

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

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

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Abbreviations

AJCC: American Joint Committee on Cancer

ALM: acral lentiginous melanoma

AOCD: American Osteopathic College of Dermatology

BAC: bacterial artificial chromosome

BRAF: v-raf murine sarcoma viral oncogene homolog B1

CAV1: caveolin 1

CCND1: cyclin D1

CDK: cyclin dependent kinase

CFS: common fragile site

CGH: comparative genome hybridization

CI: confidence interval

CNI: copy number index

CSD: chronically sun-damaged

DAPI: diaminofenilindol

del: deletion

DGV: Database of Genomic Variants

DTIC: dacarbazine

ECL: enhanced chemiluminescence

EDTA: ethylenediaminetetraacetic acid

EU-12: members of the European Union after 2004

EU-15: members of the European Union before 2004

EU-27: all the members of the European Union

FDR: false discovery rate

FFPE: formalin-fixed paraffin-embedded

FISH: fluorescence *in situ* hybridization

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

ha: high level amplification

HRP: horseradish peroxidase

IHC: immunohistochemistry

KCl: potassium chloride

KIT: v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog

la: low level amplification

LMM: lentigo maligna melanoma
LN: lymph node
MM: malignant melanoma
nd: no data available
NM: nodular melanoma
NRAS: neuroblastoma RAS viral (v-ras) oncogene homolog
MDB: WHO European mortality database
PBS: phosphate buffered saline
PVDF: polyvinylidene difluoride
OD: optical density
OR: odds ratio
qRT-PCR: quantitative reverse-transcription polymerase chain reaction
RGP: radial growth phase
RIPA: Radio-Immunoprecipitation Assay
ROC: receiver operating characteristic
RT: room temperature
SCC: squamous cell carcinoma
SCID: severe combined immunodeficiency
SD: standard deviation
SDR: standardized mortality rate
SNP: single nucleotide polymorphism
SSM: superficial spreading melanoma
TES: testin; testis derived transcript
TMA: tissue microarray
TNM: tumor – lymph node – metastasis
TSG: tumor suppressor gene
UCSC: University of California, San Francisco
UV: ultraviolet
UVA and UVB: ultraviolet A and B
VGP: vertical growth phase
WB: western blot
WHO: World Health Organization
WT: wild type

Introduction

Malignant neoplasms are the second on the list of leading causes of death worldwide following closely the cardiovascular diseases. According to the Eurostat regional yearbook 2011, Hungary is at the first place among the EU-27 based on the death rate caused by cancers, preceding Poland and the Czech Republic [1]. Malignant melanoma is one of the most aggressive skin cancer with a constantly increasing incidence worldwide [2]. According to the AOCD (American Osteopathic College of Dermatology, dermatologic disease database, 2012) statistics, the rate of this increment is approximately 5% every year. Featuring serious public health issues, the high mortality of the disease is primarily due to the resistance to chemotherapy and aggressive metastatic capacity of melanomas. Therefore, the early recognition and the exact diagnosis are of great importance in the disease outcome. Data from the World Health Organization (WHO) European Mortality Database 2013 show that the standardized melanoma mortality rate (SDR) of Hungary is above the European Union (EU) average (Figure 1). Furthermore, a mild increase can be shown in the last 30 years mainly among the more than 40-year-old males [3]. Similarly to other tumor types, accumulation of several genetic and genomic alterations can be observed in melanoma development and progression. Detailed understanding of the molecular background of tumor initiation and progression may help us to develop new therapeutic approaches beside the deeper understanding of the causes of aggressive behavior [4].

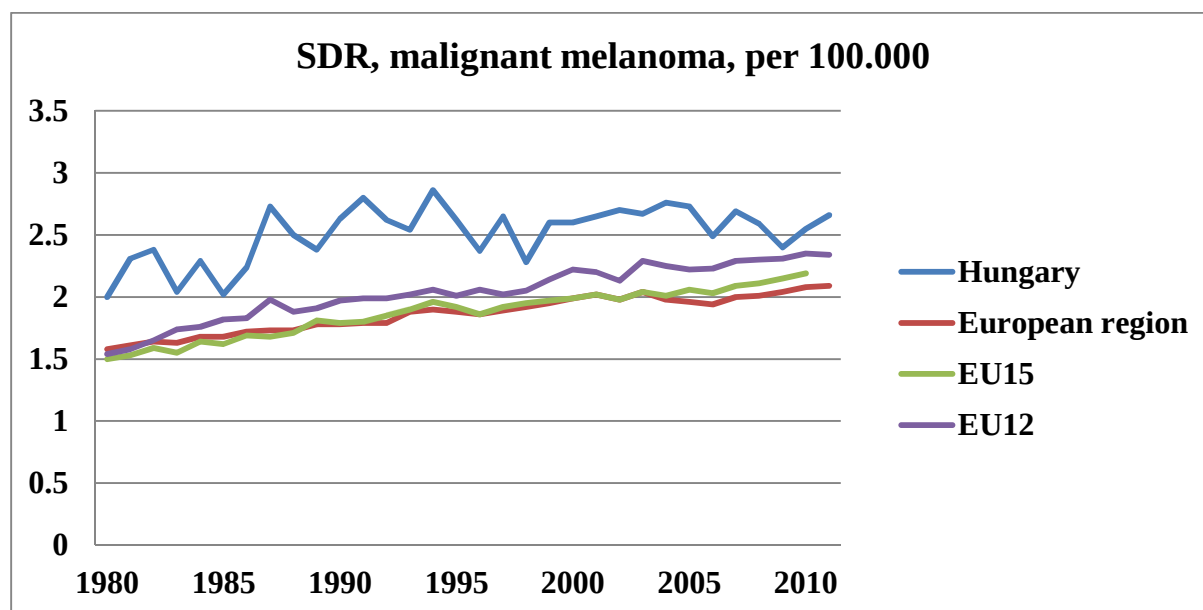


Figure 1. Standardized melanoma death rates in Hungary comparing to the EU and the European region in the last 30 years [3].

Our research group has a more than 15-year experience in the analyses of molecular alterations of malignant melanomas. We use state-of-the-art molecular biologic techniques to understand the associations between the genetic alterations and gene expression changes of primary tumors and the clinical behavior of melanomas. Because the most patients suffering from melanoma die due to the metastatic progress rather than the primary tumor itself, examination of the background of metastasis formation is extremely important [5].

In my doctoral thesis I used several kinds of molecular biologic methods, such as FISH (fluorescence *in situ* hybridization), real-time quantitative RT-PCR (qRT-PCR), tissue microarray with immunohistochemistry, western blot and mutation analysis techniques to examine the genetic alterations and gene expression changes of human malignant melanomas. Interphase FISH was applied to determine the copy number alterations of 7q31 locus and cyclin D1 (CCND1) oncogene at the single cell level. TaqMan qRT-PCR approach, immunohistochemistry and western blot were used to determine the mRNA and protein levels of testin (TES), caveolin 1 (CAV1) and CCND1 genes. Finally, we determined the v-raf murine sarcoma viral oncogene homolog B1 (BRAF) and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) mutation states by applying a PCR based technique with melting curve analysis. Alterations were correlated with the clinical-pathological parameters of primary tumors.

Background

Human malignant melanoma

Malignant melanoma, originated from melanocytes is considered one of the most aggressive skin cancers, being responsible for approximately 20% of all cases [6]. Being the most life-threatening neoplasm of skin melanoma is responsible for almost all skin cancer deaths [7, 8]. Primary melanomas are characterized by an aggressive and heterogeneous phenotype. Despite the intensive research has been employed to study this disease, melanoma prognostication is still under question. Transformed melanocytes usually maintain their pigment production ability, causing brownish lesions on the skin. However, in some cases, melanoma cells lose their ability to produce melanin during malignant transition [9]. Melanoma development and progression can be divided into morphologically and biologically different phases (Figure 2) [10, 11]:

1. Typical nevus: congenital or newly developed, but symmetrical nevus
2. Dysplastic nevus: morphologically changed, atypical nevus
3. *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
6. Metastatic melanoma: lesion forming local and/or distant organ metastases

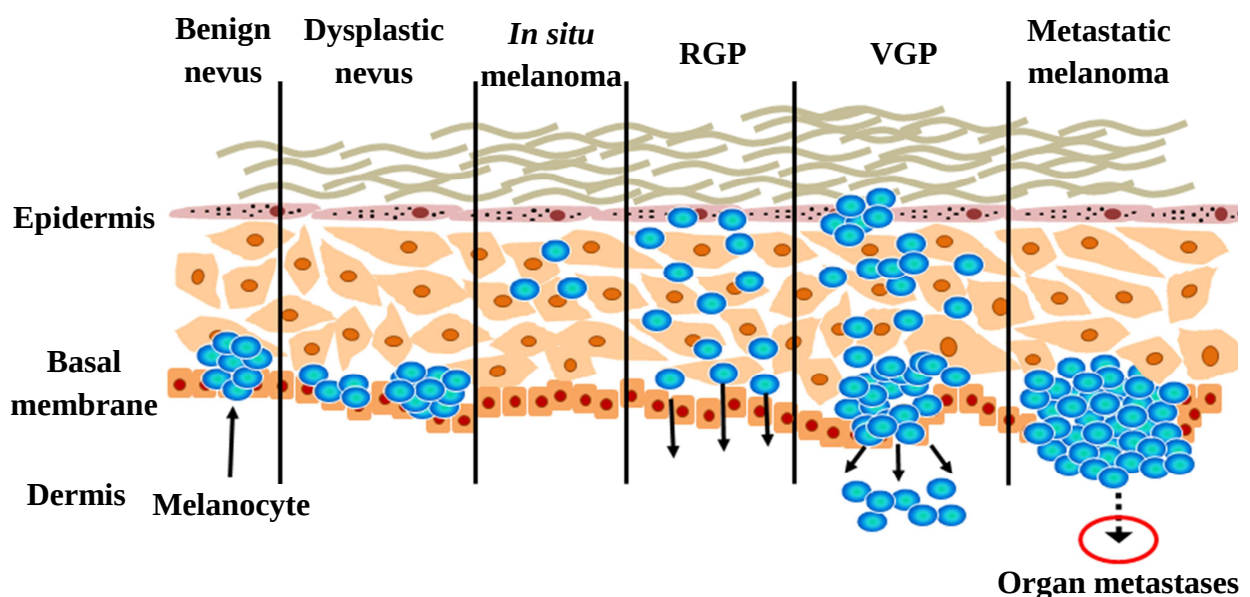


Figure 2. Possible biologic steps of the evolution and progression of malignant melanoma.
[previously unpublished figure]

Most melanomas develop *de novo* (ranged from 70% to 90%) [12]. The first step in tumor genesis is the unbalanced ratio of keratinocytes to melanocytes. This phenomenon promotes the latter cell type following hyper- and dysplasia of melanocytes, which creates a precursor lesion. The process leads directly towards the appearance of *in situ* melanomas in the upper layer of the epidermis and radial growth phase with microinvasion to the dermis. During the vertical growth phase, melanoma cells widely penetrate into the dermis and dermal adipose tissue. RGP – VGP transition is thought to be the key step of the development of an aggressive melanoma with metastatic potential. Therefore, the thickness of primary tumor is considered to be the most important parameter of melanoma prognostication [13, 14]. While the behavior of RGP melanoma cells is usually influenced by the exogenous growth factors of surrounding cells, and do not form tumor in immunodeficient mice [15], VGP melanoma cells are not dependent from the neighboring keratinocytes and fibrocytes, developing a growth factor- and anchor-independent growth. Melanoma cells in vertical growth phase are able to induce tumor formation in immunodeficient mice, and features an aggressive metastatic behavior both in human and animal models [10].

Melanoma etiology and the main risk factors

Malignant melanoma features multifactorial etiology and arises through the interplay of both genetic and environmental factors [16]. 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 [17, 18]. 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 [18, 19]. 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 [7, 20]. The intensive solar radiation increases direct photon (UVB) absorption by DNA of the epidermal cells causing direct damage of the molecule, which ultimately leads to the development of skin cancer. Cellular repair mechanisms usually correct these photoinductive damages of DNA, but the incorrect repair results in formation and the accumulation of gene alterations leading to tumor

development [7]. In contrast to UVB, UVA rays penetrate more deeply into the skin, reaching also the basal layer of the epidermis and dermal fibroblasts. UVA has an indirect effect on DNA and primarily causes oxidative damage of the molecule which is potentially mutagenic [7, 20]. However, sunburn damage is more dangerous than the chronic sun exposure. There are epidemiologic evidences that more than five sunburns in a lifetime can cause a significantly higher risk for melanoma [18]. Beside the natural UV radiation, the usage of artificial sources such as popular tanning beds and solariums causes a 1.2 to 4.7 times higher annual dose of UVA radiation than is received directly from the sun [7]. Several studies conducted in the last 10 years show that people who began using tanning devices before 30-35 years of age were 75% more likely to develop melanoma [17, 21].

In addition to UV exposure, heritable traits such as skin, hair and eye color, the presence of large numbers of nevi (benign, atypical or giant congenital), and familial history of melanoma with at least 3 affected relatives are also important risk factors for disease development [18, 22]. Owing to the last years' extensive research about the molecular aspects of melanoma development and progression, a separate chapter is devoted to summarize the current knowledge about this topic.

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 [23-25].

Major histological subtypes and distinct clinical-pathological manifestations of invasive melanomas

More than forty years ago, the current WHO clinical and histological classification scheme was described for cutaneous melanoma distinguishing four main subtypes [19, 26, 27]:

1. superficial spreading melanoma (SSM)
2. lentigo maligna melanoma (LMM)
3. nodular melanoma (NM)
4. acral lentiginous melanoma (ALM)

Despite the tweaks to the classification scheme, these basic findings form the foundation for melanoma diagnosis and staging today [28]. SSM is the most common subtype responsible for approximately 75% of all melanoma cases. It is often derived from nevus as an

asymmetric edged and variegated colored lesion which grows superficially. The second most frequent subtype is NM (10% - 20% of melanoma patients) where the often amelanotic tumor cells immediately grow vertically, forming a more aggressive lesion with higher metastatic frequency. Because the tumor pigmentation and edge can be smoothed, it is easy to mix up with other skin diseases such as basal cell carcinoma, seborrheic keratosis or benign nevus. The slowly growing LMM frequently occurs on skin exposed to chronic solar radiation such as face, and shows a direct correlation with UV-radiation. This subtype is the most typical in the elder age groups. Finally, ALM, which is the most common subtype of cutaneous melanoma among the non-Caucasian population, develops on the skin of palm and sole, under the nails or perhaps on the genital and oral mucosa. Due to its localization, ALM is often recognized in a later stage causing a worse prognosis.

Besides the classic subdivision, nowadays, another scheme is emerging based on the molecular characteristics of tumors [19, 29-31]. This scheme distinguishes two types of melanoma on non-glabrous skin: 1.) melanomas on non-chronically sun-damaged skin (non-CSD) with frequent BRAF mutations, along with the loss of chromosome 10q (including PTEN) and no KIT (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) 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, despite expressing frequent alterations of KIT similarly to the acral subtype, were proposed as a separate entity based on the patterns of chromosomal aberrations.

Classification of cutaneous melanoma, the TNM staging system (AJCC 7th edition)

Considering the main factors which influence tumor prognosis, a new and uniform staging system, the TNM (tumor - lymph node - metastasis) system was established in January 2002 based on the recommendations of a number of international organizations [32]. The main advantage of this classification method is the introduction of sentinel lymph node biopsy which reduces the radical, much disputed and often unnecessary removal of local lymph nodes to prevent metastasis formation. TNM classification has been changed several times

since its introduction [23, 24]. Its main principles, regarding melanoma, are summarized in Table 1 and 2.

Table 1. TNM staging for cutaneous melanoma [23]

Primary tumor (T)		
<i>Classification</i>	<i>Thickness (mm)</i>	<i>Ulceration status / mitoses</i>
Tis	NA	NA
T1	≤1.0	a: Without ulceration and mitosis <1/mm ² b: With ulceration and mitosis ≥1/mm ²
T2	1.01-2.00	a: Without ulceration b: With ulceration
T3	2.01-4.00	a: Without ulceration b: With ulceration
T4	>4.00	a: Without ulceration b: With ulceration
Regional nodes (N)		
<i>Classification</i>	<i>No. of metastatic nodes</i>	<i>Nodal metastatic burden</i>
N0	0	NA
N1	1	a. Micrometastasis ^a b. Macrometastasis ^b
N2	2-3	a. Micrometastasis b. Macrometastasis c. In-transit metastases/satellites without metastatic nodes
N3	4+ metastatic nodes, or matted nodes, or in-transit metastases/satellites with metastatic nodes	
Distant metastases (M)		
<i>Classification</i>	<i>Site</i>	<i>Serum LDH</i>
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c ^c	All other visceral metastases	Normal
	Any distant metastases	Elevated

Abbreviations: NA = not applicable; LDH = lactate dehydrogenase; Tis = *in situ* melanoma

^adiagnosed after sentinel lymph node biopsy; ^bdefined as clinically detectable nodal metastases confirmed pathologically; ^cpatients with metastases to any other non-pulmonary visceral metastasis or any distant metastasis including lung with elevated serum LDH.

Table 2. Anatomic stage groupings for cutaneous melanoma [23]

Clinical staging ^a				Pathologic staging ^b			
	T	N	M		T	N	M
0	Tis	N0	M0	0	pTis	N0	M0
IA	T1a	N0	M0	IA	pT1a	N0	M0
IB	T1b	N0	M0	IB	pT1b	N0	M0
	T2a	N0	M0		pT2a	N0	M0
IIA	T2b	N0	M0	IIA	pT2b	N0	M0
	T3a	N0	M0		pT3a	N0	M0
IIB	T3b	N0	M0	IIB	pT3b	N0	M0
	T4a	N0	M0		pT4a	N0	M0
IIC	T4b	N0	M0	IIC	pT4b	N0	M0
III	Any T	N > N0	M0	IIIA	pT1-4a	N1a	M0
					pT1-4a	N2a	M0
				IIIB	pT1-4b	N1a	M0
					pT1-4b	N2a	M0
					pT1-4a	N1b	M0
					pT1-4a	N2b	M0
					pT1-4a	N2c	M0
				IIIC	pT1-4b	N1b	M0
					pT1-4b	N2b	M0
					pT1-4b	N2c	M0
					Any pT	N3	M0
IV	Any T	Any N	M1	IV	Any pT	Any N	M1

Abbreviations: Tis = *in situ* melanoma

^aincludes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.

^bincludes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial (ie, sentinel node biopsy) or complete lymphadenectomy. Pathologic stage 0 or stage IA patients are the exception; they do not require pathologic evaluation of their lymph nodes.

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 [19, 22, 33].

It is well-known that approximately 10% of melanoma patients have a familial predisposition associated with a more than 2-fold increase in the risk of tumor development [22, 34]. The major germline alterations are proved to be single-base mutations and deletions of the CDKN2A (cyclin-dependent kinase inhibitor 2A) gene at the 9p21 locus, contributing to 10% to 40% of familial cases depending on the geographical location. Mutations harboring the CDK4 (cyclin-dependent kinase 4) gene, and some unidentified genes on chromosomes 1p22 and 20q11.22 have also been observed [22, 35, 36]. Recent studies focus on the melanoma susceptibility based on single nucleotide polymorphisms (SNP), and revealed some loci such as 16q24.3 (MC1R - melanocortin 1 receptor), 20q11.22 (MYH7B/PIGU/ASIP), 11q14.3 (TYR - tyrosinase), 5p13.2 (SLC45A2), 9p21 (p14/p16), 11q22.3 (MMP1 - matrix metalloproteinase 1) and 4q11-q12 (KIT) or genes involved in the DNA repair or oxidative stress pathways, which are significantly associated with a higher risk of cutaneous melanoma [37-42].

Several signaling pathways, involving a number of tumor suppressor genes (e.g. PTEN (phosphatase and tensin homolog), TES, CAV1 or 9p21 locus coding the p16 and p14 regulatory proteins) and oncogenes (e.g. BRAF, NRAS, MITF (microphthalmia-associated transcription factor), cKIT, cMET, CCND1 or EGFR) have been identified as key regulator in *de novo* melanoma progression by somatic mutations, gene copy number alterations and expression changes including the epigenetic events of the different pathway members. Some of them are already known such as the p16INK4a-CDK4/6-RB and p14ARF-MDM2-p53 pathways contributing to cell cycle deregulation and the sensitization by p16 error to oxidative stress by UV-induced DNA damage [36, 43, 44]. The RAS/MAPK (mitogen-activated protein kinase) or PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)/AKT (v-akt murine thymoma viral oncogene homolog) 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 [8, 22, 36]. Besides, the canonical WNT (wingless-type MMTV integration site family) - β -catenin and MSH (proopiomelanocortin)-PKA (protein kinase A)-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

(nitric oxide synthase 2, inducible) cascade contributes to the elimination of reactive oxygen and nitrogen species [22]. The molecular complexity of signaling pathways is represented on Figure 3.

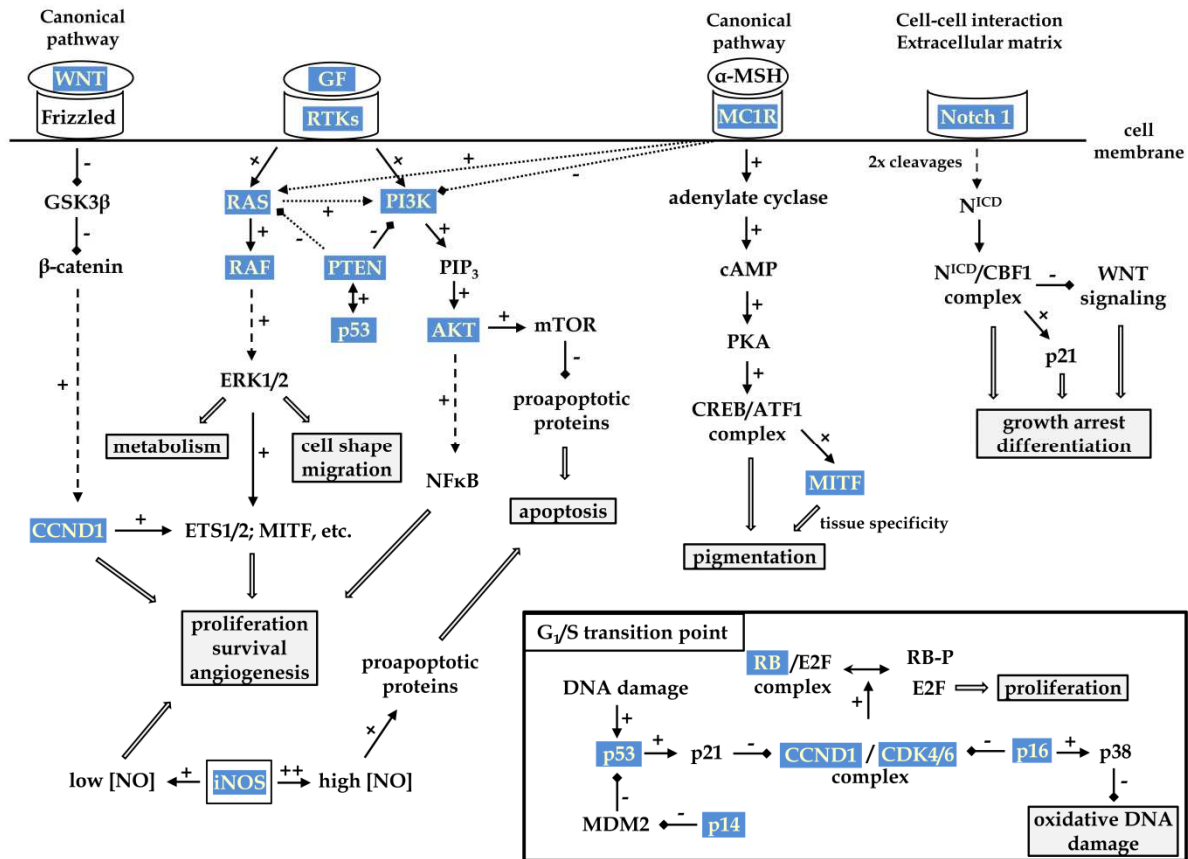


Figure 3. Schematic representation of the signaling pathways affected in melanoma progression [22]. Members showed (epi)genetic and/or expressional alterations are highlighted with blue.

Our previous array CGH (comparative genome hybridization) studies revealed many chromosomal regions altered in aggressive melanomas [45, 46] such as 7q31 and 11q13 loci (Figure 4A and B). Nevertheless, several other segments show chromosomal gains (e.g. 1q, 3p, 6p, 7, 8q, 11q, 12q14, 17q, and 20q) or losses (e.g. 1p, 6q, 9p, 10q, 11q, 17p, and 21q) harboring oncogenes or tumor suppressor genes [8]. The 7q31 region contains the FRA7G common fragile site which is neighbored by several genes like CAV1 and TES as possible targets of genetic alterations during tumor genesis similarly to the FHIT (fragile histidine triad) and WWOX (WW domain containing oxidoreductase) genes [47, 48]. CAV1 as the main component of membrane lipid rafts can play a significant role in the vesicular transport, cholesterol homeostasis, receptor internalization and the regulation of signal transduction

through interactions with several proteins, including EGFR (epidermal growth factor receptor), H-RAS (Harvey rat sarcoma viral oncogene homolog) and eNOS (nitric oxide synthase 3, endothelial) [49]. 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 [49-53]. TES is an important member of focal adhesions, influencing tumor cell motility, and behaves as a suppressor molecule during tumor genesis [54-57].

The 11q13 locus contains the CCND1 oncogene, which plays a significant role in the highly regulated G₁/S phase transition of the cell cycle via binding CDK4/6 kinases and consequently inactivating the retinoblastoma protein. The transcriptional activation of CCND1 is primarily carried out by tyrosine kinase receptors and the Ras–Raf–MEK–ERK signaling cascade [58-60]. 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 [61, 62].

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.

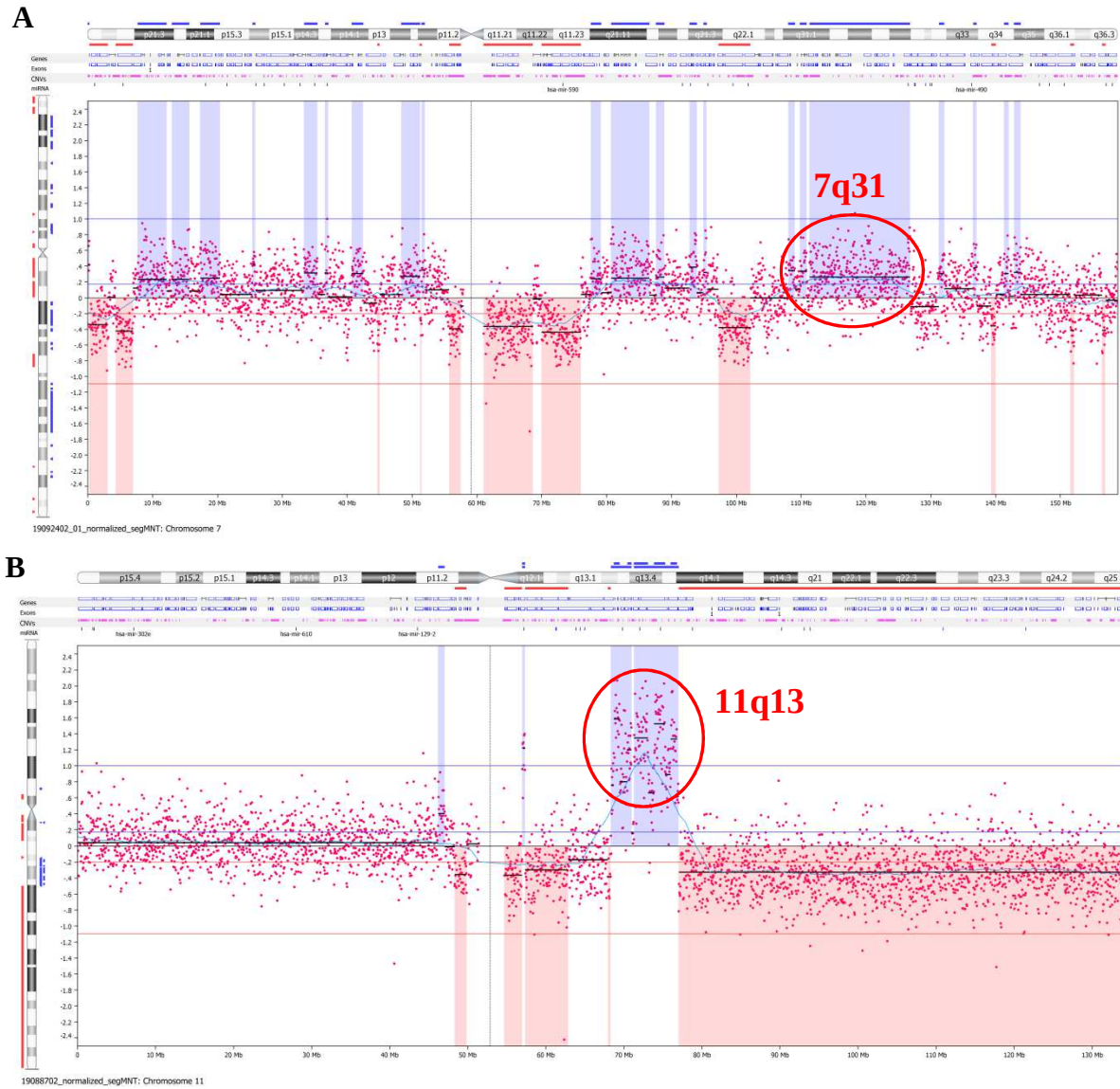


Figure 4. Frequent alterations are found by array CGH on chromosome 7 and 11. Ideograms of representative samples show copy number gain on the 7q31 (A) and 11q13 (B) chromosomal regions. Blue color represents copy number gain, red color represents copy number loss on both schematic chromosomes and ideograms. Annotation tracks on the upper side indicate genes, exons, CNVs (copy number variations from The Center for Applied Genomics' Database of Genomic Variants), and miRNA data when it was available. The y-axis indicates the median log ratio value of the probes in the given segment of the sample. The x-axis indicates the distance in Mb from the end of the p-arm.

[previously unpublished figure]

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 [63]. 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% [64]. High dose interferon-based adjuvant systemic therapy and adjuvant radiation treatment in given conditions (e.g. multiple positive lymph nodes and desmoplastic neurotropic melanoma) have also been applied in high-risk melanomas to combat the disease with more or less success [64, 65]. However, over the past few years, there have been 3 novel agents approved for the treatment of melanoma by the US Food and Drug Administration with many limitations, but providing an excellent basis for the future therapies [64, 66]:

1. **vemurafenib (PLX4032)**, a BRAF inhibitor for unresectable or metastatic melanomas bearing 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

Davies et al. (2002) published at first in Nature that BRAF is harbored by an activating missense mutation at codon 600 in more than 65% of malignant melanomas, causing a valine to lysine transition (V600E) in the kinase domain. The V600E mutation is account for approximately 80% of all BRAF mutations [67]. Vemurafenib introduced by Sala et al. (2008) as PLX4032, is a highly selective BRAF inhibitor, and is capable of silencing mutant BRAF (V600E) with little effect on the wild-type protein. This breakthrough discovery could significantly reduce the likelihood of side-effects [68]. 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 the acceleration of the growth of cutaneous squamous cell carcinomas (SCC) and keratoacanthomas in about 20% of treated patients. Fortunately, these tumors are easily removed with local excision [66, 69].

Whereas ipilimumab is an immunostimulatory antibody against the negative costimulator antigen-4 of cytotoxic T-lymphocytes (CTLA4), therefore, consequently increases the T-cell activity and promotes their antitumor activity. Ipilimumab is presumed to be a potential combination or monotherapy treatment of melanoma and several other cancers [70]. 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 [66, 71].

There are many other targeted therapies in clinical trials which may have an impact in the future melanoma treatment. These include the imatinib, an oral tyrosine kinase inhibitor against activated c-kit, the lapatinib against the mutant ERBB4, the anti-VEGF therapies or the improvement of immunotreatments (e.g. HD IL2 or anti-PD-L1, which is a negative regulator of the immune system by which melanoma is thought to evade it) perhaps in combination with molecular targeted therapies [64, 66].

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 [23, 24]. 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. The clinical-pathological parameters of the tumors are summarized in Table 3 and 4.

The average age of patients with primary melanoma was 61.9 ± 13.3 years (range: 33-87 years) in the 7q31 study and 60 ± 14 years (range: 23-93 years) in the CCND1 study. The average survival after surgery for the primary tumor until the end of the 7q31 study was 4.1 ± 3.0 years (range: 0.04-14.5 years) and until the end of the CCND1 study was 3.9 ± 2.8 years (range: 0.04-12.6 years).

Table 3. Clinical-pathological parameters of primary melanomas analyzed in the 7q31 study

Variables	No. of tumors detected by		
	FISH ¹	qRT-PCR	IHC ²
All patients	75	33	60
Histological subtype			
Superficial spreading	43	21	34
Nodular	32	12	26
Gender			
Female	34	17	39
Male	41	16	21
Age (years)			
20-50	22	9	11
≥50	53	24	49
Breslow thickness (mm)³			
≤2	21	16	29
2-4	13	5	8
>4	41	12	23
Location of primary tumor			
Extremity	32	19	30
Trunk	35	11	22
Head	8	3	8
Metastasis formation⁴			
Absent	20	16	31
Present	48	17	25
Patient's survival⁴			
Alive	28	23	35
Death	40	10	21
Ulceration			
Absent	30	17	34
Present	45	16	26
Location of metastasis⁵			
Cutaneous and/or lymph node	16	6	9
Distant organ	18	5	9
Multiple ⁶	14	6	7

¹Fluorescence *in situ* hybridization; ²immunohistochemistry; ³thickness categories are based on the current staging system; ⁴patients with at least a 5-year follow-up period were included; ⁵metastasis of the examined primary tumors; ⁶the primary melanoma formed metastases in both the skin/lymph node and in a distant organ.

Table 4. Clinical-pathological parameters of primary melanomas analyzed in the CCND1 study

Variables	No. of tumors detected by			
	FISH ¹	qRT-PCR	IHC ²	mutation analysis of BRAF and NRAS genes
All patients	76	28	54	55
Histological subtype				
Superficial spreading	48	19	27	31
Nodular	28	9	27	24
Gender				
Female	45	16	35	28
Male	31	12	19	27
Age (years)				
20-50	25	8	12	18
≥50	51	20	42	37
Breslow thickness (mm)³				
≤2	30	14	23	19
2-4	20	3	9	11
>4	26	11	22	25
Location of primary tumor				
Extremity	40	16	27	27
Trunk	29	10	22	21
Head	7	2	5	7
Metastasis formation⁴				
Absent	35	13	30	21
Present	40	15	24	34
Patient's survival⁴				
Alive	43	19	34	29
Death	32	9	20	26
Ulceration				
Absent	39	15	29	25
Present	37	13	25	30
Location of metastasis⁵				
Cutaneous and/or lymph node	13	6	9	13
Distant organ	16	4	8	14
Multiple ⁶	11	5	7	7

¹Fluorescence *in situ* hybridization; ²immunohistochemistry; ³thickness categories are based on the current staging system; ⁴patients with at least a 5-year follow-up period were included; ⁵metastasis of the examined primary tumors; ⁶the primary melanoma formed metastases in both the skin/lymph node and in a distant organ.

Melanoma cell lines and cell culturing

The following human melanoma cell lines with different biological properties were used in our experiments:

- **WM35:** derived from the radial growth phase, non-metastatic superficial spreading melanoma of a 24-year-old woman. Breslow thickness of the scapula/neck localized tumor was 0.69 mm [72, 73].
- **WM983A:** derived from the vertical growth phase, aggressive metastatic (lymph node) primary melanoma of a 54-year-old man. Breslow thickness of the abdomen localized tumor was 25 mm [74, 75].
- **WM983B:** derived from the lymph node metastasis of the previous patient's primary tumor [76].
- **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 line was derived from A2058 line xenografted to nude mice. Whereas HT168M1 was originated from the *in vivo* adaptation of HT168 to the lien of immunosuppressed mice [77].
- **HT199:** derived from the radial growth phase, metastatic nodular melanoma of a 23-year-old woman. The tumor was able to form liver metastasis in SCID mice [78].

All cell lines were maintained at 37°C (5% CO₂) in RPMI 1640 medium supplemented with 10% fetal bovine serum and 300 mg/l L-glutamine and antibiotics. Monolayer adherent cells were treated with 0.2% trypsin/EDTA solution (0.53mM) and washed twice with PBS (pH 7.2). Cell pellet was then treated with 0.75mM KCl (hypotonic treatment) for 20 min at 37°C, dropped on slides and fixed with Carnoy's fixative (3:1 methanol:aceticacid). 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 using 1 µg of tumor and normal reference DNA labeling by random priming with Cy3-dUTP and Cy5- dUTP, respectively. Labeled DNA was mixed with 100 µg of Cot-1 DNA. Sample hybridization to the BAC arrays, imaging setup, and preliminary analysis using Spot and Sproc software were performed at the UCSF Comprehensive Cancer Center (San Fransico, CA, USA). DNA spots

were automatically segmented, local background was subtracted, and the intensity ratio of the two dyes for each spot was calculated by $\log_2$ -transformed modeling. Spots for which the $\log_2$ SD of the triplicates was more than 0.2 were discarded from the further analysis [45]. All BAC clones were mapped to the human genome (February 2009) using the UCSC genome browser site (<http://genome.ucsc.edu/>). In the course of filtering, BAC clones located on sex chromosomes or have overlaps with polymorphisms in DGV (Database of Genomic Variants) database were excluded. Analysis of Copy Errors algorithm in CGH Explorer software 3.2 (Department of Informatics, The Faculty of Mathematics and Natural Sciences, Blindern, Oslo, Norway) was applied with a false discovery rate (FDR) of less than 0.01 to determine gains and losses of each regions. Ratio thresholds for high copy gains (more than 0.55) and homozygous deletions (less than -0.8) in aCGH data were used based on literature data [79].

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). A total of 500µl blood sample in special medium supplemented with heparin was incubated at 37°C for 72 hours in a sterile 5% CO₂ thermostat following by 4-hour incubation with 2 drops of 10µg/ml colchicine (Sigma-Aldrich, Inc., St. Louis, MO, USA) in a final concentration of 0.09µg/ml. After centrifugation, the cell pellet was resuspended in 0.5 ml supernant, subjected to hypotonic treatment (5ml 75mM KCl, room temperature), and incubated for 20 minutes at room temperature following by a second centrifugation step and resuspension. Modified Ibraimov solution (92% distilled water, 5% acetic acid and 3% methanol) was then added to the sample and centrifuged it. Finally, we treated the samples with 5 ml fresh-made Carnoy’s fixative and repeated this latest step till we got a cytoplasm-free suspension, which was dropped onto wet slides. The slides were air-dried at room temperature 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

FISH 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, with slight modifications [80]. The probe mixture and target cells were denatured simultaneously in a hybridization chamber (HYBrite) for 2 minutes at 73°C, and then hybridized overnight at 37°C. Aspecifically or non-binding DNA probes were removed by washing 3-times at 45°C with pH 7.0 washing buffer (50% formamid, 10% 20x SSC and 40% distilled water) for 10 minutes. Nuclei were labeled with 200 ng/ml diaminophenylindole (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 Metasystems GmbH, Altlussheim, Germany). Copy number index (CNI) and categories of the 7q31 locus and CCND1 copy number alterations were defined as follows [81]:

1. Deletion: copy number of locus/gene is less than the copy number of corresponding chromosome centromere in more than 10% of cells.
2. Apparent amplification: copy number of the chromosome is equal to the locus/gene copy number, but more than 2 (chromosome amplification causes gene amplification).
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 more than 10% of cells.

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

RNA extraction and real time qRT-PCR

RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) was used to isolate total RNA from fresh melanoma tissues. The obtained RNA was quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was determined using 2100 BioAnalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). Samples with integrity lower than 7 were excluded. TaqMan one-step reverse-transcription PCR (RT-PCR) was used to determine the mRNA levels of the CAV1, TES, BRAF, NRAS and CCND1 genes. Each reaction contained 150 ng of total sample RNA and was run in triplicate on an ABI-PRISM 7000 instrument (Life Technologies Corporation, Carlsbad, CA, USA) with the following conditions: reverse transcription (48°C 30 sec), polymerase activation (95°C 10 min) and 40 cycles of amplification (annealing at 95°C for 15 sec and synthesis at 60°C for 1 min). PCR Master Mix and TaqMan assays (Table 5) were obtained from Life Technologies.

Table 5. TaqMan assays used in real-time qRT-PCR analysis

Gene name	Assay number	Target region
CAV1	Hs00184697_m1	exon 1-2 boundary
TES	Hs00210319_m1	exon 6-7 boundary
CCND1	Hs00277039_m1	exon 2-3 boundary
BRAF	Hs00269944_m1	exon 1-2 boundary
NRAS	Hs00180035_m1	exon 4-5 boundary
GAPDH	Hs99999905_m1	within exon 4

PCR data were analyzed using the Livak method ($2^{-\Delta\Delta C_t}$), with glyceraldehyde-3-phosphate dehydrogenase as the reference (endogenous control) gene and nevi collected from three different individuals and melanocyte as calibrator samples [82].

Tissue microarrays [83] and immunostaining

Tissue sections (4 μ m) 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 of 4 μ m 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 1 mM boiling citrate buffer (pH 6, 3 min). Applied primary antibody solutions are indicated in Table 6.

Table 6. Primary antibody solutions used for immunohistochemistry

Targeted protein	Clone	Host organism	Isotype	Dilution
CAV1	N-20	Rabbit	Polyclonal IgG	1:200
TES	AA-7	Mouse	Monoclonal IgG	1:50
CCND1	SP4	Rabbit	Monoclonal IgG	1:25
HMB45	-	Mouse	Monoclonal IgG	1:200

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 (1 h at RT), 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 a 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 [84]. 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 (Figure 5 and 6). Scores for triplicate cores were consolidated to a single value per case. In the case of CCND1 we also used the quickscore method, which considers both the staining positivity and the percentage of positive cells (Table 7) [85].

Table 7. Quickscores used for evaluation of immunohistochemical results [85]

Score for proportion of stained cells	Score for intensity of staining
1 – from 0% to 4%	0 – no staining
2 – from 5% to 19%	1 – weak staining
3 – from 20% to 39%	2 – moderate staining
4 – from 40% to 59%	3 – strong staining
5 – from 60% to 79%	
6 – from 80% to 100%	

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, the 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, the 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.

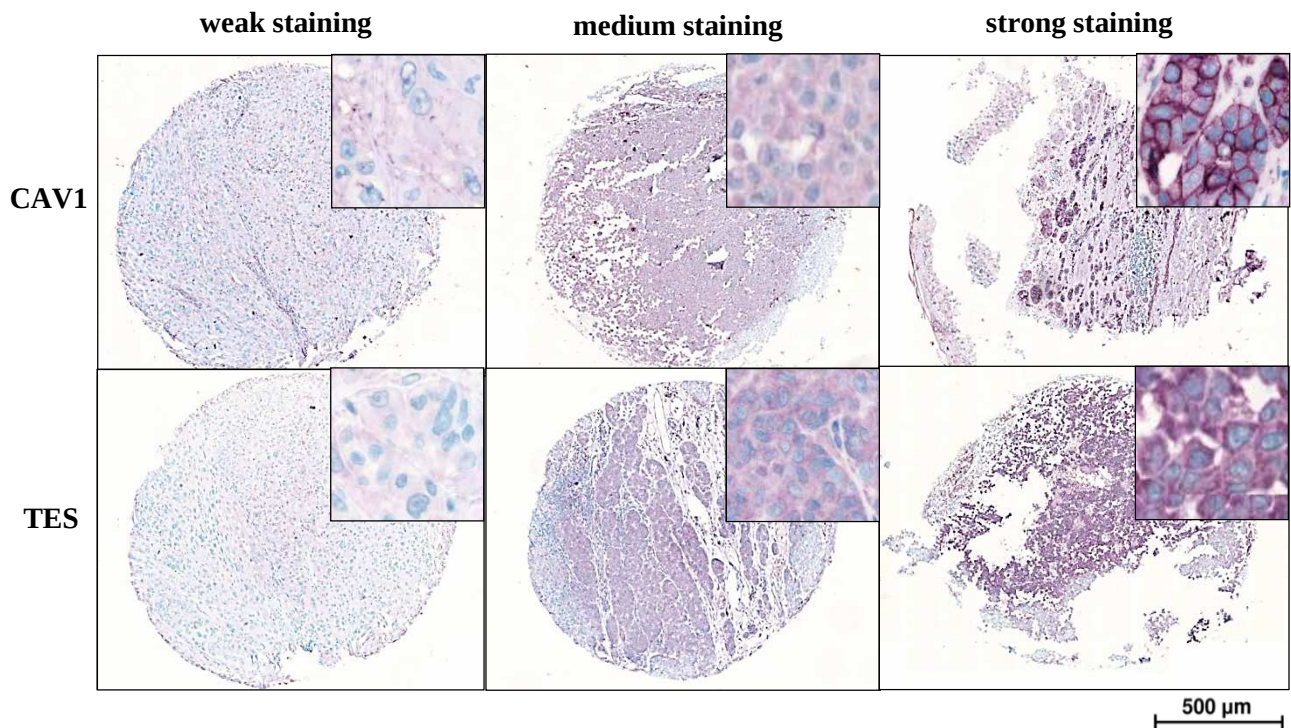


Figure 5. Scoring system for immunostaining intensity of CAV1 and TES in primary melanomas. The staining intensity of CAV1 and TES proteins in melanoma samples with weak (1+), medium (2+) and high (3+) level protein expression. CAV1 and TES proteins were stained by VIP (purple color) and cell nuclei were stained by methylgreen (blue color).

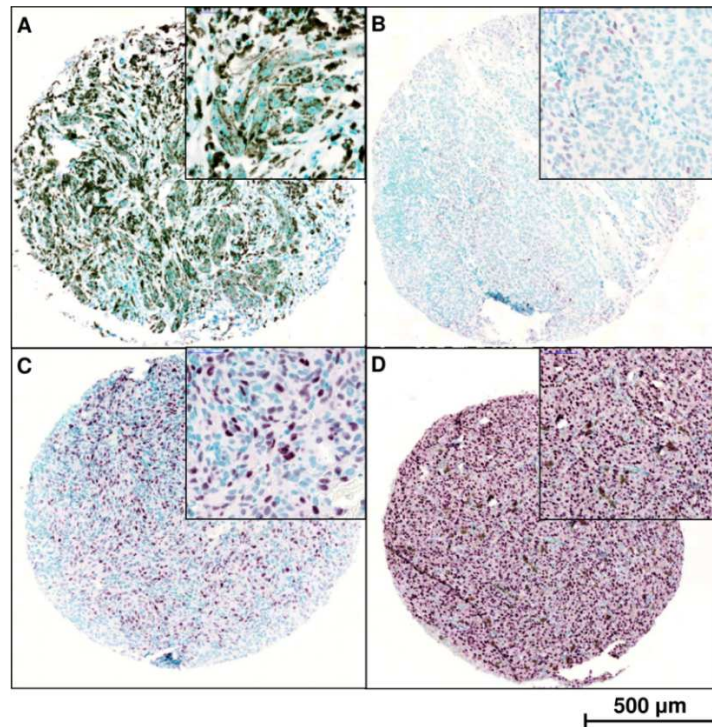


Figure 6. Scoring system for the immunostaining intensity of CCND1 in primary melanomas. The staining intensity of CCND1 in melanoma samples with no (a), weak (b), medium (c), and high (d) levels of protein expression. Only nuclear staining was considered to be specific. CCND1 protein was stained by VIP (purple color), cell nuclei were stained by methylgreen (blue color) and brown color is derived from the melanin within the tumor tissue.

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. Approximately 3×10^6 cells per cell line were harvested in RIPA (Radio-Immunoprecipitation Assay) 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 the following specific primary antibodies (Table 8.).

Table 8. Primary antibody solutions used for western blot analysis

Targeted protein	Clone	Host organism	Isotype	Dilution
CAV1	N-20	Rabbit	Polyclonal IgG	1:400
TES	AA-7	Mouse	Monoclonal IgG	1:200
CCND1	SP4	Rabbit	Monoclonal IgG	1:200
GAPDH	H-12	Mouse	Monoclonal IgG2a	1:200

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^{V600} and NRAS^{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), as described previously [86]. Sequences of primers and probes used for mutation analysis are the followings:

• BRAF^{V600E, V600K}

forward primer: 5'-CTCTTCATAATGCTTGCTCTGATAGG-3'

reverse primer: 5'-TAGTAACTCAGCAGCATCTCAGG-3'

sensor probe: 5'-AGCTACAGTGAATCTCGATGGAG-3'

anchor probe: 5'-GGTCCCATCAGTTTGAACAGTTGTCTGGA-3'

- NRAS^{Q61L, Q61K}

forward primer: 5'-CACCCCCAGGATTCTTACAGA-3'

reverse primer: 5'-GATGGCAAATACACAGAGGAAGC-3'

sensor probe: 5'-ATACAGCTGGACAAGAAAGAG-3'

anchor probe: 5'-AGTGCCATGAGAGACCAATACATGAGCA-3'

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 (copy number index) or $\log_2$ (mRNA expression 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

Copy number alterations of chromosome 7 and 7q31 locus in primary melanomas and melanoma metastases

Seventy-five primary melanoma samples were analyzed by FISH (Table 3.). Centromere 7 and 7q31 signals were counted simultaneously in all samples. Representative images of FISH results are shown in Figure 7A-D. The copy number of cep7 and 7q31 varied from 1 to more than 10 copies/cell. Chromosome 7 aneusomy was observed in 79% of primary melanomas, whereas 95% showed 7q31 copy number alterations. The copy number index (CNI) was between 1.8 - 13.0 for chromosome 7 and between 1.2 - 13.3 for 7q31 (Figure 7.).

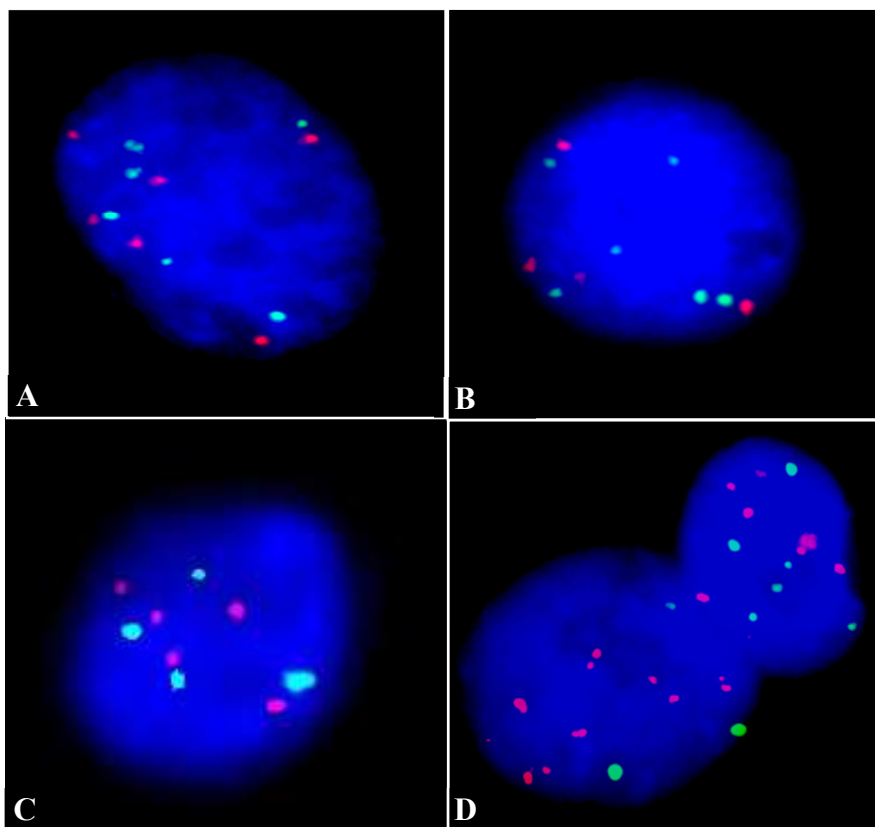


Figure 7. Copy number distributions of the 7q31 region in interphase melanoma cells. A chromosome 7 centromere-specific probe was labeled with SpectrumGreen (green signals), and a 7q31 locus-specific probe was labeled with SpectrumOrange (red signals). Nuclei were stained with DAPI (blue fluorescence). 7q31 apparent amplification in a metastatic SSM melanoma (A), 7q31 deletion in a non-metastatic SSM melanoma (B), low-level 7q31 amplification in a metastatic NM melanoma (C) and high-level 7q31 amplification in a metastatic NM melanoma (D).

RESULTS

Amplification of 7q31 was associated with chromosome 7 polysomy ($p < 0.001$), whereas deletion was accompanied by chromosome 7 di- or monosomy (Table 9.).

Table 9. Significant association (p -value ≤ 0.001 ; two-sided Fisher's exact test) between alterations of the 7q31 locus copy number and chromosome 7 ploidy

Chromosome 7 ploidy	7q31 locus copy number category					
	No. of samples (%) ¹	Deletion N (%)	No alteration N (%)	Amplification status		
				Apparent N (%)	Low level N (%)	High level N (%)
Monosomy	7 (9.3)	6 (85.7)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)
Disomy	17 (22.7)	3 (17.7)	4 (23.5)	0 (0.0)	9 (52.9)	1 (5.9)
Polisomy	51 (68.0)	5 (9.8)	0 (0.0)	4 (7.8)	34 (66.7)	8 (15.7)

¹Frequency is given within a copy number category of chromosome 7.

Extra copies of 7q31 relative to the copy number of chromosome 7 were observed in 71% (53/75) of primary tumors, whereas only 19% (14/75) exhibited deletion in this region. High-level amplification of 7q31 was observed in nine cases (12%). Except for one case, these tumors were in a more advanced stage with an aggressive phenotype, and eight out of nine melanomas metastasized mainly to distant locations (brain and lung, Table 10).

Table 10. Clinical-pathological parameters of primary melanomas with high-level 7q31 locus amplification (N=9)

Sample no.	7q31/7c ratio	TNM stage ¹	Histological subtype	Localization of the primary tumor	Survival ²	Metastasis	Ulceration
1	1.02	T4bN3M1c	NM ³	extremities	5.9	brain, lung, LN ⁴	yes
2	1.25	T4bN0M0	SSM ⁵	trunk	2.4	lung	yes
3	1.71	T1aN0M0	SSM	trunk	alive ⁶	no	no
4	1.82	T4bN0M0	NM	extremities	0.7	LN	yes
5	2.35	T4bN0M0	NM	trunk	5.9	brain	yes
6	2.38	T3bN0M1c	SSM	extremities	2.4	brain, lung	yes
7	2.39	T4bN2bM0	NM	head	alive	lung, LN	yes
8	2.48	T4bN0M0	NM	trunk	4.9	brain	yes
9	2.62	T3bN0M0	SSM	extremities	1.4	lung	yes

¹TNM stage at the time of surgery of primary tumor; ²survival in years after surgery; ³nodular melanoma; ⁴lymph node; ⁵superficial spreading melanoma; ⁶more than 5 years

We examined the 7q31 locus genetic alterations in 7 primary melanomas and their corresponding lymph node metastasis (Figure 8.). No significant differences in the 7q31 copy number were noticed between the primary tumor and its corresponding metastasis, except for

three tumor pairs: numbered as 1, 5 and 7. In case of pairs No.1 and 5 the 7q31/7c ratio was significantly increased in the metastatic tumor, whereas in pair No.7 both the primary tumor and its metastatic lesion showed a high ratio. Metastases of tissue No. 5 and 7 exhibited a higher 7q31 copy number, whereas in case of tumor No. 1 the decrease of chromosome 7 copy numbers resulted in an increased ratio in the metastasis, but similar pattern of 7q31 was found in primary tumor and its metastasis.

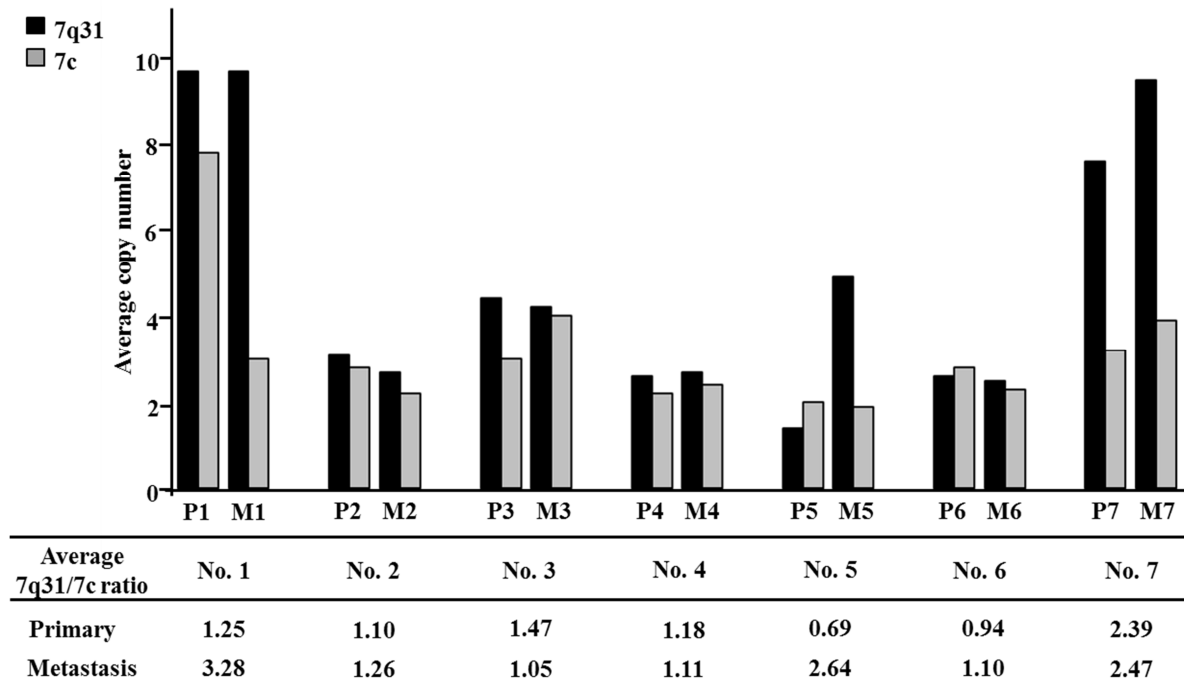


Figure 8. Average copy number of the 7q31 region and chromosome 7 in primary cutaneous melanomas (P) and their corresponding lymph node metastasis (M). Numbers included in the table below the graph represent the 7q31/7c ratio for the tumor pairs.

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

Examining the correlation of the average locus copy number with the different clinical-pathological parameters, we found a significant association with metastasis formation, tumor surface ulceration, patients' survival and age. The calculated 7q31 locus copy number index was significantly higher in primary tumors with metastatic property ($p=0.012$; average CNI= 4.6 ± 2.6 vs. 3.1 ± 1.5) and ulcerated surface ($p=0.002$; average CNI= 4.7 ± 2.6 vs. 3.0 ± 1.3). Patients older than 50 years were characterized as having a slightly higher number of 7q31 copies compared to patients in the younger age group ($p=0.038$; average CNI= 4.4 ± 2.5 vs. 3.1 ± 1.6).

CAV1 and TES mRNA and protein expression in primary melanomas

Thirty-three primary melanomas were available for gene expression analysis of the CAV1 and TES genes. Table 11 summarizes the results of the gene expression analysis. CAV1 mRNA level was significantly lower in thick tumors (>4.0 mm, $p=0.002$). TES mRNA levels were different in the primary tumors depending on the location of the corresponding metastasis ($p=0.011$). TES overexpression was found only in samples with cutaneous or lymph node metastasis (6/17). Tumors that formed cutaneous and/or lymph node and distant organ metastasis (6/17) were characterized by TES mRNA downregulation. Lesions with only lung, brain, bone and liver metastasis (5/17) had similar TES mRNA level as nevus samples.

Table 11. Correlation between the relative CAV1 and TES mRNA levels and the clinical-pathological parameters of primary melanomas

Variables		No. of tumors analyzed	CAV1	p^1	TES	p
Subtype ²	SSM	20	4.706 ± 0.413	0.207	1.640 ± 0.413	0.870
	NM	13	2.947 ± 1.687		1.494 ± 0.486	
Gender	Female	17	3.025 ± 1.182	0.292	1.545 ± 0.413	0.986
	Male	16	5.062 ± 3.674		1.622 ± 0.480	
Age	<50 years	9	1.259 ± 0.562	0.414	1.792 ± 0.624	0.677
	≥ 50 years	24	5.046 ± 2.531		1.504 ± 0.364	
Breslow thickness	<4.0 mm	21	5.651 ± 2.844	0.002	1.966 ± 0.429	0.063
	≥ 4.0 mm	12	1.147 ± 0.882		0.911 ± 0.348	
Metastasis ³	Absent	16	2.619 ± 0.697	0.067	2.092 ± 0.514	0.191
	Present	17	5.416 ± 3.602		1.135 ± 0.344	
Survival ³	Alive	23	3.010 ± 0.961	0.125	1.956 ± 0.418	0.057
	Dead	10	6.475 ± 5.925		0.778 ± 0.236	
Location of metastasis	Lymph node /cutaneous	6	3.960 ± 3.384	0.702	1.887 ± 0.901	0.011
	Distant organ	5	12.130 ± 11.910		1.098 ± 0.207	
	Multiple	6	1.274 ± 0.561		0.415 ± 0.116	

¹Mann-Whitney-Wilcoxon or Kruskal-Wallis test; ²SSM: superficial spreading melanoma, NM: nodular melanoma; ³within a 5-year follow up period; significant p-values are indicated in bold

We could examine the protein expression levels of CAV1 and TES genes in 60 primary melanomas using tissue microarray. Thick (>4.0 mm) and advanced-stage (Clark IV-V) melanoma samples (n=6) showed high intratumor staining heterogeneity regarding the morphologically different cell areas (i.e., foam cells or spindle cells). The other lesions were homogenous both histologically and with respect to protein staining. The examined nevus

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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 ($p=0.038$; Table 12). 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.

Table 12. CAV1 (N=51) and TES (N=55) protein staining distributions of primary melanomas regarding clinical-pathological parameters

Variables			No. of samples (%)	Staining category				p ¹
				0	1+	2+	3+	
Subtype								
CAV1	SSM		26 (51)	5 (19)	0 (0)	12 (46)	9 (35)	0.099
	NM		25 (49)	5 (20)	5 (20)	7 (28)	8 (32)	
TES	SSM		29 (53)	10 (35)	7 (24)	9 (31)	3 (10)	0.196
	NM		26 (47)	3 (12)	11 (42)	9 (34)	3 (12)	
Breslow thickness (mm)								
CAV1	<4.0		29 (57)	7 (24)	0 (0)	13 (45)	9 (31)	0.038
	≥4.0		22 (43)	3 (14)	5 (23)	6 (27)	8 (36)	
TES	<4.0		32 (58)	9 (28)	9 (28)	11 (35)	3 (9)	0.482
	≥4.0		23 (42)	4 (17)	9 (39)	7 (31)	3 (13)	
Metastasis²								
CAV1	no		24 (51)	7 (29)	0 (0)	9 (38)	8 (33)	0.053
	yes		23 (49)	2 (9)	5 (22)	9 (39)	7 (30)	
TES	no		27 (52)	9 (33)	5 (19)	10 (37)	3 (11)	0.128
	yes		25 (48)	4 (16)	12 (48)	6 (24)	3 (12)	
Patient's survival²								
CAV1	no		27 (57)	6 (22)	1 (4)	10 (37)	10 (37)	0.327
	yes		20 (43)	3 (15)	4 (20)	8 (40)	5 (25)	
TES	no		31 (60)	8 (26)	8 (26)	12 (38)	3 (10)	0.409
	yes		21 (40)	5 (24)	9 (43)	4 (19)	3 (14)	
Ulceration								
CAV1	no		27 (53)	6 (22)	0 (0)	12 (45)	9 (33)	0.089
	yes		24 (47)	4 (17)	5 (21)	7 (29)	8 (33)	
TES	no		29 (53)	8 (28)	6 (21)	12 (41)	3 (10)	0.206
	yes		26 (47)	5 (19)	12 (46)	6 (23)	3 (12)	

¹two-sided Fisher's exact test; ²within 5-year follow up period

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 (Table 13). The results clearly show that amplification of the locus is not or weakly correlated with mRNA and protein expressions of CAV1 and TES genes. The alterations of CAV1 and TES mRNA expressions had an impact on protein levels in some cases. However, we did not find any correlation between the genetic alterations, gene and protein expression levels.

Table 13. Comparison of 7q31 locus copy number status and the expression level of CAV1 and TES genes in cutaneous melanoma

Tumor no.	7q31 CNI	Main 7q31 copy number category	% of cells with 7q31 alteration	Relative mRNA level		Immunohistochemical score	
				CAV1	TES	CAV1	TES
10	2.1	Deletion	97.2	0.153	0.092	2+	1+
11	2.6	Low level ampl.	39.5	2.528	0.484	2+	1+
12	3.5	Low level ampl.	50.0	0.634	2.174	1+	1+
13	3.6	Low level ampl.	69.3	0.076	0.137	1+	1+
14	3.6	Low level ampl.	90.3	0.526	0.096	2+	3+
15	4.5	Low level ampl.	98.5	0.607	1.663	2+	3+
16	4.9	Low level ampl.	91.4	59.760	0.607	2+	0
17	5.0	Low level ampl.	99.1	0.139	0.107	3+	2+
8	6.4	High level ampl.	70.7	0.212	0.762	2+	2+
1	13.3	High level ampl.	100.0	0.223	0.319	1+	1+
18	1.7	Deletion	37.1	nd	nd	1+	1+
19	2.6	Deletion	68.0	nd	nd	2+	1+
20	3.3	Apparent ampl.	84.0	nd	nd	0	1+
21	3.4	Low level ampl.	74.4	nd	nd	nd	2+
22	nd	nd	nd	0.076	1.503	3+	2+
23	nd	nd	nd	1.418	0.128	2+	2+
24	nd	nd	nd	3.693	0.690	3+	nd

nd: no data available

In order to get clearer view of this phenomenon we 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 (the cell line data are summarized on Figure 9).

All cell lines exhibited heterogeneous copy number distribution for chromosome 7 centromere and 7q31 copy numbers. Low-level amplifications and deletions were present in all cell lines with different frequencies. Locus deletion was observed with high frequency in

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three (WM983B, HT199 and WM35) and low level amplification was present in two (WM983A and HT168), whereas high level amplification was detected in two cell lines (A2058 parental cell line and HT168M1 cell line selected in vivo from the A2058 with a high liver metastatic capacity).

The relative mRNA expression of the CAV1 gene was similar in the WM35, WM983A, WM983B, HT199 and HT168 melanoma cell lines (Figure 9). 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 expression in the WM983A cell line (Figure 9). No CAV1 protein expression was observed in the HT168M1 cell line.

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 (Figure 9).

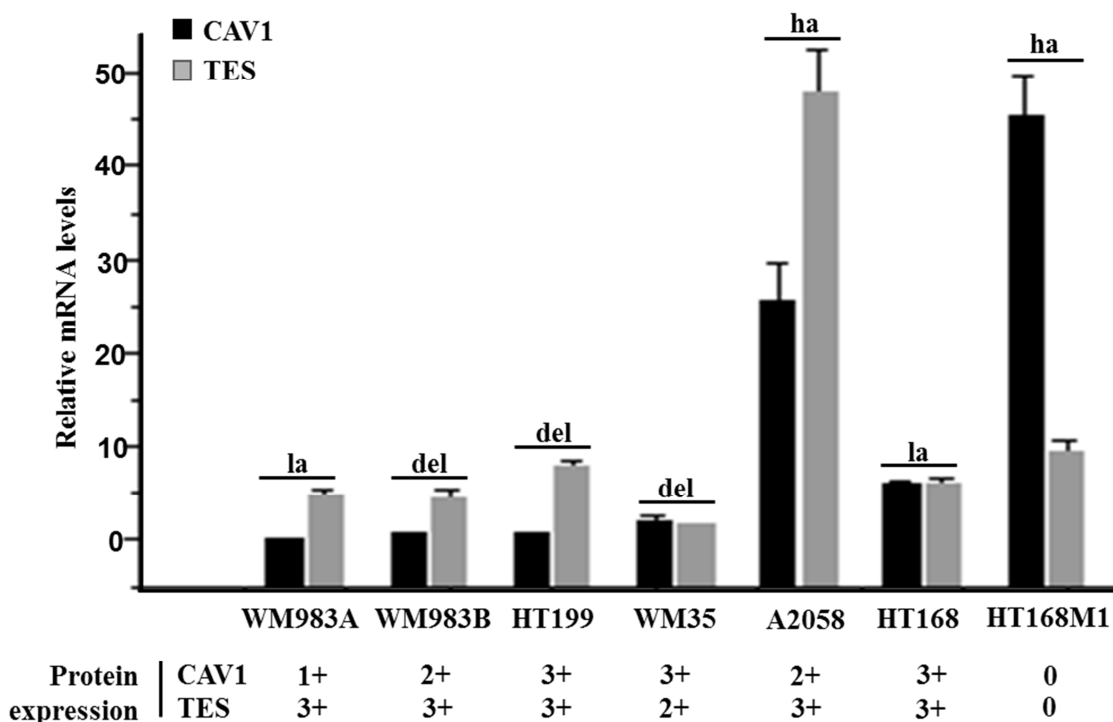


Figure 9. Relative mRNA and protein expression in human melanoma cell lines. The main 7q31 copy number categories are labeled on the top of the columns: la = low level amplification, del = deletion, ha = high level amplification. Error bars represent $\pm$ SD. The lower part of the figure summarizes the staining intensities of the CAV1 and TES proteins: no staining (0), weak (1+), medium (2+) and strong (3+).

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In case of three melanoma cell lines (WM35, HT199 and WM983B) we confirmed protein expression results obtained by immunohistochemistry using western blot analysis (Figure 10).

Cell line	GAPDH WB trace	CAV1 WB trace	CAV1/GAPDH	TES WB trace	CCND1/GAPDH
WM35	1.410	1.254	0.889	0.891	0.632
HT199	2.203	2.339	1.062	1.542	0.670
WM983B	1.727	0.154	0.089	1.522	0.881

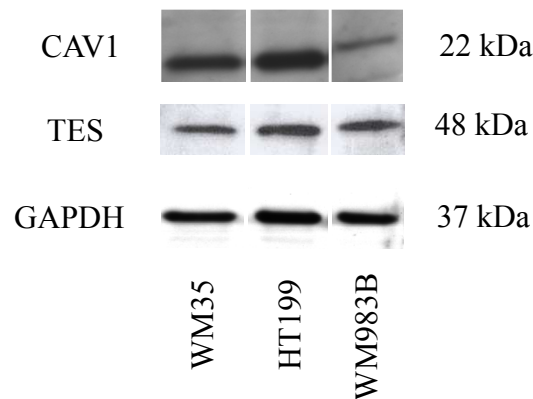


Figure 10. CAV1 and TES protein expression in human melanoma cell lines (WM35, HT199 and WM983B) with different biological properties. WB trace: CAV1 or TES protein levels after quantification of the western blot results. The density was obtained based on the optical density (OD) multiplied by the area occupied (mm).

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

A total of 76 primary melanoma samples were analyzed by FISH (Table 4). Chromosome 11 and CCND1 signals were counted simultaneously in all samples. Representative images of the FISH results are shown in Figure 11A-D. The copy numbers of chromosome 11 and CCND1 varied from 0 to more than 10 copies per cell. Chromosome 11 aneusomy was observed in 66% of primary melanomas, whereas 83% of samples showed CCND1 copy number alterations. The calculated copy number index (CNI) was between 1.3 and 4.9 for chromosome 11 and between 1.5 and 15.0 for CCND1, and there was a significant association between the copy numbers of CCND1 and the chromosome 11 centromere ($p=0.008$; Table 14).

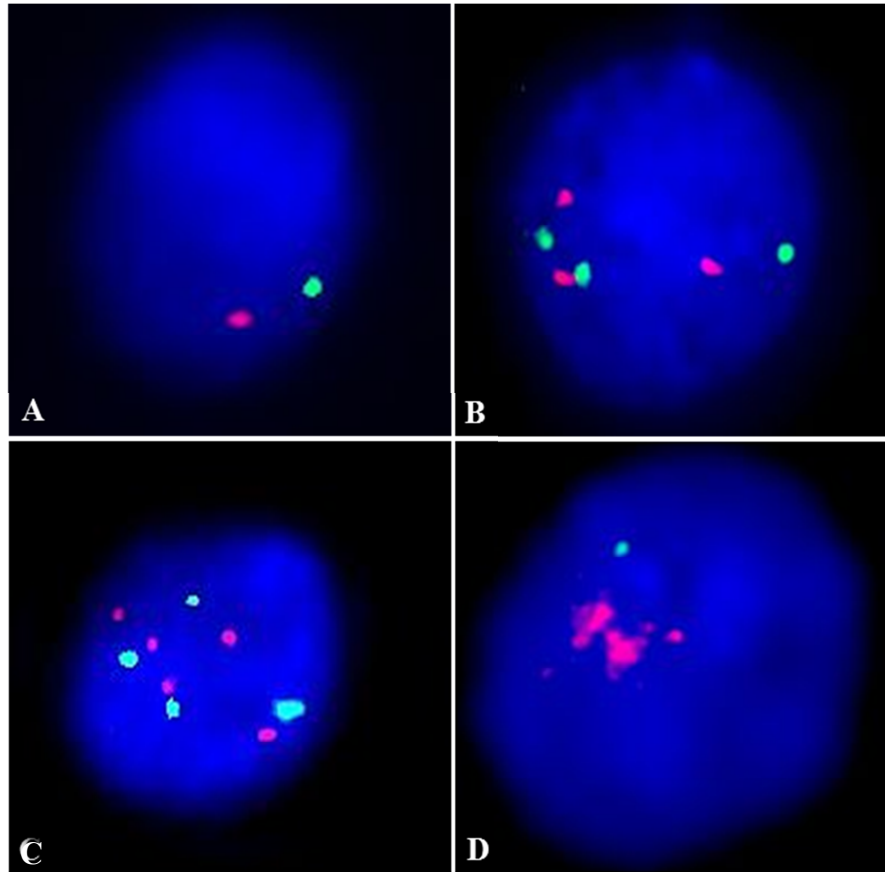


Figure 11. Copy number distributions of *CCND1* gene in interphase melanoma cells. A chromosome 11 centromere-specific probe was labeled with SpectrumGreen (green signals), and a *CCND1* gene-specific probe was labeled with SpectrumOrange (red signals). Nuclei were stained with DAPI (blue fluorescence). *CCND1* deletion in a metastatic NM melanoma (A), apparent amplification in a non-metastatic SSM melanoma (B), low-level amplification in a non-metastatic SSM melanoma (C) and high-level amplification in a metastatic NM melanoma (D).

Table 14. Significant association ($p=0.008$; two-sided Fisher's exact test) between alterations of the *CCND1* gene copy number and chromosome 11 ploidy

Chromosome 11 ploidy	No. of samples (%) ¹	<i>CCND1</i> gene copy number category				
		Deletion N (%)	No alteration N (%)	Amplification status		
				Apparent N (%)	Low level N (%)	High level N (%)
Monosomy	8 (10.5)	7 (87.5)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
Disomy	26 (34.2)	5 (19.2)	8 (30.8)	0 (0.0)	11 (42.3)	2 (7.7)
Polisomy	42 (55.3)	12 (28.6)	4 (9.5)	4 (9.5)	16 (38.1)	6 (14.3)

¹frequency is given within a copy number category of chromosome 11.

Extra copies of CCND1 relative to chromosome 11 were observed in 51% (39/76) of primary tumors, whereas 32% of primary tumors (24/76) exhibited a CCND1 gene deletion. A high level of CCND1 amplification was observed in eight cases (11%). With the exception of one case where the patient died for a reason other than melanoma, these tumors were metastatic and showed ulcerated surfaces. Through searching for correlations between the average CNI and different clinical–pathological parameters, we found a significant association of the average CNI with the location of the primary tumor, location of the metastasis, and tumor surface ulceration. The calculated CCND1 CNI was significantly lower in primary tumors located at the extremities ($p=0.043$; Table 15) and without ulceration ($p=0.048$; Table 15) and was increased in primary melanomas with brain, lung, liver, and spinal metastases and similar observation was made for multiple metastases ($p=0.029$; Table 15).

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Table 15. Correlations between the CCND1 copy number index and clinical–pathological parameters of primary melanomas

Variables	No. of tumors analyzed	Avg. CCND1 copy number	CI ¹	p ²	Avg. chr 11 copy number	CI	p
Subtype				0.304			0.563
SSM	48	4.2	2.5-5.8		2.5	2.2-2.8	
NM	28	3.5	2.1-4.8		2.6	2.2-2.9	
Gender				0.191			0.843
Female	45	4.2	2.2-6.1		2.6	2.2-3.1	
Male	31	3.5	2.3-4.7		2.5	2.2-2.8	
Age				0.919			0.919
<50 years	25	4.4	1.7-7.2		2.5	2.1-2.8	
≥50 years	51	3.5	2.5-4.6		2.6	2.3-2.9	
Breslow thickness (mm)				0.277			0.673
<2.0	30	4.1	0.4-8.5		2.3	1.5-3.1	
2.0-4.0	20	2.9	2.0-3.8		2.5	2.0-2.9	
>4.0	26	4.2	2.6-5.8		2.6	2.3-3.0	
Location of primary tumor				0.043			0.032
Extremities	40	3.5	2.7-4.3		2.7	2.3-3.1	
Trunk	29	4.0	1.4-6.7		2.2	2.1-2.3	
Head	7	4.2	0.7-7.6		2.9	2.2-3.7	
Ulceration				0.048			0.110
Absent	39	2.4	2.1-2.7		2.2	2.0-2.5	
Present	37	4.2	2.9-5.5		2.7	2.4-2.9	
Metastasis³				0.059			0.210
Absent	35	2.5	2.2-2.8		2.3	2.2-2.5	
Present	40	3.7	2.7-4.7		2.5	2.3-2.8	
Patient's survival³				0.160			0.173
Alive	43	3.7	2.0-5.4		2.5	2.1-2.9	
Dead	32	3.8	2.5-5.1		2.6	2.3-2.9	
Location of metastasis⁴				0.029			0.025
LN ⁵ /Cutaneous	13	2.7	2.0-3.4		2.5	2.0-2.9	
Distant organ	16	3.8	1.9-5.6		2.4	2.1-2.7	
Multiple ⁶	11	5.0	2.4-7.5		2.9	2.4-3.5	

¹CI confidence interval (95 %); ²Mann–Whitney–Wilcoxon or Kruskal–Wallis tests; ³Patients with at least a 5-year follow-up period were included; ⁴Metastasis of the examined primary tumors; ⁵Lymph node; ⁶The primary melanoma formed metastases in both the skin/lymph node and in a distant organ; Significant p values are indicated as bold

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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 based on ROC analysis) had a 1.6-fold higher risk of death from the disease, though this association was not significant ($p=0.278$, Figure 12) probably due to the small amount of data.

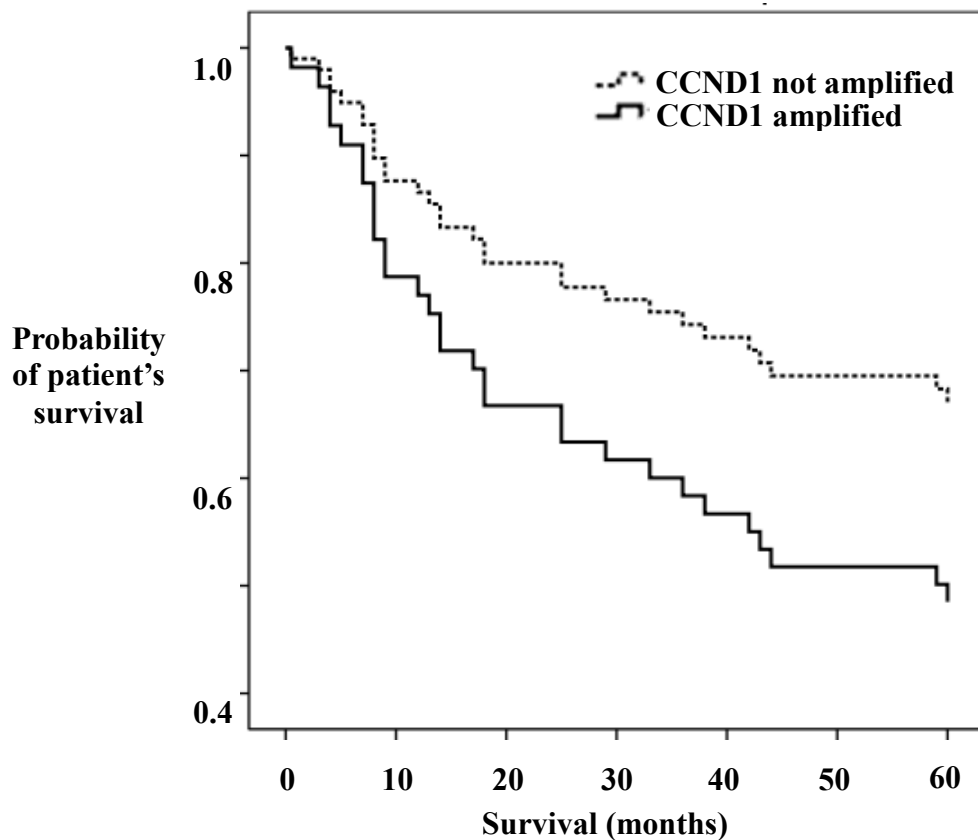


Figure 12. Association of *CCND1* genetic alterations with patients' survival. Kaplan–Meier survival curves of *CCND1*-amplified (continuous line) and unamplified (dotted line) primary melanoma samples. The Cox regression coefficient (B) was 1.6, with a significance of $p=0.278$. Cutoff value (2.5) between the two defined groups was determined by ROC analysis correcting for ulceration and metastasis formation

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

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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. Table 16 summarizes the data of average copy numbers of CCND1 and chromosome 11 in primary melanomas with (N=32) or without (N=23) BRAF or NRAS mutations.

Table 16. Average CCND1 copy numbers in primary melanomas with (N=32) or without (N=23) BRAF or NRAS mutations

Sample no.	chronic sun exposure ¹	metastasis formation ²	localization of metastasis	ulceration	BRAF ^{V600} mutation status	NRAS ^{Q61} mutation status	CCND1 copy number index
1	—	—	—	—	V600E	WT ³	2.0
2	—	+	liver	—	V600K	WT	2.3
3	+	—	—	—	V600E	WT	2.1
4	—	—	—	+	V600E	WT	2.2
5	—	+	brain	+	V600E	WT	2.1
7	+	+	LN ⁴ , lung, brain	+	V600E	WT	4.1
9	—	+	brain	+	V600K	WT	3.3
10	+	+	LN	+	V600E	WT	5.3
11	—	+	brain	+	V600E	WT	15.0
12	+	—	—	—	V600E	WT	4.1
13	—	+	LN	—	V600E	WT	2.1
15	—	—	—	+	V600E	WT	3.2
24	+	—	—	—	V600R	WT	3.0
25	—	—	—	—	V600E	WT	1.7
26	—	+	LN	+	V600E	WT	2.1
27	—	+	liver	—	V600E	WT	2.4
28	—	+	brain, liver	+	V600E	WT	3.0
29	—	+	brain	+	V600E	WT	2.5
30	—	—	—	+	V600E	WT	3.5
31	—	+	LN	+	V600E	WT	2.6
32	—	+	lung, brain	+	V600E	WT	6.8
33	—	+	liver	+	V600E	WT	2.2
34	—	+	LN, liver	+	V600E	WT	2.3
35	—	—	—	—	V600E	WT	2.7
6	+	+	LN, lung, brain	+	WT	Q61K	3.3
8	+	+	LN, cutaneous,	+	WT	Q61K	4.4
14	+	—	—	—	WT	Q61R	2.1
36	—	+	lung	+	WT	Q61K	8.2
37	+	+	LN	+	WT	Q61K	2.6
38	—	—	—	—	WT	Q61K	3.5
39	—	+	LN	—	WT	Q61R	2.2
40	+	+	LN, cutaneous	—	WT	Q61L	2.5

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Table 16. continuous

Sample no.	chronic sun exposure	metastasis formation	localization of metastasis	ulceration	BRAF ^{V600} mutation status	NRAS ^{Q61} mutation status	CCND1 copy number index
16	—	—	—	—	WT	WT	1.9
17	+	+	LN	+	WT	WT	1.9
18	—	+	LN	+	WT	WT	2.0
20	+	+	LN	+	WT	WT	4.5
21	—	—	—	—	WT	WT	1.8
22	—	+	LN	—	WT	WT	2.8
23	+	—	—	—	WT	WT	5.9
41	—	—	—	—	WT	WT	2.1
42	+	—	—	+	WT	WT	2.5
43	+	—	—	—	WT	WT	2.6
44	—	+	lung, brain	+	WT	WT	2.0
45	—	—	—	—	WT	WT	2.4
46	—	+	LN	—	WT	WT	2.0
47	—	+	LN	—	WT	WT	2.0
48	—	—	—	—	WT	WT	2.4
49	—	—	—	—	WT	WT	3.8
50	—	+	cutaneous, lung	+	WT	WT	15.0
51	+	+	liver	+	WT	WT	1.5
52	+	+	brain	+	WT	WT	2.3
53	+	+	LN, lung	+	WT	WT	9.1
54	—	—	—	—	WT	WT	1.7
55	+	+	LN, liver	+	WT	WT	5.0
56	—	+	lung, liver, bone	+	WT	WT	1.9

¹primary tumor is located on the face, lower arm or shin; ²within the 5-year follow-up period; ³wild type; ⁴lymph node metastasis

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

A 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 ($p=0.013$, Figure 13).

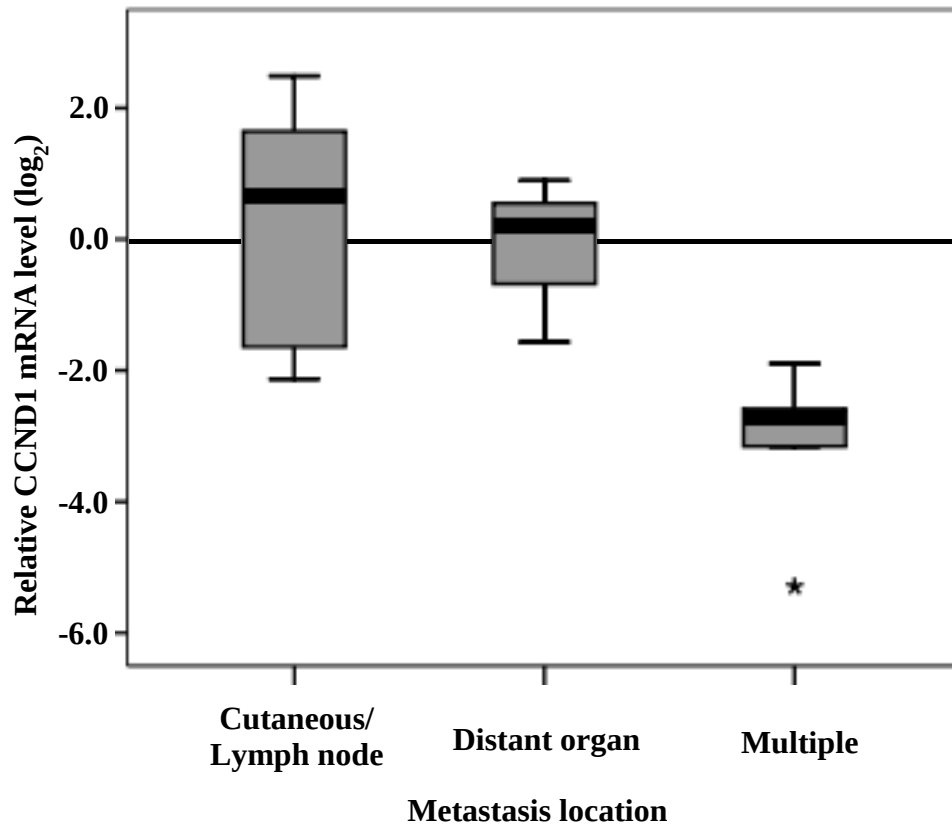


Figure 13. Log₂-transformed CCND1 mRNA levels in melanomas relative to control nevi with respect to the location of metastasis. Multiple metastases were defined as when the primary melanoma formed metastases in the skin, lymph nodes, and in a distant organ. The vertical line at zero indicates the normal mRNA level; the average CCND1 mRNA level of tissue samples within a given group is the same as that of the average level in control nevi

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.

CCND1 expression changes were moderately correlated with the BRAF ($B=0.553$, $p=0.002$, Figure 14A) and NRAS ($B=0.678$, $p<0.001$, Figure 14B) mRNA levels.

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Furthermore, the expression of BRAF showed a remarkably strong association with the expression of NRAS ($B=0.832$, $p<0.001$, Figure 14C).

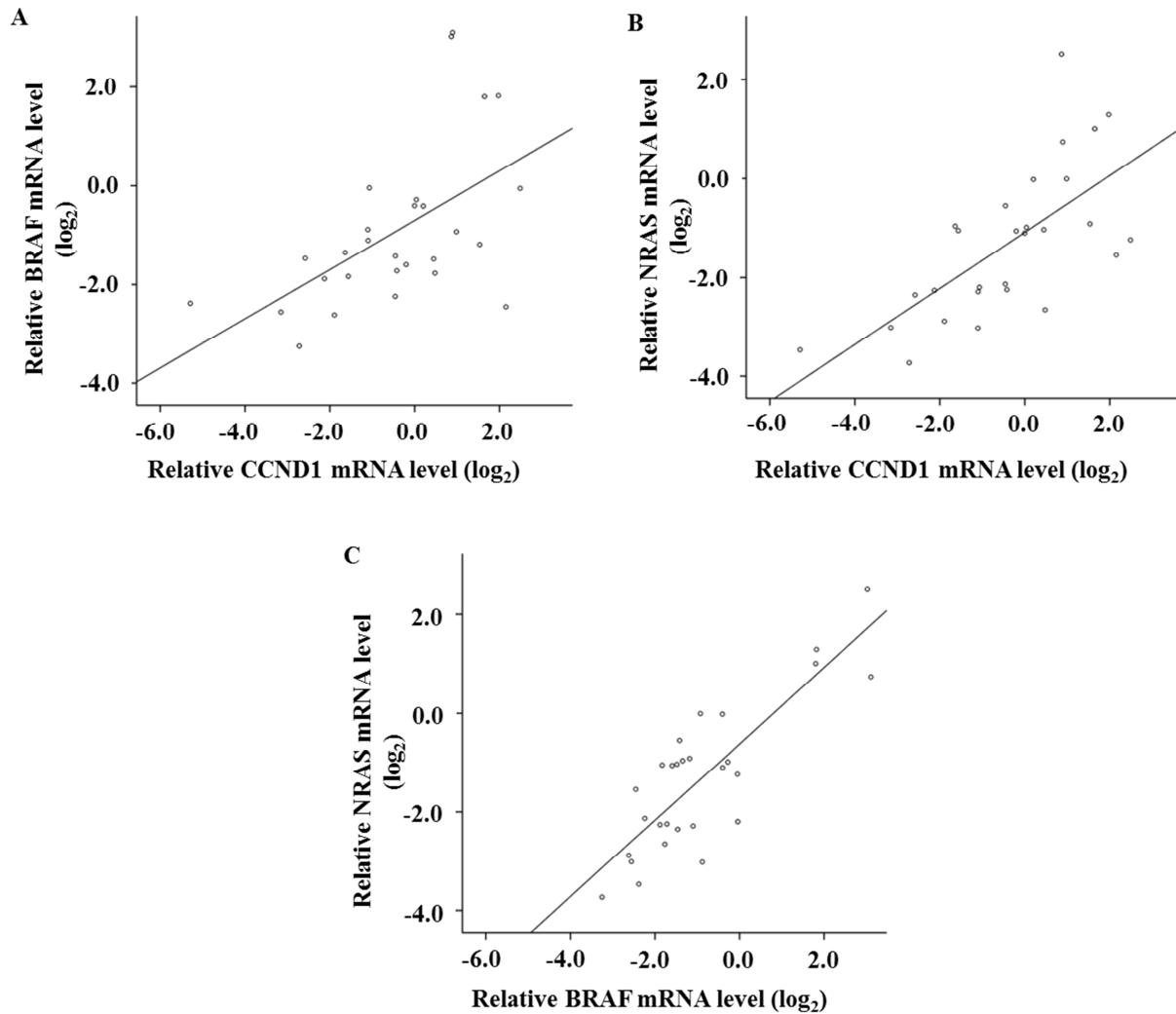


Figure 14. Correlation of CCND1, BRAF, and NRAS mRNA levels in primary melanomas. (A) Pearson correlation between the log₂-transformed relative mRNA levels of CCND1 and BRAF genes ($B=0.553$, $p=0.002$). (B) Pearson correlation between the log₂-transformed relative mRNA levels of CCND1 and NRAS genes ($B=0.678$, $p<0.001$). (C) Pearson correlation between the log₂ transformed relative mRNA levels of BRAF and NRAS genes ($B=0.832$, $p<0.001$)

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 (Table 17). Examining the association between the CCND1 protein level and mRNA expression, we did not find a significant correlation.

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Table 17. *CCND1 mRNA levels in primary melanomas with or without BRAF and NRAS mutations*

Sample no.	BRAF ^{V600} mutation status	NRAS ^{Q61} mutation status	relative CCND1 mRNA level
1	+	WT ¹	0.465
2	+	WT	1.151
3	+	WT	0.729
4	+	WT	0.727
5	+	WT	0.338
6	+	WT	0.152
7	+	WT	0.026
8	+	WT	0.167
9	+	WT	1.858
10	+	WT	0.229
11	+	WT	4.447
12	+	WT	1.363
13	WT	+	0.321
14	WT	+	1.822
15	WT	+	0.46726
average CCND1 mRNA level (±SD)			0.951 ± 1.133
16	WT	WT	1.977
17	WT	WT	3.130
18	WT	WT	0.870
19	WT	WT	0.269
20	WT	WT	5.608
21	WT	WT	0.999
22	WT	WT	2.898
23	WT	WT	1.392
average CCND1 mRNA level (±SD)			2.143 ± 1.715

¹wild type

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

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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, Table 18). Melanoma samples presenting a greater thickness (>4.0 mm) and metastasis, and causing the death of the patient 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 (Figure 15). Western blot analysis confirmed the immunohistochemical results (Figure 15).

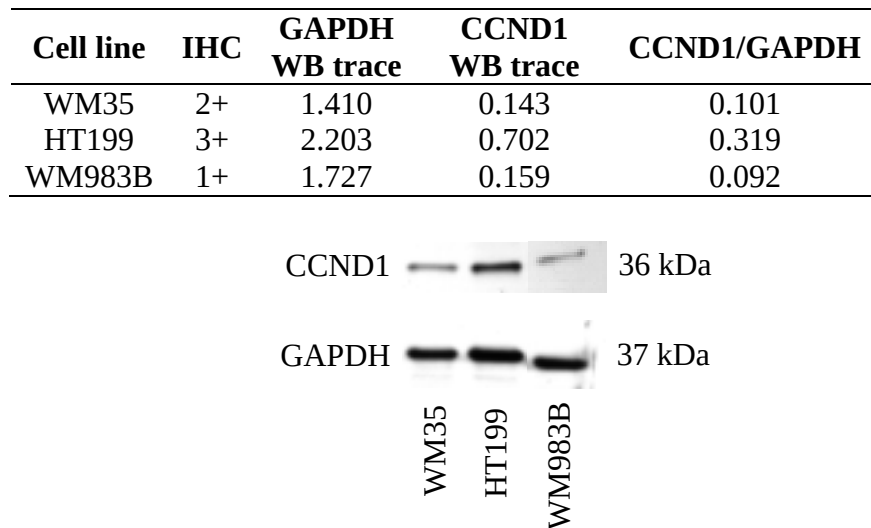


Figure 15. CCND1 protein expression in human melanoma cell lines with different biological properties. IHC: immunohistochemistry. The staining intensities for the CCND1 protein are reported as follows: no staining (0), weak (1+), medium (2+) and strong (3+). WB trace: CCND1 protein level after quantification of the western blot results. The density was obtained based on the optical density (OD) multiplied by the area occupied (mm).

RESULTS

Table 18. CCND1 protein staining distributions and quickscores of primary melanomas with respect to clinical-pathological parameters

Parameters	No. of tumors (%)	Staining intensity (%)				p-value ¹	Q _{AXB} ² category (%)			p-value
		0	1+	2+	3+		0	1	2	
Subtype						0.083				0.108
Superficial spreading	27 (50.0)	4 (14.8)	9 (33.3)	9 (33.3)	5 (18.6)		14 (51.9)	9 (33.3)	4 (14.8)	
Nodular	27 (50.0)	3 (11.1)	6 (22.2)	4 (14.8)	14 (51.9)		9 (33.3)	7 (25.9)	11 (40.8)	
Gender						0.481				0.480
Female	35 (64.8)	4 (11.4)	12 (34.3)	7 (20.0)	12 (34.3)		17 (48.6)	9 (25.7)	9 (25.7)	
Male	19 (35.2)	3 (15.8)	3 (15.8)	6 (31.6)	7 (36.8)		6 (31.6)	7 (36.8)	6 (31.6)	
Age						0.535				1.000
<50 years	12 (22.2)	3 (25.0)	2 (16.7)	3 (25.0)	4 (33.3)		5 (41.7)	4 (33.3)	3 (25.0)	
≥50 years	42 (77.8)	4 (9.5)	13 (31.0)	10 (23.8)	15 (35.7)		18 (42.8)	12 (28.6)	12 (28.6)	
Breslow thickness						0.047				0.157
<2.0 mm	23 (42.6)	3 (13.0)	6 (26.1)	10 (43.5)	4 (17.4)		10 (43.5)	10 (43.5)	3 (13.0)	
2.0 – 4.0 mm	9 (16.7)	1 (11.1)	4 (44.4)	1 (11.1)	3 (33.4)		5 (55.5)	1 (11.1)	3 (33.4)	
>4.0 mm	22 (40.7)	3 (13.6)	5 (22.7)	2 (9.1)	12 (54.6)		8 (36.4)	5 (22.7)	9 (40.9)	
Ulceration						0.169				0.453
Absent	29 (72.2)	4 (13.8)	8 (27.6)	10 (34.5)	7 (24.1)		13 (44.8)	10 (34.5)	6 (20.7)	
Present	25 (27.8)	3 (12.0)	7 (28.0)	3 (12.0)	12 (48.0)		10 (40.0)	6 (24.0)	9 (36.0)	
Metastasis ³						0.028				0.021
Absent	30 (55.6)	4 (13.3)	9 (30.0)	11 (36.7)	6 (20.0)		14 (46.7)	12 (40.0)	4 (13.3)	
Present	24 (44.4)	3 (12.5)	6 (25.0)	2 (8.3)	13 (54.2)		9 (37.5)	4 (16.7)	11 (45.8)	
Survival ³						<0.001				0.026
Alive	34 (63.0)	4 (11.8)	10 (29.4)	13 (38.2)	6 (20.6)		15 (44.1)	13 (38.2)	5 (17.7)	
Death	20 (37.0)	3 (15.0)	5 (25.0)	0 (0.0)	12 (60.0)		8 (35.0)	3 (15.0)	10 (50.0)	
Location of metastasis ⁴						0.054				0.146
Cutaneous /Lymph node	9 (37.5)	0 (0.0)	1 (11.1)	1 (11.1)	7 (77.8)		1 (11.1)	2 (22.2)	6 (66.7)	
Distant organ	8 (33.3)	2 (25.0)	1 (12.5)	0 (0.0)	5 (62.5)		3 (37.5)	1 (12.5)	4 (50.0)	
Multiple ⁵	7 (29.2)	1 (14.3)	4 (57.1)	1 (14.3)	1 (14.3)		5 (71.4)	1 (14.3)	1 (14.3)	

¹Two-sided Fisher's exact test; ²multiplicative quickscore; ³within a 5-year follow up period; ⁴metastasis of the examined primary tumors;

⁵the primary melanoma formed metastases in both the skin/lymph node and in a distant organ. Significant p-values are indicated as bold.

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 [63, 87]. 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% [88]. 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 [89]. 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 [90, 91].

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 a 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 [92-95]. 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 thus contribute to their fragility [96, 97]. 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 [98-100].

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 [101, 102]. 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 [47, 48, 103]. Similarly to others, our research group has 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 [48]. 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 [47, 104]. Accumulation of intrachromosomal amplicons might responsible for drug-resistance of cancers [92]. 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 [105-107].

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 [108]. 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 [109, 110]. 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 [111]. Furthermore, a recent melanoma study provided evidence for the possible anti-metastatic function of the CAV1 protein [112]. 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 [113, 114]. 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 [108] 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 [115]. 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 [116].

Downregulation of TES, which has previously been observed in head and neck squamous cell carcinomas [117], gastric cancers [118] and breast cancers [57] at both mRNA and protein levels, was frequently found in our primary melanoma samples. Although TES did not exhibit a 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 [119]. Furthermore, an error in the epigenetic regulation of genes could also affect the mRNA and protein levels [103, 120].

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 [121]. 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 [122]. 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 [63].

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 [90, 123, 124]. 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 [29]. 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 [31]. 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 [23]. 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 [125]. 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 [119]. 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 [126]). 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 [127]. 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 [128]. 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 [122].

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 [122]. 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 [129]. 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 [130].

Another explanation for this result could be the posttranscriptional degradation of CCND1 mRNA regulated by the recently discovered miR-193b micro RNA [131-133].

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.

Summary

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.

Analysis of the 7q31 region:

1. Significant associations were found between the copy number alterations of 7q31 and 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.

Analysis of the CCND1 gene:

1. 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.
2. 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.
3. 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.

Összefoglalás

A disszertációban összefoglalt vizsgálatok célja volt olyan gének (CCND1, BRAF, NRAS) illetve lokuszok (7q31) genetikai és gén expressziós eltéréseinek vizsgálata, melyek szerepet játszhatnak humán melanomák progressziójában. Célunk volt továbbá a fent említett eltérések és a primer daganatok klinikai-patológiai jellemzői közötti összefüggések átfogó analízise is.

A 7q31-es régió vizsgálata:

1. A 7q31 kópiaszám eltéréseinek primer melanomákban és áttétekben végzett FISH analízise szignifikáns összefüggést mutatott ki a kedvezőtlen klinikai kimenetellel.
2. A lokuszon elhelyezkedő CAV1 és TES gének mRNS expressziós analízise csökkent CAV1 kifejeződést tárt fel vastag melanomákban, és a TES mRNS szint csökkenését a többszörös áttétképző primer mintákban; míg azok a léziók, melyek csupán kután vagy nyirokcsomó metasztázissal rendelkeztek, emelkedett TES mRNS szinttel jellemezhetők.
3. A CAV1 fehérje szint szignifikánsan magasabb vékony primer tumorokban, míg a TES fehérje expresszió nem mutatott összefüggést egyetlen vizsgált paraméterrel sem.
4. A 7q31 amplifikáció nem vagy csak gyenge korrelációban állt a CAV1 és TES gének mRNS és fehérje expressziós változásaival.

A CCND1 gén vizsgálata:

1. A gén kópiaszám eltérések analízise primer melanomákban szignifikáns összefüggést tárt fel a kedvezőtlen klinikai kimenetellel. Ezt az összefüggést befolyásolta a BRAF és NRAS gének mutációs státusza a napsugárzásnak való kitettség esetében.
2. CCND1 mRNS szint csökkenés figyelhető meg azokban a primer tumorokban, melyek többszörös áttétképzéssel jellemezhetők; és a gén expresszió korrelációt mutat mind a BRAF és NRAS mRNS szintekkel, mind pedig ez utóbbi gének mutációs státuszával.
3. A CCND1 protein fokozott expressziója áttétképzéssel és rövidebb túléléssel társul, valamint a fehérje szint összefüggést mutat a Breslow tumor vastagsággal is.

Eredményeinket összefoglalva, a 7q31-es lokusz és CCND1 gén amplifikációja prognosztikai jelentőséggel bírhat kután melanomák kedvezőtlen klinikai kimenetelével kapcsolatban. Továbbá, az eredmények azt mutatják, hogy a CAV1, TES és CCND1 gének expressziós eltérései is számos szempontból befolyásolhatják a tumor biológiai viselkedését.

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Publications



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Subject: Ph.D. List of Publications

Candidate: Laura Vízkeleti

Neptun ID: LG7VT5

Doctoral School: Doctoral School of Health Sciences

List of publications related to the dissertation

1. Vízkeleti, L., Ecsedi, S., Rákossy, Z., Orosz, A., Lázár, V., Emri, G., Koroknai, V., Kiss, T., Ádány, R., Balázs, M.: The role of CCND1 alterations during the progression of cutaneous malignant melanoma.
Tumor Biol. 33 (6), 2189-2199, 2012.
DOI: <http://dx.doi.org/10.1007/s13277-012-0480-6>
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2. Vízkeleti, L., Ecsedi, S., Rákossy, Z., Bégány, Á., Emri, G., Tóth, R., Orosz, A., Szöllősi, A., Méhes, G., Ádány, R., Balázs, M.: Prognostic relevance of the expressions of CAV1 and TES genes on 7q31 in melanoma.
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Mod. Pathol. 22 (10), 1367-1378, 2009.
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List of other publications

5. Rákossy, Z., Ecsedi, S., Tóth, R., **Vízkeleti, L.**, Hernandez-Vargas, H., Lázár, V., Emri, G., Szatmári, I., Herceg, Z., Ádány, R., Balázs, M.: Integrative genomics identifies gene signature associated with melanoma ulceration.
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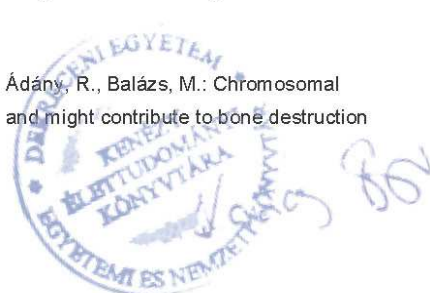
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Presentations at conferences

1. Rakosy Z, **Vizkeleti L**, Begany A, Adany R, Balazs M: poster: Amplification of the EGFR gene and the 7q31 locus are associated with metastasis formation of malignant melanomas; 30th FEBS Congress and 9th IUBMB Conference 2-7 July, 2005, Budapest, Hungary
2. 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
3. Ecsedi S, **Vizkeleti L**, Rakosy Z, Juhasz A, Adány R, Balazs M: poster: Analysis of chromosomal alterations in interphase cells in cholesteatomas; Hungarian Cancer Society XXVI. Congress 10-12 Nov, 2005; Budapest, Hungary
4. 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
5. Rakosy Z, **Vizkeleti L**, Ecsedi S, Begany A, Adany R, Balazs M: poster: Analysis of gene expression pattern of primary malignant melanomas using microarray technique; V. Hungarian Cell Analytical Conference; 4-6 May, 2006, Budapest, Hungary
6. **Vizkeleti L**, Ecsedi S, Rakosy Z, Begany A, Adany R, Balazs M: poster: Alterations of 7q31 locus in human malignant melanomas; V. Hungarian Cell Analytical Conference; 4-6 May, 2006, Budapest, Hungary
7. Ecsedi S, **Vizkeleti L**, Rakosy Z, Juhasz A, Adany R, Balazs M: poster: Cytogenetically analysis of cholesteatomas with different biological behavior; V. Hungarian Cell Analytical Conference; 4-6 May, 2006, Budapest, Hungary
8. Balazs M, Lazar V, Rakosy Z, **Vizkeleti L**, Ecsedi S, Begany A, Emri G, Adany R: oral: Array CGH and fluorescence in situ hybridization analyses reveal new genomic alterations in malignant melanoma; MAF10 10th Conference on Methods and Applications of Fluorescence, 9-12 Sept, 2007, Salzburg, Austria

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Keywords

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Tissue microarray
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Appendix