SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Genetic and epigenetic alterations of invasive melanoma cells

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF HEALTH SCIENCES

DEBRECEN, 2020

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The Examination takes place at the Library of Department of Internal Medicine Bldg.A Faculty of Medicine, University of Debrecen at 11:00 on 26th August 2020

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The PhD Defense takes place at the Lecture Hall of In Vitro Diagnostic Centre, Faculty of Medicine, University of Debrecen at 13:00 on 26th August 2020

INTRODUCTION

Malignant melanoma is considered as one of the most aggressive human cancers with high metastatic propensity into different organs. Although it is less common compared to the other type of skin cancers, it accounts for the majority of skin cancer death. Global incidence of melanoma is about 230,000 new cases per year, with 55,000 deaths, however, the estimated age-standardized incidence (cases per 100 000 residents) varies widely. Recently, the rapid rise in melanoma incidence appears to be decreasing, specifically among younger age groups, it has been found that the long-term incidence is still increasing globally, including Hungary as well. Epidemiological studies have indicated the relationship between ultraviolet-B (UVB) radiation exposure and melanoma development; moreover, sunburns in childhood are associated with the highest risk. Despite the increasing treatment possibilities, early diagnosis and complete surgical excision still remain the best treatment for melanoma patients.

Cutaneous malignant melanoma and metastatic spread

Cutaneous melanoma develops from melanocytes, which originate from highly motile neural crest progenitors that migrate through the skin during embryonic development. The differentiation of melanocytes and their progenitor cells, melanoblasts, is controlled by a complex network of transcription factors, including microphtalmia-associated transcription factor (MITF), which transcription is activated by the synergistic act of SOX10 and PAX3, and drives the expression of several genes essential for melanogenesis.

Metastasis formation is a multi-step process that includes (1) tumour cell invasion, (2) intravasation to vessels, (3) survival in the circulatory system, (4) extravasation and (5) proliferation, leading to metastatic colonization. To efficiently metastasize, invasive melanoma cells change their cytoskeletal organization and alter their

contacts with the extracellular matrix (ECM) and the surrounding stroma. In order to invade the stroma, tumour cells have to pass through the basement membrane.

Melanoma cell plasticity

The ability of melanoma cells to pass through the ECM and invade the surrounding tissues requires the transition of typical epithelial histologic features including apical-basolateral polarization, basement membrane integrity, and cell-cell adhesion to cells of invasive phenotype. Tumour cells have different strategy of movement. Apart from the collective invasive strategy, where cells invade as multicellular units by forming strands or sheets, individual tumour cells can also migrate through tissue compartments separated by the basement membrane. The main types of individual invasion are the elongated/mesenchymal and the rounded/amoeboid modes.

Several studies have been carried out in the last decades focusing on the signalling pathways in association with invasive cell's plasticity. Rho GTPase signalling is one of the main molecular pathways of interest in this field, as Rho-family GTPases are key regulators of cytoskeletal motility, actomyosin contractility, adhesion, proliferation and survival. Amoeboid type of movement is induced by Rho-ROCK and Cdc42 signalling generating actomyosin contractility, while Rac1 signalling is involved in the elongated movement through actin assembly.

Genetic alterations in invasive melanoma

Melanomagenesis can be associated with different somatic mutations affecting the MAPK signalling pathway activation, including KIT^{L576P}, $NRAS^{Q61K}$ or $BRAF^{V600E}$ mutations, or loss of tumour suppressor genes, e.g., *PTEN*, *P14ARF*, or *P16INK4a*, involved in cell cycle regulation. However, the association of mutations and/or copy number alterations with the invasive capacity of melanoma cells remain incompletely understood. The gain-of-function mutation of *BRAF* gene leads to increased proliferation and survival by constitutive activation of the ERK

signalling. Furthermore, *BRAF*^{V600E} mutation is also involved in invasion and metastasis development of melanoma.

According to a proposed model of melanoma progression, the cascade is initiated by mutations that trigger the MAPK signalling (*BRAF*, *NRAS*), while *PTEN* and *TP53* mutations were found only in advanced primary melanomas. The model indicates the role of biallelic inactivation of *CDKN2A* in the invasive phenotype of melanoma.

Gene expression signature of invasive melanoma

According to gene expression patterns of melanoma tissues, it can be categorized into different groups with different metastatic potential. One of these signatures (the so-called proliferative phenotype) is characterized by the overexpression of *MITF* and other melanocytic genes (e.g., *TYR*, *DCT*, and *MLANA*) along with several neural crest-related factors (e.g., *SOX10*, *TFAP1A*, and *EDNRB*).

On the other hand, the second signature (the so-called invasive phenotype) downregulates the aforementioned proliferative gene signature and exhibits the upregulation of genes involved in extracellular matrix remodelling (TGFß-type signalling), and in the epithelial-to-mesenchymal transition (EMT) (e.g., *ZEB1*, *COL5A1*, *SERPINE1*, and *WNT5*). The invasive signature of the tumour is associated with lower rates of proliferation and high motility. Moreover, switch between invasive and proliferative phenotype has been described, which is a possible result of microenvironmental changes during melanoma progression.

The gene signatures distinct for proliferative and invasive cellular states were also described and extended into the gene regulatory landscape. Indeed, the proliferative cellular state is characterized by the expression of SOX10 and MITF transcription factors as master regulators of the melanoma-proliferative cell state. On the contrary, invasive cells exhibit TEAD and AP1 expression.

DNA methylation pattern related to invasive behaviour

Global hypomethylation has been observed in several different types of cancer, including melanoma. On the other hand, DNA hypermethylation at the transcriptionally active gene regions and promoters resulting in silencing of tumour suppressor genes. The proliferative vs. invasive transcriptomic signature is highly correlative with either permissive or repressive chromatin states underlying the importance of epigenetics regulation in the acquisition of invasive cellular state.

Recent improvements in epigenome-wide (EWAS) DNA methylation methods are allowed for the identification of potential biomarkers that could be exploited in clinical settings. Of note, several genes of the melanocyte lineage differentiation pathway were found to be methylated such as *KIT, PAX3, SOX10*, different members of the HOX family genes and *MITF*. Importantly, EWAS on melanomas have more often focused on the metastatic tumours and therefore, the DNA methylation changes accompanying the early molecular invasion events remain to be elucidated.

OBJECTIVES

The focus of this study was to discover the molecular background of melanoma the molecular background of melanoma invasion, as an initial step of metastasis formation. We aimed to study the DNA methylation landscape and its effect on gene-expression during early invasion using *in vitro* selection of invasive melanoma cell subpopulation established from primary melanomas.

In our study we aimed to

1. investigate the relationship between copy number (CN) alterations and the invasiveness of melanoma cells using array (CGH) analyses, and to identify invasiveness related recurrent genetic regions.

2. define genetic alterations associated with *BRAF* and *NRAS* mutations, and compare copy number changes associated with these mutations in the invasive cell lines.

3. establish selected invasive subpopulations of melanoma cells separated from the original cell lines in order to further analyse the invasion-related genomic alterations using genetic and methylation profiling of the selected invasive cells and integrate the methylation alterations with the gene expression profiles that may render cellular plasticity towards increased invasion.

4. characterize copy number or methylation alterations of the candidate genes related to invasiveness in melanoma tumour tissue samples.

MATERIALS AND METHODS

Cell culture

Experiments were performed in primary tumour-(WM35, HT199, WM1789, WM793B, WM3211, WM1361, WM902B, WM39, WM278, WM983A, WM1366, WM3248) and metastatic tumour derived melanoma cell lines (WM1617, WM983B, A2058, HT168, M24, M24met). The cells were cultured in RPMI 1640 medium (Lonza Group Ltd, Basel, Switzerland) or MCDB153-L15 medium (Sigma-Aldrich Co. LCC, St Louis, Missouri, USA) supplemented with 5–10% foetal bovine serum (Gibco, Carlsbad, California, USA) at 37°C in an atmosphere containing 5% CO2.

In-vitro invasion assay

The invasive potential of the melanoma cell lines was determined using BD Biocoat Matrigel invasion chambers (pore size: 8 μm, 24-well; BD Biosciences, Bedford, Massachusetts, USA). The upper chamber was filled with 500 μl cell suspension in serum-free media (5×10^4 cells/well). Medium containing 10% FBS was applied to the lower chamber as a chemoattractant. After the cells were incubated for 24 h at 37°C, cells in the lower layer were fixed with methanol and stained with hematoxylin–eosin. The invaded cells were counted using a light microscope in seven different visual fields at 200X magnification and the data are presented as the means ±SD of three independent experiments.

Selection of invasive cells

The invading cells in the lower chamber of the BD Biocoat Matrigel chamber were treated with 0.5% trypsin/0.2% EDTA solution (Sigma-Aldrich Inc., St. Louis, MO, USA) for recovery from the membrane. Selected invasive cells were cultured using standard protocol until DNA and RNA isolations.

Cell proliferation assay

Cell proliferation rate was determined using the WST-1 assay (Sigma-Aldrich Inc., St. Louis, MO, USA) according to the manufacturer's guidelines. Absorbance was measured at 450 nm using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA), while the reference absorbance was set at 700 nm.

Array CGH and data analysis

Genomic DNA was isolated using the G-spin Genomic DNA extraction kit (Intron Biotechnology Inc., Seongnam, Korea) according to the manufacturer’s protocol. DNA samples were hybridized to Cytochip ISCA 8 × 60 arrays (BlueGnome Ltd, Cambridge, UK). Array data were analysed using BlueFuse Multi v2.2 software (BlueGnome Ltd) and Nexus Copy Number 6.1 software (BioDiscovery Inc.,

Hawthorne, California, USA). To detect gains and losses, ±0.3 log2 ratio thresholds were set, while 0.6 for high CN gains and −1.0 for homozygous deletions were adjusted. Significantly different CN events between invasive and non-invasive melanoma cell lines, and between selected invasive subpopulations and the original cell lines were identified using two-sided Fisher's exact test.

Genome wide DNA methylation analysis

For methylation studies, bisulphite modification was performed on 600 ng of DNA using EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). We confirmed the quality of modification by PCR (HotStarTaq Master Mix kit; Qiagen GmbH, Hilden, Germany) using modified and unmodified primers for the GAPDH gene. To determine the DNA methylome profile, Illumina Infinium II Human Methylation 450K (HM450K) BeadChip assay was used (Illumina, San Diego, CA, USA). The data pre-processing and all analyses were performed using several Bioconductor packages in R v.3.2.2. methylation beta values were logarithmically transformed to M values before parametric statistical analyses, as recommended. DNA methylation changes were considered significant with an FDR-adjusted p value less than 0.05.

Correlation between gene expression and DNA methylation

RNA was isolated using RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacture's protocol. To assess gene expression at genome-wide levels, we purchased Affymetrix Human Gene 1.0 microarrays (Affymetrix Inc., Santa Clara, CA, USA). The raw CEL files were imported to R v.3.2.2 using the Oligo package. We calculated the Pearson's correlation coefficients to correlate the gene expression log2 fold changes to the DNA methylation changes (Δß) in the genes belonging to the DMPs.

The Cancer Genome Atlas (TCGA) data analysis

Detailed genomic analysis using array CGH data of melanoma samples was completed with the Skin Cutaneous Melanoma (SKCM) dataset: The Cancer Genome Atlas (TCGA, Provisional). The results are based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov). On the other hand, we downloaded Illumina Methylation 450K data available for SKCM containing 437 tissues (88 primary and 349 metastatic melanoma samples) from the TCGA-GDC data portal (https://portal.gdc.cancer.gov/) by using the GDCquery and GDCprepare functions of the TCGAbiolinks R package.

TaqMan Copy Number Assay

Real time quantitative PCR (qPCR) method was used on 18 melanoma cell lines to confirm the array CGH results. Copy numbers of *GLIPR1*, *COL1A2* and *RELN* genes were assessed using pre-designed TaqMan® Copy Number Assays. All assays were performed with TaqMan® Universal PCR Master Mix according to the manufacturer's protocol (Applied Biosystems Inc., USA).

Real time quantitative PCR analysis

The relative expression level of the selected genes was determined by quantitative real-time PCR using LightCycler® 480 Real-Time PCR System (Roche Diagnostics, GmbH, Mannheim, Germany).

Statistical analysis

SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The Shapiro–Wilk test was used to evaluate the normality of the data. Pearson's correlation coefficient was calculated to correlate the array CGH and qPCR data. Mann-Whitney-Wilcoxon test was used to compare the mRNA expression of non-invasive cell lines to invasive ones. $P < 0.05$ was considered statistically significant.

RESULTS

Genomic profiling of melanoma cell lines

Array CGH analysis

We performed array comparative genomic hybridization (CGH) analyses using the CytoChip ISCA array to identify chromosome copy number alterations in 18 human melanoma cell lines originated from primary- (n=12) and metastatic (n=6) tumours. A high degree of CN instability was identified across the genomes of all cell lines, involving CN gains in 1q, 6p, 7, 8q, 17q, 20 and 22q and CN losses in 6q, 9p and 10p. We observed CN alterations of several melanoma-related genes including *NEDD9, EGFR, BRAF* and *MYC* genes.

Invasive behaviour related recurrent genetic regions in melanoma cell lines

Comparison of the genomic alterations between *in vitro* invasive (n=4) and non-invasive (n=8) primary tumour derived cell lines and metastatic cell lines (n=6) revealed that copy number changes on 7q and 12q chromosomal regions appeared specifically in cell lines with invasive behaviour and were not detected in non-invasive and metastatic tumour derived cell lines. Additionally, the gain of 5p13 and 8q24 were present not solely in invasive cell lines but occurred in metastasis-derived cell lines as well, harbouring *GDNF* (5p13.1), *GPAA1*, *PLEC* and *SHARPIN* (8q24.3) as invasion-related genes.

To validate our array CGH data, real time quantitative PCR method was performed using TaqMan® Copy Number Assays, and a good concordance was found between the data derived from array CGH and qPCR methods.

Copy number alterations of candidate genes in melanoma tumour samples

In order to define the relevance of the invasion associated alterations in native melanoma tissues, we applied the Skin Cutaneous Melanoma dataset (TCGA, Provisional). We focused on candidate genes (*GDNF, GPAA1, PLEC* and *SHARPIN*) that exhibited copy number alterations in both invasive and metastatic

melanoma cell lines. Notably, these genes exhibited copy number alterations exclusively in the metastatic tissues but not in the primary lesions. Interestingly, *GPAA1*, *PLEC* and *SHARPIN* genes were co-amplified in 26 metastatic melanomas out of the 366 samples. *GDNF* was found to be amplified to a lesser extent, in 14 metastatic tissues.

BRAF and NRAS mutation in association with invasion

Cell lines with $BRAF^{V600E}$ mutation had a higher frequency of 7q gain, including the BRAF gene located on 7q34. Other CN alterations associated with $BRAF^{V600E}$ mutation were gains of 1q and 6p, whereas loss of 9p and 10p occurred more frequently in cell lines with wild-type *BRAF*. Additionally, loss of 19p12 was seen only in $BRAF^{V600E}$ mutated cell lines. On the other hand, loss of 4q was associated with NRAS mutations.

Invasive cell lines with $BRAF^{V600E}$ mutation (WM983A, HT199) showed copy number gain of 6p25.3-p22.3 targeting *RREB1* (6p25) and *NEDD9* (6p24) genes, whereas gain of 7p was characteristic for invasive cell lines with wild-type *BRAF* gene (WM3211, WM1366).

Phenotypic characterization of selected invasive cells

To further analyse the invasion-related genetic and epigenetic alterations in melanoma cells, we established four invasive cell subpopulations (WM983A-INV, WM1366-INV, WM3211-INV and WM793B-INV) using Matrigel coated invasion chambers. Invasive cells were flatter, and more spindle-shaped than the original cell lines

Based on further invasion experiments, the selected cell lines had significantly higher invasive potential compared to the original cell lines

The proliferation rate was lower of the invasive cell lines than the original cell lines, however, the difference was not statistically significant.

Genetic profile of the selected invasive cells

Array CGH analyses were performed on the 4 selected invasive cell lines (WM983A-INV, WM3211-INV, WM1366-INV and WM793B-INV) and compared to the original cell lines to identify specific invasion related chromosomal alterations. CN alterations located on 1p, 2q and 8q were present at higher frequencies in the *in vitro* selected invasive cells compared to the original cell lines.

Methylation profile of the selected invasive cells

Invasion related methylation changes in selected invasive cells

Altogether, we identified 1,554 regions with significant differences at the methylation levels between the invasive and the original cell lines. Globally, hypermethylated DMRs (n=1,216) were more predominant than the hypomethylated DMRs (n=33) with a total of 8,733 and 165 CpG sites, respectively. We applied more stringent criteria to determine significant DMRs with increased Δβmean >10% between the invasive and the original cell lines. As a result, we identified the 416 DMRs with 1,982 DMPs (corresponding to 384 genes) with hypermethylation in the selected invasive population, and only one DMR with 3 DMPs (corresponding to one gene) were hypomethylated.

The top significant DMR presented with 15 differentially methylated probes and showing more than 20% included the *BAALC* gene with its corresponding non-coding RNA (ncRNA) pair, *BAALC-AS2*. We also identified genes with well-known functions in melanomas (e.g., *MITF, CYP27A1* and *GRIA2*) among the significant DMRs. We performed pathway analysis using EnrichR web application on our predefined gene list ranked by the Δβmean methylation differences and we found that hypermethylation mostly affected the neural crest differentiation pathway (WP2064; *NOTCH3, PAX7, HEY2, MITF, FGFR2, FGFR3, RHOB, MSX2, TLX2*, and *ZIC5* genes) and the regulation of actin cytoskeleton pathway (WP51; e.g., *MOS*, *GSN*, *ACTN1*, *WASF2*, and *VAV1* genes).

Integration of methylation and gene expression profiles

To investigate the functional relevance of the DNA methylation changes observed in the selected invasive cells, we performed integrative analysis of the DNA methylation data and gene expression alterations. We identified a total of 886 significantly correlated CpG sites corresponding to 392 individual genes between DNA methylation and gene expression, of which 220 showed negative, whereas 172 genes exhibited positive correlation. Interestingly, CpG island shore hypermethylation was associated with decreased expression level in case of four DMRs corresponding to *RHOB, ID4, ST8SIA1*, and *GRIA2* genes.

By applying more stringent criteria for the correlation analysis (genes with 1-fold expression differences between the invasive and the original cell lines were correlated to DMPs of Δβmean >10%), majority of the genes were negatively correlated, i.e. hypermethylation was associated with decreased gene expression (*IL12RB2, LYPD6B, CHL1, SLC9A3, BAALC, FAM213A, SORCS1, GPR158, FBN1*, and *ADORA2B.* On the other hand, a few genes exhibited positive correlation between hypermethylation and increased gene expression (*MCC, PTCHD4, EGFR, RBP4* and *FAR2*). Additionally, two hypomethylated genes revealed significant correlation with either upregulation (*NNMT*) or downregulation (*NBPF8*) of gene expression.

Invasion related methylation changes in melanoma tumour samples

To validate our results of methylation changes observed in the selected invasive cells, we compared our findings with publicly available melanoma dataset of the TCGA-SKMC cohort. Firstly, we determined methylation changes presented in the TCGA metastatic melanomas (n=349) versus tissues of primary sites (n=88). 28 genes out of our differentially methylated 385 genes showed overlap to the TCGA metastatic melanomas.

Several of the overlapping genes between the 2 datasets have already well-established role in invasion and metastasis formation, of which includes *TP73, HOXD13, PAX6, ITPKA, NR2F2, SLC17A7, SPTBN1, AHNAK, CCL23, NFE2L3,*

and *SLC9A*. Furthermore, 10 out of the 28 genes seem to have a role in the transcriptomic reprogramming during early invasion: the methylation changes of *CBFA2T3, TP73, CTSK, NAV2, PAX6, ARHGAP22, SDK1, ATP11A, RASA*3, and *SLC9A3* showed significant correlation with gene expression changes.

Furthermore, we identified 448 differentially methylated genes (corresponding to 1269 probes) seem to have a role during early invasion represented by the Clark staging system. Of note, 18 out of the 385 genes in our dataset show overlap with the TCGA (e.g., *MECOM, CHD5, TRIM55, FZD6, TPBG*, and *TRPC4*).

Comparing our data with the TCGA, the most interesting finding is the hypermethylation of *ARHGAP22* and *NAV2* genes that were commonly presented in locally invasive primary melanomas as well as during metastasis.

Expression of DNMTs, UHRFs and TETs in selected invasive cells

We analysed the relative mRNA expression of the DNA methyltransferases (DNMT1, DNMT13A, and DNMT13B), the ubiquitin-like protein containing PHD and RING finger domains 1 and 2 (UHRF1 and UHRF2) and the TET methylcytosine dioxygenase enzymes (TET1 and TET2), all playing a crucial role in the maintaining and removing of epigenetic marks. We observed that each of the selected invasive cells had decreased *DNMT1* and *DNMT3B* expression compared to the original cell lines. *UHRF1* and *UHRF2* genes also showed downregulation in invasive cells compared to the original cell lines. Additionally, the expression levels of *TET1* and *TET2* were also remarkably lower in two of the four invasive cells than in the original cell lines. Unexpectedly, *TET2* down-regulation was associated with hypermethylation at the *TET2* gene promoter region in the invasive subpopulation.

DISCUSSION

Cytogenetic heterogeneity resulting from chromosomal instability is a major driving force of melanoma progression. Invasion is one of the first steps of metastasis formation in primary tumours; however, insufficient data are available on the genetic and epigenetic alterations involved in this initial process in melanoma.

Based on our invasion assay experiments on melanoma cell lines, we were able to group the cell lines according to their invasive capacity. We found several CN alterations that were uniquely detected in the invasive cell lines. Although alterations of these genes have already been reported in a variety of invasive tumours; but we were the first to describe that the structural and functional alterations of these genes have fundamental role in melanoma invasion.

Through DNA copy-number profiling, we also aimed to determine genetic changes related to the *BRAF* and *NRAS* mutation status of melanoma cell lines. The comparison of CN alterations in invasive *BRAF* mutant and wild-type cell lines resulted in that gain of 6p25.3-p22.3 was present specifically in invasive cell lines with *BRAF* mutation, including *NEDD9* (6p24) and *RREB1* (6p25) genes. The copy number change of *RREB1* is a well-known alteration in melanoma, this gene has a very important role in melanoma diagnosis, it is a target of multiprobe FISH to differentiate cutaneous nevi from melanoma.Amplification of the *NEDD9* gene also correlates with melanoma metastasis by promoting elongated movement and invasion of melanoma cells. It is important to note, that alteration of *NEDD9* in association with BRAF mutation was not described previously.

We also aimed to select invasive cells in vitro from the original cell lines and analysed their invasion-associated DNA methylation changes, which followed by functional analysis of the observed changes at mRNA expression level. A number of studies have indicated that several tumour suppressor genes are silenced by DNA methylation in malignant melanoma compared to normal melanocytes or nevi. MITF (microphthalmia-associated transcription factor) has been extensively

studied in the context of master-regulator of melanin-production, suppression of invasion and regulation of the proliferative phenotype in melanoma cells. Its methylation change was also observed in melanoma brain metastases, suggesting its role not only in invasion property, but also in metastasis formation. In accordance with previous studies, selected invasive melanoma cells showed hypermethylation of MITF that may directly affect MITF expression, giving a functional role of the detected epigenetic change. Additionally, pathway analysis of hypermethylated genes in the invasive cells revealed a significant enrichment of the neural crest differentiation pathway (WP2064).

Altered gene expression in correlation with methylation changes in potential DNA methylation biomarkers of melanoma (e.g., *TFI2, HCK, MGMT* and *TP73*) were also observed in the selected invasive cells. The methylation changes of the aforementioned genes have been described in association with advanced clinical stage, shorter overall survival and the presence of metastasis, and it seems that, according to our results, these genes have a potential role in the earlier invasion steps of primary melanoma cells.

While previous studies have shown that DNA hypermethylation at gene promoters is associated with the silencing of gene expression, recent studies have shown that the methylation of the gene body is positively correlated with transcription. Similar to these observations, hypermethylation in the gene body potentially plays a role in the upregulation of *EGFR* and *RBP4* genes in the selected invasive cell populations. The role of *EGFR* expression in melanoma cells is controversial, it was described as not associated with the outcome of melanoma and not related to specific pathological features, however, a high percentage of primary and melanoma samples shows EGFR positivity.

To obtain insights into the possible clinical relevance of the DNA methylation changes identified in our in vitro invasion model, we compared our results to the publicly available 450k TCGA-SKCM datasets involving more than 400 melanoma samples with the vast majority being metastatic melanomas and the representative for heterogeneous anatomic locations.

Based on this analysis we identified several methylation changes that can have functional role in melanoma tumour samples, including *HOXD13*, being the member of the well-known master regulators of developmental processes, and involved both in oncogenesis and tumour suppression. We recognized further 8 differentially methylated members of the HOX gene family including *HOXA5*, *HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXC5*, *HOXC9*, and *HOXD11*. It is suggested that methylation pattern of homeobox genes can be specific to melanoma cells, and it is a possible approach to use epigenetic biomarker panels including homeobox genes in diagnosis, prediction and prognosis.

The most interesting finding between our results and the TCGA melanoma data is the hypermethylation of *ARHGAP22* and *NAV2* promoter regions that are commonly presented in locally invasive primary melanomas as well as during metastasis. Both *ARHGAP22* and *NAV2* (neuron navigator 2) have been identified to be involved in cell migration of different tumour types including melanoma. NAV2 has several functional domains, which play key roles in the regulation of cytoskeletal remodelling and cell migration facilitating tumour invasion and metastasis. Furthermore, a recent study suggested that *NAV2* might contribute to melanoma invasion by epithelial–mesenchymal transition through the GSK-3β/β-catenin-SNAI2 pathway. *ARGHAP22* is a member of Rho GTPases that regulate the cytoskeleton-dependent processes during migration and invasion. Silencing of *ARHGAP22* results in increased number of elongated cells in melanoma cell lines,and can regulate the mesenchymal-amoeboid transition. The switch between mesenchymal and amoeboid types of movement, allowing metastatic tumour cells to adapt their morphology and movement in different microenvironments. Our results indicate the relevance of methylation mediated gene expression changes of *ARHGAP22* and *NAV2* during the invasion of primary tumours, and also during invasion-related melanoma progression.

MAIN STAEMENTS AND RESULTS

The main purpose of the doctoral thesis was to identify the association between genomic alterations (copy number changes, DNA methylation and gene expression alterations) and the invasiveness of melanoma cells.

Investigation of the relationship between copy number (CN) alterations and the invasiveness of melanoma:

- We demonstrate that gain of the *GDNF* (5p13.1), *GPAA1*, *PLEC* and *SHARPIN* (8q24.3) genes were significantly more frequent in invasive cell lines compared to the non-invasive ones, which were also found in cell lines originated from metastases, suggesting their role in melanoma metastasis formation.

Comparison of copy number changes associated with BRAF and NRAS mutations of the invasive cell lines:

- We found that gain of the 6p25.3-p22.3 locus occurred specifically in invasive cell lines with *BRAF* mutation, including *NEDD9* (6p24) and *RREB1* (6p25) genes.

Analyses of the invasion related genetic and epigenetic alterations in melanoma cells of the selected invasive cells:

- Our data revealed predominantly hypermethylated genes in the invasive cells.
- Pathway analysis of hypermethylated genes in the invasive cells revealed a significant enrichment of the neural crest differentiation pathway including *MITF* gene.
- Integrative analysis of the methylation and gene expression profiles resulted in a cohort of hypermethylated genes with decreased expression.
- Hypermethylation in the gene body potentially plays a role in the upregulation of *EGFR* and *RBP4* genes in the selected invasive cell populations.

Validate the candidate genes with copy number alteration or methylation changes related to invasiveness in melanoma tumour samples:

- The hypermethylation of *ARHGAP22* and *NAV2* promoter regions were commonly presented in locally invasive primary melanomas as well as during metastasis.

In conclusion, our classification of copy number alterations associated with melanoma invasiveness may provide novel candidate genes for further functional studies, as well our results indicate the relevance of hypermethylated pattern in invasive melanoma cells, which might associate with the early invasion steps of melanoma.

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Subject: PhD Publikációs Lista

Candidate: Viktória Koroknai
Neptun ID: EBWBZY
Doctoral School: Doctoral School of Health Sciences
MTMT ID: 10044575

List of publications related to the dissertation

1. **Koroknai, V.**, Szász, I., Hernandez, V. H., Fernandez, J. N., Cuenin, C., Herceg, Z., Vízkeleti, L., Ádány, R., Ecsedi, S., Balázs, M.: DNA hypermethylation is associated with invasive phenotype of malignant melanoma.
Exp. Dermatol. [Epub ahead of print], 2019.
DOI: http://dx.doi.org/10.1111/exd.14047
IF: 2.868 (2018)

2. **Koroknai, V.**, Ecsedi, S., Vízkeleti, L., Kiss, T., Szász, I., Lukács, A., Papp, O., Ádány, R., Balázs, M.: Genomic profiling of invasive melanoma cell lines by array comparative genomic hybridization.
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DOI: http://dx.doi.org/10.1097/CMR.0000000000000227
IF: 2.615

List of other publications

3. Szász, I., **Koroknai, V.**, Kiss, T., Vízkeleti, L., Ádány, R., Balázs, M.: Molecular alterations associated with acquired resistance to BRAFV600E targeted therapy in melanoma cells.
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In: The Retinoblastoma Protein. Ed.: Pedro G. Santiago-Cardona, Springer Science+Business Media, New York, NY, 85-100, 2018.

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Total IF of journals (all publications): 19,677
Total IF of journals (publications related to the dissertation): 5,483

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

16 January, 2020

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

ACKNOWLEDGEMENTS

First and foremost, I wish to thank my supervisor Professor Margit Balázs, who has introduced me into the world of cancer research, and helped and guided my research during both my undergraduate and graduate research studies, and providing me the opportunity to learn a wide variety of molecular genetic techniques and different types of analysis.

I would like to express my thankfulness to Professor Róza Ádány for providing me the opportunity to work in the MTA-DE Public Health Research Group, as well as for all of her support during PhD study.

I am thankful for my colleagues István Szász, Tímea Kiss and Szilvia Ecsedi for their always generous help and their critique during my experiments.

I would like to acknowledge my colleagues of Department of Preventive Medicine for their help during my experiments, especially to Györgyné Kovács for her assistance.

Finally, I would like to say special thanks to my family for their support and patient..

This work was supported by the National Research Development and Innovation Fund (grant numbers K-112327) and GINOP-2.3.2-15-2016-00005 projects. The project is co-financed by the European Union under the European Social Fund and European Regional Development Fund and supported by the ÚNKP-19-3 New National Excellence Program of the Ministry for Innovation and Technology.