BIOLOGICAL ROLES AND PROGNOSTIC RELEVANCES OF GENE ALTERATIONS IN HUMAN MALIGNANT MELANOMAS

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The Examination takes place at the Bldg. A, Department of Internal Medicine, Medical and Health Science Center, University of Debrecen
19th December 2013, 11 am

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Medical and Health Science Center, University of Debrecen
19th December 2013, 1 pm
Introduction

Human malignant melanoma

Malignant melanoma, originated from melanocytes is considered one of the most aggressive skin cancers responsible for approximately 20% of all cases. Being the most life-threatening neoplasm of skin melanoma is responsible for almost all skin cancer deaths. Primary melanomas are characterized by an aggressive and heterogeneous phenotype. Despite the intensive research, melanoma prognostication is still under question. Transformed melanocytes usually maintain their pigment production ability, causing brownish lesions on the skin. Melanoma progression can be divided into morphologically and biologically different phases: 1.) typical nevus: congenital or newly developed, but symmetrical nevus, 2.) dysplastic nevus: morphologically changed, atypical nevus, 3.) \textit{in situ} melanoma: noninvasive, the malignant tumor is still confined to the upper layers of the epidermis, 4.) radial growth phase (RGP): radially growing lesion on the epidermal surface with microinvasion into the dermis, 5.) vertical growth phase (VGP): vertically growing lesion into the deeper tissues and 6.) metastatic melanoma: lesion forming local and/or distant organ metastases.

The current WHO clinical and histological classification of cutaneous melanoma distinguishes four main subtypes: 1.) superficial spreading melanoma (SSM) as the most common subtype responsible for approximately 75% of all melanoma cases, 2.) nodular melanoma (NM), the second most frequent subtype, 3.) lentigo maligna melanoma (LMM) frequently occurring on skin exposed to chronic solar radiation and 4.) acral lentiginous melanoma (ALM) developing on the skin of palm and sole, under the nails or perhaps the genital and oral mucosa. Besides the classic subdivision, nowadays, another scheme is emerging based on the molecular characteristics of tumors. This scheme distinguishes two types of melanoma on non-glabrous skin: 1.) melanomas on non-chronically sun-damaged skin (non-CSD) with frequent BRAF, along with the loss of chromosome 10q (including PTEN) and no KIT mutations or mutations in the NRAS gene alone, and 2.) melanomas on CSD skin with infrequent BRAF mutations and KIT mutations in approximately 20%. Melanomas developing on glabrous skin or under the nails were considered as acral melanomas based on the genetic features such as oncogene amplifications and mutations or amplification of KIT in 40% of cases. Finally, mucosal melanomas were proposed as separate entity based on the patterns of chromosomal aberrations.
Considering the main factors which influence tumor prognosis, a new and uniform staging system, the TNM (tumor - lymph node - metastasis) system was established based on the recommendations of a number of international organizations. The main advantage of this classification method is the introduction of the sentinel lymph node biopsy which reduces the removal of local lymph nodes to prevent metastasis formation.

**Melanoma etiology and the main risk factors**

Malignant melanoma is featured by multifactorial etiology and arises through the interplay of both genetic and environmental factors. Owing to the intensive research in the last decades, several risk factors have been revealed as important exogenous or endogenous factor for the development of malignant melanoma. Exogenous factors include the ultraviolet (UV) exposure, rising constantly due to the ozone depletion and usage of tanning beds and the multiple sunburns in a lifetime. Endogenous factors include errors in natural photoprotection and DNA damage repair mechanisms, photosensitive drugs and cosmetic ingredients, a family history or DNA polymorphisms and somatic mutations. Acute sun exposure leading to sunburn plays a crucial and supporting role in most melanoma tumors, and is correlated with the fair-skinned population. Such exposures during childhood may be particularly important. The environmental human carcinogen present in sunlight is the ultraviolet (UV) irradiation, mainly the short-wavelength UVB (280-320 nm) component. UVA (320-400 nm) has a smaller contribution to melanoma genesis. Subsequent to the development and recognition of the disease, prognosis depends on the Breslow thickness, tumor surface ulceration, number of infiltrated lymph nodes and infiltration rate (micro- or macro metastasis). Prolonged survival and successful therapies are usually more characteristic of women.

**Molecular background of malignant melanoma**

Malignant melanoma is featured by a high genomic heterogeneity. Several molecular pathways and the complexity of their interaction are involved in disease development and progression. This characteristic suggests that no individual genetic or molecular alteration is crucial in these processes per se. The accumulation of such alterations in combination with the crosstalk between the tumor cells and their microenvironment are involved in the generation of a specific set of clinical-biological outcomes. These findings suggest that molecular features may help to refine the currently existing classification schemes.

It is well-known that approximately 10% of melanoma patients have a familial predisposition. The major germline alterations are proved to be the single-base mutations and
deletions of CDKN2A, contributing to 10% to 40% of familial cases depending on the geographical location. Mutations harboring the CDK4 gene, and some unidentified genes on chromosomes 1p22 and 20q11.22 have also been observed. Recent studies focus on the melanoma susceptibility based on single nucleotide polymorphisms (SNP), and revealed some genes involved in DNA repair or oxidative stress pathways, which are significantly associated with a higher risk of cutaneous melanoma.

Several signaling pathways have been identified as key regulator in \textit{de novo} melanoma progression. Some of them are already known such as p16INK4a-CDK4/6-RB, p14ARF-MDM2-p53 pathways contributing to cell cycle deregulation and the sensitization by p16 error to oxidative stress by UV-induced DNA damage. The RAS/MAPK or PI3K/AKT pathways are the main targets of the receptor tyrosine-kinase signaling with a crucial role in melanoma genesis, regulating tumor proliferation and survival. Through molecular interactions between the foregoing cascades they can also enhance tumorigenesis, cellular growth, chemoresistance, invasion, migration and cell cycle dysregulation. Besides, the canonical WNT-\(\beta\)-catenin and MSH-PKA-MITF signaling also contribute to melanoma survival/proliferation and pigmentation, respectively. Some other pathways are recently recognized as important. The Notch1 signaling has a mixed tumor suppressor and oncogene effect on cancer progression, depending on the cell-cell interactions and the extracellular environment. Moreover, the iNOS cascade contributes to the elimination of reactive oxygen and nitrogen species.

Our previous array CGH studies revealed many chromosomal regions altered in aggressive melanoma tumors such as 7q31 and 11q13 loci. The 7q31 region contains the FRA7G common fragile site neighbored by several genes like CAV1 and TES as possible targets of genetic alterations during tumorigenesis. CAV1 as the main component of membrane lipid rafts can play a significant role in many aspects of the cell life through interactions with other proteins, including EGFR, H-RAS and eNOS. However, its role in cancer progression is ambiguous because CAV1 can have both apoptotic and anti-apoptotic behavior, depending on the tumor type and stage. TES is an important member of focal adhesions, influencing tumor cell motility, and behaves as a suppressor molecule. The 11q13 locus contains the CCND1 oncogene, which plays a significant role in the G\(_1\)/S phase transition of the cell cycle. The transcriptional activation of CCND1 is primarily carried out by tyrosine kinase receptors and the Ras–MAPK signaling cascade. In addition, the PI3K–Akt–mTOR cascade; stimulation
through several cytokines, transcription factors, and extracellular matrix proteins; and reduced protein degradation also have an important role in the increased CCND1 levels.

Identification of novel genetic alterations, their concerted action with epigenetic alterations in regulating gene expression, and signaling pathways contributing to cancer progression and malignant transformation could provide new opportunities for cancer treatment.

**Current and novel targeted therapies in melanoma**

The most effective therapy against melanoma is still the surgical resection of primary tumors, followed by the observation of a sentinel lymph node. Dacarbazine (DTIC) became the first chemotherapeutic agent approved for the treatment of metastatic melanoma with a response rate of 15%-25%, but the complete response was only 5%. High dose interferon-based adjuvant systemic therapy and adjuvant radiation treatment in given conditions have also been applied in high-risk melanomas to combat the disease with more or less success. However, over the past few years, there have been three novel agents approved for treatment of melanoma by the US Food and Drug Administration with many limitations, but providing an excellent basis for the future therapies:

1. **vemurafenib (PLX4032)**, a BRAF inhibitor for unresectable or metastatic melanomas bearing $\text{BRAF}^{V600E}$ mutation
2. **ipilimumab**, an immune stimulatory agent for treatment of unresectable or metastatic melanomas
3. **pegylated interferon alpha-2b** for treatment of stage III melanomas

Vemurafenib (PLX4032) is a highly selective BRAF inhibitor, and is capable of silencing mutant BRAF with little effect on the wild-type protein. Unfortunately, vemurafenib therapy is limited by a relatively short duration (averages around 6 months) due to the early resistance to the drug, and has an important toxic effect by acceleration of the growth of cutaneous squamous cell carcinomas (SCC) and keratoacanthomas in about 20% of treated patients. Whereas ipilimumab is an immunostimulatory antibody against the negative costimulator antigen-4 of cytotoxic T-lymphocytes (CTLA4), and presumed to be a potential combination or monotherapy treatment of melanoma and several other cancers. However, the response rate and improvement in overall survival is relatively modest, and many adverse events can be administered, including immune-related enterocolitis, hepatitis, and dermatitis. There are many other targeted therapies (e.g. imatinib, lapatinib, anti-VEGF treatments or the improvement of immunotreatments) in clinical trials which may have an impact in the future melanoma treatment, perhaps in combination with molecular targeted therapies. However,
despite recent advances in immunotherapy and targeted therapy, treatment options are still limited. Therefore, the discovery of agents with novel mechanisms of action would be urgent to be able to treat this challenging malignancy.
Objectives

The cytogenetic heterogeneity and aggressive metastatic behavior of human cutaneous melanomas result in serious problems not just in the classification of histologically ambiguous primary tumors or differentiation of melanomas from other lesions, such as Spitz, blue or proliferating congenital nevus, but in the prognostication and effective cancer treatment as well. Deeper understanding of the molecular alterations behind tumorigenesis and progression and evaluating the known molecular targets will be crucial in predicting subsets of melanoma patients with particular biologic features who may respond favorably to a therapy that specifically targets a characteristic molecular signature. Therefore, our research focused on the identification of alterations that are characteristic for melanomas with different biologic behavior. We also aimed to evaluate alterations with possible diagnostic and prognostic value.

Specific objectives of the recent study were the followings:

Analysis of the 7q31 region:

1. to evaluate genetic alterations of 7q31 locus using interphase FISH in human primary melanomas and corresponding lymph node metastasis, and in melanoma cell lines with different biologic behavior, and to correlate the results with clinical-pathological parameters of primary tumors.

2. to define mRNA and protein level changes of CAV1 and TES genes both located at 7q31 in primary lesions and cell lines by qRT-PCR and immunohistochemistry on tissue microarrays, and to correlate the results with clinical-pathological parameters of primary tumors.

3. to define the possible connection between locus alterations and gene expression changes.

Analysis of the CCND1 gene:

1. to evaluate genetic alterations of the CCND1 oncogene in primary melanomas using interphase FISH, and to correlate the results with clinical-pathological parameters of primary tumors and mutation state of BRAF and NRAS genes.

2. to define mRNA and protein expression changes of CCND1 in primary lesions by qRT-PCR and immunohistochemistry on tissue microarrays, and to correlate the results with clinical-pathological parameters of primary tumors, besides the expression and mutation state of BRAF and NRAS genes.
Materials and methods

Melanoma tissue samples
Melanoma tissues were obtained from the Department of Dermatology at the University of Debrecen (Debrecen, Hungary) from patients who did not undergo therapy prior to surgical removal of their primary lesions. This study was approved by the Regional and Institutional Ethics Committee of the University of Debrecen and was performed according to the relevant regulations. Written informed consent was obtained from the patients. Lesions were diagnosed on the basis of formalin-fixed paraffin-embedded (FFPE) tissue sections stained with haematoxylin-eosin and classified based on the latest edition of TNM staging system. Examinations of the 7q31 locus and CCND1 were not performed at the same time, thus the studied sets of melanoma samples were not in complete overlapping.

Melanoma cell lines and cell culturing
The following human melanoma cell lines were used in our experiments:

- **WM35**: derived from the RGP, non-metastatic superficial spreading melanoma of a 24-year-old woman. Breslow thickness of the scapula/neck localized tumor was 0.69 mm.
- **WM983A**: derived from the VGP, aggressive metastatic primary melanoma of a 54-year-old man. Breslow thickness of the abdomen localized tumor was 25 mm.
- **WM983B**: derived from the lymph node metastasis of the previous patient’s primary tumor.
- **A2058-HT168-HT168M1**: Parts of a melanoma model system. The original A2058 cell line is derived from the amelanotic melanoma lymph node metastasis of a 43-year-old man. HT168 was derived from A2058 xenografted to nude mice. Whereas HT168M1 was originated from the *in vivo* adaptation of HT168 to the lien of immunosuppressed mice.
- **HT199**: derived from the RGP, metastatic nodular melanoma of a 23-year-old woman. The tumor was able to form liver metastasis in SCID mice.

All cell lines were maintained in RPMI 1640 medium supplemented with fetal bovine serum, L-glutamine and antibiotics. Monolayer adherent cells were treated with trypsin/EDTA solution and washed twice with PBS (pH 7.2). Cell pellet was then treated with KCl (hypotonic treatment), dropped on slides and fixed with Carnoy’s fixative. The slides were stored at -20°C until use. All chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich, Inc., St. Louis, MO, USA). WM35, WM983A and WM983B cell lines were obtained from
the Coriell Cell Repositories (Coriell Institutes, Camden, NJ, USA), A2058 cell line was obtained from the American Type Culture Collection (LGC Standards GmbH, Wesel, Germany), while HT168, HT168M1 and HT199 melanoma cell lines were generous gifts from the National Institute of Oncology (Budapest, Hungary).

Array comparative genomic hybridization
Array CGH experiments were performed on HumanArray 3.1 (University of California, San Francisco Cancer Center array Core, USA). Labeled DNA was mixed with Cot-1 DNA. Hybridization, imaging setup, and analysis were performed as described elsewhere.

Chromosome preparation from normal lymphocytes
Chromosome spread of normal lymphocytes from healthy donors was prepared using EuroClone Chromosome kit „P” (EuroClone S.p.A., Milano, Italy) according to the manufacturer’s protocol. Cytoplasm-free suspension was dropped onto wet slides, air-dried and stored at -20°C.

DNA specific probes
DNA specific probes applied in FISH experiments were the followings:
- Chromosome 7 centromere-specific / 7q31 locus specific DNA probes
- Chromosome 11 centromere-specific / CCND1 gene specific DNA probes
DNA specific probes were obtained from Vysis, Inc. (Abbott Molecular, Des Plaines, IL,USA). Centromere-specific probes were labeled with Spectrum Green conjugated dUTP (green fluorescence), whereas locus and gene specific probes were labeled with Spectrum Orange conjugated dUTP (red fluorescence). Nuclei were counterstained with diaminofenilindol (DAPI, blue fluorescence) dissolved in antifade solution (Vectashield, Vector USA). Reliability of the DNA specific probes was tested on chromosome spreads derived from normal lymphocytes.

Fluorescence in situ hybridization
Experiments were performed on imprint preparations produced from fresh-frozen tumors using slides treated with 3-aminopropyltrimethoxy-silane (Sigma-Aldrich Inc., St. Louis, MO, USA) according to the manufacturer’s protocol. The probe mixture and target cells were denatured simultaneously and hybridized in a hybridization chamber (HYBrite). Aspecifically or non-binding DNA probes were removed by washing buffer. Nuclei were labeled with
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DAPI in antifade solution. Fluorescence signals were scored in approximately 100-200 cells per specimen using a fluorescence microscope (ZEISS Axioplan, Carl Zeiss, Jena, Germany). Three-color images were captured using a digital imaging analysis system (ISIS Metasystem GmbH, Altlussheim, Germany). Copy number index (CNI) and categories of the 7q31 locus and CCND1 copy number alterations were defined as follows:

1. Deletion: copy number of locus/gene is less than the copy number of corresponding chromosome centromere in ≥10% of cells.
2. Apparent amplification: copy number of the chromosome is equal to the locus/gene copy number, but more than 2.
3. Low-level amplification: gene copy number is maximum 5-times higher than the copy number of the chromosome, but does not exceed 10 copies per cell.
4. High-level amplification: gene copy number is more than 5-times higher than the copy number of the chromosome, and exceeds 10 copies per cell in ≥10% of cells.

CNI was defined as total number of fluorescent signals / number of cells counted in one sample. For statistical interaction and logistic regression analyses of CCND1 results, we defined two groups based on the calculated CNI: no CCND1 amplification (CNI ≤ 2.5) and CCND1 amplified (CNI > 2.5). The optimal cutoff value was defined by ROC curve analysis.

RNA extraction and real time qRT-PCR

Total RNA was isolated using RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and quantified by NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). TaqMan one-step RT-PCR was used to determine the mRNA levels of CAV1, TES, BRAF, NRAS and CCND1. Each reaction was run in triplicate on an ABI-PRISM 7000 instrument (Life Technologies Corporation, Carlsbad, CA, USA). Reverse transcriptase, Master Mix and Assays were obtained from Life Technologies. Data were analyzed using Livak method, with GAPDH as reference gene and nevi collected from three different individuals or cultured melanocyte as calibrator samples.

Tissue microarrays and immunostaining

Tissue sections of the original blocks were first stained with haematoxylin-eosin and then reviewed by pathologists to select the area to be punched automatically using a TMA Master (Carl Zeiss, Jena, Germany). Most cases were represented using three 1-mm tumor cores taken from the original blocks. Serial sections were excised from the TMA blocks and used...
for immunohistochemical investigations following haematoxylin-eosin validation of the sample spots. After deparaffinization and blocking of endogenous peroxidases and non-specific binding sites, we retrieved antigens in boiling citrate buffer (pH 6). Anti-HMB45, anti-CAV1 and anti-TES primary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), whereas anti-CCND1 antibody was obtained from Thermo Fisher Scientific (Waltham, MA, USA). After incubation with the primary antibodies, the samples were stained using the Envision/HRP detection system (DAKO Inc., Glostrup, Denmark) and the VIP peroxidase substrate kit (Vector Laboratories LTD, Peterborough, UK). Nuclei were counterstained with methyl green (Vector Laboratories LTD, Peterborough, UK). Primary antibodies were omitted in negative controls. Positive controls were as follows: A541 human lung cancer cell line and endothelial cells as controls for CAV1, Jurkat cells as control for TES and MCF7 human breast cancer cell line as control for CCND1. The slides were digitized using MiraxScan slide scanning device (Carl Zeiss, Jena, Germany). Digital images were displayed using MiraxViewer software (Carl Zeiss, Jena, Germany) and evaluated by two dermatopathologists who were blind with respect to the sample data. Reaction positivity of each of the examined proteins (CAV1, TES and CCND1) was scored on a four-grade scale: 0, no staining; 1+, definite but weak staining; 2+, moderate staining; and 3+, strong staining. Scores for triplicate cores were consolidated to a single value per case. In the case of CCND1 we also used the quickscore method, considering both the staining positivity and the percentage of positive cells. Additive quickscore is defined by the addition of category A (proportion of stained cells) to category B (intensity of staining), whereas category A is multiplied by category B in the multiplicative quickscore method. Tumors were then categorized into groups with scores from 0 to 2. Using the additive quickscore method, scores were assigned to groups as follows: 0, scores 1-3; 1, scores 4-6; and 2, scores 7-9. In the multiplicative quickscore method, scores were assigned to groups as follows: 0, scores 0-6; 1, scores 7-12; and 3, scores 13-18. As the different quickscore approaches showed a strong positive correlation (R=0.996, p<0.0001, Spearman correlation), we used the multiplicative method for further analysis because the additive approach did not permit negative results, which we found in 13% (7/54) of cases, and the scoring range was wider for the multiplicative method.
Protein extraction from melanoma cell lines and western blot analysis

Three melanoma cell lines with different biologic behavior (WM35, HT199 and WM983B) were subjected to western blot analysis. Cells were harvested in RIPA buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). The total protein concentrations were measured using the Bradford assay (Bio-Rad Hungary Ltd., Budapest, Hungary). The isolated proteins were then stored at -80°C. Denatured and reduced (2-β-mercaptoethanol) samples were separated on 5-14% gradient SDS-polyacrylamide precast gels, semi-dry transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Hungary Ltd., Budapest, Hungary) and labeled with specific primary antibodies. Anti-GAPDH (used as a loading control), anti-CAV1 and anti-TES primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), whereas anti-CCND1 antibody was obtained from Thermo Fisher Scientific (Waltham, MA, USA). For signal detection, a peroxidase-conjugated secondary antibody was added, followed by exposure using enhanced chemiluminescence (ECL, GE Healthcare, IL, USA). The immunoblots were quantified using a GS-800 Calibrated Densitometer (Bio-Rad Hungary Ltd., Budapest, Hungary), and protein levels were compared.

Mutation analysis of BRAF and NRAS genes

G-Spin Genomic DNA Extraction Kit (iNtRON Biotechnology, Kyunggi, Korea) was used to isolate genomic DNA from fresh-frozen melanoma tissues. The obtained DNA was quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer. DNA quality was determined by 1.2% agarose gel electrophoresis. Mutation analysis of BRAF\textsuperscript{V600} and NRAS\textsuperscript{Q61} was performed with a LightCycler real-time PCR system (Roche Diagnostics, GmbH, Mannheim, Germany) via melting curve analysis using fluorescent probes (TIB Molbiol, Berlin, Germany). The sensor probe was conjugated by fluorescein, whereas the anchor probe was conjugated by LCRed640. The results were confirmed by direct sequencing of PCR products (BIOMI Kft, Gödöllő, Hungary).

Statistical analysis

SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and R-2.15.0 were used for statistical analysis. The Shapiro-Wilk test was applied to control the normality of the data, and Leven’s test was used to control the equality of variances. Because of the abnormal distribution of the data, non-parametric tests were applied for statistical analyses. When necessary, data were normalized by reciprocal (CNI) or \log_2 (mRNA level) transformation. Receiver operating characteristic
(ROC) curve analysis was used to determine the optimal cutoff value of CCND1 CNI used in survival tests. The model included the known melanoma prognostic factors such as ulceration and metastasis formation. Fisher’s exact, Kruskal-Wallis, Mann-Whitney-Wilcoxon tests, and logistic regression were used to compare the 7q31 and CCND1 alterations to the corresponding chromosomes (7 and 11, respectively) copy numbers and the clinical-pathological data of the primary tumors. Statistical interactions (univariate analysis of variance) were also examined to determine whether the mutational state of BRAF or NRAS modifies the influence of CCND1 gene alterations on the clinical-pathological data of the primary tumors. To analyze the relationship between the gene expression data of CAV1, TES or CCND1 and the clinical-pathological parameters of melanoma patients, we used the Kruskal-Wallis and Mann-Whitney-Wilcoxon tests. To correlate the mRNA levels of CCND1, BRAF and NRAS, Pearson’s correlation coefficient was calculated. The Spearman test was used to define the correlation between the additive and multiplicative quickscore methods. The Kaplan-Meier, Log Rank and Cox regression tests were used for survival analysis. A p value of less than 0.05 was considered statistically significant. In agreement with literature norms, changes in the gene expression level of more than two-fold were considered to represent under- or overexpression.
Results

CAV1 and TES genes on 7q31 as candidate prognostic markers of human melanomas

Correlation of 7q31 alterations and the clinical-pathological parameters of primary tumors

Seventy-five primary melanoma samples were analyzed by FISH. The calculated 7q31 locus CNI was correlated with the copy numbers of chromosome 7 (p<0.001), and was significantly higher in primary tumors with metastatic property (p=0.012) and ulcerated surface (p=0.002). Patients older than 50 years were characterized as having a higher number of 7q31 copies compared to patients in the younger age group (p=0.038).

CAV1 and TES mRNA and protein expression in primary melanomas

Thirty-three primary melanomas were available for mRNA expression analysis of the CAV1 and TES genes. CAV1 mRNA level was significantly lower in thick tumors (p=0.002), whereas TES mRNA levels were different in the primary tumors depending on the location of the corresponding metastasis (p=0.011).

We could examine the protein expression levels of CAV1 and TES genes in 60 primary melanomas using tissue microarray. The examined nevus samples did not show CAV1 and TES positivity. Melanoma tissues in general showed strong membrane and weak, diffuse cytoplasmic CAV1 immunostaining. The TES protein often formed plaques in the membrane, however, weak plasma positivity was also observed. CAV1 protein expression was detected in 83% (41/51) of primary lesions. The remaining 10 samples did not show any CAV1 immunostaining. Breslow thickness was the only parameter showing different CAV1 protein levels between the two groups (p=0.038). Thicker melanomas have a reduced CAV1 expression; however, it is important to note that a subpopulation of the thinner lesions exhibited no protein staining. TES expression was found in 76% (42/55) of primary lesions. However, we did not find any significant associations between the TES protein expression and the clinical-pathological parameters of the primary melanomas.

Correlation between 7q31 genetic alterations and mRNA and protein expression changes of CAV1 and TES in primary melanomas and melanoma cell lines

In case of 14 primary melanomas we had the possibility to analyze the 7q31 copy number alterations and the expression levels for both CAV1 and TES genes in the same tumor
samples. The results clearly show that amplification of the locus is not or weakly correlated with mRNA and protein expressions of CAV1 and TES genes.

We also analyzed 7 melanoma cell lines (WM35, WM983A, WM983B, HT199, A2058, HT168 and HT168M1) with different biological behavior for 7q31 locus copy number aberrations, mRNA and protein (CAV1 and TES) expressions. Low-level amplifications and deletions were present in all cell lines with different frequencies. Locus deletion was observed with high frequency in three (WM983B, HT199 and WM35), low level amplification was present in two (WM983A and HT168), whereas high level amplification was detected in two cell lines (A2058 and HT168M1). The relative mRNA expression of CAV1 was similar in the WM35, WM983A, WM983B, HT199 and HT168 cell lines. A significant association between high level amplification of the locus and the relative mRNA level was observed for two aggressive cell lines (A2058 and HT168M1). All cell lines showed CAV1 protein expression, but the level was different. High protein expression was found in the invasive but not metastatic WM35 and the metastatic WM983B, HT199, A2058, HT168 cell lines and low level expression in WM983A. No CAV1 protein expression was observed in HT168M1. The mRNA level of TES was higher in the cell lines compared to the mRNA expression in melanocytes and was associated with strong protein expression as detected by immunohistochemistry. In case of three melanoma cell lines (WM35, HT199 and WM983B) we confirmed protein expression results using western blot analysis.

**CCND1 as a prognostic marker of human cutaneous melanoma**

**CCND1 copy number alterations in primary melanomas and association with the mutations of BRAF and NRAS genes**

Seventy-six primary melanoma samples were analyzed by FISH. The calculated CCND1 CNI was correlated with the copy numbers of chromosome 11 (p=0.008), and was significantly lower in primary tumors located at the extremities (p=0.043) and without ulceration (p=0.048) and increased in primary melanomas with distant organ or multiple metastases (p=0.029).

Logistic regression analysis also showed an increase of CCND1 amplification in melanomas with ulceration [odds ratio (OR) =2.934; 95% confidence interval (CI) =1.155–7.454; p=0.024] and multiple metastases (OR=11.538; 95% CI=1.297–102.654; p=0.028). Analysis of the association between CCND1 copy number alterations and survival after correction of the known predictors of poor outcome such as ulceration, Breslow thickness, and metastatic potential revealed that patients with CCND1-amplified melanoma (CNI >2.5
RESULTS

Based on ROC analysis) had a 1.6-fold higher risk of death from the disease, though this association was not significant (p=0.278) probably due to the small amount of data.

BRAF mutations at codon 600 were present in 24 out of 55 primary melanomas (44%). Of these, 21 lesions (88%) carried V600E mutation, 2 tumors were V600K mutant (8%), and only one melanoma carried V600R mutant (4%) BRAF gene. NRAS mutations at codon 61 were present in 8 out of 55 primary melanomas (15%). Of these, five lesions (63%) carried Q61K mutation, two tumors were Q61R mutant (25%), and only one melanoma carried Q61L mutation (2%). No sample was found with both BRAF and NRAS mutations in the same tumor. We handled the different types of BRAFV600 and NRASQ61 mutations together because of the relatively low number of cases within one type and the fact that mutations affecting the same molecular pathway were mutually exclusive.

Examination of the interactions between CCND1 copy number alterations and mutations in BRAF and NRAS revealed no common effect on any of the clinical–pathological parameters. After correction for BRAF and NRAS mutation status, we found that CCND1 amplification was still associated with a higher frequency of ulceration (OR=3.185; 95% CI=1.025–9.896; p=0.045) and multiple metastases (OR=7.817; 95% CI=0.753–81.175; p=0.085). However, the latter association was not significant, probably due to either the wide confidence interval or the relatively low sample number involved. Melanomas showing CCND1 amplification but without BRAF and NRAS mutations developed 4.4 times more frequently on sun-exposed skin (95% CI=1.195–16.491, p=0.026).

**CCND1 mRNA expression in primary melanomas**

Gene expression analysis of CCND1 was performed in 28 primary melanomas. A greater than twofold decrease in the CCND1 mRNA level was found in 39% (11/28) of these melanomas, whereas overexpression was observed in only 18% (5/28) of the examined samples. In determining the relationship between gene expression levels and clinical–pathological parameters, we found that the pattern of CCND1 mRNA expression significantly affected the location of metastasis formation (p=0.013). Samples with multiple metastases (33%) were characterized by reduced CCND1 mRNA levels, whereas tumors that formed only cutaneous and/or lymph node metastases (40%) showed a moderate increase in CCND1 expression, and melanomas with lung, brain, bone, or liver metastases (27%) did not show differences in CCND1 mRNA levels compared to nevus samples.
RESULTS

CCND1 expression changes were moderately correlated with the BRAF (B=0.553, p=0.002) and NRAS (B=0.678, p<0.001) mRNA levels. Furthermore, the expression of BRAF showed a remarkably strong association with the expression of NRAS (B=0.832, p<0.001). The CCND1 mRNA level was significantly associated with the mutation state of BRAF and NRAS genes (p=0.009, two sided exact Mann–Whitney–Wilcoxon test). Samples without BRAF or NRAS mutations exhibited an approximately 2.3-fold higher CCND1 expression level relative to the melanomas bearing BRAF or NRAS mutations. Examining the association between the CCND1 protein level and mRNA expression, we did not find a significant correlation.

CCND1 protein expression in primary melanomas and melanoma cell lines

We examined CCND1 protein expression levels in 54 primary melanomas and three human melanoma cell lines exhibiting different biological behaviors. Melanomas showed strong nuclear and, in some cases, weak plasma CCND1 expression, but only nuclear CCND1 staining was considered to be specific. We determined that 87% of the melanoma samples were positive for CCND1. High intratumor heterogeneity was not characteristic of the examined tissues. CCND1 positivity showed a significant correlation with Breslow tumor thickness, metastasis formation, and patient survival (p=0.047, p=0.028, and p<0.001, respectively). Melanoma samples presenting a greater thickness (>4.0 mm), metastasis, and exitus showed stronger CCND1 staining. Examining the association between the CCND1 protein level and average CNI, no significant correlation was found.

Three melanoma cell lines with different biological behaviors were also analyzed for CCND1 protein expression using immunohistochemistry and western blot techniques. Strong CCND1 protein expression characterized the HT199, which is an aggressive cell line derived from a primary tumor. In contrast, the invasive but non-tumorigenic (in nude mice) WM35 cell line exhibited less CCND1 protein expression. The melanoma metastasis-derived WM983B line exhibited the lowest CCND1 protein level. Western blot analysis confirmed the immunohistochemical results.
Discussion

Although the incidence of malignant melanoma has increased over the last few decades, the accuracy of clinical diagnosis is limited, and surgical resection in combination with observation of a sentinel lymph node is still the most effective therapy against this malignancy. Beside the increasing incidence, occurrence of melanoma can also be remarkable in younger age groups. Early detection and surgical removal of primary lesion are currently the only chance for the permanent cure of melanoma patients. Patients’ survival decrease dramatically with the appearance of metastasis, and in the presence of distant metastases the five-year survival falls below 10%. It is well known that similarly to other solid tumors, cutaneous melanoma is a genetic disease caused by the accumulation of several genetic alterations during cancer development and progression, affecting regulatory mechanisms of genome integrity on the molecular level, which is consequently resulted in the activation of oncogenes and inactivation of tumor suppressor genes. Therefore, understanding the molecular alterations during tumor progression may contribute significantly to the classification, particularly in cases of histologically ambiguous or difficult-to-identify primary melanomas, moreover, may improve melanoma diagnosis and prognostication or may become an effective therapeutic target in the future.

Associations between the alterations of 7q31 locus and melanoma progression

In this study one of our aims was to define 7q31 locus copy number aberrations in relatively large number of primary melanoma samples using FISH, and correlate the genetic results with the clinical-pathological parameters of patients. The strength of this study is that beside the copy number changes at site of the large fragile site, FRA7G, we also determined the mRNA and protein expression levels of the important locus-related genes (CAV1 and TES) surrounded the fragile site and defined their role during melanoma progression. Common fragile sites (CFSs) are non-randomly positioned, large (megabase or more) regions in all individuals’ genome showing site-specific gaps, breaks on metaphase chromosomes after partial inhibition of DNA synthesis. These sites are parts of the normal chromosomal structure and normally stable in somatic cells. However, CFSs and associated genes are frequently deleted, rearranged or amplified in many tumor cells reflecting their importance in genome instability of cancers. Their genetic instability is due to their special structure: these sequences are G-band characteristics within an R-band site. This discrepancy between CFSs and their flanking region may affect the control of DNA replication and chromatin condensation, and
DISCUSSION

thus contribute to their fragility. Interestingly, some obvious similarities like large size or parallel gene expression profile between CFSs suggest that these sites and their associated genes may play an important role in a normal protective response of cells to environmental and cognitive stress.

FRA7G is a common aphidicolin-inducible fragile site at 7q31.2 locus, which is the fourth most frequently expressed one after FRA3B (containing FHIT tumor suppressor gene), FRA16D (containing WWOX tumor suppressor gene) and FRA6E. This chromosomal region has an extent of 544 kb containing several genes like TES which is prone to viral integration, and CAV1 which is often amplified in some solid tumors. Similarly to others, our research group have noticed the deletion and also amplification of the 7q31-qter in cancer cells by high-throughput techniques. Locus deletion may be resulted by discrete homozygous intra-locus deletions or translocations. Whereas gene amplifications on FRA7G possibly via breakage-fusion-bridge mechanism are essential features of advanced cancers and have prognostic as well as therapeutic significance in clinical cancer treatment. Accumulation of intrachromosomal amplicons might responsible for drug-resistance of cancers. We proposed that the instability-induced alterations of the FRA7G, disturbing the normal function of the associated genes, contribute to melanoma progression observed as FHIT and WWOX tumor suppressor genes (TSGs) in many human cancers through genetic and epigenetic mechanisms, such as promoter hypermethylation or histone hypoacetylation.

Using FISH, we found deletions and different levels of amplification of the 7q31 locus. Deletion was associated with a favorable prognosis in contrast to the amplification that resulted in a poor clinical outcome and made primary tumors liable to form metastases. Increased CAV1 protein level statistically showed a high association with tumor thickness in several cases. The influence of TES expression during melanoma progression requires more detailed investigation.

As previously found, CAV1, a multifunctional scaffolding protein has a controversial role in the development of several human tumors. The upregulation of CAV1 gene expression was associated with an elevated level of CAV1 protein, and had an influence mainly on tumor thickness in our melanoma samples. On the other hand, downregulation of CAV1 was also observed in a subpopulation of thinner samples and melanoma cell lines with poor clinical outcome suggesting that CAV1 may play a dual role in melanoma progression. Furthermore, the lack of CAV1 protein in the HT168M1 cell line, despite the high CNI and mRNA level, raises an issue that it would be worthwhile to extensively examine the epigenetic processes
affected this molecule. These are important observations, because it has recently been tested that CAV1 protein is a potential biomarker for melanoma prognosis, since it contributes to tissue invasion and metastasis formation. The reduced expression of CAV1 may be associated with an increased proliferative ability of tumor cells or the loss of tumor cell apoptosis through caspase-3 consequently leading to the development of metastatic disease. Furthermore, a recent melanoma study provided evidence for the possible anti-metastatic function of the CAV1 protein. Total loss of the protein may result in decreased tumor neovascularisation, tumor suppression through the inhibition of cytokine receptor signaling or diminished tumor cell motility through the regulation/stabilization of focal adhesion domain organization. It is also possible that CAV1 protein has a dose-dependent effect on cancer cells. Another reason might be that CAV1 has multiple interacting partners; therefore, the alterations of these interacting molecules may have a significant impact on CAV1 function during cancer progression as it can be found in thyroid cancers where Galectin-3, an accurate marker for the diagnosis of differentiated thyroid cancer, can override the tumor suppressor function of CAV1. Gal-3, in concert with CAV1, is able to promote focal adhesion turnover, tumor cell migration and invasion. Finally, a recent study showed highly important difference in the function of the two caveolin 1 variants: the full-length form CAV1 α (wild-type CAV1) and the truncated form CAV1 β, which latter variant lack of a Tyr-14 region at the N terminus. In the case of CAV1 α, the phosphorylation of Tyr-14 can facilitate mitochondrial apoptosis, and consequently increases the sensitivity of breast cancer cells to paclitaxel. Therefore, CAV1 composition of the tumor cells may have an effect on drug resistance.

Downregulation of TES, which has previously been observed in head and neck squamous cell carcinomas, gastric cancers and breast cancers at both mRNA and protein levels, was frequently found in our primary melanoma samples. Although TES did not exhibit significant association with metastasis formation, the mRNA level was significantly altered between lesions with different preference of site to form metastasis, and might have a predictive value in the future. These observations support the idea that TES may play an important role in primary melanomas. However, further studies, including metastatic melanomas, are required to clarify the exact role of TES in cancer progression.

The deletion of 7q31 locus was followed by the downregulation of the CAV1 and TES genes in some cases, but we did not find significant associations. One reason could be the relatively small available set of paired samples to perform the correlation analysis. Another reason can be that we observed only heterozygous 7q31 deletion; therefore, the remaining
copy may make uninterrupted mRNA synthesis possible. However, amplification might not affect the entire locus, and the size of the altered region is difficult to determine. It is also difficult to define whether the alterations are included in a definite gene. Furthermore, an error in the epigenetic regulation of genes could also affect the mRNA and protein levels.

In conclusion, 7q31 amplification is associated with unfavorable prognosis and reduced CAV1 protein level could have an impact on melanoma cell invasion. Whereas TES may play a part in the anchoring of circulating tumor cells, defining the location of metastasis. This study shows that in the future it is worthwhile to examine deeper the role of these genes in melanoma genesis. Because cancer development is a complex process that alters a large number of genes and molecular pathways, it is also important to consider these alterations on the basis of their own complexity.

Associations between the alterations of CCND1 gene, BRAF and NRAS mutation status and primary melanoma progression

Gast et al. have identified two distinct molecular pathways in melanomas: the first group involved melanomas presenting BRAF and NRAS mutations in combination with alterations in the CDKN2A and PTEN tumor suppressor loci, a molecular profile that was mainly connected to sun-exposed areas of the skin; the second group included lesions bearing amplifications of a number of oncogenes, such as MYC or CCND1, together with loss of the 13q and 16q regions. These newly revealed molecular characteristics can potentially provide crucial guidance in predicting subsets of melanoma patients who may respond favorably to therapies that specifically target a characteristic molecular signature.

CCND1 gene copy number changes together with RREB, MYC and chromosome 6 copy number alterations are widely tested as diagnostic and classification tools of melanocytic lesions applying FISH method. Previous genetic studies have been conducted to address the role of CCND1 in melanoma progression and have frequently found CCND1 gene amplifications in acral melanomas. Furthermore, lesions on skin with chronic sun-induced damage showed CCND1 copy number gains compared to tumors from skin without such damage, which was inversely correlated with BRAF mutations. As CCND1 is an important transcriptional target of several well-known and novel molecular pathways affected in melanoma progression, the aim of the present part of our study was to examine copy number alterations and expression changes in the CCND1 gene in primary cutaneous melanomas also in relation to the mutational and expression status of the BRAF and NRAS genes. We aimed
to evaluate whether CCND1 amplification could be a prognostic factor in cutaneous melanoma independently from the mutation status of BRAF and NRAS genes.

According to the American Joint Committee on Cancer, Breslow tumor thickness and tumor surface ulceration are the most effective independent predictors of survival. Our results showed that CCND1 genetic alterations and expression changes strongly affect metastatic potential, Breslow thickness, survival, ulceration, and the mode of metastasis formation, with the last association being shown for the first time in the present study. Melanomas with multiple metastases showed a significant increase in CCND1 copy number and decrease in CCND1 mRNA level. Although in the case of mRNA expression, further examinations are needed because of the relatively low number of samples analyzed. Predicting the preferred site of metastasis for a given tumor on the basis of molecular alterations could have a remarkable prognostic impact in the future. Examination of the correlation between CCND1 genetic alterations and mRNA and protein expressions did not show significant associations. One reason could be the relatively small available set of paired samples to perform the correlation analysis. On the other hand, we did not observe homozygous deletion of CCND1 gene; therefore, the remaining copy may make uninterrupted mRNA synthesis possible. However, amplification might not affect the entire gene or the extra copies of CCND1 may not function properly. Finally, it would be also worthwhile to examine the epigenetic processes affecting CCND1 expression.

The protein expression of CCND1 showed a significant association with Breslow thickness in our set of tissue samples (CCND1 protein expression was increased in thick lesions, as Oba et al. have also found). However, the distribution was heterogeneous within the different thickness categories and the significance was disappeared regarding not just the staining positivity, but the number of positive cells as well (Quick score evaluation). To clarify these results, we conducted experiments in melanoma cell lines with different biological properties. These experiments revealed that the aggressive primary tumor-derived HT199 cell line, which shows a high colonization capacity, expressed stronger CCND1 protein positivity compared to the invasive, but non-tumorigenic (in nude mice) WM35 line and the metastasis-derived WM983B cell line. Detailed studies have previously shown that a progressive increase in the CCND1 protein level was exhibited during malignant transformation of the melanocytic lesions, whereas a relative decrease in its protein expression was detected in advanced-stage cancers. We observed decreased CCND1 mRNA and protein levels in primary melanomas with multiple metastases, reflecting the role of
CCND1 in the early stages of melanoma progression. Another reason for the changes in CCND1 expression levels could be the role of CCND1 in the interplay between cancer cell proliferation and migration, similar to its role in breast cancer. Cells in a steady state (G0/G1 phase) exhibit an enhanced migratory capacity compared to proliferating ones in which CCND1 has a major driving role. However, CDK-independent modulation of tumor cell migration through direct regulation of several transcription factors unrelated to the main role of CCND1 in the regulation of proliferation has also been observed. Therefore, as an integral part of a complex multi-molecular mechanism involved in the development of melanoma, CCND1 may exert its effect through precisely controlled molecular interactions.

In relation to the mutation states of BRAF and NRAS genes, CCND1-amplified tumors without such alterations developed more frequently on sun-exposed skin, and CCND1 mRNA level was also higher in samples with wild-type BRAF and NRAS. These results support the idea that the abovementioned molecular alterations may develop in different ways, defining distinct melanoma subgroups. Furthermore, CCND1 amplification and mutations in BRAF or NRAS were not mutually exclusive, and the CCND1 mRNA expression level showed only a moderate degree of correlation with BRAF and NRAS mRNA levels in our specimens, indicating that CCND1 and the upstream molecules BRAF and NRAS may also have an independent effect on prognosis or might cooperate with each other to enhance for instance drug resistance. We did not detect any significant correlation in our tumor dataset between the changes in CCND1 mRNA expression and CCND1 protein expression. This finding may be due in part to deregulation of protein degradation, which is enhanced and regulated by the ROC1 protein. Thus, decreased expression of ROC1 can increase the CCND1 protein level, without mRNA overexpression being involved. Another explanation for this result could be the posttranscriptional degradation of CCND1 mRNA regulated by the recently discovered miR-193b micro RNA.

Although CCND1 genetic alterations and gene expression changes may have an impact on melanoma progression, it would be important to examine its role in a larger set of cutaneous melanoma to clarify the borderline associations. It would be also useful to compare the CCND1 alterations of primary tumors and their corresponding metastatic pairs. Finally, the high intratumoral heterogeneity characteristic for melanoma is also a huge problem and may hide some important associations.
In recent years several research groups conducted studies to gain deeper insight into the molecular background of malignant melanoma. However, due to the high complexity of the disease the achievements are limited and several questions have remained. Therefore, identification of biological markers that are able to improve the prognosis of aggressive behavior would be essential. Based on our results the alterations of both CCND1 and 7q31 including the expression changes of the locus-related CAV1 and TES genes could have a prognostic relevance in melanoma progression and tumor cells’ ability to form metastasis. Finally, the interaction found between the CCND1 gene alterations and activating mutation of BRAF and NRAS emphasizes the importance of systematic view of malignant melanoma.
Main statements and results

The main purpose of the doctoral thesis was to examine the genetic alterations and gene expression changes of candidate genes (CCND1, BRAF, NRAS) and loci (7q31) with a possible role in human melanoma progression, and to define the associations between these alterations and the clinical-pathological parameters of primary melanomas.

1. Analyzing the copy number alterations of 7q31 in primary melanomas and melanoma metastasis, we found significant associations with poor clinical outcome.

2. mRNA expression analysis of CAV1 and TES genes located on 7q31 locus showed a decreased CAV1 mRNA level in thick lesions, and decreased TES mRNA level in samples formed multiple metastases, whereas elevated TES mRNA expression was exhibited by primary melanomas formed only cutaneous or lymph node metastases.

3. CAV1 protein level was significantly higher in thin primary tumors. Whereas TES protein expression did not show significant associations with any of the examined clinical-pathological parameters.

4. Amplification of 7q31 is not or weakly correlated with the mRNA and protein expressions of CAV1 and TES genes.

5. Interphase FISH analyses of the copy number alterations of CCND1 gene in primary melanomas revealed significant correlations with poor clinical outcome, which was influenced by the mutation status of BRAF or NRAS genes in the term of sun exposure.

6. CCND1 mRNA level decreased in lesions with multiple metastases and was correlated with both the mRNA levels and mutation state of BRAF and NRAS genes.

7. CCND1 protein expression was associated with Breslow thickness, metastasis formation, and shorter survival time.

In conclusion, amplification of 7q31 and CCND1 could have a prognostic relevance in cutaneous melanoma predicting unfavorable outcome. Furthermore, our data show that expression alterations of the CAV1, TES and CCND1 genes possibly affect many aspects of tumor behavior.
List of publications related to the dissertation

   DOI: http://dx.doi.org/10.1007/s13277-012-0480-6  
   IF: 2.143 (2011)

   DOI: http://dx.doi.org/10.2741/501

   DOI: http://dx.doi.org/10.5772/152339

   Mod. Pathol. 22 (10), 1367-1378, 2009.  
   DOI: http://dx.doi.org/10.1038/modpathol.2009.109  
   IF: 4.406
List of other publications

   DOI: http://dx.doi.org/10.1371/journal.pone.0054958
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   DOI: http://dx.doi.org/10.1097/CMR.0b013e328352dc98
   IF: 2.187 (2011)

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Presentations at conferences


Rakosy Z, Vizkeleti L, Begány A, Adany R, Balazs M: poster: Amplification of the 7q31 locus is a frequent event in malignant melanoma and associated with extra copies of EGFR gene; ECCO 13 EACR Congress, 30 Oct – 3 Nov, 2005; Paris, France

Rakosy Z, Vizkeleti L, Treszl A, Adany R, Balazs M: oral: Amplification of the EGFR gene (7p12) and the 7q31 locus are associated with metastasis formation of malignant melanomas; Hungarian Cancer Society XXVI. Congress 10-12 Nov, 2005, Budapest; Hungary


