern by hierarchical clustering using an average linkage and Pearson's correlation. Volcano plot filtering (volcano plot allows the relationship between fold-change and statistical significance) and parametric one way ANOVA with a p value cut off of 0.05 were applied for identification of differentially expressed genes in the groups with different clinical parameters. For multiple testing correction the Benjamini and Hochberg false discovery rate was used.

### **Pathway Analysis**

Functional analysis of differentially expressed genes that showed greater than 2 fold changes ulcerated melanomas versus melanomas without ulcerated surface (1095 genes) was done using the Ingenuity Pathways Analysis software (IPA, Ingenuity Systems, [www.ingenuity.com](http://www.ingenuity.com)). We also applied the Database for Annotation Visualization and Integrated Discovery (DAVID, <http://david.abcc.ncifcrf.gov>) Gene Functional Classification Tool and DAVID Functional Annotation Clustering Tool for functional analysis of the genes. This program was designed as a server-client application.

## **Array comparative genomic hybridization (aCGH)**

Genomic DNA extracted from melanoma tissue applying Gspin Genomic DNA extraction kit (Intron Biotechnology, Sangdaewon-Dong, Korea) according to the manufacturers' instructions.

Array CGH experiments were performed on HumArray 3.1 microarrays using the Cancer Center Array Core Service (University of California, Cancer Center, San Francisco). The array contains 2.464 BAC and P1 clones printed in triplicates covering the genome at roughly 1.4 Mb resolution. The threshold for gain or loss was set to  $\log_2$  ratio of + or - 0,25 (mean +/- 3SD). All clones above this threshold as a result of the control hybridization were left out from the analysis. Thus, the final set, on which all of the analyses were performed, contained 2.220 clones. In addition, high-level amplifications were defined as a  $\log_2$  ratio >1.5 and homozygous deletions as  $\log_2$  ratio less than -0.90.

## **Correlation of aCGH and expression data**

The web-based MatchMiner program was applied to identify genes represented in the array CGH. Using the program we could match the FISH clone IDs of aCGH to the appropriate Affymetrix microarray probe sets. For genes that were represented by more than one probe sets on the Affymetrix array, we used the sequence that gave the highest correlation between oligonucleotide array expression data. That procedure yielded a total of 16 unique genes from the array CGH data.

## **Quantitative real-time PCR**

Validation of the expression of selected genes were performed using quantitative real-time PCR (qRT-PCR) was performed on all melanoma samples using ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, USA). Reverse transcription (RT) was carried out on 600 ng total RNA using the High Capacity cDNA Archive Kit, according to the protocol of the supplier (Applied Biosystems). TaqMan Low Density Array with pre-designed TaqMan® Gene Expression Assays (Applied Biosystems) was employed to perform qPCR for 94 genes. Multiple house keeping genes ( $\beta$ -actin (Hs99999903\_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs99999905\_m1) were used as controls for the accurate normalization gene expression data. For all qPCR reactions standard concentration of assays and Universal TaqMan® PCR mastermix were applied, according to manufacture's instructions (Applied Biosystems). Amplification plot and predicted threshold

cycle (Ct) values were obtained with the sequence Detection Software (SDS 2.1, PE Applied Biosystems). In order to measure the EGFR gene expression level accurately, we generated a normalization factor (Nf), that is the geometric mean of  $\beta$ -actin and GAPDH genes. The data analysis was performed using the GeneSpring 7.3 software.

### **EGFR mutation analysis**

Twenty-seven primary melanomas were analyzed for the presence of hotspot mutation on exon 19 of the EGFR gene by Light-Cycler melting point analysis. Genomic DNA was extracted from melanoma tissue applying Gspin Genomic DNA extraction kit (Intron Biotechnology, Sangdaewon-Dong, Korea) according to the manufacturers' instructions. A 170-bp genomic fragment of exon 19 was amplified using 2  $\mu$ l FastStart Mastermix (Roche, Germany), 4  $\mu$ M MgCl<sub>2</sub>, 0,5  $\mu$ M forward primer (50- TCTGGATCCCAGAAGGTGAG-30), 0,5  $\mu$ M reverse primer (50-CAGCTGCCAGACATGAGAAA-30) 0,2  $\mu$ M 30 FL-labeled hybridization probe (50-GCA ACATCT CCGAAAGCCAA-30FL) and 0,2 IM 50 LC Red640 labeled hybridization probe (50- LCRed640-GGAAAT CCT CGATGTGAGTTTCTG C-30pH).

### **Immunohistochemistry**

Six micron sections were cut from selected frozen melanoma tissues (Leica CM1850). The selection of tissues for the detection of EGFR protein expression was based on the EGFR gene copy number pattern detected by interphase FISH. We selected tissues that were characterized by low or high level gene amplification. Tissue sections were fixed in 4% paraformaldehyde according to standard protocol. Detection of EGFR protein was performed with two different monoclonal antibodies (Erbix1 humanized, Merck KgaA, 64271 Darmstadt, Germany and anti EGFR monoclonal antibody (mAb) 528 (IgG2a) produced by the hybridoma cell line 528: a.k.a. HB-8509, ATCC, Manassas, VA and purified using protein A affinity chromatography), both recognizing the extracellular domain of the EGF receptor. When simultaneously with the EGFR protein labeling, staining of the CD63 (heterogeneously glycosylated-melanoma-associated antigen) protein was performed a directly labeled humanized anti-EGFR (Erbix1) and a monoclonal anti-CD63 was used. Tissue sections were first incubated with FITC-labeled humanized EGFR specific antibodies (dilution 1:100) in a moist chamber overnight at 4°C. After washing, sections were blocked with 1% BSA in PBS and incubated with anti-CD63 antibody (dilution 1:50 Vector Laboratories, USA) for 1

hr at 4°C. The CD63 antibody was labeled with antimouse-Cy5-IgG (dilution 1:100) at RT for 60 min. The labeled sections were washed with PBS two times. Nuclei were counterstained with DAPI in antifade solution. For the evaluation of EGFR expression, membranous staining of tumor cells was scored from 0 to 3+ as follow: 0, no staining; 1+, faint, partial membrane staining; 2+, weak complete membrane staining; 3+, intense complete membrane staining in >10% of cells.

### **Labeling of cells for flow cytometry**

Cells were trypsinized in 3 ml of 0.05% (w/v) trypsin 0.02% (w/v) EDTA solution, resuspended in medium containing 10% FBS and washed three times (10 min, 600 x g) in phosphate buffered saline (PBS, 7.2). All chemicals were of reagent grade and obtained from Sigma-Aldrich.  $1 \times 10^6$  cells were aspirated by gently pipetting in 50  $\mu$ l PBS supplemented with bovine serum albumin (BSA) and labeled with 10–20  $\mu$ g directly labeled anti EGFR monoclonal antibody (mAb 528) at 4°C for 1 hr. After labeling, cells were washed 3 times in PBS and fixed in 500  $\mu$ l of 1% formaldehyde-PBS.

For flow cytometric measurements of phospho-EGFR the cell lines were stimulated with 10  $\mu$ g/ml EGF for 10 min. on 37°C where indicated. For measurements of phospho-EGFR cells were fixed in 3.7% formaldehyde-PBS for 30 min. The fixed cells were washed twice with PBS-0.1 M Tris and labeled with a saturating concentration (50  $\mu$ g/ml) of unlabeled primary antibody (antiphospho-EGFR, Cell Signaling Technology, Danvers, MA) followed by secondary labeling with A488-conjugated GaMIg in 0.1% Triton X-100, 0.1% BSA-PBS for 30 min on ice. After labeling, the cells were washed twice with PBS then fixed in 300 ml 1% formaldehyde.

### **Flow cytometric detection of EGFR expression**

The quantitative estimation of receptor expression levels were carried out on FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). A total of 20,000 cells were recorded in the FL-1 channel (excitation at 488 nm, detection through a 530 nm band pass filter) in linear mode. The mean of the corresponding autofluorescence distribution curve was subtracted from the mean of the measured fluorescence distribution curves.

## **Western blot analysis**

Whole cell lysates were prepared in lysis buffer (20 mM Tris– HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1% Triton X-100) supplemented with phosphatase (1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF) and protease inhibitors (P1860, Sigma-Aldrich). The melanoma and the A431 cell lines were stimulated with 10 µg/ml EGF for 10 min on 37C where indicated. Immunoprecipitation of EGFR was carried out with monoclonal antibodies against ErbB1 (528) on ice for 1 hr followed by incubation with Sepharose 4B Fast Flow Protein G beads (Sigma-Aldrich) on ice for 1 hr. The anti EGFR-528 antibody was produced from their respective hybridoma supernatants obtained from ATCC and purified using protein A affinity chromatography. Immunoprecipitates were resolved on 10% polyacrylamide gels and blotted to PVDF membranes. Anti-EGFR Mab, clone F4 (Sigma-Aldrich) and phospho-EGFR (Cell Signaling Technology, Danvers, MA) antibodies were used for primary labeling of membranes followed by peroxidase-conjugated goat anti-mouse IgG and enhanced chemiluminescence detection (Amersham, Freiburg, Germany).

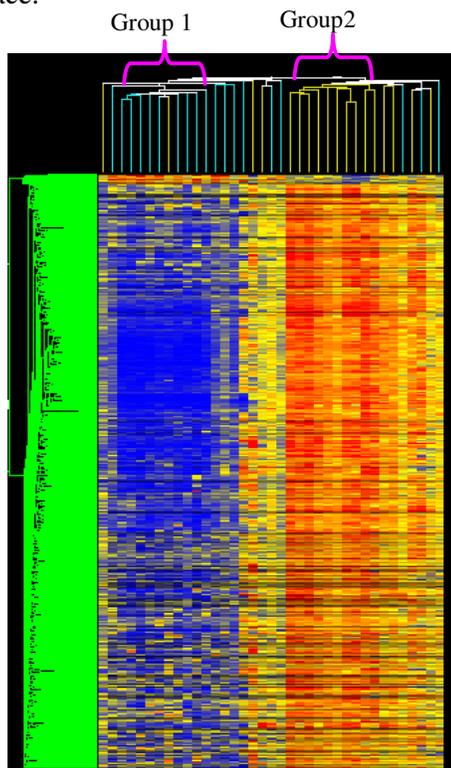
## Results

### Gene expression alterations in human malignant melanomas

#### *Gene expression patterns of primary melanomas*

We used the available highest throughput Affymetrix microarray platform containing 46,700 probe sets on 37 primary melanomas to study gene expression changes and molecular signature associated of clinical parameters of melanoma. The primary lesions were divided into groups according to the clinicopathological parameters of the samples. Volcano plot (p-value cutoff 0.05 with Benjamini and Hochberg False Discovery Rate) was applied in order to identify genes differentially expressed (greater than 2-fold) between the melanoma groups. The analysis showed that only the ulceration and metastases formation displayed characteristic significant change in gene expression. There was a good agreement between the two analysis. This might be explained that out of 37 melanomas seven primary tumors that formed metastases within two years have ulcerated surface also.

The Volcano plot analysis identified 1095 significantly differentially expressed genes that were associated with ulcerated melanomas. Majority of these genes (1021) was found to be down regulated, whereas these genes were upregulated in melanomas without ulcerated surface.



**Figure 1.** Hierarchical clustering analysis of 37 melanoma samples. Hierarchical cluster analysis shows the gene expression pattern within the data of the melanomas. Samples are displayed vertically, genes are displayed horizontally. A dendrogram of relatedness of the samples is at the top in blue ulceration samples, in yellow sample without ulceration. The color in each cell of the table represents the median adjusted expression value of each gene. The color scale used to represent the expression ratios is shown on the right, with yellow indicating increased expression relative to the median and blue decreased.

In order to further characterize melanoma expression profiles, hierarchical cluster analysis was performed based on the 1095 significant expressed genes. This Tree view revealed two main tumor groups within primary melanomas (Fig.1). The left cluster contained 20 tumors (16 lesions with ulcerated surface), whereas the other cluster had 17 primary lesions (14 non-ulcerated and 3 ulcerated melanomas). The majority of the analyzed genes were down regulated in almost all melanomas belonging to the left cluster.

The set of overexpressed genes (n=74) in the more-aggressive melanoma cluster include many genes with reported functional roles in cell cycle regulation and proliferation, such as melanoma antigen 6, interleukin 8 and CXCL5. Osteopontin (OPN) was the most highly expressed genes (fold change: 5.62) among the 74 genes in melanomas with ulcerated surface. The defensin beta, a keratin 16 and IL1F7 were the most highly up-regulated genes in less aggressive groups.

### ***Gene expression signature of metastases melanoma***

Gene expression pattern of six melanoma metastases were also analyzed by microarray. We examined differential gene expression between primary and metastases melanomas using parametric Welch t-test p-value cutoff 0.05 with multiple testing correction: Benjamini and Hochberg False Discovery Rate. We did not find any genes that demonstrate altered expression between conditions.

Therefore we compared gene expression profiles of metastases melanomas with the primer lesions using the previously employed hierarchical clustering protocol based on 1095 differentially expressed genes related ulceration. The gene expression pattern of the metastases was found to be more similar to that of the more-aggressive melanomas than the less-aggressive melanomas. Four of six metastases were included in more-aggressive melanomas, all of them are lymph node metastases. Almost all differentially expressed genes were found to be down-regulated genes in aggressive melanoma cells versus less aggressive.

### ***Pathway Analyses:***

Functional Annotation of upregulated genes was done using Database for Annotation Visualization and Integrated Discovery (DAVID) web-accessible programs. Out of the 74 overexpressed genes 67 could be identified by DAVID, out of them twenty-two genes belong to well known KEGG pathways. Three genes: CCL3L3, CCR1, EPOR are involved in cytokine-cytokine receptor interaction, in addition genes belong JAK-STAT, TGF-beta and Toll-like pathways are also found to be overexpressed in aggressive lesions..

We applied detailed analysis using Ingenuity Pathway Analysis software and DAVID programs to identify networks and interactions of 1021 significantly down regulated genes from the supervised comparison (more than two fold,  $p \leq 0.05$ ).

Twenty-six networks were identified by IPA analysis based on the functional role of the genes. This gene signature is comprised of critical mediators of hair and skin development and function, organ development, dermatological diseases and conditions cellular development, cellular growth and proliferation, cardiovascular system development and function cell morphology, cellular assembly and organization, cell-to-cell signaling and interaction. We also demonstrated that these genes are mainly involved in the p53, Nf-kB, WNT/  $\beta$ -catenin pathways.

#### ***Array CGH data and gene expression changes***

Array CGH analysis was also performed for eleven primary and two metastases melanomas to evaluate the possible relation between the difference of gene expression levels and the alterations of the genome revealed by CGH microarray. The 1.4 MB BAC Array (HumArray3.2) was applied which contains 2.464 clones spotted in triplicate.

Out of genes with increased expression levels in aggressive melanomas our aCGH platform only represents specific BAC clone (No. RP11-120N14) for SULF-1 gene are located 8q13.2-q13.3 Amplifications of SULF-1 (intensity level:  $\log_2=0.3$ ; threshold level:  $\log_2=0.25$ ;) was observed in 3 cases by aCGH, however was not high enough to consider as the unambiguous marker of excess of this chromosome region. As CGH gives information on copy number patterns of cell populations' pool, it is necessary to validate the alteration of 8q13.2 with FISH analysis.

Genes with decreased level of expression are located mainly in chromosome 1 (1p21-p22.3, 1p36, 1q21, 1q36), in chromosome 6 (6p21, 6q21-q23), in 10q, 11q, and 15q22. It was revealed  $\log_2 < -0.25$  intensity level in three specimens. The intensity rate which reflects heterozygous deletion ( $\log_2 = -0.5$ ) were recorded in the chromosomal region of 18q11.2 coding LAMA3 and 15q22.2 coding RORA. There was no relation between decreased gene expression and genome alterations in the remaining melanomas.

#### **Taqman Low Density Array (TLDA) analysis**

The qPCR basic Taqman Low Density Array approach was applied for validating our expression array having been performed in 35 primary melanoma samples. We chose 65 genes on the basis of the microarray results and 29 other genes published in the literature to be

associated with the progression of the disease. Glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) and  $\beta$ -actine genes were used as controls. The qPCR experiments consequently failed in case of 7 genes in all of the specimens. In general, our qPCR results showed good correlation with the data of the expression microarray. When compared gene expression with ulceration we detected the minimum of two fold of expression change in 47 genes which was significant in the case of 25 genes.

## **EGFR gene copy number alterations in primary melanomas**

### ***EGFR gene-and chromosome 7 copy number alterations in primary melanomas***

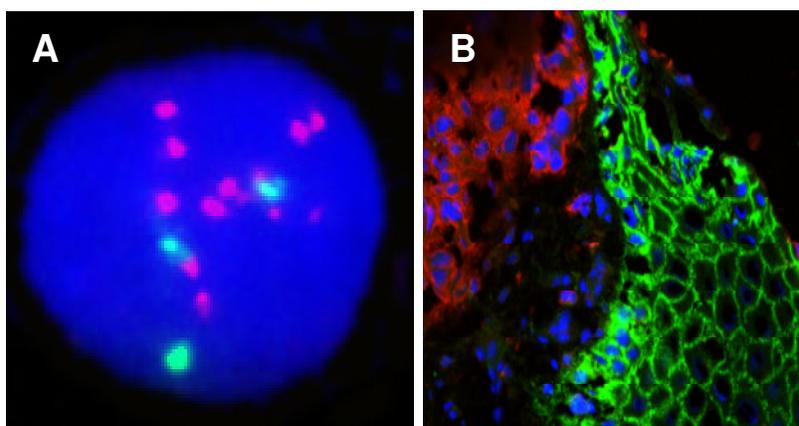
Aneusomy of chromosome 7 was seen in 70% of lesions. The cep7 copy number index was between 1.7 and 6.0. The EGFR gene copy number index was between 1.0 and 8.9. Copy number alteration of the EGFR gene was seen in 64 primary melanomas (79%). Most of the tumors exhibited low level amplification (EGFR/cep7 copy number ratio  $<5$ ). In 13 tumors we found cells with extremely high amplification pattern; however, highly amplified cells exceeding more than 10% of all cells, were detectable only in 6 lesions. Amplification of the EGFR gene was associated with polysomy 7 ( $p < 0.0001$ ).

### ***Correlation of EGFR mRNA expression, EGFR protein expression and EGFR gene copy number alterations***

Gene expression data were available for 16 primary melanomas. Seven lesions belonged to the EGFR-nonamplified, 5 to the low-, 3 to the apparent- and 1 to the highly amplified group. Five of these tumors showed increased EGFR mRNA levels greater than threefold of the average mRNA level of the 7 EGFR-nonamplified melanomas (used as reference). One sample with apparent EGFR gene amplification showed more than fivefold increase for the mRNA level; however, the protein expression was relatively low in this sample. On the other hand, a lesion with low mRNA level exhibited intermediate expression for the EGFR protein.

EGFR protein expression was evaluated by immunohistochemistry. Selection of melanoma samples was based on the FISH results, lesions with apparent, high and low level EGFR gene amplifications, gene deletions and normal gene copies were selected. To visualize the EGF-receptor protein and to discriminate the melanoma cells from the surrounding cells,

directly labeled humanized anti-EGFR antibody and monoclonal anti CD63 antibody followed by fluorescently labeled secondary antibody were used on the same tissue section. EGFR staining was found all over the epidermal layer in normal skin samples and lack of the CD63 labeling was observed under the epidermis layer in the control samples. In contrast, strong intensity for the CD63 marker and no or weak EGFR immunoreactivity was present in the tumor tissues, the epidermis layer of the tissues showed marked EGFR membrane specific staining (Fig. 2). Samples that showed normal copies for cep7 and EGFR did not express EGFR protein. Samples with intermediate level of EGFR amplifications exhibited weak or moderate EGFR expression.



**Figure 2.** Confocal immunofluorescent image showing cell surface distribution of the EGFR (green fluorescence) and intracellular distribution of the CD63 protein (red fluorescence) on frozen section of melanoma tissue. Nuclei were labeled with the blue fluorescent dye, DAPI.

High level amplification was not associated with EGFR protein expression. Overall, we could not find strong correlation between EGFR gene copy number alterations and the level of EGFR protein expression in our melanoma sample collection.

We also examined the somatic mutation of the tyrosine kinase domain in exon 19 of the EGFR gene by melting curve analysis using LightCycler. DNA was available in 27 primary tumors, but no mutation was seen in any of the analyzed samples within this sequence.

#### ***Correlation of EGFR gene copy alterations with clinicopathological parameters***

*Patients' age and gender:* Extra copies of the gene were more frequently detectable in the older group; however, it did not differ significantly from patients 50 or younger. Deletion and

low level amplification of the EGFR gene was significantly higher in tumors removed from male patients when compared to female patients ( $p = 0.05$ ).

*Histological subtypes.* Normal copy number, deletion, apparent- and low level amplification of the gene was present with similar frequency in both nodular and superficial spreading melanomas. Highly amplified EGFR gene copy numbers (with a copy number ratio of EGFR/cep7 >5) were detected in 5 nodular tumors, whereas similar amplification was seen in only one SSM lesion. When we calculated the average EGFR gene copies we found that it was significantly higher in the nodular subtype than in the superficial spreading subtype ( $p = 0.023$ ).

*Tumor thickness.* Correlation between tumor thickness and the incidence of EGFR alteration was also detectable. The average number of EGFR gene copies was significantly higher in tumors that were thicker than 4.00 mm compared to the thinner tumors ( $p < 0.0001$ ). Low level and apparent amplification of the gene were found in all thickness groups, but high level amplification was seen only in tumors thicker than >4.01 mm ( $p = 0.05$ ). Similar correlation was detected when comparing Breslow thickness to the EGFR gene copy index; however, the relationship between elevated EGFR gene copies and tumor thickness were stronger ( $p < 0.0001$ ).

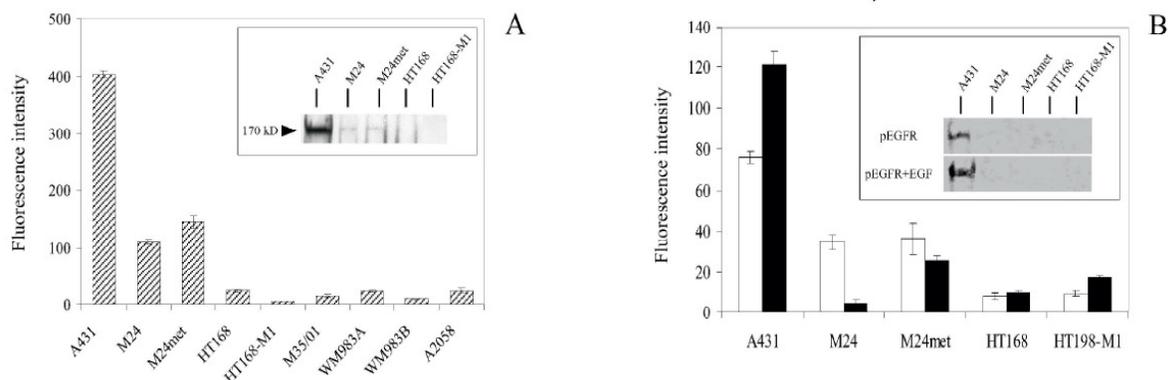
*Metastasis formation and patient survival.* Loss of EGFR copy related to chromosome 7 centromeric signals was seen in 5 lesions, 4 out of these 5 tumors later formed metastases. The EGFR gene copy number index in tumors that formed metastasis within 5 years after the diagnosis of the primary lesion was significantly higher than in tumors that did not metastasize during the follow up period ( $p = 0.038$ ). The exitus of patients was also associated with higher gene copy number index, although it did not reach a significant level when compared to data of patients who were alive after 5 years ( $p = 0.151$ ). High level amplification was only observed in the metastasizing tumors. Overall, the outcome of patients whose primary tumors had highly amplified or extra copies of the EGFR gene was poor.

#### ***Chromosome 7 and EGFR gene copy number alterations, cell surface EGFR expression and phospho-EGFR level in melanoma cell lines***

Eight melanoma cell lines were analyzed for EGFR copy numbers alterations by double-target FISH assay. All cell lines showed heterogeneous distributions for chromosome 7 and EGFR

gene copy numbers, signals ranged between 2 and 7 for cep7 and 1 to more than 20 for the EGFR gene. Low level amplification was present in all cell line with a frequency between 16 and 75%. High level amplification was detected in three cell lines (M24met, WM983A and WM983B), but the frequency of the amplified cells were below 15%.

High level cell surface expression of the EGFR protein was detected in the A431 cells but negligible EGFR was expressed in most of the melanoma cell lines as detected by flow cytometry and Western blot analysis. Only the M24 and its metastatic M24met subline showed moderate level of the EGFR protein (Fig. 4a). We also tried to detect the activated EGFR protein (pEGFR) with and without EGF stimulation in melanoma cell lines with different EGFR gene copy numbers. The M24 cell lines, which were polysomic for chromosome 7 and exhibited extra EGFR copies, showed low level pEGFR and after EGF stimulation the intracellular level of the pEGFR did not increase (Fig. 4b). In contrast, the positive control A431 cells, known to have highly amplified EGFR gene, displayed two times higher pEGFR and after EGF stimulation the pEGFR level increased significantly, as detected by flow cytometry and confirmed by Western blot analysis (Fig. 4b insert).

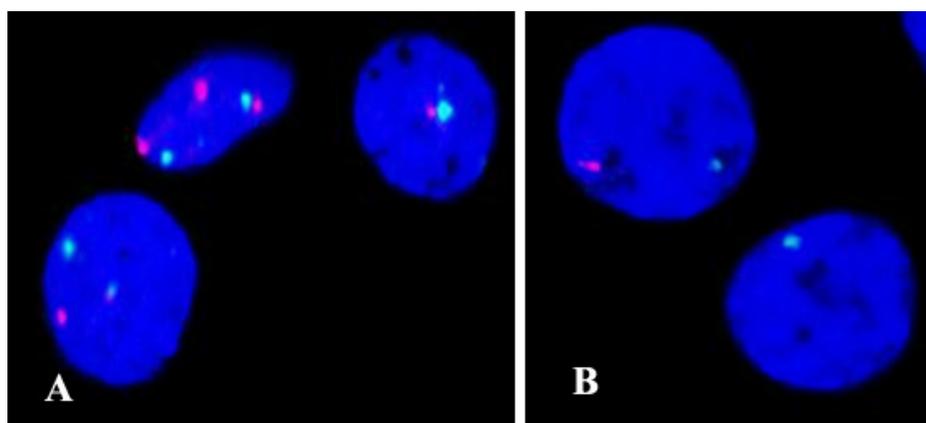


**Figure 4.** EGFR expression in melanoma and A431 cell lines. (a) Cell surface EGFR expression on the A431 and melanoma cells detected by flow cytometer. The insert shows the results of the Western blot analysis of total EGFR levels on A431 and on selected melanoma cell lines. (b) Phospho-EGFR (pEGFR) levels in unstimulated and EGF stimulated A431 and melanoma cell lines detected by flow cytometer. Empty bars represent the fluorescence intensity of cells without stimulation and black bars shows the pEGFR after EGF stimulation. The insert display the results of Western blot analysis with and without EGF stimulation.

## Characterization of the 9p21 copy number alterations in human melanoma by fluorescence *in situ* hybridization

Deletion of chromosome 9 was present in both early and late stage melanomas. Loss of CEP 9 was more characteristic for the superficial spreading subtype (SSM, 44%) when compared to the nodular subtype (NM, 24%). Polysomy of chromosome 9 (fluorescent signals/cell > 2) was seen in 25 lesions (31 %). The polysomic cell population in these tumors was above 20% and no monosomic cell population for CEP 9 was observed. The incidence of polysomic cell population exceeded 50 % in 16 tumors. Only one melanoma exhibited more than 6 signals/cell for CEP 9. Polysomy of chromosome 9 was present at the same frequency (31 %) in both histological subtypes.

Deletion of the 9p21 locus was detected in 68 lesions (83.9 %) making chromosome 9 loss evident in 22 (27.1 %) melanomas. Clear homozygous deletion, affecting 63-83% of melanoma cells, was infrequent; only 7 melanomas (8.6 %) belonged into this category. The most characteristic 9p21 alteration was the relative loss of 9p21 signals over chromosome 9 centromeric signals, which could be revealed in 46 primary tumors (56.8 %). The presence of homozygous deletion and relative loss of 9p21 within the same sample were detected in 15 lesions. Extra 9p21 copies were observed in six melanomas. Only 7 samples did not exhibit alterations for the 9p21 locus.



**Figure 4.** 9p21 alterations in sporadic malignant melanoma samples. 1A) Three cells with different patterns of chromosome 9p21 alterations are displayed. Two cells are disomic for chromosome 9 (green signals), one of these cells exhibits deletion for 9p21 (appears only one red signal in this nucleus), the other one shows three copies of the 9p21 locus (three red spots) and the third cell shows one CEP 9 and one 9p21 signal. 1B) A melanoma cell exhibiting only CEP 9 signal and no signal is visible for the 9p21 which is the sign of a homozygous deletion. Another cell exhibits losses for both probes.

Figure 4 demonstrates the characteristic 9p21 alterations in primary melanomas as detected by FISH. In contrast with chromosome 9, we could not find difference between the frequency of 9p21 copy number alterations and histological subtypes of tumors. Homozygous deletion was detected at a higher frequency in NMs. 9p21 gain was detected in both groups. Deletion of

9p21 was present in all Breslow categories. Interestingly, no homozygous deletion was detectable by FISH in thin lesions (Breslow thickness < 2 mm), but the incidence of 9p21 loss was the highest in this category (70.6%). Six of the eight tumors that were localized on the head of the patients showed loss at higher frequency when compared to other tumor sites, but this difference did not reach statistical significance.

Overall, no statistical differences were detected in the deletion frequency of 9p21 loss and clinical parameters (Fisher's exact test) in sporadic primary melanomas.

## Discussion

Our main purpose was to investigate the genetic and gene expression changes during the melanoma progression, and to correlate the alteration to the clinicopathological parameters of tumors.

1. Correlation of gene expression changes of primary melanomas and clinicopathological variables of lesions, significant association was only present related to the ulceration and metastases formation of tumors.
  - The Volcano plot analysis identified 1095 significantly differentially expressed genes are associated with ulceration. Major part of differentially expressed genes (1021) was found to be down regulated in ulcerated melanomas versus less aggressive group, whereas these genes were upregulated in melanomas without ulcerated surface
  - Osteopontin (OPN) was the most highly expressed genes in aggressive melanomas. The defensin- $\beta$ , was the most highly up-regulated genes in less aggressive melanomas. Both genes activate the Nf- $\kappa$ B transcription factor.
  - This gene signature is comprised of critical mediators of hair and skin development and function, organ development, dermatological diseases and conditions cellular development, cellular growth and proliferation, cardiovascular system development and function cell morphology, cellular assembly and organization, cell-to-cell signaling and interaction. We also demonstrated that these genes are mainly involved in the p53, ERK/MAP, Nf- $\kappa$ B, WNT/  $\beta$ -catenin pathways.
  - Comparing the results of aCGH and microarray, there was no correlation between decreased gene expression and genome alterations in the melanomas
2. Gene expression pattern of lymphnode metastases was found to be more similar to that of the more-aggressive melanomas than the less-aggressive melanomas.

3. We found that elevated copy number of EGFR gene is associated with poor prognosis in primary melanomas. amplified
  - Deletion and low level amplification was significantly higher in tumors removed from male patients compared to female patients.
  - The average number of EGFR gene copies was significantly higher in tumors that were thicker than 4.00 mm compared to the thinner tumors.
  - The EGFR gene copy number index in tumors that formed metastasis within 5 years after the diagnosis of the primary lesion was significantly higher. Outcome of patients whose primary tumors had highly amplified or extra copies of the EGFR gene was poor.
  - Significant correlation was also detected between the ulceration of the tumor surface and the elevated EGFR gene copies.
  
4. The correlation between the gene amplification status and the level of mRNA and protein expression was not linear.
  - Samples exhibited weak or lack of EGFR protein expression without strong correlation between EGFR gene copy number alterations and the level of EGFR protein expression.
  
5. No mutation was seen in any of the analyzed samples within tyrosine kinase domain in exon 19 of the EGFR gene.
  
6. Our FISH results demonstrate that, the loss of 9p21 is a frequent alteration in primary melanomas.
  - 9p21 deletion is present in early and late stages of the disease at similar frequency.
  - Beside on chromosome 9 and 9p21 losses we also detected copy number gain of CEP 9 and 9p21.
  - Comparing the 9p21 status of melanomas and the patients' clinical parameters we could not find strong correlation.