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Characterization of distinct molecular genetic alterations in *BRAF* and *NRAS* mutated human primary melanoma

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The Examination takes place at the Department of Pathology, Medical and Health Science Center, University of Debrecen, on 30.05.2013 at 11h.

Head of the Defense Committee:	Zoltán Nemes, D.Sc.
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Members of the Defense Committee:	László Puskás, D.Sc.
	Balázs Dezső, Ph.D.

The Ph.D. Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Medical and Health Science Center, University of Debrecen, on 30.05.2013 at 14h.

INTRODUCTION

Melanoma is one of the most aggressive forms of skin cancer and its incidence is on the rise worldwide, it shows the second fastest growing incidence rate among lethal cancers. As soon as the first distant metastasis appears, the disease becomes one of the most aggressive and chemoresistant tumors, therefore the early recognition of cutaneous melanoma is of crucial importance in saving lives. Among the environmental factors that play an important role in the development of malignant melanoma, ultraviolet exposure (UV) appears to be the major determinant. Apart from its beneficial effect UV radiation also has strong genotoxic effects that lead to DNA damage. It directly produces specific DNA damage particularly pyrimidine dimers that triggers changes in the conformation of DNA but through an indirect mechanism it also induces oxidative stress-mediated mutations in the genome. Melanoma develops as a result of accumulated molecular alterations in several important genetic pathways of the melanocyte and as a result of loss of the tight-knit regulation from keratinocytes leading to a continuous production of melanin. These abnormalities promote uncontrolled cell proliferation and escape of melanoma cells from programmed cell death in response to DNA damage. However it is thought that tumor cell evolution is an enormously diverse, unpredictable, stochastic process; on the other hand, it is also proved that during tumor genesis, different chromosomal abnormalities occur either singly or in combination, in a distinctly nonrandom pattern. It is also supported by a hypothesis that epigenetic and genetic alterations in tumors are clonally selected in a fashion that is not independent. Cells in neoplasms compete for resources, such as oxygen and glucose, as well as space. Thus, tumor cells that acquired mutations that increased their fitness will spread in the neoplasm. In this way, a population of mutant cells, called a clone, can expand in the neoplasm and distant organs.

The human genome sequencing, the high-resolution microarray technology and the available advanced bioinformatics tools have allowed a new revolutionary discipline called genomics to emerge. The growth of genomics and the resulting boom in development of novel associated technologies have created a tremendous global impact in the better understanding of several disease processes so as to form a basis for the development of novel diagnostic and prognostic tools and the development of targeted therapeutic approaches. Over the past ten years molecular genomic studies led to the discovery of hundreds of target molecules and the successful implication of novel personalized targeted treatment therapies. These drugs are now a component of therapy for many common malignancies, including breast (*HER2*

targeted therapy, trastuzumab) and colorectal (*EGFR* targeted therapy) cancers.

There is increasing evidence that melanoma often display constitutively activated MAPK pathway through *BRAF* or *NRAS* mutations. These mutations arise early during melanoma pathogenesis, but it is also clear that isolated mutations are not sufficient to initiate human melanoma *in vivo*. However, nowadays it is still an open question which additional mutations play a crucial part in the development and progression of *BRAF* or *NRAS* mutated primary melanoma subtypes. Based on the presence and the absence of these mutations tumors can be classified into three groups: 1) $BRAF^{mut}$ 2) $NRAS^{mut}$ 3) WT (wild type for both loci) primary melanomas. Previous results from melanoma cohorts interestingly show that mutations in the *BRAF* and *NRAS* genes are almost always mutually exclusive. Recent advances in the understanding of the prevalence and patterns of *BRAF* mutation in melanoma have already led to impressive results in clinical trials of kinase inhibitor therapies for this disease. Despite the robust early clinical efficacy, the antiproliferative effect of these kinase inhibitors is limited which means that identifying concomitant genetic alterations may help enhance the response and decrease the resistance to kinase inhibitors.Therefore during our study we aimed to better characterize the possible association of *BRAF* and *NRAS* mutations with marked genetic alterations and perform correlation analysis to define how these chromosomal changes act jointly within the different mutation subgroups. Another focus of our analysis was to explore a possible set of gene copy number alterations that have important impacts on dysregulation of the EGF/MAPK pathway along with the *BRAF* mutation. These additional oncogenic events which are associated with *BRAF* or *NRAS* mutations can provide rational additional targets for a combination therapy with kinase inhibitors.

The 11q13 chromosomal region, based on our and others result, frequently harbors amplification in the majority of primary melanomas. The Cyclin D1 (*CCND1*) gene has been widely considered to be a target gene in the region because its overexpression is commonly observed in several human cancers, including breast, head and neck, and bladder cancers. Beside the *CCND1*, several other oncogenes and/or cancer-related genes reside within this amplicon core, such as *TAOS1, FGF3*, *FGF4, FGF19* and *EMS1*. However, the possible role of these candidate genes within the 11q13 amplicon has not yet been investigated in primary melanoma. Therefore another objective of our study was to investigate gene copy number alterations in this 11q13 amplicon in primary and metastatic melanomas by performing Q-PCR analysis and to elucidate the possible relationship between the presence of the BRAF and NRAS mutation and their clinicopathological parameters.

AIMS

Based on literature data *BRAF* and *NRAS* mutations arise early during melanoma pathogenesis and it is proven that they are preserved throughout tumor progression and contribute to invasive melanoma but only in conjunction with other mutations.

During our study we aimed to explore the characteristic genetic alterations in *BRAF* and *NRAS* mutated primary melanomas and to investigate the gene copy number alterations in the 11q13 amplicon core which was previously observed as a common large regional gain by our array CGH analysis.

Our major objectives were:

I. to elucidate chromosomal regions that differs in copy number between *BRAF*, *NRAS* mutated primary melanomas ($BRAF^{mut}$, $NRAS^{mut}$) and tumors with wild type for both loci (WT),

II. to reveal chromosomal alterations that coexist more often in the different subtypes of melanoma ($BRAF^{mut}$ or WT melanomas),

III. to explore a set of gene copy number alterations that has significant impacts on dysregulation of the MAPK pathway along with the *BRAF* mutation,

IV. to investigate gene copy number alterations between and within different signalling pathways in genetically different melanoma subgroups,

V. to develop a sensitive Q-PCR method for determining the amplification pattern of six candidate genes (*TAOS1, FGF3, FGF19, FGF4, EMS1* and *CCND1*) that map to the 11q13 amplicon core,

VI. to elucidate the combined incidence of the *BRAF* and *NRAS* mutation and the oncogenes located in the 11q13 amplicon core and their associations with clinicopathological parameters.

MATERIALS AND METHODS

Melanoma samples

The melanoma tissue samples were collected in the Department of Dermatology, Medical and Health Science Center, University of Debrecen (Debrecen, Hungary). The study was approved by the Regional and Institutional Ethics Committee, Medical and Health Science Center, University of Debrecen, and conducted according to regulations.

Forty seven primary tumors were subjected to extract DNA for array CGH analysis. Moreover, gene copy number alterations of the 11q13 amplicon were analyzed in 68 primary and 6 metastatic melanomas by Q-PCR. Tumor DNA was extracted using the G-spin Genomic DNA Extraction Kit following the instructions of the manufacturer (Macherey-Nagel, Düren, Germany). Only high quality DNA template was accepted with an absorbance ratio range between 1.79-1.9 (260nm/280nm) and 1.7- 2.5 (260nm/230nm) as measured by NanoDrop 1000 instrument (NanoDrop Technologies, Wilmington, Delaware USA). Our sample set contained relatively more frequent primary tumors with bad prognostic factors such as NM subtype (40%), higher (>4mm) Breslow thickness (45%) and the presence of ulceration (60%).

Detection of BRAF and NRAS mutations

Analysis of mutations in the *BRAF* codon 600 and in the *NRAS* codon 61 was performed on LightCycler real time PCR System (Roche Diagnostics, GmbH, Mannheim, Germany) by melting curve analysis using fluorescent probes. All primers and probes were purchased from TIB Molbiol (Berlin, Germany). The accuracy of the method was confirmed by direct sequencing (BIOM Kft., Gödöllő, Hungary) of PCR products that showed deviation from the wild type a genomic DNA melting peak.

Array CGH experiments

Array comparative genomic hybridization (array CGH) is a useful tool in detecting gains and losses of different chromosomal regions and it is based on the approach of *in situ* hybridization. This method was developed to survey DNA copy-number variations across a whole genome. It has a significantly higher resolution than karyotyping. During the process differentially labelled test (i.e. tumor) and reference (i.e. normal individual) genomic DNAs along with unlabelled human cot-1 DNA are cohybridized to known DNA substrates (BAC clones) of which the physical chromosome position is known. Microarray slides are then read by laser scanner and the ratio of the fluorescent intensities along the arrayed DNA elements provide a locus by locus representation of the relative DNA copy-number variation.

Array CGH experiments were performed on HumArray 3.1 in the University of California, San Francisco Cancer Center Array Core. This array contains 2464 bacterial artificial chromosomes (BAC) and P1 clones, printed in triplicates and covering the genome at roughly 1.4 Mb resolution. The acquired microarray images were analyzed by Spot and Sproc software.

Array CGH data analysis

All BAC clones were mapped to the human genome using data provided by the UCSC genome browser site (http://genome.ucsc.edu/). From data processing, all X- and Y-chromosome clones were excluded. BAC clones, which are known to have genomic variants according to the Database of Genomic Variants (The Centre for Applied Genetics, Toronto, Canada, http://projects.tcag.ca/variation/) were omitted. The $\log_2$ transformed data were analysed with the CGH-Explorer software 3.2 using a False Discovery Rate (FDR) of <0.01. For the subsequent identification of high level gains and homozygous deletions in array CGH data, ratio thresholds (>0.55 (> ~5 copies) and <-0.8, respectively) were used as described in previous studies.

Fisher's exact test was applied to identify BAC clones or regions that differ in copy number between tumor subgroups. In order to increase our power for identifying regional changes in copy number between tumor subgroups, log2 was averaged ratios over windows of five consecutive BAC clones and used a two-sample t statistic to compare the average log2 ratio for the tumor subgroups for each window. An FDR correction procedure was used to adjust for multiple comparisons and denote these resulting P values as adjusted P values.

For the identification of chromosomal alterations that coexist more often in $BRAF^{mut}$ or WT melanomas a standard Pearson correlation was used. BAC clones were selected for this analysis if either the difference in the BAC's gain or loss percentages between the tumor subgroups ($BRAF^{mut}$, $NRAS^{mut}$ and WT) was >40% or if its adjusted P value from the categorical or the windowed analysis was < 0.3. All the analyses were performed in the open source statistical computing environment "R" (http://www.r-project.org/).

Investigating genetic alterations within and between different signalling pathways

Data of the different gene signaling networks was downloaded from the SignaLink database (http://signalink.org/). To assess gene alterations in these eight signaling networks we estimated the copy number changes of each of these pathway genes using the closest BAC clone within 2Mb. We considered a signaling interaction to be altered if the copy-number change of at least one of the participating genes was classified as a gain or loss by array CGH analysis, and then the average frequency of altered signaling interaction was simply calculated within and between different pathways for a particular tumor subgroup.

Random Forest analysis

Random forest analysis is getting more and more widely used in the field of the evaluation of microarray experiments because it has several characteristics that make it ideal to investigate

these data sets, such as a) when the number of variables is much larger than the number of observations and b) when datasets contain a large number of noisy variables. Our aim was with this method to explore a relevant subset of gene copy number alterations that have important impacts on the dysregulation of the EGF/MAPK pathway along with the BRAF mutation (*BRAF* gene is one of the key activators of the EGF/MAPK pathway) with this method. The Random Forest package of the R-statistical programming language (http://www.r-project.org/) was applied to calculate the Random Forest classification and importance measures on the array CGH data related to the MAPK pathway genes. At the end of the analysis a cross-validation was performed (with those most important 15 variables that came up from the analysis) to calculate the accuracy of the Random Forest classification.

Cluster analysis

For hierarchical clustering, the $\log_2$ transformed data were submitted for each BAC clone using the Pearson correlation as similarity metric and centroid linkage clustering with the Cluster 3.0 software [138]. Results were displayed using the TreeView program

Real-time quantitative PCR analysis

The primers were designed for the genes using Primer Express 2.0.0 software (Applied Biosystems, Foster City, USA) and Primer3 (Whitehead Institute, Cambridge, USA). To avoid the secondary structures, the web-based MFOLD version 3.2 software (http://www.bioinfo.rpi.edu/applications/mfold/) was used.

Reactions were carried out using a Power SYBR-Green PCR Master Mix-et (Applied Biosystems) and an ABI Prism 7000 sequence detector (Applied Biosystems). The amplification mixtures (25 µL) contained 1 µL template DNA (~10 ng/µL), 12.5 µL Power SYBR-Green PCR Master Mix (Applied Biosystems) and 100-300 nM primer. The reactions were performed under the following conditions: 10 minutes of polymerase activation at 95°C then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Quantification was performed using the Pfaffl method. We aimed to quantify the relative amounts of the target genes, all located in the 11q13 amplicon core, using two reference genes for normalization, GNS (12q14.3) and UBE2E1(3p24.2). To check the validity of the genes used as reference genes (GNS/UBE2E1), we determined the copy number ratio of the genes in 14 normal control DNA samples and 74 tumor samples. To calculate the PCR amplification efficiency (E= efficiency) we calculated the slope value from the three separated standard curves for the target genes and reference genes as well. Instead of interpolating unknown samples from a standard curve, the haploid copy number solely based on the observed C_T

values has been calculated.

$$Ratio = \frac{\left(1+E_{\text{target gene}}\right)^{-\Delta C_{T\ \text{target gene}}}}{\sqrt{\left(1+E_{\text{reference gene1}}\right)^{-\Delta C_{T\ \text{reference gene1}}} \times \left(1+E_{\text{reference gene2}}\right)^{-\Delta C_{T\ \text{reference gene2}}}}}$$

Where

$E_{\text{target gene}}$ = efficiency of the PCR reaction for the target gene,

$E_{\text{reference gene}}$ = efficiency of the PCR reaction for the reference gene,

$\Delta C_{T\ \text{target gene}}$ = difference in threshold cycle value between test sample and calibrator sample for the target gene and

$\Delta C_{T\ \text{reference gene}}$ = difference in threshold cycle value between test sample and calibrator sample for reference gene.

To determine whether copy numbers of the investigated genes are significantly different from the controls, a tolerance interval (TI) was set up for the relative gene copy number, using the mean SD of the ΔC_T values for target and reference genes in 12-14 normal DNA isolated form healthy individuals.

RESULTS AND DISCUSSION

Mutation frequencies of *BRAF* and *NRAS* oncogenes in primary melanomas

BRAF codon 600 mutations at codon 600 were found in 37% and *NRAS* codon 61mutations in 19% of the primary melanomas but both mutations were never simultaneously present.
Paired primary and metastatic tumor were available for the analysis in four cases. In three out of the four cases we found mutations in the primary tumors (one carrying a BRAFV600E mutation, two harboring a NRASQ61K mutation) and also in the corresponding metastatic lesions. *NRAS* codon 61 mutations were significantly more frequent in tumors originating from chronically sun-exposed sites (p=0.005). The strand opposite the *NRAS* codon 61 (CAA) may provide some explanation for this pattern because it contains a TT sequence, so this site is a prime candidate for UV damage. Our results are in concordance with other studies and support the idea that these mutations are preserved throughout melanoma progression.

Frequent chromosomal alterations in primary cutaneous melanoma cancer samples identified by array CGH

Tumor DNAs from 47 frozen tissue primary melanoma samples were analysed with array

CGH (see Table 2 for more details). Based on our analysis the most frequent large (>5MB) amplification was identified on the 1q, 6p, 7q31, 8q11- 8q12, 8q21 - 8q24, 11q13, 15q22-15q25, 17q25, 19p13, 20p11 - 20q13, 22q11 - 22q13 chromosomal regions and deletions on the 1p36 - 1p35, 2q, 4q, 5q22 - 5q23, 6q, 11q14.1 - 11q24.2, 13q14 - 13q31 and 17p13 - 17q21 chromosome regions and on the 9 and 10 chromosomes. We observed that the average frequency of copy number changes was observed to be higher in tumors harbouring *BRAF* mutation than in WT tissues. (Mann-Whitney test; p=0.04 and p=0.01, for gains and losses, respectively).

The frequency patterns of copy number changes for each different mutated tumor group across the entire genome were also analysed. It was observed that *BRAF* mutated tumors show higher frequency of segmental chromosome 7 gains and chromosome 10 losses which always included *BRAF* (7q34) and *PTEN* (10q23.3). This observation points to a conclusion that *BRAF* mutation is not just an early genetic event of melanoma but it has also significant effect during melanoma progression. Unsupervised hierarchical clustering of a large region (~40MB) at the 10q chromosomal arm also revealed that their deletion patterns are linked to high-grade tumors and to the presence of *BRAF* mutation. It has been implied in several recent studies that the inactivation of the *PTEN* tumor suppressor gene is a key genetic event in melanoma. On the other hand, whereas extended deletions on chromosome 10 suggest a novel theory wherein a broad range of chromosome 10 losses in conjunction with *BRAF* activation and *PTEN* inactivation may be involved in polygenic melanoma tumorigenesis. The 7q36.3 region harboring the *PTPRN2* gene was found to be one of the most frequent high-level amplifications (14% of primary melanomas) and was also linked to the presence of *BRAF* mutation. The product of this gene acts as a signaling molecule involved in cell growth, differentiation, mitosis and oncogenic transformation. In a recent study, the downregulation of a set of genes, including the *PTPRN2*, was able to suppress metastasis of breast cancer cells to different organ sites. Furthermore, loss of 11q23.3-11q25, which carries the well-established tumor suppressor gene named *OPCML*, that negatively regulates receptor tyrosine kinases, was mainly associated primary melanoma harbouring *NRAS* mutation. Moreover the loss of one allele at 17p13.3 was also observed frequently in *NRAS* mutated melanomas. This tumor suppressor locus on 17p has already been observed to carry two candidate tumor suppressor genes *HIC1* and *OVCA1*. It was also suggested that the reduction to hemizygosity of 17p13.3 resulted in cell cycle deregulation and promote tumorigenesis. Additionally, the group of tumors with *BRAF* or *NRAS* mutations also frequently showed deletions and amplification in the 9p including the loci of *CDKN2A* (9p21.3) and in the loci of *E2F3* (6p22.3), respectively.

Primary melanoma without *BRAF* or *NRAS* mutations was primarily characterized by amplification on the 17q24-17q25 and deletions on the 4q23-q25 chromosomal regions.

Correlation analysis of gain or loss changes in primary melanomas

Pairwise correlation analysis of the subset of $BRAF^{mut}$ and WT tumors revealed chromosomal alterations that coexist more often together in these groups of tumors. There were positive strong correlations in $BRAF^{mut}$ primary melanomas (correlation coefficient >0.7) between loss in 1p-4q, loss in 1p-14q and loss in 4q-11q23; and gain in 7q-20p12 chromosome regions. Relatively large regions of negative correlation (correlation coefficient <-0.7) were seen in WT tumors between changes in copy number of BAC clones such as: a) loss in 4q13 - gain in 14q and 17q chromosome regions, gain in 7q - loss in 17p. Furthermore, strong positive correlation was observed c) with copy number gain in the 14q-17q25 in WT primary melanomas.

Identification of gene signature associated with the BRAF mutation in the MAPK pathway

Because the *BRAF* oncogene is one of the key activators of the MAPK pathway, we performed a focused analysis of this signaling pathway to investigate whether there are any gene signatures in this pathway that are related to the *BRAF* mutation. Using the random forest classifier among the EGF/MAPK pathway genes, we identified a signature of 15 genes that was highly predictive for a *BRAF*-positive mutation status. Frequent coamplification of well-known oncogenes (*EGFR*, *PI3K*, *JNKK2*) and several less-known genes (*SHC1*, *PEA15*, *ELK4*) were also found to be most likely associated with the presence of the *BRAF* mutation. With this method, a particular deletion pattern involving three well-known cancer genes, such as *PTEN*, *HVH-3* and *JNK1* were identified and found to be associated with *BRAF* mutation. *PTEN* is a well-known tumor suppressor gene, but little data has been published on the role of *HVH-3* and *JNK1* in melanoma tumorigenesis. According to the literature, the expression of *HVH-3* resulted in both the specific inactivation of *ERK2* and it has been proven that silencing the *ERK2* mRNA inhibits tumor growth in vivo. There is also further evidence for the role of *JNK1* gene in tumorigenesis: failure of the function of *JNK1* could facilitate tumor formation and $JNK1^{-/-}$ mice developed spontaneous intestinal tumors

Frequent changes of signaling interactions in primary melanomas harboring *BRAF* mutation

We considered a signaling interaction to be altered if the copy-number change of at least one

of the participating genes was classified as a gain or loss by array CGH analysis. Signaling interaction between the MAPK and JAK networks was observed to be extensively altered in the $BRAF^{mut}$ tumors compared to the $BRAF^{wt}$ lesions. Additionally, interactions within the EGF-JAK, JAK-IGF and EGF-IGF pathways are more frequently altered (>20%). This analysis further supported the potentially important role of the HH pathway in $BRAF^{mut}$ primary melanomas.

Determining the amplification pattern of genes on the 11q13 chromosomal region and their associations with clinicopathological parameters

Based on our previous array CGH analysis we selected the 11q13 region as a frequent alteration of primary melanomas to define gene copy alterations in details. Therefore a sensitive Q-PCR assay was designed for the *CCND1*, *TAOS1, FGF3, FGF19, FGF4* and *EMS1* gene copy number determination. We found that 32% of the primary melanomas exhibited amplification of the *CCND1* gene. The *CCND1* gene amplification pattern was further compared in the primary and its metastatic tumor in four cases: the *CCND1* gene was amplified in all the primary lesions but we could detect *CCND1* amplification only in 1 metastasis.

Samples with *CCND1* amplification (22 primary melanomas) were further characterized for the *TAOS1, FGF3, FGF4*, *FGF19* and *EMS1* gene copy number alterations by Q-PCR. Among these samples, 18% showed amplification for all of these additional five genes and in 46% of tumors *CCND1* was co-amplified with *TAOS1*. However there were no associations between *CCND1* gene alterations and clinicopathological parameters. The co-amplification of *CCND1* with *TAOS1* was the most frequent event and had a significant association with the presence of ulceration (p=0.017), a clinical feature that can predict poor prognosis. *TAOS1* has been described as a possibly important gene that might drive the 11q13 amplification in oral squamous cell carcinoma. However the amplification pattern of the genes located on the 11q13 chromosomal region did not show significant association with the prevalence of *BRAF* or *NRAS* mutations, their coamplification pattern were more frequently ($p<0.01$) found in thick (>9 mm Breslow thickness) melanomas.

In addition, we observed that an increased *CCND1*gene copy number in conjunction with either *BRAF* or *NRAS* activation mutations was significantly more common in primary tumors with ulcerated surfaces (P=0.028).

Based on our recent results we assume that coamplification of these candidate genes in the 11q13 region or carrying a *CCND1* alteration along with either the activating *BRAF* or *NRAS*

mutation may be more important for prognosis in subgroups of aggressive melanomas than the presence of these alterations alone.

SUMMARY

Human malignant melanoma is one of the most aggressive forms of skin cancer with an exceptionally bad prognosis. Melanoma often display constitutively activated MAPK pathway through *BRAF* or *NRAS* mutations. It is also known that these mutations are almost never simultaneously present and they appear at early stages and preserved throughout tumor progression, although it is proved that these alterations alone are insufficient to cause tumor progression. Therefore the first aim of our study was to properly evaluate the prevalence of these mutations and those distinct genetic alterations which can properly differentiate these subtypes of primary melanomas with a) *BRAF*, b) *NRAS* mutation and c) WT (wild type for both loci). Mutation analysis in the *BRAF* and *NRAS* oncogene was performed by melting curve analysis using fluorescent probes. High-resolution array comparative genomic hybridization (array CGH) was used to assess genome-wide analysis of DNA copy number alterations.

Based on our results we can conclude that:

- Melanomas with the presence of *BRAF* mutation exhibited more frequent losses on 10q23-q26 and gains on chromosome 7 and 1q23-q25. Loss on the 11q23-q25 sequence was found mainly in conjunction with *NRAS* mutation. Hierarchical cluster analysis revealed that the presence of *BRAF* mutation is associated with deletion pattern on 10q chromosome and is frequently observed in lesions with advanced clinical stages.

- Correlation analysis revealed chromosomal alterations that coexist more often in the different subgroups of primary melanomas ($BRAF^{mut}$, WT).

- In order to investigate the specific dynamic activities among eight different signaling pathways we used a novel database (SignaLink) and estimated the copy number changes of these pathway genes. The result highlighted the frequent alterations of genes involved in the protein-protein interactions between the MAPK-JAK pathways in *BRAF* mutated primary melanomas.

- Using Random Forest analysis we also found a gene alteration signature in the MAPK pathway that was commonly related to the presence of the *BRAF* mutation in our melanoma cohorts.

- After developing an accurate Q-PCR method for determining the co-amplification pattern of six candidate genes that reside in the 11q13 amplicon core, we found that coamplification of these candidate genes or the *CCND1* amplification along with either *BRAF* or *NRAS* mutations might be more important for prognosis than the presence of these alterations alone.

Even though both *BRAF* and *NRAS* function as key molecules along the EGF/MAPK pathway; they may cooperate with different oncogenic events during melanoma development. Based on these results we were able to prove the existence of marked differences in the genetic pattern of the *BRAF* and *NRAS* mutated melanoma subgroups that might suggest that these mutations contribute to malignant melanoma in conjunction with distinct cooperating oncogenic events. In general, it is an interesting phenomenon suggesting that these mutations provide probably the 'guiding force' for these tumors and it also suggests that there are alternative genetic pathways to melanoma. These additional oncogenic events which are associated with *BRAF* or *NRAS* mutations can provide rational additional targets for a combination therapy with kinase inhibitors.

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List of original publications

This thesis is based on the following original publications:

Lázár V., Ecsedi S., Szöllosi AG., Tóth R., Vízkeleti L., Rákosy Z., Bégány A., Adány R., Balázs M. Characterization of candidate gene copy number alterations in the 11q13 region along with BRAF and NRAS mutations in human melanoma. Mod Pathol., 2009 Oct;22(10):1367-78.
IF: 4.406

Lázár V., Ecsedi S., Vízkeleti L., Rákosy Z., Boross G., Szappanos B., Bégány A., Emri G., Ádány R., Balázs M. Marked genetic differences between BRAF and NRAS mutated primary melanomas as revealed by array comparative genomic hybridization. Melanoma Res. 2012 Jun;22(3):202-14.
IF: 2.187 (2011)

Other original publications:

Barok, M., Balazs, M., **Lazar, V.,** Rakosy, Z., Toth, E., Treszl, A., Vereb, G., Colbern, G.T., Park, J.W., Szollosi, J. Characterization of a novel, trastuzumab resistant human breast cancer cell line. Frontiers in bioscience 2010. 2:627-640.
IF: - (2011)

Vízkeleti L., Ecsedi S., Rákosy Z., Orosz A., **Lázár V.,** Emri G., Koroknai V., Kiss T., Ádány R., Balázs M. The role of CCND1 alterations during the progression of cutaneous malignant melanoma.Tumour Biol., 2012 Sept 23., [Epub ahead of print]
IF: 2.143 (2011)

Rakosy Z, Ecsedi S, Toth R, Vizkeleti L, Herandez-Vargas H, **Lazar V**, Emri G, Szatmari I, Herceg Z, Adany R, Balazs M. Integrative genomics identifies gene signature associated with melanoma ulceration. PLoS One. 2013;8(1):e54958. Epub 2013 Jan 30.
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List of publications related to the dissertation

1. **Lázár, V.**, Ecsedi, S., Vízkeleti, L., Rákosy, Z., Boross, G., Szappanos, B., Bégány, Á., Emri, G., Ádány, R., Balázs, M.: Marked genetic differences between BRAF and NRAS mutated primary melanomas as revealed by array comparative genomic hybridization.
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2. **Lázár, V.**, Ecsedi, S., Szöllősi, A., Tóth, R., Vízkeleti, L., Rákosy, Z., Bégány, Á., Ádány, R., Balázs, M.: Characterization of candidate gene copy number alterations in the 11q13 region along with BRAF and NRAS mutations in human melanoma.
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List of other publications

3. Rákosy, Z., Ecsedi, S., Tóth, R., Vízkeleti, L., Herandez-Vargas, H., **Lázár, V.**, Emri, G., Szatmári, I., Herceg, Z., Ádány, R., Balázs, M.: Integrative genomics identifies gene signature associated with melanoma ulceration.
PLoS One. 8 (1), e54958, 2013.
DOI: http://dx.doi.org/10.1371/journal.pone.0054958
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4. Vízkeleti, L., Ecsedi, S., Rákosy, Z., Orosz, A., **Lázár, V.**, Emri, G., Koroknai, V., Kiss, T., Ádány, R., Balázs, M.: The role of CCND1 alterations during the progression of cutaneous malignant melanoma.
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DOI: http://dx.doi.org/10.1007/s13277-012-0480-6
IF:2.143 (2011)

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Posters and Presentations

***Lázár V.**, Singh GP., Nagy I., Horváth B., Hrtyan M., Busa-Fekete R., Bogos B., Méhi O., Csörgő B., Pósfai Gy., Fekete G., Szappanos B., Kégl B., Pál Cs. & Papp B.* Exploring the network of antibiotic cross-resistance and collateral sensitivity interactions during bacterial evolution. 2012 GRC Drug Resistance Conference, 29 July-3 August 2012, Stonehill College in Easton, MA US

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