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ONCOGENIC DRIVERS OF CUTANEOUS MALIGNANT MELANOMA AND THEIR ROLE IN PROGRESSION

PhD thesis

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1 The list of Abbreviations

ACT	adoptive cell therapy
ALM	acral lentiginous melanoma
AML	acute myeloid leukemia
ARF	tumor suppressor gene known as $p14^{ARF}$ in human and $p19^{ARF}$ in mouse
BCR-ABL	breakpoint cluster region - Abelson murine leukemia viral oncogene homolog
BLAST	Basic Local Alignment Search Tool
bp	base pair
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BRCA2	breast cancer 2, DNA repair associated
c	codon
C	cytidine nucleobase
cAMP	cyclic adenosine monophosphate
CDKN2A	cyclin dependent kinase inhibitor 2A
CDK4	cyclin dependent kinase 4
CNS	central nervous system
CNV	copy-number variation
CPD	cyclobutane pyrimidine dimer
CRAF	v-Raf murine sarcoma viral oncogene homolog C
CTLA4	cytotoxic T-lymphocyte antigen 4
DALY	disability-adjusted life year
DNA	deoxyribonucleic acid
DOPA	dihydroxyphenylalanine
e	exon
ERK	extracellular signal-regulated kinase
FDA	U.S. Food and Drug Administration
FFPE	formalin fixed paraffin embedded
FGFR	fibroblast growth factor receptor
FLT3	FMS-like tyrosine kinase-3 receptor
GBD	Global Burden of Disease
GIST	gastrointestinal stroma tumor
H	MAF category high for samples bearing more than 40% mutant allele
HRAS	Harvey rat-sarcoma viral oncogene homolog

IFN-α	interferon-α
IL-2	interleukin-2
JAK/STAT	Janus kinase/signal transducers and activators of transcription pathway
KIT	cellular homolog of the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog, stem cell factor receptor
KRAS	Kirsten rat-sarcoma viral oncogene homolog
L	MAF category low, for less than 15% mutant allele
LDH	lactate dehydrogenase
LMM	lentigo maligna melanoma
LOH	loss of heterozygosity
M	MAF category medium for 15–40% mutant allele
MAF	mutant allele fraction
MAPK	mitogen-activated protein kinase pathway
MC1R	melanocortin receptor 1
MEK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MITF	microphtalmia-associated transcription factor
MSH	melanocyte-stimulating hormone
mU	million units
NCBI	National Center for Biotechnology Information
NF1	neurofibromin 1
NM	nodular melanoma
NRAS	neuroblastoma rat-sarcoma viral oncogene homolog
NSCLC	non-small cell lung cancer
OS	overall survival
PD-1	programmed death-1 receptor
PDL-1	programmed death-ligand 1
PDGFRA/B	platelet derived growth factor receptor A/B
PI3K	phosphatidylinositol-3′-kinase pathway
PTEN	phosphatase and tensin homolog
RAF	v-Raf murine sarcoma viral oncogene homolog
RAS	rat-sarcoma viral oncogene homolog
RET	glial cell-line derived neurotrophic factor receptor
RFLP	restriction fragment length polymorphism

ROS	reactive oxygen species
SSM	superficial spreading melanoma
T	thymine nucleobase
TC%	percentage of tumor suspected nuclear morphology for all examined nuclei
T-VEC	talimogen laherparepvec
UV, UVR	ultraviolet radiation
VEGFR	vascular endothelial growth factor receptor
XP	xeroderma pigmentosum

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2 Introduction

Until the end of the 19th century infectious diseases were the most common cause of mortality. With the improvement of living standards, noncommunicable diseases (chronic diseases) became the most frequent cause of death [1]. Today cardiovascular diseases, malignancies, respiratory diseases and diabetes account for more than 70 percent of mortality worldwide [2]. These illnesses are driven by factors that include rapid unplanned urbanization, unhealthy lifestyle and ageing of the population. Cancer (defined by the National Cancer Institute as a collection of disease in which abnormal cells can divide and spread to nearby tissues) is the second leading cause of death globally. As the definition suggests, cancer can arise in many parts of the body. Skin cancer, the most frequently occurring cancer, has the lowest mortality rate compared to other types of tumors [2]. One of the well-known risk factors of skin cancer is ultraviolet (UV) radiation [3], which is subdivided into UVA and UVB wavelengths and is part of the electromagnetic spectrum that reaches the earth from the sun. UVB - ranging between 290-320 nm - is the main cause of skin reddening and sunburn [4]. It plays a key role in damaging the skin's cellular DNA: excessive UV radiation produces genetic mutations that can lead to skin cancer [3]. Stratospheric ozone plays a fundamental role in protecting living beings from exposure to harmful levels of UV radiation.

Amongst skin cancers, malignant melanoma is a relatively rare neoplasm, but it accounts for the highest mortality rate within this group. Its incidence is continuously rising. In the United States, patients in the 65-74 age group are the most commonly affected [3]. The behavior of this tumor is rather unpredictable and even the thinnest primary tumor carries the risk of distant metastasis. Although regression might occur, the patient may die during metastatic progression, which process may even take decades. While the most common form of melanoma is cutaneous, it can also arise from mucosal surfaces, the uveal tract and the leptomeninges [5]. Owing to the various etiopathogenetic factors, biological behavior, differences in underlying genetic changes and prognosis, treatment of this neoplasm is challenging despite the widespread therapeutic options available.

The burden of skin cancer in Hungary is among the highest in Europe and the disease is responsible for the highest cancer-related overall mortality in men. Among those aged 20–39 years, the incidence of melanoma is forecasted to precede colorectal cancer by 2030 [6], which in 2018 was the second most common cancer type in both sexes, in all age groups [7].

Environmental factors and normal cellular processes – such as proliferation – cause constant damage to the DNA of normal cells. Although most damage is repaired, a small portion may be converted to fix mutations. The vast majority of malignant neoplasms are sporadic and occur due to the accumulation of somatic mutations in key genes: gain-of-function mutations (upregulation) in genes which take part in the cell differentiation, proliferation, inhibition of apoptosis (called oncogenes) and loss-of-function mutations (downregulation) in proapoptotic genes (called tumor suppressor genes). Accordingly, oncogenes contain driver mutations that deregulate the control of normal cell functions leading to growth advantage for the malignant clone [8].

By means of targeting single oncogenes (targeted therapy), a new era has arrived as regards antitumor treatment. This therapy can produce dramatic response rates in selected patients based on the presence of driver mutations (personalized therapy). Imatinib was the first targeted therapy, which is an oral multiple tyrosine kinase inhibitor administered to patients with chronic myeloid leukemia. Nowadays, imatinib is used in gastrointestinal stromal tumors (GIST) as well as in melanoma patients, since oncogenic mutation in the cellular homolog of the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog - also known as stem cell factor receptor (KIT gene) - was described in these neoplasms, even though in case of melanoma the KIT gene is only the third on the list of possible mutant oncogenes [9, 10]. The most common driver oncogene in melanoma is v-Raf murine sarcoma viral oncogene homolog B (BRAF), which similarly to KIT, is also a tyrosine kinase inhibitor causing constitutive activation of the mitogen-activated protein kinase (MAPK) pathway [11] in approximately 50% of skin melanomas. Vemurafenib was the first selective BRAF inhibitor applied, leading to encouraging results in case of BRAF V600E mutant metastatic melanomas [12]. Unfortunately, almost all patients treated with BRAF inhibitor in monotherapy develop progressive disease usually within less than a year, its use is therefore recommended in combination with mitogen-activated protein kinase (MEK) inhibitors, since double blocking is achieved within the MAPK pathway [13]. The second most frequent driver oncogene in melanoma is the neuroblastoma rat-sarcoma viral oncogene homolog (NRAS), which is present in 15-20% of all cases. Although activating mutations in rat-sarcoma viral oncogene homolog (RAS) oncogenes are extremely frequent, found in approximately one third of all human cancers, no targeted treatment of the RAS oncogene is available today. MEK inhibitors and immunotherapy can possibly prove to be useful in the future [14].

In case of melanoma, the leading cause of death is often not the primary tumor itself, but the metastasis, however our biological and genetic knowledge on melanoma and generally on cancer is based mostly or exclusively on the primary tumor. Metastasis is a heterogeneous biological entity ranging from locoregional recurrences to lymphatic- or visceral metastases. Fatal progression affects crucial visceral organs. Even with the newly approved targeted therapies and immunomodulating drugs, the long-term survival of patients with metastatic disease remains poor. One possible reason for this is that we usually have no comprehensive information on the progressing disease. For example, there are hardly any data on the possible organ-specific metastatic drivers in melanoma [15].

In our research, we investigated the mutant allele fraction changes in BRAF and NRAS genes during visceral progression and we studied the molecular epidemiology of KIT mutation in skin melanoma compared to a small mucosal melanoma cohort.

In the introduction of my doctoral thesis, I shall present the literature review of melanocyte biology and melanogenesis, the classical clinical forms of the tumor and the up to date TNM classification system of skin melanoma. Thereafter, based on molecular genetic analysis, I shall present the molecular classification of cutaneous malignant melanoma, followed by the epidemiological data and a detailed summary of the relationship between UV radiation (main predisposing factor) and melanoma. Finally, I would like to discuss the prevention and treatment options.

2.1 Melanocyte biology

Melanoma is a malignant tumor that arises from uncontrolled proliferation of pigment-producing cells, so-called melanocytes. Melanocytes are derived embryologically from pluripotent stem cells of the neural crest. They mostly migrate to and differentiate within the epidermis, although they can also reach other extra-cutaneous pigment-containing sites, including the choroidea, the gastrointestinal and genitourinary mucosal membranes and the leptomeninges [16, 17]. The focus of my PhD work is skin melanoma, I therefore wish to concentrate on the description of the melanocytes of the skin.

The skin is the largest organ of the body covering the entire external surface. It is the initial barrier against pathogens, UV light, chemicals and mechanical injury. It also regulates the amount of water released into the environment and helps to control temperature. Histologically the skin is made up of three layers: epidermis (epithelium), dermis (connective tissue) and hypodermis (subcutaneous adipose tissue). The layers of

the epidermis include the stratum basale (the deepest portion of the epidermis), stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum.

The stratum basale, also called stratum germinativum, is separated from the underlying connective tissue by a basement membrane. The cells of stratum basale are attached to this by hemidesmosomes. The cells found in this layer are cuboidal or columnar and are mitotically active stem cells constantly producing keratinocytes.

Melanocytes migrate to the stratum basale of the epidermis and are located between the keratinocytes. Their product, melanin is responsible for the pigment content of the skin. Melanin is produced by a specific organelle called melanosome, by converting tyrosine to dihydroxyphenylalanine (DOPA) by the enzyme tyrosinase. UVB light stimulates melanin production, which is protective against UV radiation. Melanosomes are then transferred to keratinocytes from the long processes extending to the neighboring epidermal cells. This process involves the phagocytosis of tips of melanocyte processes by keratocytes [18].

Cutaneous melanoma contains transformed melanocytes. These cells usually maintain their ability to produce melanin pigment, causing brownish lesions on the skin. Cutaneous melanoma generally evolves through three clearly discernible progression stages. First, transformed melanocytes proliferate above the epidermal basement membrane. This is the *in situ* or epidermal radial growth phase. Later, the cells invade the papillary dermis (the micro-invasive radial growth phase) and finally they acquire the capacity to invade and thus become a malignant tumor (the invasive vertical growth phase) [19].

2.2. Clinical types of melanoma

The vast majority of melanomas are cutaneous, only 4-5% of all primary melanomas arise outside of the skin (ocular melanoma, mucosal melanoma). Four major histopathological subtypes of primary cutaneous melanoma have been distinguished. The most common forms include the superficial spreading melanoma (SSM), the nodular melanoma (NM), the lentigo maligna melanoma (LMM) and the acral lentiginous melanoma (ALM). Less common forms are the spitzoid, desmoplastic form, usually located on the head or neck of elderly patients and the nevoid melanoma. This classification is based solely on the clinical appearance and does not provide any information on the future prognosis [20]. Such information is indicated by the TNM staging system, currently used worldwide and endorsed by the American Joint Committee on Cancer (AJCC).

SSM is the most common type of melanoma in fair-skinned individuals. Approximately seventy percent of diagnosed melanomas are SSM cases occurring most frequently

between 30 to 50 years of age. SSM can develop on any part of the body, but is most frequently detectable on the trunk of men and the lower extremity of women. It usually begins asymptomatically as a brown to black macule with color variations and irregular borders. Typically, after a relatively long horizontal growth phase (that may last for several years during which the tumor is limited to the epidermis), a rapid vertical progression occurs. Clinically this phase is characterized by the development of a papule or nodule. About half of these melanomas arise from a pre-existing nevus.

NM cases account for approximately 15% to 30% of all melanomas and are diagnosed most frequently in patients in their 60s. This form of the disease may appear as a blue to black, or sometimes pink to red-colored nodule. NMs typically lack the pre-existing horizontal growth phase and are likely to arise as a de novo vertical growth phase tumor. Hence, it is not surprising that they are often diagnosed at a more advanced stage when they are thicker and are therefore generally associated with a poor prognosis.

LMM cases are most often found on the chronically sun-damaged skin of adults, appearing on the arms, legs, face, neck and other areas exposed to the sun. The risk of this type of melanoma may increase with age by reason of prolonged sun exposure.

ALM is a term used to describe melanomas arising from the palms, soles, and nail beds. This form accounts for only 2–3% of all cutaneous melanomas, but is the most common subtype in Africans and Asians. Mechanical stress and trauma in medical history are well-known risk factors for the development of ALM, but the association with a preexisting nevus is unusual. The ALM survival rate is 10–20%, which rate is lower than for the common melanoma forms, such as SSM or NM, but this bad prognosis is mainly associated with socioeconomic factors that contribute to delayed diagnosis rather than the behavior of the tumor itself [20].

Extracutaneous forms of melanoma include ocular melanoma, which can be further subdivided into conjunctival and uveal melanoma. The prognostic features and treatment of this tumor is clearly different from those of the cutaneous forms with entirely different etiology, epidemiology, biology, genetics and clinical aspects [21].

Another extracutaneous melanoma form is mucosal melanoma, which is the rarest subtype, accounting for only about 1.3% of all melanomas. This form emerges from melanocytes located in the mucous membranes of the respiratory, gastrointestinal and genitourinary tract. The risk of the latter type is associated with UV radiation. The head and neck are the most common locations, involving the nose, paranasal sinuses, oral cavity, pharynx and larynx. Together these account for more than half of all mucosal

melanomas. Most studies report a similar distribution of mucosal melanomas between men and women with the notable exception of genital tract melanomas, which are more common in women. The incidence of mucosal melanomas also varies among races. For example, a greater proportion (8%) of all melanomas are mucosal in Japanese patients as compared to the Caucasian population (1%). The hidden location, thus the delayed diagnosis and the rich vascularization of the mucous membrane are factors that contribute to a poorer prognosis when compared to cutaneous melanoma. The average 5-year overall survival rate (OS) is only 25% [20].

Personalized medicine - also known as precision medicine - is a medical practice that divides patients with the same diagnosis into distinct groups in which interventions, treatments, or other medical decisions are designed for the individual case and based on each patient's predicted response to the disease. The concept requires an adequate knowledge of the molecular and cellular mechanisms of the disease as well as the availability of proper diagnostic and therapeutic techniques. Implementation of such a model for melanoma requires an update of its classification system [20].

2.3. TNM classification system of skin melanoma

It has been observed that cancer survival rates are higher in cases where the malignancy is localized than in cases where the disease has extended beyond the organ of origin. The stage of illness is very important at the time of diagnosis to find out the most effective course of treatment and to standardize patient care and research activities. The AJCC staging system is the common language of cancer. It takes into consideration tumor size (T), loco-regional dissemination to the lymph nodes (N) and occurrence of any distant metastasis (M).

The staging of primary melanoma is based on tumor thickness (Breslow) as well as tumor ulceration. Thin melanomas (0.1–1 mm in Breslow scale) have lower risk for metastasis and thus better prognosis compared to thicker melanomas (>1 mm).

The seventh edition of the AJCC Melanoma Staging System has been widely adopted since its first and original publication in 2009 and its implementation in 2010. The current edition was published in 2018 (8^{th} edition) and includes the following key changes: first, tumor thickness measurements to be recorded to the nearest 0.1 mm instead of 0.01 mm; second, definitions of T1a and T1b are changed (T1a, <0.8 mm without ulceration; T1b, 0.8-1.0 mm with or without ulceration or <0.8 mm with ulceration), with abolishing mitotic rate as a T category criterion; third, pathological (but not clinical) stage IA is updated to include T1b N0 M0 (formerly pathologic stage IB); fourth, the N category

descriptors “microscopic” and “macroscopic” for regional lymph node metastasis are revised as “clinically occult” and “clinically apparent”; fifth, prognostic stage III groupings are based on N category criteria and T category criteria (particularly, primary tumor thickness and ulceration) and expanded from 3 to 4 subgroups (stages IIIA-IIID); sixth, definitions of N subcategories are improved, with the presence of microsatellites, satellites, or in-transit metastases now categorized as N1c, N2c, or N3c based on the number of tumor-involved regional lymph nodes, if any; seventh, descriptors are added to each M1 subcategory designation for lactate dehydrogenase (LDH) level (LDH elevation no longer upstages to M1c); and eighth, a new M1d description is added for central nervous system (CNS) metastases [22].

2.4. Molecular classification of melanoma

2.4.1 Germline mutations as genomic drivers of melanoma

Similar to any other tumor, melanoma is caused by a complex interaction between genetic predisposition and environmental exposure [23].

Genetic predisposition is considered to be responsible for the formation of nearly 10% of cutaneous malignant melanomas [24] and approximately 1-2% of cases are strongly familial [25].

Any kind of skin cancer in the personal anamnesis increases the chance of melanoma formation. From 5 to 10% of melanoma patients a history of melanoma is found in one of their family members. A positive family history for melanoma with at least one affected relative increases the risk for this neoplasm by 2.2-fold [26].

Familial malignant melanoma is the most common genetic syndrome predisposing to melanoma [27]. This heterogeneous group of disorders is characterized by multiple occurrences of malignant melanomas within a family: by definition with the involvement of two or more first-degree relatives. Family history studies suggest multifactorial polygenic inheritance. Furthermore, a high incidence of pancreatic cancer has also been reported in these families, sometimes in association with breast cancer [28].

Other rare genetic syndromes associated with melanoma are *familial atypical multiple mole melanoma syndrome-pancreatic cancer* (FAMMM-PC) [29], *dysplastic nevus syndrome* [30], and *melanoma-astrocytoma syndrome* [31].

In addition, melanoma along with other tumors may appear in hereditary cancer syndromes such as *Lynch syndrome, Li-Fraumeni syndrome* and *Muir-Torre syndrome* [32].

Cyclin dependent kinase inhibitor 2A (CDKN2A) (9p21, OMIM 600160), the most significant high-risk melanoma susceptibility gene was described in 1994. This was the first of many similar genes. It encodes the p16 protein that inhibits the activity of cyclin D1-cyclin-dependent kinase 4/6 complex, the function of which is to promote cellular proliferation. Thus, CDKN2A is a tumor suppressor gene inhibiting cell division [33]. Germline mutation frequencies in the CDKN2A gene show substantial variations among members of melanoma families. There is a strong correlation between the number of melanoma cases in the family and the number of CDKN2A gene alterations [34]. The penetrance varies in a wide scale (30% - 91%) and depends on the geographic origin and on age. It is also possible that UV radiation increases the penetrance of the CDKN2A mutations [35]. It should be pointed out, however, that CDKN2A alterations have been found only in the minority of familial melanoma cases.

Other genes have also been implicated in the pathogenesis of melanoma [36, 37, 38]. The tumor suppressor gene known as $p14^{ARF}$ in humans and $p19^{ARF}$ in mice (ARF) shares exon 2 with CDKN2A encoding the transcript, $p14^{ARF}$, which is involved in the regulation of the cell cycle and apoptosis [36]. Mutations in the DNA repair associated breast cancer gene 2 (BRCA2) (13q12, OMIM 600185) predispose to a range of cancer types, including but not limited to malignant melanoma [38]. Cyclin dependent kinase 4 (CDK4) was also suggested to be associated with melanoma (12q13, OMIM 123829) [37].

The melanocortin receptor 1 (MC1R) gene (16q24, OMIM 155555) codes the receptor for melanocyte-stimulating hormone (MSH). Certain germline allelic variants of the gene (Arg151Cys, Arg160Trp, Asp294His) found in fair-skinned and red-haired individuals were found to be associated with an increased risk of melanoma. The MC1R gene also increases penetration of CDKN2A mutations [39].

Xeroderma pigmentosum (XP) is a disease characterized by high sensitivity to sunlight and the development of cutaneous tumors at an early age. The malignancies can include melanoma, basal cell carcinoma and squamous cell carcinoma of the skin. Several XP susceptibility genes have been described, including XPA (OMIM 278700), XPC (OMIM 278720) and XPD (OMIM 278730) all of which are involved in UV-damaged DNA repair [40].

2.4.2 Somatic mutations as genomic drivers of melanoma

As compared to Darwin's theory of evolution, carcinogenesis is based on two fundamental processes, the continuous acquisition of more-or-less random mutations in the cells and natural selection acting on the resultant phenotypic diversity [8].

Melanoma has one of the highest number of somatic mutations among solid tumors and in certain cases the specific mutational signature indicates that they are related to UV radiation. Most mutations are simply neutral (passengers) [41], whereas others are crucial in the development of melanomas (drivers). The most frequent "driver" mutations are activating mutations of the BRAF, NRAS and KIT oncogenes. On the contrary, inactivating mutations of the oncosuppressor genes are less frequent. Within this group CDKN2A and NF1 mutations are often followed by p53 and phosphatase and tensin homolog (PTEN) mutations [15].

Apart from mutations, genetic studies have identified other possible mechanisms as well regarding the evolution of melanoma. For example, gene amplification, loss of heterozygosity and microheterogeneity can also affect oncogene functions [15].

Considering the genetic alterations in melanoma, the most frequently involved are the signaling pathways – especially the growth factor receptor pathways. Melanocytes and thus malignant melanoma are mostly driven by the KIT signaling pathway, which is responsible for lineage-specific activities (Figure 1.). The most common driver mutations of melanoma belong to this signaling pathway. The major driver is BRAF followed by NRAS and KIT is third on the list [41].

The BRAF gene encodes cytoplasmic serine/threonine kinase. Mutations can be detected in 40–60% of patients with advanced melanoma, resulting in the constitutive activation of the MAPK pathway. Proteins of this pathway, which include RAS, v-Raf murine sarcoma viral oncogene homolog (RAF), MEK and extracellular signal-regulated kinase (ERK), play key roles in proliferation, differentiation, survival and cell death. The BRAF gene is positioned on chromosome 7q34. More than 30 different BRAF mutations have been identified so far and these are the most frequent mutations occurring in melanomas. It is not surprising therefore that BRAF is the most commonly targeted gene in melanoma therapy. Interestingly, BRAF mutations can also be detected in melanocytic nevi without any sign of malignancy [42]. From the BRAF mutations in melanoma, the vast majority (approximately 90%) affect codon 600 of exon 15. The most frequently occurring is a substitution at the second position of the codon (GTG>GAG), (c.1799T>A) resulting in an amino acid change from valine (V) to glutamic acid (E) (p.V600E). This mutation however is not specific for melanoma. It can also be present in colorectal adenocarcinoma, thyroid gland papillary carcinoma, non-small cell lung cancer (NSCLC) as well as malignant glioma [43-46]. The second most common BRAF mutation in melanoma is also in this position, V600K, a substitution of lysine (K) for valine (V).

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Other mutations, including V600R and D, have also been described [47]. Approximately 10% of BRAF mutations in melanoma are outside of codon 600. From this group a lysine (K) to a glutamic acid (E) at position 601 is the most common that may cause elevated kinase activity [48]. Another non-codon 600 mutation affects exon 11. This is less frequent in melanoma and - interestingly - in case of BRAF G466E mutation in this exon, a clear decrease in kinase activity is seen. This mutation, however, can still promote cellular signaling through the MEK-ERK pathway, by activating v-Raf murine sarcoma viral oncogene homolog C (CRAF), a related family member [49, 50].

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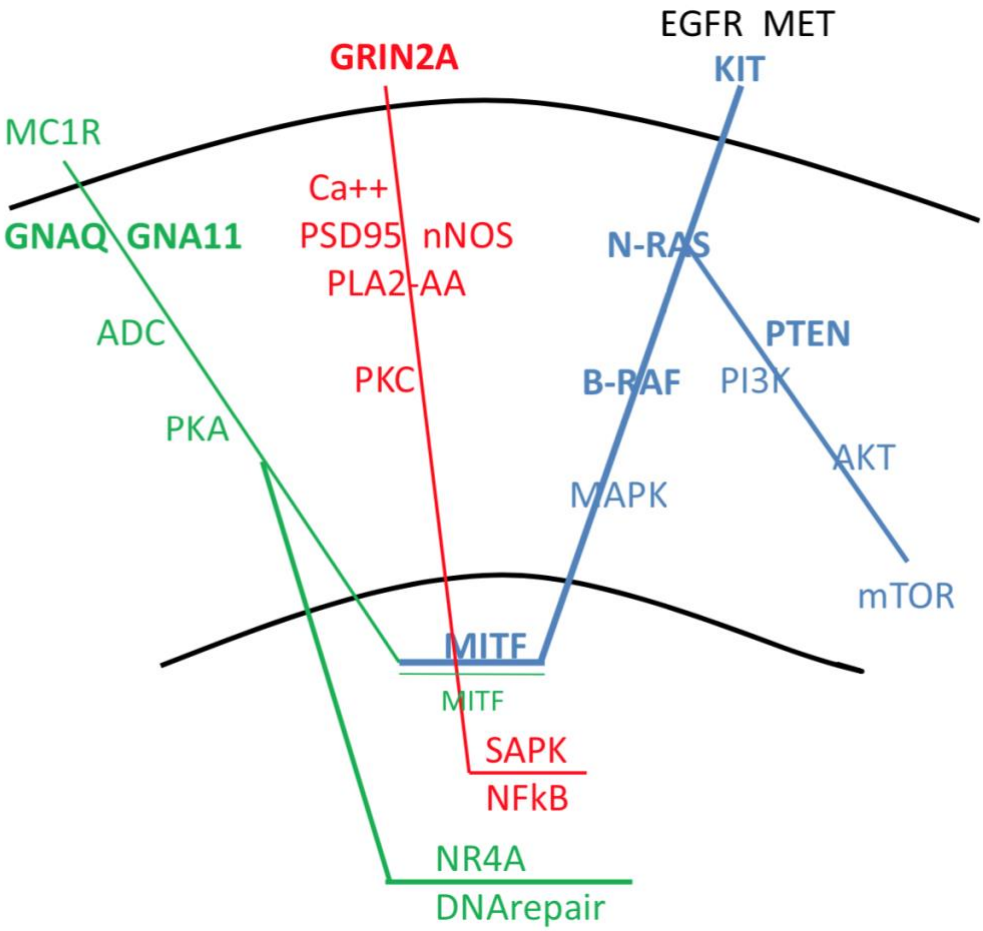

Figure 1. Genetically altered melanoma specific signaling pathways [15].

Somatic mutations in genes encoding the RAS oncogenes, can be detected in approximately one third of all human cancers. RAS oncogenes are GTPases. The RAS family consists of KRAS, HRAS and NRAS. Although KRAS mutations are the most frequent RAS mutations as regards all human malignant diseases [51], in melanoma the most commonly mutated isoform is NRAS. The best characterized subgroup of BRAF wild type melanomas are these NRAS mutant melanomas. NRAS activating mutations are found in 15-20% of all melanomas. The mutations typically occur at codons 61 and 12 [14]. NRAS-mutated melanomas most frequently occur in elderly patients in the body regions subjected to chronic sun damage [52]. Histologically, these melanomas are more aggressive compared to other subtypes and the lesions tend to be thicker with higher mitotic activity. There is also an increased chance of lymphatic metastases [53].

KIT is a member of the transmembrane receptor tyrosine kinase family. It regulates cell development, growth and differentiation. The protein is composed of five extracellular immunoglobulin domains, a single transmembrane region, an inhibitory cytoplasmic juxtamembrane domain and a split cytoplasmic kinase domain separated by a kinase insert segment [54]. The ligand – stem-cell factor – binds to the extracellular domain of the receptor. This causes receptor dimerization and activation of the intracellular tyrosine kinase domain through autophosphorylation of specific tyrosine residues [55]. MAPK, phosphatidylinositol-3′ -kinase pathway (PI3K) and Janus kinase/signal transducers and activators of transcription pathway (JAK/STAT) are the downstream signal transduction pathways activated by the receptor. KIT is third on the list of mutant oncogenes in skin melanoma. The mutation can involve several exons, including exons 9, 11, 13, 17 and 18 [56]. According to a study by Hodi et al., in certain melanoma forms, such as LMM, ALM or mucosal melanoma, the KIT oncogene was shown to be mutated in about 33%. ALM and mucosal melanomas are more common in the Asian population, consequently, a higher frequency of KIT alterations can be detected in this geographical region than in the Caucasians population [57]. KIT mutations can also be present in other cancer types: in about 70% of GIST cases [9], in the majority of mastocytosis cases [58] as well as in progressive acute myeloid lekemia (AML) cases [59]. The mutation profile in various tumors can differ significantly. In the latter two cases exon 8 mutations are the most common [58, 59]. There is a considerable overlap in the mutation spectra of KIT mutations found in GISTs and in melanoma. Mutation of the extracellular domain of the KIT receptor (exon 9) is only occasionally seen in melanoma [57, 60, 61]. KIT mutant melanomas (mostly melanomas on chronically sun-damaged skin, acral and mucosal

melanomas), as another spectrum of BRAF mutant melanomas, tend not to arise in association with melanocytic nevi and the UV-induced alterations are less evident in the acral or mucosal melanoma forms [62].

Neurofibromin 1 (NF1) acts as a negative regulator for RAS by converting the active RAS-GTP to the inactive RAS-GDP form. Therefore, NF1 is a tumor suppressor gene [63, 64]. NF1 somatic mutations are found in many cancer types- and in nearly 14% of melanomas [41, 64]. Mutations in NF1 are more commonly observed in desmoplastic melanoma and tumors arising on chronically sun-exposed skin of older patients [63, 65].

2.5 Epidemiology of melanoma

Cutaneous melanoma is by far the most common melanoma subtype, accounting for more than 90% of cases [66]. Although melanoma makes up only 4-5% of skin cancers, it is responsible for 71-80% of mortality [43, 67, 68]. Unfortunately, the incidence of cutaneous melanoma is continuously rising worldwide, faster than any other tumor [69]. There is a 3-6% increase in the number of new cases each year in the Caucasian population. In 1935 melanoma was diagnosed in 1 out of 1500 persons, today the disease affects one out of 50 people. Interestingly, in Africans, Asians, and Hispanics a relatively stable incidence has been observed over the past 30 years. There are huge differences in incidence rates between different populations. Skin melanoma is 10 times more common in the white population as compared to Africans [70]. Regarding age of patients, the incidence is relatively high in white females from the age of 15 years. In case of white males there is a slow and steady increase in incidence between 15 to 45 years of age, followed by a prominent rise at older ages. In Africans an increase in the incidence of cutaneous melanoma is observed only after 55 years of age [67, 71].

Beside incidence and mortality rates, other metrics are also used to describe the overall effect of melanoma on a given population. One example is the disability-adjusted life years (DALYs), which combines morbidity and mortality statistics. By definition, one DALY equals 1 year of healthy life lost. The Global Burden of Disease (GBD) study is a scientific effort to quantify and compare the magnitude of health loss resulting from diseases and risk factors according to age, sex and geography over time. The study presented statistics for melanoma in 2015 from 21 regions of the world, publishing collected data from almost 200 countries, including Hungary [72, 73].

Based on the data of this study, in 2015 the global incidence of melanoma was 351 880 cases. A total of 59 782 deaths were attributed to melanoma and the disease was responsible for 1 596 262 DALYs. The age-standardized DALY rates related to

melanoma were 27 in men and 19 in women worldwide and the rates were greater in males than in females in almost all regions studied. However, there are a number of limitations to the use of DALY. The staging system of melanoma is not taken into account, although survival rates certainly depend on tumor thickness. Moreover, there is significant progress taking place particularly in regard to advanced melanoma treatment, therefore the disability caused by the tumor is ever changing, which makes DALY comparisons over time problematic [73].

The greatest incidence rates were reported from five regions of the world: Australasia, North America, Western Europe, Central Europe and Eastern Europe. Reasons for the high incidence of melanoma in Australasia have been well documented and include three major factors: (1) predominantly fair-skinned population, (2) high solar ultraviolet radiation, (3) cultural emphasis on tanning [73, 74]. In the 1990s Australia initiated an aggressive and extensive skin cancer awareness campaign [74, 75]. Following the guidelines of the International Agency for Research on Cancer, Australia became the second nation in the World to ban UV-emitting tanning devices classified as class I "carcinogenic to humans" [73]. Interestingly, among the five countries with the highest age-standardized incidence of melanoma, only two are located in Australasia (New Zealand, Australia). The other three are European countries (Norway, Sweden and the Netherlands). The relatively high risk of melanoma in the Scandinavian population, despite the low ambient UV radiation, is most probably attributed to the high-risk phenotype (fair skin, hair and eye color) and a tanning culture preferring sunny holidays and regular indoor tanning [73, 76-78].

As far as the Hungarian data are concerned, melanoma is the 11th most common cancer in Hungary. Compared to global data, where it lays in the 19th place, it is definitely more common [7] and the incidence is continuously rising. The most affected age is between 65-69 years. The cumulative melanoma incidence based on the data of the Hungarian Cancer Registry is presented in Table 1. Incidence, prevalence and mortality are presented in Table 2 [79].

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Table 1. Cumulative melanoma incidence from Hungary between 2000-2016, Hungarian Cancer Registry [79].

Age (year)	0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80-84	85-	***Sum***
Man	6	10	33	86	195	336	577	730	834	934	1344	1756	1974	2102	2075	1579	1016	601	**16188**
Woman	6	12	39	134	376	715	944	1172	1236	1415	1536	1789	1909	1951	1877	1568	1171	760	**18609**

Table 2. Incidence, prevalence and mortality of melanoma in Hungary in 2018, Globocan [7].

New cases				***Deaths***				***5-year prevalence (all ages)***	
No.	***Rank***	***%***	***Cum. risk.***	***No.***	***Rank***	***%***	***Cum. risk.***	***No.***	***Prop.***
1724	11	2.4	1.08	351	22	1.1	0.19	5561	57.40

Although it is not necessarily true for all subtypes, environmental UV exposure seems to be a major risk factor for melanoma as evidenced by genetic and epidemiologic studies [43, 80]. Three specific UV exposure patterns are known to increase the risk of melanoma.

(1) Intermittent sun exposure through the development of multiple melanocytic nevi [43]. Nevi development is influenced by skin type, sun sensitivity and the amount and type of sun exposure [81] - especially frequency and amount of intermittent sun exposure [82]. The number of nevi, either acquired or atypical, is the best predictor of individual risk for melanoma. Especially large (i.e. >5 mm) or atypical nevi (large nevi with non-uniform color and irregular borders) increase the risk of melanoma, independent of the number of smaller nevi [80].

(2) Multiple, especially blistering sun burns during childhood correlate with increased number of nevi [43, 83] and risk of malignant melanoma [81].

(3) Chronic sun exposure: chronic sun-damage in white people leads to wrinkling, guttate hypomelanosis and development of numerous solar lentigines [43, 84, 85, 86] that are well-known risk factors of melanoma.

Not surprisingly, there is also a positive correlation between artificial UV exposure and melanogenesis [87, 88]. The relatively common psoralen plus UVA phototherapy (PUVA) for example, is known to increases the risk of melanoma in time and dose dependent manner [87].

Contrary to all previous information, several data from epidemiological studies suggest that chronic low dose ultraviolet radiation can even be protective against melanoma [81].

2.6 Ultraviolet radiation and melanoma

There are numerous epidemiological studies underlining the fact that UV radiation - both solar and artificial – is a major risk factor for the development of melanoma [89]. A strong negative correlation has been demonstrated between the place of residence (latitude) and incidence, as well as mortality rates of melanoma even in case of homogeneous populations.

UV radiation is known to affect DNA integrity, cell and tissue homeostasis and induce mutations or influence the expression of a large number of genes including oncogenes and tumor suppressor genes [90].

The ultraviolet range of the electromagnetic spectrum is divided into three parts: ultraviolet C (UVC; 200 – 280 nm), ultraviolet B (UVB; 280 – 320 nm) and ultraviolet A (UVA; 320 – 400 nm). Although the shortest wavelength UVC has the highest mutagenic

potential, it does not reach the surface but is completely absorbed by the ozone layer. UVC can also be emitted by artificial light sources (arc welding lamps, germicidal lamps or lasers). Short term exposure can cause irritation and injury to the skin and the eyes. However, only limited data are available in regard to long term exposure.

Most of the UVB radiation is also absorbed by the stratospheric ozone layer. Consequently, only 5% of the UV spectrum reaching the surface belong to UVB, 95% to UVA. Despite its very small quantity, UVB radiation is more efficiently absorbed and induces damages to the skin. UVB is thought to be primarily responsible for sunburn, DNA damage and tumor genesis [90]. The mechanism of UVB related DNA damage is a direct action, nucleic acids are the primary chromophores for the absorption of the electromagnetic energy. The end results of the energy absorption are either pyrimidine (6–4) pyrimidone photoproducts (6–4PP) or cyclobutane pyrimidine dimers (CPD) between neighboring pyrimidine sites (at TT, TC CT and CC sequences) [91]. As far as carcinogenesis is concerned, CPDs are more important than 6–4 PPs, considering the fact that in human cells 6–4 PP adducts are highly reparable. On the contrary, CPDs are only slowly removed, most often by transcription coupled repair and hardly ever by genome global repair [92]. If left uncorrected, most CPDs and 6–4 PPs can lead to mutations in many genes. The most common mutations caused by UVB are often referred to as "UVB fingerprint mutations" or "UV signature mutations". These can be C to T and CC to TT transitions at bipyrimidine sites or pyrimidine runs [93]. The tandem CC to TT transitions are the most distinct of such mutations [94].

Photochemical reactions induced by UVB can also result in cross-linking of the DNA and proteins, production of pyrimidine-purine adducts of unknown role as well as increased reactive oxygen species (ROS) production [95].

BRAF is the most frequently mutated gene in melanoma. There are numerous data indicating that the mutation is connected to ultraviolet radiation. BRAF mutations are regularly detected in melanomas arising on intermittently sun-exposed sites. They are common in SSM or NM [96], less prevalent in ALM and are usually missing in melanomas of unexposed body sites, such as the mucosal melanoma, for example. This mutation is completely missing from uveal melanomas [96]. BRAF mutations are less common in melanomas on chronically sun-damaged skin, but can also be detected in non-malignant lesions, such as congenital nevi, common acquired junctional, compound, intradermal and dysplastic nevi. Based on the latter data, it is likely that BRAF mutations are an early event in melanogenesis [97, 98]. An argument against the role of ultraviolet

radiation in the formation of BRAF mutations is the fact that most mutations are a single base-pair substitution of a thymidine to an adenine, whereas a typical “ultraviolet signature mutation” is cytosine to thymine substitution [99]. In vitro experiments using synthetic oligonucleotides have demonstrated that UVB radiation can also promote T to A conversion. Therefore, even though thymine-adenine dimers are presented in a 100-fold lower yield than bipyrimidine lesions, their formation could be of relevance concerning the predominant V600E mutation on the BRAF gene [97]. Alternatively, BRAF mutations may be related to increased ROS formation generated through the process of melanogenesis or by absorption of UVA light [99]. Another theory is that sunburn can cause strong inflammatory reaction and it is the concomitant oxidative stress that leads to BRAF mutation [99]. In support of the association, melanocytic nevi and melanomas often contain tandem BRAF mutations, which are rarely found in other BRAF mutant tumors [99]. Once BRAF mutations have developed, UV exposure might further enhance melanocytic tumor progression. It seems likely that UV radiation provokes stronger proliferative response in BRAF mutant melanocytes than in wild type cells [97]. This theory is also underlined by the fact that melanomas associated with a coexistent naevus carry the same mutation [96]. Furthermore, UVB radiation was published to increase α -MSH to MC1R, leading to cAMP upregulation and the activation of BRAF through cAMP signaling, the end result being increased melanin synthesis and melanocyte proliferation [43, 97].

The p53 pathway regulates DNA replication, cell division, apoptosis and cellular senescence. The P53 gene is one of the most extensively studied genes in oncology. It can behave both as a tumor suppressor or as an oncogene, depending on whether it is functional or mutated. Although p53 mutations are common in basal cell carcinoma and squamous cell carcinoma and are relatively rare in melanoma, they often demonstrate „UV signature mutations”. Although in case of melanoma wild type p53 is inactivated in the vast majority of cases (90%), only about 10% carry disabling point mutations [100, 101, 102].

There are several possible mechanisms that are considered to protect the DNA from the harmful effects of UV light. The outermost layer of the epidermis, the stratum corneum, is made up of several layers of dead and peeling cells, thus significantly reducing the penetration of UV into the deeper layers. As mentioned earlier, UV radiation enhances melanin synthesis [43, 97] and relocalization, which is essential in the attenuation of UV induced damage. ROS can be eliminated by antioxidative enzymes. Once the DNA has

become damaged, it is the role of DNA repair systems to try to excise damaged parts. CPDs and 6–4 PPs can be removed by nucleotide excision repair, while oxidative stress induced DNA damage is repaired by the base excision repair system [103]. In line with this observation, patients with deficient DNA repair system have a higher chance of skin cancers including malignant melanoma (in sun-exposed parts of the body) [104].

UV radiation is also immunosuppressive. Regarding the skin, it influences morphology and function of the Langerhans cells, impairing antigen presentation. Furthermore, the negative effects are not restricted to the irradiated surface, a systemic immunosuppression can also be observed. The following mechanisms are thought to take part in the suppression of immune system: DNA damage, plasma membrane injury and trans to cis isomerisation of urocanic acid [43].

Interestingly, as far as melanomas are concerned, not only the amount of UV radiation, but also the pattern can be important in estimating the risk. Intermittent exposure to high-intensity sunlight - recreational tanning often with sunburn - significantly increases the possibility of melanoma formation on the trunk and the extremities. These body parts are usually covered by clothing. However, when melanomas of uncovered parts of the skin were studied (eyelids, ear, face, scalp and neck), an elevated risk was found only in the event of continuous UV exposure (e.g. occupational exposure of outdoor workers) [105].

To summarize, the precise mechanism of melanoma genesis and the exact role of UV radiation in this process is currently unknown. The possible effects of UV light are complex and have some paradoxical features. The distribution of melanoma on the different parts of the body, statistics about ethnic origin, place of residence and migration all strongly suggest that solar radiation plays a role in the etiology of the disease. The most sensitive human oncogene for UV is BRAF, which is mutated in approximately 60% of cases. However, the most common Val600Glu T→ A is not a typical UV induced base change that usually occurs at dipyrimidine sites. Furthermore, the UV signature mutations that are frequently found in non-melanoma skin cancers (squamous cell carcinoma and basal cell carcinoma) are usually not detected in melanoma. These mutations generally affect genes, such as p53, PTEN and PTCH, which are rarely involved in melanoma. Finally, melanomas may also arise on sun-protected areas of the skin and in internal organs (e.g. esophagus, colon, cervix), where they develop independent of UV exposure and are most probably induced by genetic factors [43].

2.7 Melanoma and skin cancer prevention

The risk of melanoma – as in case of other cancer types - can largely be reduced by education of the public. Prevention is especially important in case of individuals who are at high risk: with high number of pigmentary nevi (large and atypical nevi), blue/green eyes, fair/red hair, pale skin with freckles (skin phototypes I, II) [24, 105]. Certain genetic factors and disease history (prior history of skin cancer, family history of melanoma, genetic disorders with an increased risk of skin cancers and immunosuppression) as well as excessive and intermittent sun exposure also increase the risk.

As concerns primary prevention, it is crucial to limit UV exposure. People should avoid outdoor activities around solar noon. Proper sun protective clothing is advised: loose-fitting clothes covering most of the skin, wide-brimmed hats, neck protection and sunglasses. Sunscreen should be used repeatedly since it is easily washed off or rubbed off by clothes. It should also be taken into account that certain surfaces reflect UV radiation, such as water and snow, and despite popular belief, trees and umbrellas do not offer complete protection. Finally, patients need to be educated with respect to self examination as well as when to see a dermatologist [4].

Besides natural sunlight, there are other known risk factors for melanoma. These include tanning beds (artificial UV source) and certain chemicals such as arsenic [4], which should also be avoided. Furthermore, many medications increase photosensitivity, and therefore the risk of skin cancers. Such well known drugs are tetracyclines (especially doxycycline), thiazide diuretics, sulfonamides, fluoroquinolones, nonsteroidal anti-inflammatory drugs and retinoids. It is also important therefore to take medication into account when the risk is estimated [4].

There are publications suggesting that antioxidants and vitamins (vitamin A, vitamin C, vitamin E, selenium, fatty acids and resveratrol, among others [4]) might be useful in the chemoprotection of skin cancers. These may be used adjunctively, but should not be recommended solely.

Several countries organize so-called “melanoma days” in order to call further attention to the increasing risk of skin cancers, informing the public about early signs and symptoms of melanoma to enhance early self-detection. Furthermore, a possibility for screening of moles by a dermatologist is also given to participants free of charge. Besides the Euromelanoma Screening Day, which is a pan-European prevention campaign, numerous local campaigns are also organized in Hungary, of which I am a regular participant. There are three main purposes of these events 1) to provide free screening to individuals, 2) to

detect early-stage melanomas or skin cancers and identify patients with risk factors which require closer follow up and 3) to inform the public through mass media campaigns about the early signs of melanoma and the harmful effects of sunlight on the skin.

2.8 Therapy

2.8.1 Molecularly targeted therapy

Prior to 2010 the available therapy for advanced melanoma was limited. The general prognosis was poor, with a median OS of 6–9 months. Less than 20% of patients survived 2 years [106]. These data can mostly be contributed to the fact that melanoma is genetically resistant to chemo- and radiotherapies. In the absence of other therapeutic options, palliative chemotherapy with dacarbazine (usually 1000 mg per square meter of body-surface area intravenously every 3 weeks) was the standard care for decades – with extremely limited success. A meta-analysis in 2007 found that the response to this chemotherapy was only 15% and did not improve OS [106].

In the past decade, there has been a breakthrough in the management of advanced melanoma. The development of highly effective, targeted therapy and immunotherapy has revolutionized patient care [107].

Discovery of the somatic missense activating mutation in the BRAF gene was soon followed by the development of targeted inhibitors of BRAF protein, namely, vemurafenib (PLX4032, ZELBORAF® 960 mg per os twice daily) and dabrafenib (GSK2118436, TAFINLAR® 150 mg per os twice daily). Both drugs have passed phase I-III clinical trials and are nowadays regularly used is melanoma therapy.

Original publications in 2011 (vemurafenib vs dacarbazine) and 2012 (dabrafenib vs dacarbazine) demonstrated consistent results. Both drugs showed an excellent initial response rate (approximately 50% in BRAF V600E mutant melanoma patient). Unfortunately, the positive response lasted only for an average of 6-7 months [108 109]. In 2012, considerable progress in the response rate and duration was reported in a trial, in which dabrafenib was given in combination with trametinib (GSK1120212, MEKINIST® 2 mg per os once daily). Trametinib is a MEK inhibitor downstream of BRAF in the MAPK pathway. Similarly, nowadays vemurafenib is also given in combination with another MEK inhibitor, cobimetinib (COTELLIC® 60 mg per os daily for the first 21 days of each 28-day cycle). By the end of 2014, combination therapy with BRAF and MEK inhibitors became a gold-standard worldwide standard of care for patients with BRAF-mutated locally advanced, unresectable or metastatic cutaneous

melanoma. In 2018, the U.S. Food and Drug Administration (FDA) approved another BRAF+MEK inhibitor combination, encorafenib (BRAFTOVI® 450 mg daily per os) and binimetinib (MEKTOVI®, 45 mg twice per day per os) [110]. As regards the side effects, pyrexia is more likely to occur with combination therapy, whereas the number of proliferative skin lesions seems to be reduced although the change is statistically non-significant [111].

Unlike in case of BRAF mutations, treatment options for NRAS mutant melanomas are scanty. No effective small-molecule inhibitors have been approved to date specifically targeting NRAS. MEK inhibitors have demonstrated modest response in a phase II trial [14], with evident improvement in progression free survival, but without a clear OS benefit. Melanomas with NRAS mutations have better response rates to immunotherapies – especially immune checkpoint inhibitors – than other melanoma subtypes. This suggests that immunotherapy may be an effective treatment option for these cases [14].

KIT mutant melanomas could be treated with available KIT inhibitors, however, response rates depend on the exact position of the mutation [112]. Imatinib was the first small molecule selective inhibitor of KIT developed, also inhibiting platelet derived growth factor receptor A/B (PDGFRA/B) and BCR-ABL fusion protein. It is effective in most KIT mutations (except exon 9), but not in case KIT is amplified. Clinical trials with imatinib (GLIVEC® 400 mg once per day) have demonstrated only moderate and short responses (3–4 months) [56]. Sunitinib (SUTENT® 50 mg once per day, 4 weeks on/2 weeks off) is an oral multitargeted receptor tyrosine kinase inhibitor that targets stem cell factor receptor KIT, PDGFRA/B, FMS-like tyrosine kinase-3 receptor (FLT3), the vascular endothelial growth factor receptors (VEGFR-1, VEGFR-2, VEGFR-3) and the glial cell-line derived neurotrophic factor receptor (RET). With sunitinib the outcomes are generally better if the primary mutation is in exon 9 rather than in other mutations. This is true both for GIST and for melanoma [113]. There are some ongoing trials with other KIT inhibitors, such as dasatinib and nilotinib [112].

In theory, NF1 mutant melanomas can be targeted with tyrosine kinase inhibitors (for example imatinib), MEK inhibitors (trametinib) and mTOR inhibitors (sirolimus). Clinical and preclinical trials have been conducted, but so far only vemurafenib + trametinib and dabrafenib + cobimetinib combinations have been approved by the FDA [63, 65].

2.8.2 Immunotherapy

There are many reasons why melanoma is considered to be an immunogenic tumor. Histologically, melanoma often shows strong lymphocytic infiltration that may be responsible for the partial or even the total regression of the tumor. Furthermore, the non-specific stimulation of the immune response by cytokine therapy was the only effective available treatment option for melanoma before the introduction of molecular targeted therapy [114].

The first type of immunotherapy approved in the treatment of melanoma was high-dose interleukin-2. Interleukin-2 stimulates T lymphocytes and natural killer cells. However, the required high dose produced serious side effects including severe hypotension, pulmonary edema, systemic edema with significant weight gain and renal insufficiency, rash and fatigue. Intralesional IL-2 treatment seems to be a promising alternative without systemic toxicity [114]. As mentioned earlier, NRAS mutations usually coincide with a better response to IL-2 therapy [115].

Adoptive cell therapy (ACT) uses ex vivo cultured autologous lymphocytes derived from either resected metastasis or from peripheral blood of the patients. High dose chemotherapy needs to precede cell transfer to eliminate immune regulatory elements that could affect homing and activity of transferred cells [116].

The success of immunotherapy depends on the antitumor T-cell activity. For the activation of T-lymphocytes the tumor antigen must be presented by dendritic cells.

Discovery of the importance of tumor-T cell-dendritic cell interaction led to the development of a new class of anti-cancer drugs (immune checkpoint inhibitors). In the last ten years a series of possible drugs has emerged targeting critical regulatory elements of this interaction. These include anti-cytotoxic T-lymphocyte antigen 4 (anti-CTLA-4) monoclonal antibodies such as ipilimumab, toll-like receptor (TLR) agonists, CD40 agonists, anti-programmed death-1 receptor (anti-PD-1) or anti-programmed death ligand 1 (anti-PDL-1) antibodies and others [117].

Among the checkpoint inhibitor-based immunotherapies are the anti-CTLA4 inhibitors. CTLA-4 is a receptor highly expressing T-lymphocytes and is a negative regulator of T-cell immune function. It inhibits T-cell responses, thus hiding tumors from inducing a host immune response [117]. In 2011, ipilimumab (YERVOY®, 10 mg/kg intravenously every 3 weeks), an anti-CTLA-4 monoclonal antibody was approved by the FDA for the treatment of unresectable or metastatic melanoma. This was the first immune checkpoint

inhibitor introduced into medical practice. It was reported to induce long term responses and improve OS [118] but only in a small subset of patients, similarly to other immunotherapies.

The PD-1 receptor is expressed on CD4+ T cells, CD8+ T cells, natural killer T cells, and B cells. PD-1 interacts with its ligands PDL-1 and PDL-2, negatively regulating immune responses and inactivating T-cells [119]. Monoclonal antibodies targeting PD-1, such as nivolumab (BMS-936558, OPDIVO®, 240 mg intravenously every 2 weeks or 480 mg intravenously every 4 weeks) and pembrolizumab (MK-3475, KEYTRUDA®, 200 mg intravenously every 3 weeks) block this immune suppressive effect. In general, PD interference shows higher response rates and a more beneficial side effect profile as compared to CTLA-4 inhibition. Furthermore, combination of anti-CTLA-4 and anti-PD-1 immunotherapy (ipilimumab and nivolumab) has also been approved for the treatment of metastatic or unresectable melanomas [120].

In 2015, the FDA approved T-VEC (talimogen laherparepvec, IMLYGIC®, intralesional injection) as the first oncolytic virus therapy for patients with Stage IIIB, IIIC or IV melanomas which could not be removed. The virus injected directly into surgically unresectable skin and lymph node lesions was found to create systemic immune response. However, this therapy has not been undoubtedly shown to improve OS or to shrink metastatic melanoma in monotherapy [121].

Despite the promising trials, a significant proportion of patients do not respond to molecularly targeted therapy and checkpoint inhibitor-based immunotherapy. This underlines the importance of proper selection criteria: response predictor biomarkers are required to improve response rates, disease-free survival and OS [122]. Selecting the right treatment for the right patient will limit the risk of potentially severe adverse events, thus greatly improving patient care.

2.8.3 Adjuvant therapy

After the surgical excision of a melanoma, the decision of whether or not to recommend further medication depends upon the risk of disease recurrence, which is influenced by stage at diagnosis, age, comorbidity and personal preferences. Additional treatment can be given for patients with high-risk melanoma (Breslow thickness of more than 4 mm or >2 mm with ulceration), lymph node metastasis or for patients with metastatic diseases who have undergone complete resection [123].

In 1995, the FDA approved interferon α -2b for the adjuvant treatment of melanoma. This treatment has become the standard of care for decades. Interferons enhance the immune response and the elimination of pathogens and tumor cells. Interferon-α (IFN-α) directly inhibits the division of malignant cells and enhances antigen recognition by increasing MHC class I protein expression. Furthermore, IFN-α inhibits oncogene and induces tumor suppressor gene expression, has notable antiangiogenic effect and regulates chemokine secretion [124, 125]. Peginterferon α-2b was introduced into the therapy in 2011 [126]. The pegylated form has a lower absorption rate in case of subcutaneous injection, shows reduced renal and cellular clearance as well as decreased immunogenicity [127]. The results of adjuvant IFN-α in high-risk melanoma patients are still controversial. Low-dose treatments – 2-3 million units (mU) of IFN-α administered 2-3 times per week for 1 to 3 years – failed to demonstrate any benefit in respect to OS [128]. Increasing the dose seems to improve the outcome. A very high dose interferon regimen involves an induction phase of 20 mU/m^2 intravenously 5 days a week for 4 weeks followed by a maintenance phase of 10 mU/m^2 subcutaneously 3 days a week thereafter. The whole treatment lasts for a year. Although this option is close to the maximally tolerated dose and has numerous and serious side effects, results are notably positive [129].

Monotherapy or combination of immune checkpoint inhibitors like anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab, pembrolizumab) revolutionized the adjuvant therapy for melanoma [123]. BRAF+MEK inhibitors could be a possible alternative for patients whose tumors contain a BRAF V600 mutation and who are unable to undergo immunotherapy, for example due to an active autoimmune disease [130].

Along with the evolving palette of available medications, surgical practices are also subject to reassessments which may have an effect on adjuvant treatment as well. The results of two large multicenter studies failed to show any OS benefit in respect to complete lymph node dissection after positive sentinel node biopsy. Thus nowadays, the new line of adjuvant therapy seems to be proper choice rather than complete lymph node dissection [131].

2.8.4 Future therapeutic prospect

Researchers are working around the clock on a variety of novel improvements for patients with metastatic melanoma.

Instead of combining MAPK signaling pathway inhibitors with each other (BARF+MEK), it may be more beneficial to combine one MAPK inhibitor with agents inhibiting the cell cycle or the PI3K-AKT pathway. The combination of MEK and CDK4/6 inhibitors is one of the most promising alternatives for the future [14].

Lenvatinib is a small-molecule tyrosine kinase inhibitor, which also has a possible potential in melanoma treatment. It blocks several important receptors, such as VEGFR1-3, FGFR1-4, PDGFRα and stem cell factor receptor. In phase I trials, favorable effects were observed in case of several types of cancer including melanoma [132].

Nanomedicine may be able to provide a number of new opportunities in melanoma treatment. Nanoparticles are extremely versatile in size, architecture, constituent biomaterials and surface modifications. They can be designed to fit a number of specific purposes. Nanotechnology is likely to have a major potential, especially in immunotherapy [65].

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3 Objectives

In the last decade several new targets and a series of new drugs have been introduced into the therapy of melanoma. The main problem today is to find the best therapeutic choice for the patient. Collecting additional data about the type and incidence of mutations would undoubtedly help such decision making.

There are no data available about the driver oncogene (BRAF, NRAS and KIT) mutation incidence from the Hungarian melanoma patient population regarding either the primary malignancy or the metastases, which could have clinical significance.

Since primary and metastatic melanomas are known to be clonally heterogeneous, it could also be important to set the BRAF inhibitor sensitivity threshold level of the mutant cell population in the tumor, similar to the HER2 therapy of breast cancer (30%) or the ALK inhibitor treatment of lung cancer (15%) [15]. Therefore, our aims in this thesis were the following:

3.1 To determine KIT and other oncogene mutation incidences in Hungarian skin melanoma cases

Our main goal was to determine the molecular epidemiology of KIT mutations in malignant melanoma in Central Europe, especially in Hungary. Only a single report can be found in the literature concerning a small cohort study from Slovenia [133]. Furthermore, we wished to summarize the widest range of exon mutation patterns and hotspots of KIT in melanoma since most studies do not give the full picture [134].

3.2 To determine the mutant allele frequency of BRAF and NRAS during metastatic progression of skin melanomas

We wished to determine the mutant allele fraction (MAF) of the driver oncogenes in the visceral metastases of malignant melanoma. In the clinical practice, only qualitative BRAF measurements are used to determine whether to treat the patient with RAF/MEK inhibitor or not. The MAF of driver oncogenes can be crucial from the aspect of targeted therapies. There is a big debate in the literature regarding melanoma in this respect [135, 136]. According to certain studies, the high percentage of BRAF MAF may predict better response to RAF/MEK inhibition [136].

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4 Methods

The studies were accomplished in strict accordance with the Declarations of Helsinki and were accepted by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (IRB, SE TUKEB 114/ 2012). Patient consent to participate was waived by the Ethics Committee of the Semmelweis University by reason that metastatic samples were collected at the time of autopsy and the previously archived primary tumor samples collected for diagnostic purposes were also used retrospectively after the death of the patients.

BRAF mutation carrying tissues - tested with RFLP – were examined by direct sequencing of the purified PCR products. Samples bearing BRAF wild type allele were screened for NRAS exon 2, 3 mutations and the double wild type (BRAF, NRAS) tumors were checked further for KIT mutations. In our studies on clonal heterogeneity and mutant allele fraction changes, tumor samples underwent exon 11,13 sequencing and in the KIT molecular epidemiology study tissues were screened for exon 9,11,13,17 and 18 mutations. Array Designer software (Premier Biosoft International, Palo Alto, CA, USA) was used for creation of primers for BRAF, NRAS and KIT and primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). For sequences of the primers see Table 3.

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Table 3. Primer sequences used for studies on KIT mutation pattern and mutant allele fraction changes.

	5'-3'
BRAF	
exon 15 sense	TTCCTTTACTTACTACACCTCAGA
exon 15 antisense	TGGAAAAATAGCCTCAATTC
NRAS	
exon 2 sense	TTGCTGGTGTGAAATGACTGAG
exon 2 antisense	ATATGGGTAAAGATGATCCGACAAG
exon 3 sense	AAACAAGTGGTTATAGATGGTGAAAC
exon 3 antisense	GTAGAGGTTAATATCCGCAAATGAC
KIT	
exon 9 sense	AAGTATGCCACATCCCAAGTG
exon 9 antisense	GGTAGACAGAGCCTAAACATCC
exon 11 sense	CAGAGTGCTCTAATGACTGAGAC
exon 11 antisense	AAGCCACTGGAGTTCCTTAAAG
exon 13 sense	CTTGACATCAGTTTGCCAGTTG
exon 13 antisense	TCCAAGCAGTTTATAATCTAGCATTG
exon 17 sense	AAAAGTTAGTTTTCACTCTTTACAAG
exon 17 antisense	CTTAATTTGACTGCTAAAATGTGTG
exon 18 sense	TCAGCAACAGCAGCATCTATAAG
exon 18 antisense	CAAGGAAGCAGGACACCAATG

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4.1 Patient selection

4.1.1 KIT molecular epidemiology study cohort

Originally 227 cutaneous melanoma cases were collected from the pathological FFPE archives of the 1st Department of Pathology and Experimental Cancer Research, the 2nd Department of Pathology as well as the Department of Dermatology, Venerology and Dermatooncology of Semmelweis University, Budapest. The selected cases were tested diagnostically between 2014 and 2018 for BRAF mutations. This skin melanoma set contained UV-induced forms (SSM, NM, LMM) as well as non-UV induced acral lentiginous (ALM) variants. From the 227 cases, the double wild type (BRAF/NRAS) samples that were checked for KIT mutations consisted of 55 primary melanomas and 24 metastases where the primary tumor was not available for analysis. The clinical data of cutaneous melanoma cases are summarized in Table 4.

For comparison we also had the opportunity to investigate a limited mucosal melanoma pool, consisting of BRAF/NRAS wild type mucosal melanomas comparable in number to the cutaneous melanoma cases. In this cohort of seventeen patients a female dominancy was observable (12 out of 17 cases). Equally distributed oral, anal and genital forms were frequently found, however, other gastrointestinal locations were also present, such as the colon, esophagus and parotis (4/17).

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Table 4. Summary of the clinical and pathologic characteristics of the skin melanoma cohort of the KIT molecular epidemiology study [134].

Whole cohort n=79 (100%)		***Primary tumor characteristics n=55 (100%)***		***Metastasis characteristics n=24 (100%)***	
Primary cutaneous melanoma	55 (70)	***Site***		***Site of the matched primary melanoma***	
Metastasis of cutaneous melanoma	24 (30)	*Head and neck*	5 (9)	*Head and neck*	0 (0)
Patient characteristics		*Trunk*	9 (16)	*Trunk*	3 (13)
Gender		*Upper extremity*	9 (16)	*Upper extremity*	2 (8)
Male	43 (54)	*Lower extremity*	32 (58)	*Lower extremity*	14 (58)
Female	36 (46)	*Data not available*	0 (0)	*Data not available*	5 (21)
Histological subtype		***Histological subtype***		***Histological subtype of the matched primary melanoma***	
ALM	*34 (43)*	*ALM*	32 (58)	*ALM*	2 (8)
Non-ALM (SSM, NM, LMM, NOS)*	*45 (57)*	*non-ALM (SSM, NM, LMM, NOS*)*	23 (42)	*non-ALM (SSM, NM, LMM, NOS*)*	22 (92)
Breslow thickness (mm)		***Breslow thickness (mm)***		***Breslow thickness (mm) of the matched primary melanoma***	
≤1.00	6 (7)	*≤1.00*	3 (5)	*≤1.00*	3 (13)
1.01-2.00	8 (10)	*1.01-2.00*	7 (13)	*1.01-2.00*	1 (4)
2.01-4.00	21 (27)	*2.01-4.00*	16 (29)	*2.01-4.00*	5 (21)
≥4.00	34 (43)	*≥4.00*	27 (49)	*≥4.00*	7 (29)

Data not available	10 (13)	*Data not available*	2 (4)	*Data not available*	8 (33)
Stage		***Stage***		***Stage***	
IA	4 (5)	*IA*	1 (2)	*IA*	3 (13)
IB	2 (3)	*IB*	2 (4)	*IB*	0 (0)
IIA	6 (7)	*IIA*	4 (7)	*IIA*	2 (8)
IIB	0 (0)	*IIB*	0 (0)	*IIB*	0 (0)
IIIA	16 (20)	*IIIA*	14 (25)	*IIIA*	2 (8)
IIIB	8 (10)	*IIIB*	5 (9)	*IIIB*	3 (13)
IVA	17 (22)	*IVA*	14 (25)	*IVA*	3 (13)
IVB	17 (22)	*IVB*	13 (24)	*IVB*	4 (16)
Data not available	9 (11)	*Data not available*	2 (4)	*Data not available*	7 (29)
Site of primary cutaneous melanoma				***Location of metastasis***	
Head and neck	5 (6)			*Lymph node*	7 (29)
Trunk	12 (15)			*Subcutaneous*	8 (33)
Upper extremity	11 (14)			*Local recidive*	3 (13)
Lower extremity	46 (58)			*Lung*	1 (4)
Data not available	5 (6)			*Gastrointestinal*	4 (16)
				Data not available	1 (4)

∗NOS: not other specified: unclassified melanoma, amelanotic melanoma, occult melanoma, data not available

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4.1.2 Mutant allele fraction changes skin melanoma cohort

Patient samples were collected from the pathological FFPE tissue archives of primary tumors and metastases of autopsy cases from (1) the 1st Department of Pathology and Experimental Cancer Research, the 2nd Department of Pathology as well as the Department of Dermatology, Venerology and Dermatooncology of Semmelweis University, Budapest, (2) the Saint George Teaching Hospital of Fejér County, Székesfehérvár and (3) the Saint Rafael Hospital of Zala County, Zalaegerszeg. The cohort of matched primary and metastatic melanoma samples contained 187 FFPE tissues and two aspiration cytology samples. A total of 189 specimens (50 primary melanomas and 139 associated metastases) were analyzed. A male dominance could be seen and the mean age was 50 years. Four stage IB melanomas showed regression and almost 50% of primaries presented ulceration from this aggressive primary tumor cohort (for patient and sample characteristics see Table 5.). The most frequent metastatic organs were the CNS, lungs and the liver, accounting for about half of the metastases, followed by adrenal gland, intestinal tract, distant skin, kidney and other rare sites (see Table 6.)

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Table 5. Clinical and pathological characteristics of the 50 primary melanomas in the study on mutant allele fraction changes [137].

Primary cutaneous melanoma	***N=50 (100%)***		
Breslow thickness (mm), range, SD	4.71 (0.25-24.00) (±3.98)	*IIC*	12 (24)
≤1.00	4 (8)	*IIIB*	*5 (10)*
1.00-2.00	7 (14)	*IV*	*4 (8)*
2.01-4.00	18 (36)	***Specific histopathological restrictions***	
>4.00	21 (42)	*ulceration*	24 (48)
Histological subtype		*regression*	4 (8)
SSM	20 (40)	*solar elastosis*	8 (16)
NM	20 (40)	*association with a coexistent naevus*	4 (8)
ALM	1 (2)	***Gender***	
LMM	1 (2)	*Male*	34 (68)
Unclassified	8 (16)	*Female*	16 (32)
Anatomic distribution		***Age at surgery (years), range, SD***	53 (22-81) (±16.14)
Trunk	21 (42)	*<50*	20 (40)
Head and neck	9 (18)	*≥50*	30 (60)
Extremities	20 (40)	***DNA concentration (ng/µl), SD***	134.61 (±115.54)
Stage at diagnosis		***OS (month), range, SD***	45 (1-144) (±35.64)
IB	4 (8)	***TC%, SD***	79.1 (±20.14)
IIA	10 (20)		
IIB	12 (24)		

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Table 6. Characterization of the metastasis cohort in the study on mutant allele fraction changes [137].

Distant haematogenous metastases	***N=139 (100%)***
Main visceral organs	78 (56)
CNS	38 (27)
Lung	23 (17)
Liver	17 (12)
Other organs	***61 (44)***
Adrenal gland	10 (7)
Intestinal tract	8 (6)
Distant skin	8 (6)
Kidney	6 (4)
Heart	5 (4)
Spleen	5 (4)
Pancreas	4 (3)
Bone marrow	4 (3)
Mesenterium	3 (2)
Thyroid gland	3 (2)
Bladder	1 ()
Submandibular gland	1 ()
Tongue	1 ()
Prostate	1 ()
Caval vein thrombus	1 ()
DNA concentration((ng/μl), SD	**209.43 (±197.47)**
TC%, SD	**78.8 (±21)**

4.2 DNA extraction

Prior to DNA isolation from FFPE blocks, all sections were stained with hematoxylin and eosin to evaluate tumor content ratio (TC%) by counting nuclei at three visual fields using 40x objectives. TC% is defined as the percentage of tumor suspected nuclear morphology for all examined nuclei. Appropriate areas were labelled and macrodissected. High Pure PCR Template Preparation Kit (Roche Holding Ltd., Basel, Switzerland) was used to extract DNA using the manufacturer's recommendations. DNA was quantified using NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

4.3 PCR

Concentration of the primers was 1 µM for each reaction. Primer sequences are shown in Table 3. AmpliTaq Gold 360 Master Mix (Applied Biosystems Life Technologies Corporation Carlsbad, CA, USA) was used for the reaction. The volume of each reaction was set to 25 µl and contained a minimum of 200 ng genomic DNA. PCR was run on Swift MaxPro Thermal Cycler (ESCO Healthcare, Singapore) under the following conditions: (1) activation at 95°C for 10 min, (2) amplification (38 cycles): denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and (3) final extension at 72°C for 5 min. Separation of PCR products (BRAF, NRAS and KIT) was accomplished on 2% agarose gel. The band was excised and DNA purified using the EZ-10 SPIN Column DNA Gel Extraction Kit (Bio Basic Inc., NY, USA).

4.4 RFLP of BRAF exon 15 PCR products

PCR amplification of exon 15 with BRAF specific primers (Table 3.) produced a 197 base pair product. This product was investigated applying RFLP by digestion with TspRI enzyme (New England Biolabs, Ipswich, MA, USA) in order to detect codon 600 mutant BRAF. Agarose gel electrophoresis (3%) was used for separation of digested products. After staining with ethidium bromide all fragments could be correctly detected according to the estimated length of separated products. V600 mutation dissolved the restriction site of the enzyme which led to a prominent band of 212 bp of the mutant allele, whereas wild type BRAF yielded DNA fragments of 125 bp.

4.5 Sanger sequencing

The sequencing reaction was performed using BigDye Terminator v1.1 Cycle Sequencing Kit following instructions of the manufacturer handbook on a 4-capillary automated sequencer (Applied Biosystems 3130 Genetic Analyzer, Life Technologies Corporation). The same primers were used as for the PCR amplification reactions (Table 3.). Before

analysis, purification of the sequencing reaction products was completed using the BigDye XTerminatorTM Purification Kit (Life Technologies Corporation). To detect mutations, the resulting sequences were compared to the NCBI Nucleotide BLAST Human Database using Chromas Lite Version 2.1 software (Technelysium Pty Ltd., South Brisbane, Australia). The sensitivity of mutant allele detection was determined as being 15%.

4.6 Pyrosequencing

Primary melanomas and corresponding metastases carrying different genotype via Sanger sequencing were reinvestigated using a higher sensitivity (>2%) CE IVD pyrosequencing technology (Figure 2.). The Therascreen NRAS Pyro Kit and Therascreen BRAF Pyro Kit (Quiagen, Hilden, Germany) were used on the PyroMark Q24 System (Qiagen) according to the manufacturer's protocol. The BRAF Kit specifically investigates codon 600. The sequencing primer for codon 601 of the BRAF gene had to be newly designed and purchased from Integrated DNA Technologies (Coralville, IA, USA). The primer sequence for BRAF codon 601 was the following (antisense): GGACCCACTCCATCG. Reactions were performed in a total volume of 25 µl, containing 5 µl of DNA, 12.5 µl of 2x PyroMark PCR MasterMix, 2.5 µl 10xCoral-Load Concentrate, 1 µl PCR Primer of BRAF or NRAS and 4 µl of water supplied with the KIT. PCR conditions were 15 min at 95°C, followed by 42 cycles of denaturation at 95°C for 20 sec, annealing at 53°C for 30 sec and extension at 72°C for 20 sec, followed by final extension at 72°C for 5 min. Ten µl of the PCR product were then subjected to the pyrosequencing reaction. Pyrogram outputs were evaluated with the PyroMarkQ24 software for the determination of the percentage of mutant allele versus wild type allele according to the relative peak heights of the matched nucleotides.

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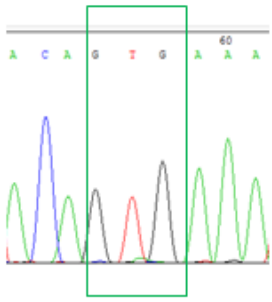
Sanger sequenogram

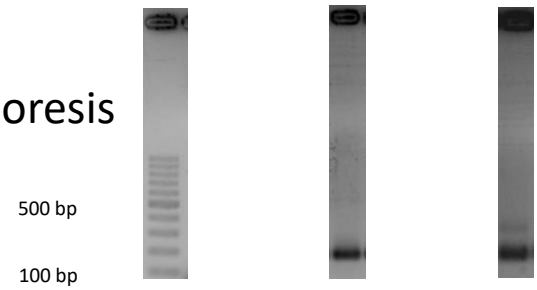
Wild genotype



BRAF gene codon 600: GTG (valine)

Agarose gele electrophoresis

BRAF V600 mutation?



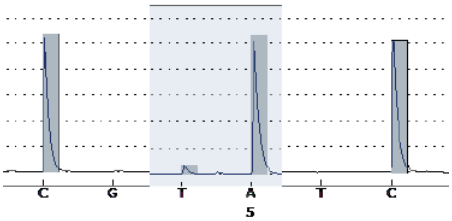
Ladder

BRAF exon 15 PCR product

Digestion products after restriction

Pyrosequencing

BRAF V600E mutation



Hystogram

Figure 2. Different methods for V600E mutation detection.

The robust Sanger sequencing showed BRAF wild genotype in case of a primary melanoma, while the matched metastases were BRAF V600E mutant. RFLP, which can detect lower amounts of mutant clones compared to the Sanger method, showed suspicious features of mutation. Following digestion, two bands were clearly visible - a stronger one at approximately 120 bp and a pale one around 200 bp suggesting incompleteness of the fragmentation at the restriction site. This suggests the presence of low-level mutations. Pyrosequencing is the most sensitive method, which is able to detect very small (>2%) amounts of the mutant clone, confirming V600E mutation.

(previously unpublished data.

4.7 MAF estimation

A percentage value was calculated for the samples referring to the mutant allele ratio based on the difference in height of the wild type and mutant curves of the Sanger sequenogram (semiquantitive method) (Figure 3.). Based on the TC%, the adjusted MAF values were defined.

It is of note that the obtained PyroMark MAF value was corrected for TC% in the given sample. Adjusted MAF value was determined by multiplying PyroMark % by 100/x % tumor DNA. Furthermore, the samples were divided into three artificial MAF categories: low (L) for less than 15% of mutant allele, medium (M) for 15–40% of mutant allele and high (H) for more than 40% of mutant allele based on the assumption that oncogenic mutation is mostly heterozygous (mutant/wild type alleles), resulting in MAF values of 50% at the basics where no further copy-number variation (CNV) changes of the two alleles are affected.

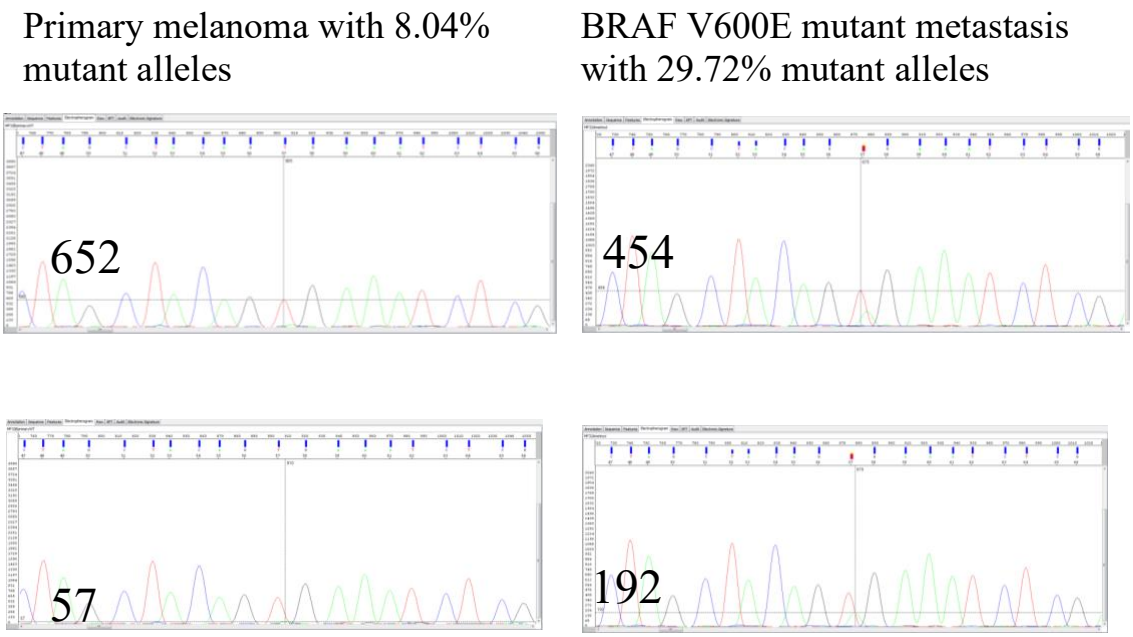


Figure 3. Mutant allele fraction calculation based on the difference in height of the wild type and mutant curves of the Sanger sequenogram.
(previously unpublished data)

4.8 Statistical analysis

SPSS statistical package 20.0 (IBM Corporation, Chicago, IL, USA) software was used for statistical analyses.

4.8.1 Study on mutant allele fraction changes

Descriptive statistics were calculated to analyze the location specific distribution of driver mutations and the frequency of mutations in primary melanomas.

For analysis of the association between the amount of mutant alleles in matched primary and metastatic samples, paired t-probe (BRAF mutant samples) and nonparametric Wilcoxon Signed Ranks test (NRAS mutant samples, owing to the low number of cases) were used. The same statistical approach was chosen to compare the amount of mutant alleles between the different locations of the metastases.

Chi square and Fisher's exact test were performed for the analysis of the correlation between the three MAF categories (L, M and H) based on MAF in primary and metastatic BRAF or NRAS mutant samples, as well as for the evaluation of the changes in mutant allele fraction during tumor progression.

The changes in allele frequency during tumor progression were characterized in four different ways: the mutant allele in metastasis compared to the corresponding primary tumor was either maintained, increased, decreased or disappeared.

4.8.2 KIT molecular epidemiology study

Statistical analysis for significance was done by χ^2 test.

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5 Results

5.1 KIT molecular epidemiology in Hungary

In order to identify the KIT mutation patterns, the molecular epidemiology study of 227 patients with skin melanoma was performed between 2014 and 2018. Further, we collected a mucosal melanoma cohort of 17 patients from Semmelweis University and George Emil Palade University of Medicine, Pharmacy, Science and Technology of Targu Mures, Romania.

5.1.1 Mutational status analysis of the melanoma cohort

In our large Hungarian melanoma cohort BRAF, the most frequently mutated oncogene, was observable in 45.4% of cases (103 out of 227 patients), which finding is in correspondence with other ethnical and geographical regions [41]. Further testing of the BRAF wild type samples (124 pieces) showed 45 NRAS mutated cases (calculated NRAS mutation rate 45/227 - 20%).

We generated a double wild type (BRAF and NRAS) skin melanoma cohort consisting of 79 cases, the summary of the clinical and pathological characteristics of which are shown in Table 4. in the Methods section. Primarily, this BRAF/NRAS wild type collection of melanoma samples contained UV-induced common skin melanoma forms, and about one third showed non-UV induced melanoma variants (ALM). Following sequencing of five exons of the KIT oncogene (exons 9, 11, 13, 17 and 18) a mutation frequency of 43.04% was observable (34 out of 79 samples). KIT mutation was found significantly more frequently in ALM as compared to UV-induced (non-ALM) common variants (58.8% versus 31.1%, p=0.014, Table 7.).

From the total of 227 cutaneous melanoma patients, the KIT mutation frequency showed an incidence rate of 15% (34 out of 227 cases). This finding corresponds to the higher range of data published worldwide [41].

In the double wild type mucosal melanoma cohort the KIT mutation incidence rate was seen to be analogous to the rates observed in case of skin melanomas (7 out of 17 patients, 41.2%) (Table 7.). It is worthy of mention that as regards three samples, in which cases the highly sensitive pyrosequencing assay was applied, BRAF/KIT double mutations were found, demonstrating the heterogeneity of primary melanomas and that these driver mutations are not necessarily mutually exclusive.

Table 7. KIT mutation frequency in BRAF/NRAS double wild type melanoma. Analysis of significance was performed by χ^2 test [134].

	Number of cases	***KIT mutation rate***
Cutaneous melanoma	79	34/79 (43.0%)
UV-induced forms (non-ALM)	45	14/45 (31.1%)
ALM	34	20/34 (58.8%) p=0.014
Mucosal melanoma	17	7/17 (41.2%)

ALM= acral lentiginous melanoma, UV-induced forms= LMM (lentigo maligna), NM (nodular melanoma), SSM (superficial spreading melanoma), NOS (not otherwise specified).

5.1.2 Involvement of KIT exons and codons

In the cohort of 34 KIT mutant cutaneous melanoma cases a total of 38 mutations were observed. In case of two patients double mutations were detected, while in one patient we observed triple mutations. Investigation of the mutations in 5 KIT exons of cutaneous melanoma revealed that analogous to GIST, the most frequently affected was exon 11 (44.7%). The second most frequently involved exon was found to be exon 9 (21.1%), followed by exon 13 (13.2%) and exon 17 (13.2%). Exon 18 was the least frequently involved (7.9%) (Table 8). Significant differences between the common UV-induced and ALM forms in case of exons 9 and 11 were not detectable. Exon 18 mutations were found only in UV-induced melanoma cases, whereas exon 13 and 17 mutations were more frequent in ALM.

On the contrary, in melanomas of the mucosal surface, exon 9 was the most frequently mutated exon (37.5%) followed by exon 13 and 17 (25% each) and exon 11 mutation was less common (Table 8.). As regards the mucosal melanoma cohort, a case was found showing double mutations of KIT exons too.

Investigation of the mutational hotspots revealed that exon 9 codons 482/491/492, exon 11 codons 559/570/572, exon 13 codon 642, exon 17 codon 822 and exon 18 codon 853 were the most frequently affected regions. It is noteworthy that mutations close to these codons were also found to be clustered in KIT mutant melanomas (Table 9.).

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Table 8. KIT exon involvement in mutations in melanoma cases [134].

KIT exon	***9***	***11***	***13***	***17***	***18***
Cutaneous (n=38)	8/38 (21.1%)	17/38 (44.7%)	5/38 (13.2%)	5/38 (13.2%)	3/38 (7.9%)
UV-induced (non-ALM) (n=15)	3/15 (20.0%)	7/15 (46.7%)	1/15 (6.7%)	1/15 (6.7%)	3/15 (20.0%)
ALM (n=23)	5/23 (21.7%)	10/23 (43.5%)	4/23 (17.4%)	4/23 (17.4%)	0
Mucosal (n=8)	3/8 (37.5%)	1/8 (12.5%)	2/8 (25%)	2/8 (25%)	0

ALM= acral lentiginous melanoma

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Table 9. Involvement of KIT codons in melanoma cases [134].

CKIT exon	***9***	***11***	***13***	***17***	***18***
UV-induced skin (n=15)	c459 c465 c471	c551 c558-561del c559 (2x) c566 c573-584del c575-581del	c641	c816	c847 c853 (2x)
ALM (n=23)	c482 c491 (2x) c492 (2x)	c557-561del c559 (2x) c570-576del (4x) c572 (2x) c576	c642 (2x) c643 c657	c815 c818 c822 (2x)	
mucosal (n=8)	c451 c482 c492	c565	c658 c660	c822 c833	

ALM= acral lentiginous melanoma, c= codon

5.2 Melanoma clonal heterogeneity and mutant allele fraction changes during progression

For the MAF analysis of driver oncogenes, metastases were collected from the autopsies of melanoma patients, which was followed by requests for the corresponding FFPE blocks of the primary melanoma from the pathological archives. The cohort consisted of 50 visceral progressing cutaneous melanoma cases originating from 50 patients, in most of which multiple metastatic organs were available. Using this cohort we were able to compare the metastases to the primary tumor and the various organ metastases to each other. A total of 189 samples were included in the analysis: 50 were primary cutaneous tumors and 139 were associated haematogenous metastases from 18 different visceral locations. In this large collection of primary-metastatic matched samples 29/50 had multiple distant metastases.

5.2.1 Mutational status analysis of the primary and metastatic melanoma cohort

Regarding driver mutations, 32 out of 50 primary melanoma cases (64%) were affected by the BRAF gene and 12 cases by any of the NRAS gene mutations (24%). None of the examined tumors carried KIT mutations. The BRAF/NRAS wild type cases were in minority (6/50, 12%) in the primary melanoma cohort. However, regarding the KIT molecular epidemiology study, the ratio of BRAF/NRAS wild type samples was higher (79/227 - 35%), thus the probability of finding KIT mutant samples was higher too.

Analyses of the metastases revealed a BRAF mutation rate of 73% (101/139), NRAS mutation rate of 17% (24/139) and a rate of 10% in triple wild type cases (14/139). The difference between the mutational status ratio of the two different groups (primary and metastatic samples) was not significant (BRAF mutant/NRAS mutant/triple wild type; p=0.25/0.29/0.70, respectively, Figure 4.)

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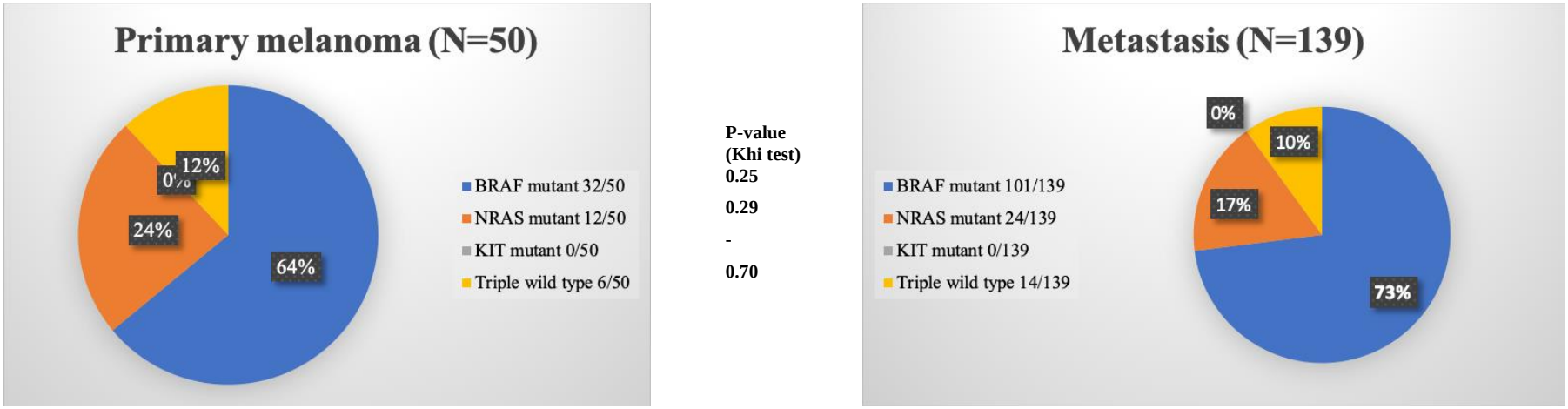

Figure 4. Molecular classification of the primary and metastatic melanoma cohort.

(previously unpublished figure)

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BRAF mutations of primary melanomas were located at codon 600 and 601. The most common V600E mutation was found in 26 out of the 32 BRAF mutant primary melanomas (81.25%) and V600K in 5 out of the 32 BRAF mutant skin melanomas (15.6%). We observed one case carrying a rare codon 601 alteration, K601E (see Figure 5.).

In the cohort of NRAS mutant cutaneous melanoma cases, Q61K and Q61R mutations were found to be equally frequent (5 cases each), in one case Q61L mutation was present (see Figure 6.), whereas another patient showed codon 12 mutation, namely G12C (see Figure 7.).

Survival (time interval from detection of the primary malignancy to death) of advanced melanoma patients as presented on the Kaplan-Meier curve was not influenced by the driver status of the primary malignancy (see Figure 8.).

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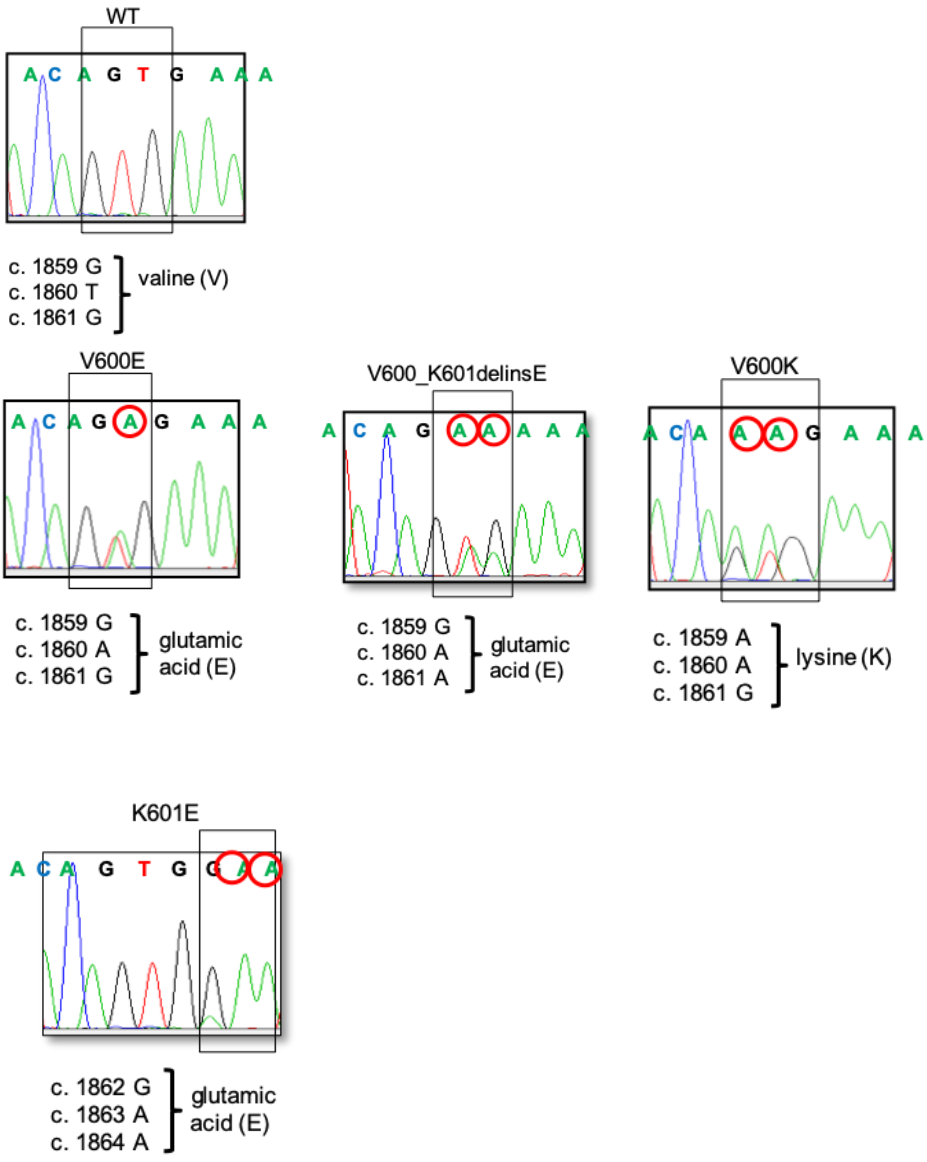

Figure 5. Detected BRAF mutations on the electrophenogram of Sanger sequencing. V600E: BRAF codon 600 mutation (GAG/GTG) encoding V600E mutant protein; V600-K601delinsE: conversion of codons 600 (GTG) and 601 (AAA) into a single codon (GAA) resulted in the insertion of glutamic acid, encoding V600E mutant protein; V600K: BRAF codon 600 mutation (AAG/GTG) encoding V600K mutant protein; K601E: BRAF codon 601 mutation (AAA/GAA) encoding K601E mutant protein. (previously unpublished figure)

DOI:10.14753/SE.2021.2456

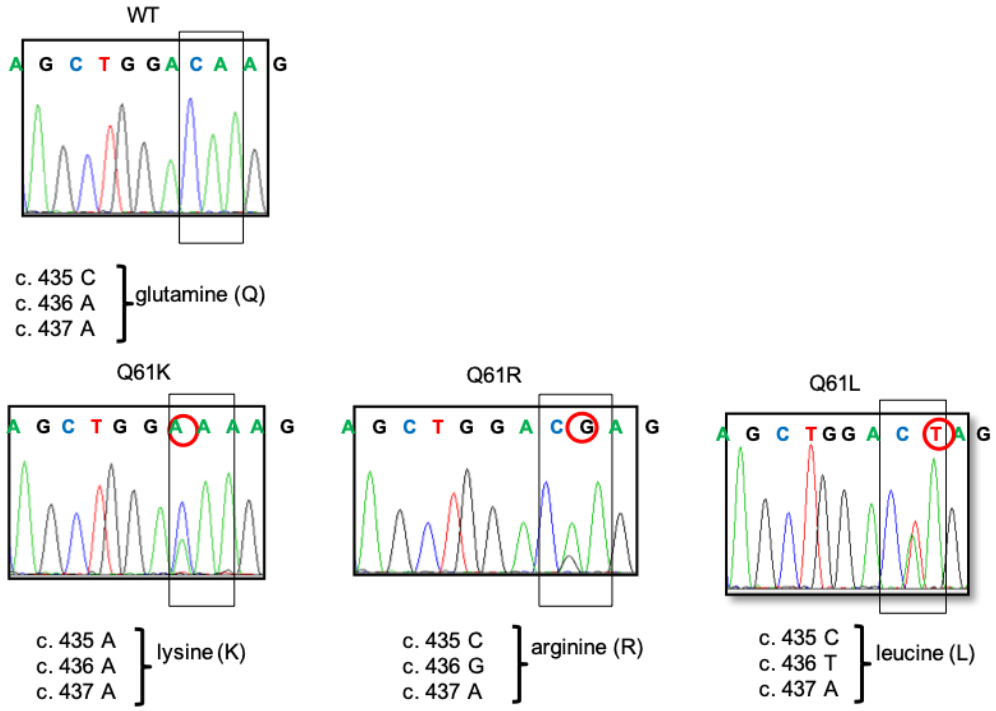

Figure 6. Detected NRAS codon 61 mutations in the electrophenogram of Sanger sequencing.

Q61K: NRAS codon 61 mutation (CAA/AAA) encoding Q61KG mutated protein;

Q61R: NRAS codon 61 mutation (CAA/CGA) encoding Q61R mutated protein;

Q61L: NRAS codon 61 mutation (CAA/CTA) encoding Q61L mutated protein.

(previously unpublished figure)

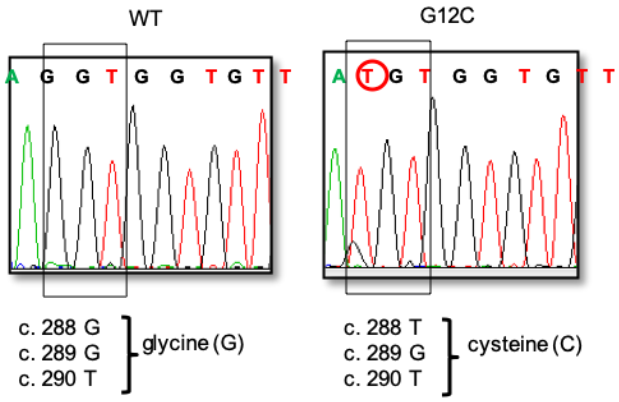

Figure 7. Detected NRAS codon 12 mutations in the electrophenogram of Sanger sequencing.

G12C: NRAS codon 12 mutation (GGT/TGT) encoding G12 C mutated protein.

(previously unpublished figure)

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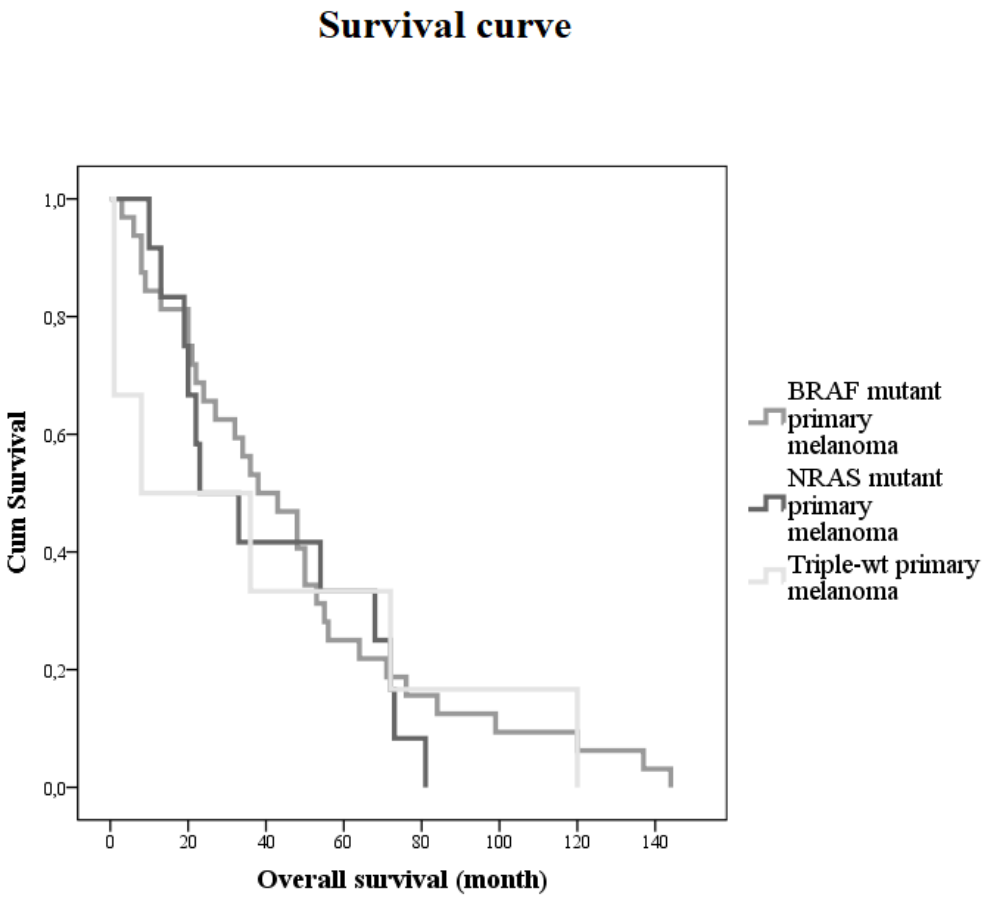

Figure 8. Kaplan-Meier survival curve based on driver oncogenic mutational status of the primary melanomas.

(previously unpublished figure)

5.2.2 Mutant allele fraction of drivers in primary and metastatic samples

The mutant allele fraction (MAF) of BRAF mutant samples was found to be in the range of 2.2–80.3%, while for NRAS-MAF the range was between 4.6–71.0%. Stunning differences were observed between the various tumor samples, however, these large variations were not affected by differences in TC%. Extremely low and high TC% cases were found to have low MAF rates or vice versa. The MAF values were also corrected for TC%.

5.2.3 Clonal selection of the oncogenic driver BRAF during tumor progression

The average MAF reading was expected to be around 50% due to the heterozygosity, however, it was observed to be below 50%. In primary melanomas BRAF-MAF was found to be 24.7+/−16.3 and NRAS-MAF to be 30.7+/−20.9. MAF of driver oncogenic mutation showed significant increase only in metastases of BRAF mutant samples (Figure 9.).

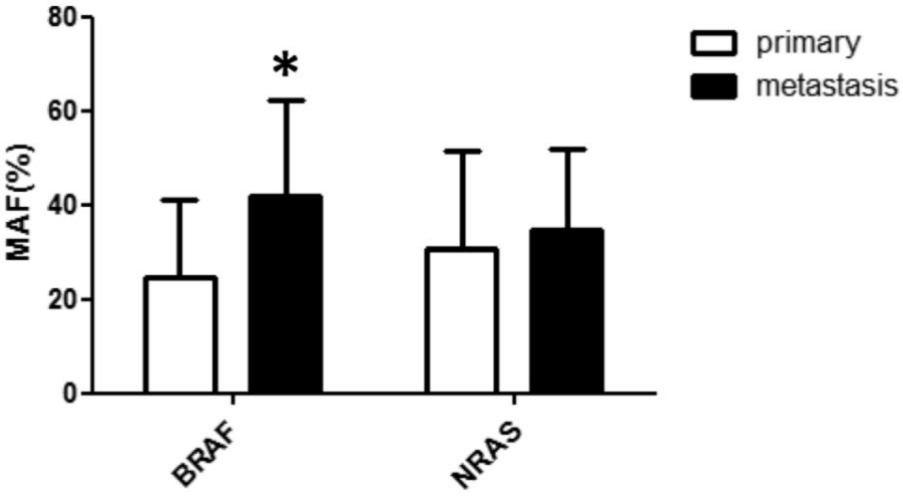

Figure 9. MAF values of BRAF and NRAS oncogenes in metastatic sites as compared with primary malignant melanomas.

Data represent mean+/-SD. ∗=p <0.05 (Wilcoxon Signed Ranks Test) [137].

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5.2.4 Organ specificity of MAF increase in BRAF mutant metastases

Analysis of BRAF mutant cases showed significant increase in MAF to be specifically at the metastatic sites of the lung, adrenal gland, intestinal tract and kidney (Figure 10A), while no significant alterations were detected in metastases of NRAS mutant melanomas as compared to the primary tumors (Figure 10B).

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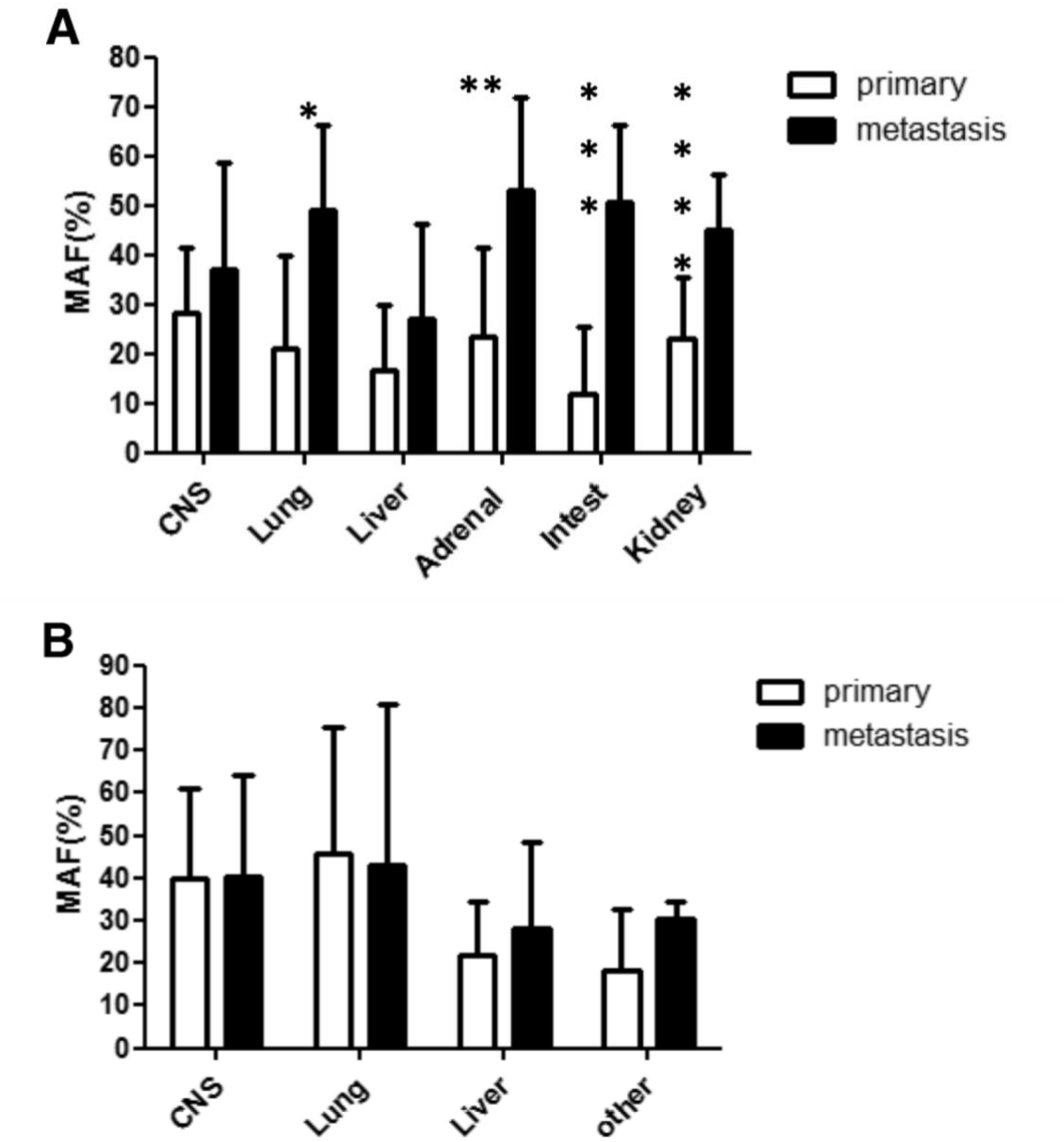

Figure 10. MAF values of driver oncogenes (**A**: BRAF **B**: NRAS) in the most frequent metastatic sites compared to primary melanomas.

Data represent mean +/-SD. The differences in BRAF mutant samples (**A**) are significant in case of the lung ∗p=0.001, adrenal gland ∗∗p=0.021, intestinal tract ∗∗∗p=0.018, kidney ∗∗∗∗p=0.043 based on Wilcoxon Signed Ranks Test. No significant alterations were detected in NRAS mutant tumors (**B**) [137].

5.2.5 Dynamic alterations of MAF during metastatic progression

Deeper analysis of individual patients showed three different patterns.

(1) There were cases where MAF of the primary melanoma was maintained in metastases (single paired samples: primary malignancy and metastasis are classified in the same arteficial MAF category and multiplex associated metastatic cases: all the metastases were classified in the same arteficial MAF category as the primary tumor, for details read Methods section 4.7, MAF estimation): BRAF mutant samples No. 1–2 shown on Figure 11A, cases No. 5–12 seen on Figure 11B and NRAS mutant cases No. 7–10 and 12 presented on Figure 12.

(2) The second pattern comprised samples where a moderate shift of MAF was detected in the metastases (maximum one step difference in the artificial low, medium or high MAF categories up or down between primary malignancy and the associated metastasis): BRAF mutant cases No. 3, 5, 10, 11 observable on Figure 11A, cases No. 2–4 shown on Figure 11B and NRAS mutant cases No. 2, 3, 5 presented on Figure 12.

(3) The third, quite different pattern exhibited extreme MAF changes in the metastases as compared to the primary malignant melanoma (high to low or low to high MAF category change): BRAF mutant cases No. 4, 6–9 shown on Figure 11A, case No. 1 shown on Figure 11B and cases 1, 4 and 7–9 seen on Figure 11C, as well as NRAS mutant cases No. 4 and 11 presented on Figure 12. These patterns were independent of the type of metastases.

Furthermore, in the 129 metastases of 44 mutant (either BRAF or NRAS) primary melanomas, the mutant allele of the driver oncogene could not be identified in two BRAF- and one NRAS mutant samples, in 4 of the 129 metastases (3.1%) affecting the spleen and the liver.

Homogeneous metastatic cases mean that all the associated metastases of a primary melanoma are rated into the same MAF category low, medium or high. Heterogeneous metastases mean that the MAF values are different between the corresponding multiplex metastases.

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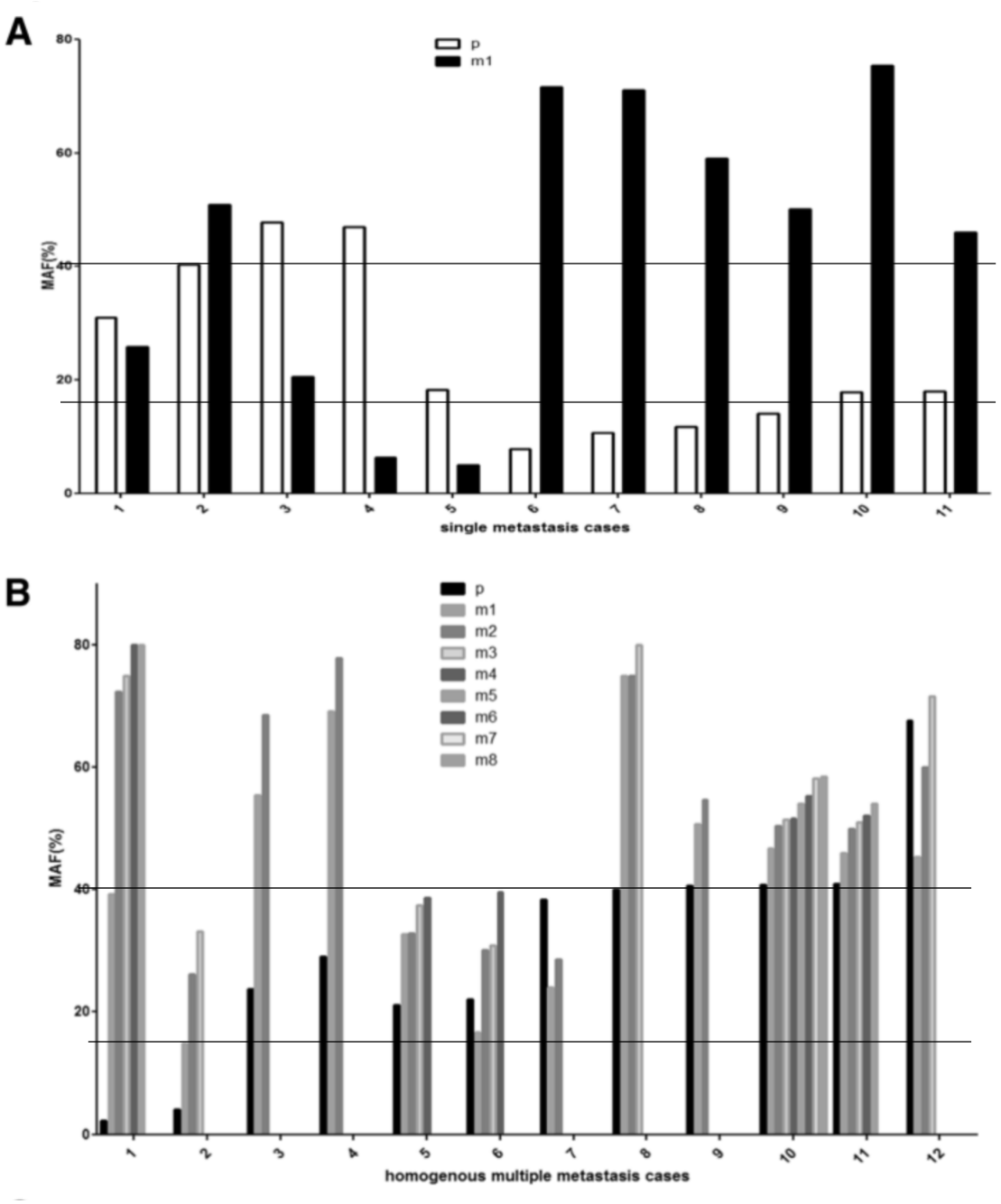

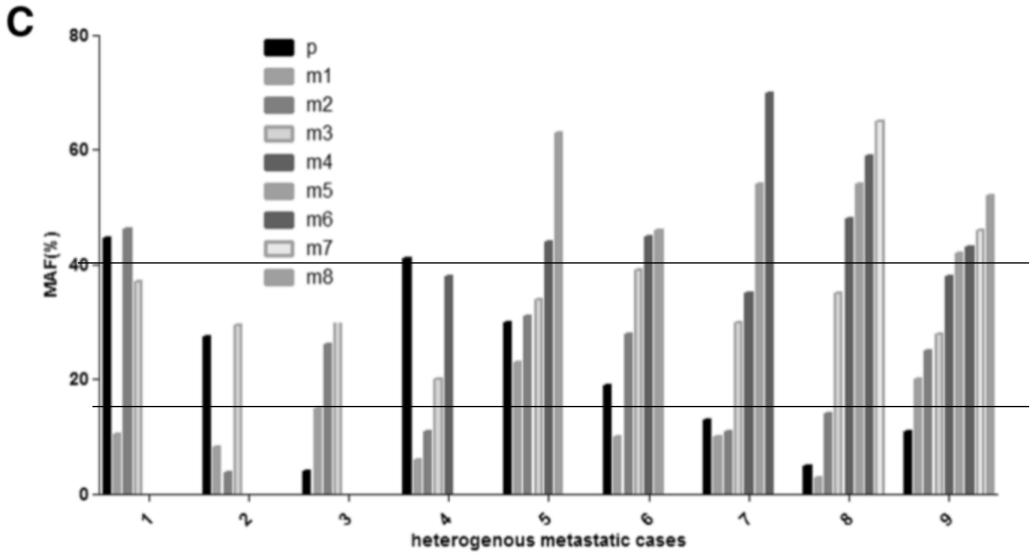

Figure 11. Case by case presentation of changes in BRAF-MAF values of melanoma metastases as compared to primary melanoma cases.

Cutoff lines show 40% MAF and 15% MAF. **A** Single metastatic cases, p= primary tumor (white bar), m1= metastatic tumor (black bar) **B** Multiple homogeneous metastatic cases **C** Multiple heterogeneous metastatic cases **B/C** p= primary (black bar), m1-8= metastasis (shades of grey) [137].

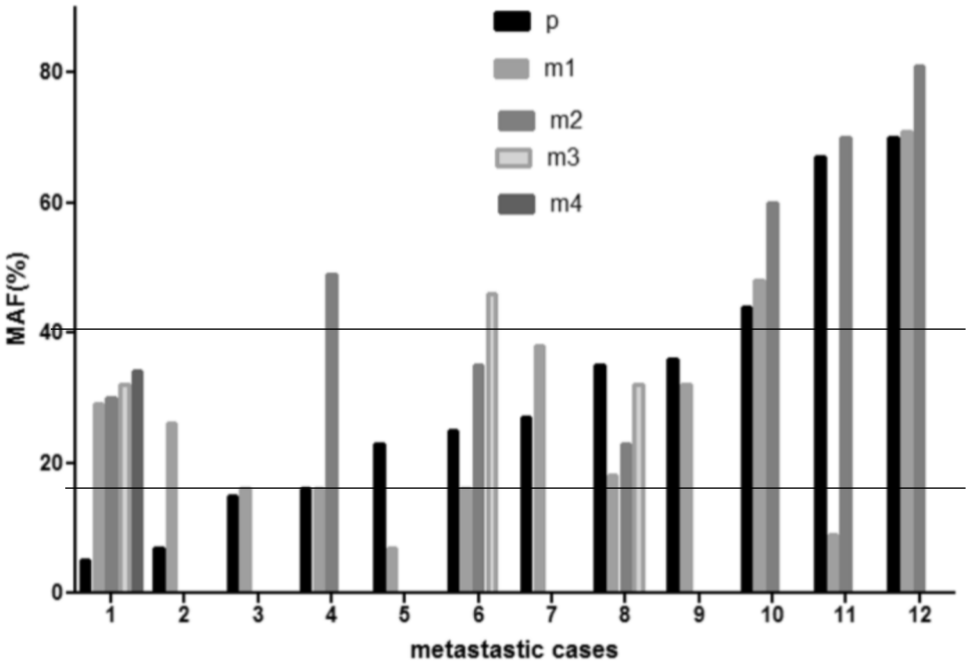

Figure 12. Case by case presentation of changes in NRAS-MAF values of primary melanomas and metastases.

Cutoff lines show 40% MAF and 15% MAF. p= primary tumor (black bar), m1-4= individual metastasis (shades of grey) [137].

5.2.6 Changes in MAF levels during metastatic progression

For better presentation of the above patterns, the individual samples were artificially grouped into three MAF categories (Methods 4.7.) based on the rational that high MAF values represent monoclonality, whereas low values assume subclonality. Roughly, regardless of the BRAF/NRAS mutational status the three MAF categories were found to be equally distributed in the primary melanomas. As shown in Tables 10 and 11, the primary to metastasis MAF alterations are clearly identifiable and it is also evident that multiple metastases in case of both oncogenic drivers are either homogeneous or heterogeneous. Homogeneous metastases - where the MAF values are highly similar to each other - are observable in 23 out of 32 BRAF mutant cases presented in Table 10, as well as in 9 out of 12 NRAS mutant cases shown in Table 11. Heterogeneous metastatic cases - where the MAF values are different between the corresponding multiplex metastases - are presented in Table 10. in 9 out of the 32 BRAF mutant cases and 3 out of the 12 NRAS mutant cases (Table 11.). Neither patterns presented statistically significant alterations in case of the two drivers. From a clinical point of view, an important finding was that extreme MAF differences in visceral metastases as compared to the primary tumor (shift from high to low or low to high MAF category) were rather frequent: in the 32 BRAF-mutant cases 6 (18.75%) from the homogeneous pairs, 4 (12.5%) from the heterogeneous pairs (Table 10., grey box) and 2 out of 12 (16.7%) in NRAS-mutant cases (Table 11., grey box). Moreover, signs of positive selection in case of BRAF mutant melanomas during metastatic progression from the primary tumor were manifest, as medium to high (6/32), low to medium (3/32) and low to high (7/32) MAF switches of metastases were more frequent (16/32, 50%) when compared with the high to medium (3/32), medium to low (3/32) and high to low (3/32) changes (9/32, 28.1%) (Table 10.).

Table 10. MAF patterns of BRAF mutant melanoma metastases compared to the primary. Patterns have been derived from Figure 11. MAF classification: high was characterized by MAF> 40%, medium referred to MAF of 15-40% while low was defined as < 15% MAF [137].

Maintained MAF profile							
p	H	M					
m	H	M					
n=	6	4					
Homogeneous metastases by MAF (Figure 11B)							
p	H	H	M	L	M	L	
m	M	L	H	H	L	M	
n=	1	1	4	5	1	1	
Heterogeneous metastases by MAF (Figure 11C)							
p	H	H	M	M	M	L	L
m	ML	HML	HM	ML	HML	ML	HML
n=	1	1	1	1	1	2	2

p= primary tumor m= metastasis n= number of cases H= high M= medium L= low. Grey box= cases where extreme MAF change (H to L or L to H) were detected in the individual metastases

Table 11. MAF patterns of NRAS mutant melanoma metastases compared to the primary. Patterns have been derived from Figure 12. MAF classification: high was characterised by MAF > 40%, medium referred to MAF of 15-40% while low was defined as < 15% MAF [137].

Maintained MAF profile			
p	H	M	L
m	H	M	L
n=	2	3	1
Homogeneous metastases by MAF (Figure 12)			
p	L	M	
m	M	L	
n+	2	1	
Heterogeneous metastases by MAF (Figure 12)			
p	L	H	H
m	HL	HL	HML
n=	1	1	1

p= primary tumor m= metastasis n= number of cases H= high M= medium L= low. Grey box= cases where extreme MAF change (H to L or L to H) were detected in the individual metastases

6 Discussion

6.1 KIT molecular epidemiology study

There are no published data to be found on the molecular epidemiology of cutaneous melanoma coming from the central European region. We therefore collected a large cohort of melanoma patients, within which cohort the BRAF mutation rate was found to be predominant (103/227 cases, 45.4%), corresponding to other geographical regions. In our sample collection, similar to another recent study [137], the NRAS mutation rate was 20%, which is also in accordance with other geographical regions [15]. Reading the melanoma literature, one might have the impression that KIT mutations in cutaneous melanoma are rare [41]. On the contrary, a contemporary meta-analysis of studies on the frequency of KIT mutations in malignant melanoma patients showed an average rate of 9.5% with considerable variations [138]. KIT mutations in melanoma have specific features, such as affecting elderly people, being associated with chronic sun damage and often being presented in mucosal and acrolentiginous forms [138]. For example, according to data coming from the neighbouring Slovenia, the KIT mutation frequency showed an extremely low value of 1.3% [133], whereas a recent analysis from another European country, Italy, revealed a KIT mutation status (UV- and non-UV melanoma forms) of around 10%, as similar data were published in France (mucosal melanomas) [139, 140]. One of the reasons for these variations is the distribution of the different melanoma forms within the investigated cohorts: mucosal/non mucosal melanomas and UV-induced or non-UV-induced forms.

A further reasonable explanation for these discrepancies could be the testing technology: in certain studies only GIST-exons were analysed, whereas in others exons 9 and 18 were also included. The results of our analysis involving the five most important exons 9, 11, 13, 17 and 18 in Hungarian cutaneous melanoma patients showed a KIT mutation rate of 15%, which is a rather high rate in comparison to the global quota. It is of note that our melanoma samples, as compared to the Italian ones, contained both UV-induced and non-UV induced forms [139]. According to our analysis on double wild type mucosal melanoma in central Europe, the KIT mutation rate was found to be similar to the skin variants. It is to be recognized, however, that in mucosal melanomas BRAF/NRAS mutations are much rarer, the KIT mutation rate is therefore higher as contrasted to skin melanomas, although due to the cohort size statistical analysis was not possible.

Another important feature is that the KIT mutation pattern of skin melanoma is similar to that of GIST (KIT-mutant prototype cancer). Mutations in exons 11 and 9 are the most

characteristic to GIST (~70% and 10%, respectively) [60, 141]. In the literature on melanoma, usually not all five exons are studies in regard to their involvement in KIT mutations. Analogous to GIST, in our cutaneous melanoma collection KIT exon 11 was the most often mutated (44.7%), showing lower frequency than in GIST. This was followed by exon 9 (21.1%) and the additional three exons (exons 13, 17 and 18), which showed similar low mutation rates, indicating increased carcinogenicity in malignant melanoma. The presented KIT mutation pattern in melanoma is complementary to the mutation pattern shown in studies from China [57].

As regards mutational hotspots, in case of GIST codons 502/503 in exon 9 were described [60], however, in our skin melanoma cohort codons 491/492 were demonstrable. It is important to note that mutations in codons 557/558 in exon 11 were found to be the same hotspots as in case of GIST [60], however, the neighbouring codon 559 occurred in melanoma cases as well. Regarding exon 13, GIST [60] and melanoma share the same target, codon 642, although in case of melanoma, the adjacent codons are also involved. The melanoma KIT mutational hotspot pattern in Hungary seems to be similar to the pattern demonstrated in China [57]. Another similarity between GIST [60] and melanoma is that in exon 17 both tumors share the same hotspot, namely codon 822, unlike in case of exon 18, where gastrointestinal stromal tumors show codon 842 mutation, which is not the case in melanomas. The variations in mutational hotspots between GIST and melanoma bear clinical relevance since KIT mutations are predictive markers for KIT-inhibitor therapies of GIST and other KIT-mutant tumors.

Oncologists have a decade long experience of therapy for GIST with KIT inhibition. In this tumor type, exon 9, 11 and 13 mutations are sensitive to imatinib and sunitinib [60, 141, 142]. Another malignancy with KIT mutation is AML, where the response to KIT inhibitor therapy is not known for exon 17 codon 816 mutations [59, 141, 142]. Thymic carcinoma can have KIT mutations as well, in which tumor type exon 9 codon 490 and exon 11 codon 553, 557, 559 and 576 mutations showed sensitivity to KIT inhibitor therapy, although exon 17 codon 820 did not [142].

In case of melanoma few clinical trials were carried out with KIT inhibitors. Regarding exon 9 mutations partial response was not detected [56, 143, 144], however, responses were frequently seen in exon 11 (codons 576, 577, 557, 559, 560) mutant melanomas [56, 143, 144]. Moreover, in exon 13 (codon 642) partial response to mutant melanoma was also detected [142, 143, 144].

6.2 Melanoma clonal heterogeneity and mutant allele fraction (MAF) changes during progression

Our analysis of the MAF profiles on a large cohort of matched primary and metastatic skin melanoma samples revealed the extreme heterogeneity of both oncogenic drivers (BRAF/NRAS), which was neither due to technical problems nor to the wide ranges of the TC%, since these factors were compensated for. Our findings are actually in line with previous studies found in the literature [145, 146, 147].

Our analysis demonstrated that MAF in case of BRAF mutant samples increased significantly during melanoma progression, suggesting a positive selection of mutant clones. On the contrary, positive selection of NRAS mutant clones in metastases was not detectable. When MAF values were grouped into three practical categories (high was characterised by MAF > 40%, medium referred to MAF of 15-40%, while low was defined as < 15% MAF), the increase in BRAF mutant clones during the metastatic process became more evident. Namely, no difference was noticed between the incidence of high, medium or low MAF variants in case of primary melanomas, while in case of the metastases high MAF variants were predominant (15/32, 46.8%) and the observance of low MAF cases was 2 out of 32 (6.25%).

Higher than 50% (monoclonality in practice) or lower than 15% MAF (subclonality) categories were common findings both in primary melanoma as well as in metastatic samples. The extraordinarily high MAF can be related to the amplification of the mutant gene or LOH of the wild type allele. Otherwise, the overly low MAF values can also be related to LOH of the mutant allele or amplification of the wild type allele. A current research of our group on CNV characterization will surely give feedback concerning amplification or LOH of the driver oncogenes.

Our results are the first to demonstrate that BRAF mutant melanoma clones are significantly expanded in organ-selective manner: in the lung, adrenal gland, intestine and kidney metastases but not in the CNS or liver. According to our view, organ specific genetic mechanisms are involved in the metastatic process of malignant melanomas. The results of our research group are contradictory to those published recently, according to which – based on the mutational status of the primary tumor – driver mutation (BRAF/NRAS) bearing melanomas usually give metastasis to the CNS and the liver and NRAS mutant melanomas are associated with pulmonary metastasis [148]. In our opinion the BRAF/NRAS MAF of the primary melanoma can predict organ selection during metastatic progression.

In our cohort, we had the possibility to analyse more than one hematogenous metastasis of the same BRAF or NRAS mutant primary melanoma. We found extreme inter-metastatic heterogeneity for MAF (in case of BRAF mutant samples: 28.2% and in case of NRAS mutant samples: 25%) in a remarkable proportion of multiple metastatic tumors, even though most of the metastases were homogeneous. Furthermore, in our study we observed that BRAF and NRAS mutant melanomas showed differences during the metastatic process. In 50% of the NRAS mutant melanoma patients, the metastases maintained the MAF category of the primary tumor, but this was not so typical in case of BRAF mutant patients (31.3%). Moreover, in a relative majority of BRAF mutant cases metastases shifted from low to high or high to low MAF and such an extreme switch was also detectable individually in heterogeneous multiple metastatic BRAF mutant samples, unlike in NRAS mutant tumors.

In our opinion, the presented findings can have significant impact on clinical decision making. In case of malignancies, such as melanoma, molecular targeted therapy is based on the detection of mutational status of the driver oncogene. BRAF inhibitors can be effective for BRAF mutant melanoma patients, although in a fraction of such patients the drug is not effective for unknown reasons, and even in responders drug resistance will developed sooner or later [149, 150]. One of the causes of the ineffectiveness or transient effect of BRAF inhibitor therapy could well be the extreme heterogeneity of the MAF values of BRAF, which in our study was in the wide range of 2.2–80.3%. Moreover, in cases with more than one metastasis, the inter-metastatic heterogeneity of MAF values is prominent, which could well be one of the causes for resistance to targeted therapy. This metastatic heterogeneity, however, can also be found at a lower frequency in NRAS mutant tumors.

Nowadays, in a significant proportion of patients the oncogenic driver mutation status is defined from the primaries. Some of our data may justify this tactic, since complete disappearance of the oncogenic mutations in the metastases of a mutant primary melanoma was an extremely rare event in the investigated cohort (3.1%) at our technical threshold of 2%. However, the very high diversity of mutant allele fractions in metastases cannot be predicted from the analysis of the primary tumors, therefore it would be important to test metastases whenever possible, since the metastatic disease is treated in the majority of cases and not the removed primary tumor.

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6.3 Molecular progression of melanoma

It is commonly accepted in the literature that malignant tumors are composed of genetically heterogeneous cancer cells. The natural bases of heterogeneity are the germline variances between people, the genetic imbalances of tumors, the discrepancies in incidence of somatic mutations in the different tumor types and in addition, the potential alterations in tumor microenvironment [15].

It has long been acknowledged that intratumoral genetic heterogeneity (Figure 13.) is initially recognized in solid tumors although it can also be described in hematopoetic malignancies too. Genetically and phenotypically different tumor cells have been observed within a given primary malignancy. Clonal heterogeneity can be detected at MAF levels as well as at allelic copy number variations [15].

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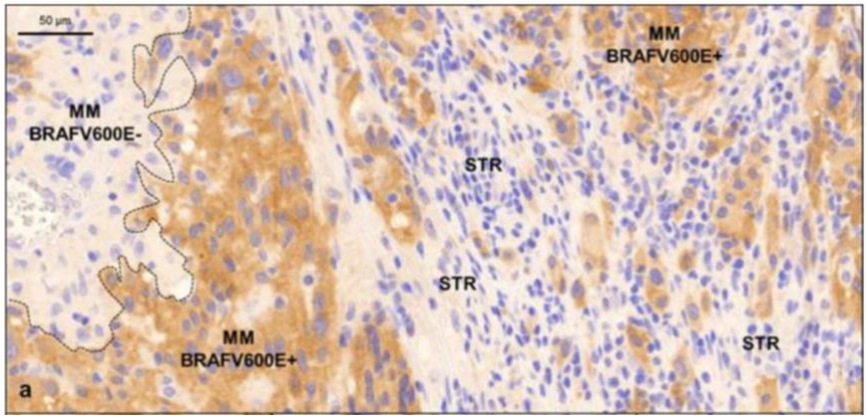

Figure 13. Intratumoral heterogeneity of IHC stained FFPE tissue sample prepared by an international research group to which I also belong. The BRAF V600E mutation carrying melanoma cells do not display immunoexpression of the mutated protein (left side of the dashed line), whereas strong immunoexpression of the mutated BRAF protein is evident to the right of the dashed line. Stromal inflammatory cells (STR) are the negative, internal controls for the staining [151].

The mutation burden is one of the highest in case of melanoma and the main carcinogenic factor is well known (UV-radiation), in contrast to other solid organ malignancies with the exception of lung cancer (smoking). Regarding this genetic palette, the phenotypic plasticity of melanoma provides genetic basis for an excessive metastatic potential. Melanomas are among the most invasive tumors represented by an exclusive full-blown organ metastatic pattern. This suggests that melanoma can metastatize to any organ and tissue by hematogenous route [15].

A special hallmark of cutaneous malignant melanoma is its homing potential, which means that it is able to give metastases to the skin, often at a later phase of tumor progression, the biologic background of which is still not clear [15].

Various models of tumor progression, including melanoma progression, have been presented to date (Figure 14.). A universal model for tumor progression is the "clonal evolution" model. This model is appropriately demonstrated in colorectal carcinogenesis [150] and reveals that several tumor cells with additional genetic changes predominate in a malignant cell population. According to this model, metastasis serves as the end stage of evolution, and the attendance of genetic changes responsible for the metastatic power of tumor cells is forecasted. The conclusion that primary tumor cell populations are constituted of cells with different metastatic potentials promotes the "clonal evolution" model. If malignant cells with aforementioned genetic alterations reside in a tiny subpopulation within the primary tumor cells, these alterations can only be noticed in metastatic tumors and are not (or hardly) identified in the matched primary tumor [152]. Indeed, some publications identified several genetic alterations that were present alone in metastatic tumors and not in the paired primary malignancies [153], supporting the authenticity of this model [154].

Further models for malignant progression and metastasis have been suggested. One of these is "the parallel evolution model," which implies the early appearance of metastasis and side-by-side growth of primary and metastatic tumors [155]. This theory was established in the publication of Schmidt-Kittler et al. [156], in which study the genetic alteration of primary breast cancer cells was not detectable in bone marrow metastases. The "parallel evolution model" is easily applicable to the metastatic pattern of several solid epithelial tumors. However, data provided by global gene expression profiling of primary tumor cells led to the idea of another tumor progression model, namely "the same-gene model" presented by Bernards and Weinberg [157] . According to this model, the genetic alterations occurring at an early stage of carcinogenesis negotiate not only a

selected replicative advantage but also a bias to metastasize on cancer cells. This theory was based on the publication of van't Veer et al. who predicted that people with breast cancer can be prognosticated by gene expression profiles of the primary malignancy [158]. Ramaswamy et al. demonstrated that a group of primary tumors mirrored metastatic tumors with respect to gene expression signature [159]. In this model, it was considered that there are no genes and genetic alterations clearly and completely involved in orchestrating the process of metastasis [154].

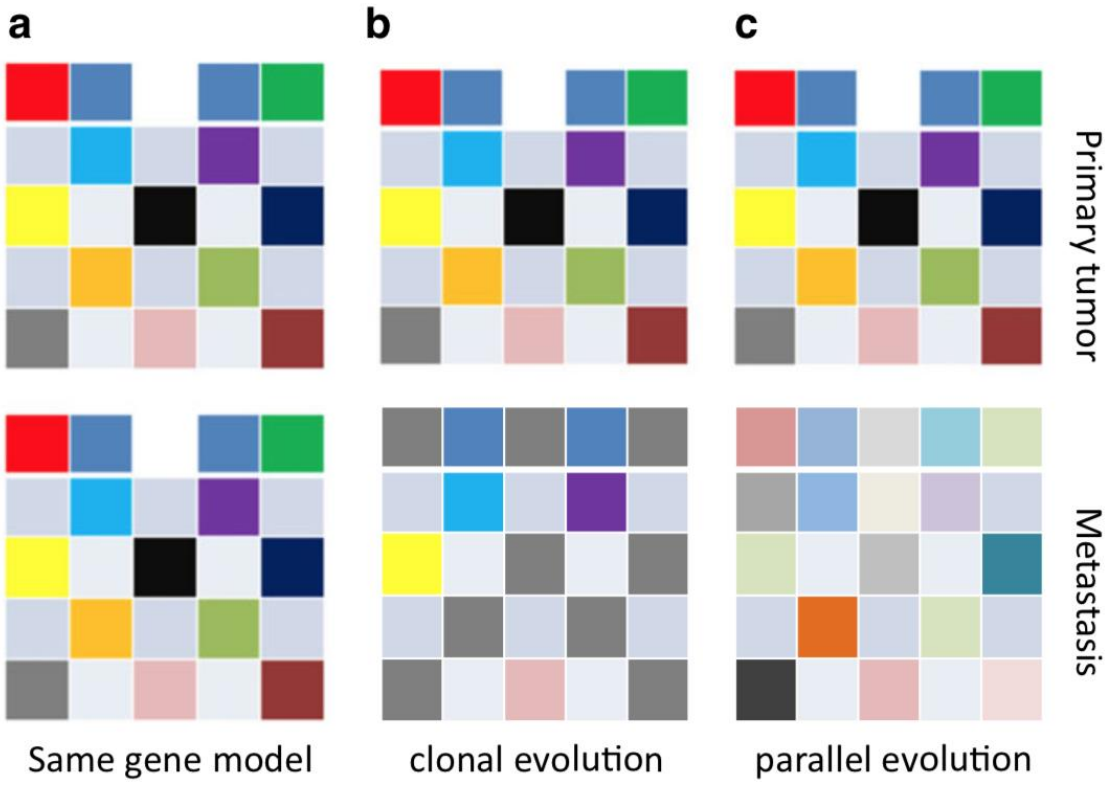

Figure 14. Models of genetic progression of metastasis. **A** Metastasis is genetically matched to the primary **B** metastasis is a clonal representative of the primary **C** metastasis is completely different from the primary [15].

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7 New observations of the PhD Dissertation

7.1 KIT molecular epidemiology of melanoma

1. We have provided evidence that in Central Europe the frequency of KIT mutations in skin melanoma is approximately 15%.

2. We have also demonstrated that in this group of melanomas KIT mutations are significantly more prevalent in the acral lentiginous form as compared to any other forms.

3. We have shown that 50% of KIT mutation carrying melanomas bear exon 11 and exon 13 mutations, presenting a significant patient population which could be treated with KIT inhibitors [112].

7.2 Clonal heterogeneity and mutant allele fractions in skin melanoma

4. We have shown that BRAF MAF, but not NRAS MAF, is significantly increased in the metastases of melanoma cases.

5. Further, we have shown that this increase can not be detected in CNS and liver metastases, suggesting the organ specific role of BRAF mutation in melanoma.

6. We have also demonstrated dynamic and unpredictable changes in MAF of melanoma metastases (both for BRAF and NRAS mutant forms) as compared to their respective primary tumors in a significant proportion of cases. Based on these data, we suggest to perform molecular testing on metastases whenever possible before therapeutic decision making.

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8 Summary

Skin melanomas are characterized by clonal heterogeneity associated with oncogenic drivers. We wished to determine the BRAF/NRAS mutant allele frequency (MAF) during tumor progression in a primary and associated visceral metastatic melanoma patient cohort consisting of 189 samples. MAF levels were corrected for TC% and classified as high (> 40%), medium (15–40%) or low (<15%). Data on the mutation rate of KIT showed high regional/ethnical variability worldwide and differences were also found in the various melanoma histotypes. Therefore, we studied 227 skin melanoma cases for KIT exon 9, 11, 13, 17 and 18 mutations.

Contrary to NRAS mutant cases, in BRAF mutant melanomas MAFs were found to be significantly increased in lung-, adrenal gland-, intestinal- and kidney metastases due to clonal selection compared to the primary tumors. Only 31.3% of BRAF mutant cases and 50% of NRAS mutant cases maintained the MAF profile of the primary in metastasis. However, in 18.7% of BRAF mutant cases low MAF primaries switched to high MAF in metastases. Investigating the inter-metastatic heterogeneity, the metastases were found to be relatively homogeneous regarding MAF. In heterogeneous BRAF mutant metastatic cases low to high or high to low MAF conversions occurred in another 12.5% of cases.

In the KIT molecular epidemiology cohort a KIT mutation frequency of 15% was observed. Exon 11 was the most frequent mutation site (44.7%) followed by exon 9 (21.1%), equally characterizing UV-induced common histotypes and ALM tumors. In our cohort of 79 BRAF/NRAS double wild type cutaneous melanoma cases, we observed a KIT mutation frequency of 43.04% with a significantly higher rate detected in ALM as compared to UV-induced common variants (58.8% versus 31.1%, p=0.014). For comparison, we investigated 17 mucosal melanomas, in which case the double wild type cohort showed a comparable KIT mutation frequency (41.2%). In the mucosal melanoma cases exon 9 was the most frequently involved exon followed by exons 13 and 17. KIT mutation hotspots were identified in the following exons: 9 (c482/491/492), 11 (c559, c572, c570), 13 (c642), 17 (c822) and 18 (c853).

Our findings suggest that in visceral metastases of malignant melanoma BRAF- or NRAS-MAFs are rather heterogeneous and cannot be predicted from data of the primary tumor. These data may have clinical significance when using targeted therapies.

Based on the finding of a relatively high mutation rate of KIT in our cohort, it seems to be justified to screen BRAF/NRAS double wild type melanoma patients for KIT mutations at least in regard to exons 11/13, irrespective of the type of melanoma.

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9 Összefoglalás

Ismert tény a szolid tumorok klonális heterogenitása. Kutatómunkám során mutáns allél frakciót vizsgáltam BRAF és NRAS gén tekintetében 50 melanomás beteg 189 szövetmintájában. Meghatároztuk továbbá a KIT gén magyarországi molekuláris epidemiológiáját másik 227 melanomás mintán 5 exonra kiterjedő szekvenálással.

A BRAF mutációt hordozó melanomák esetében szignifikánsan magasabb mutáns allél arányt találtunk a metasztázisokban, mint a primer tumorban, ez a mutáns klónok pozitív szelekciójára enged következtetni. Szervi lokalizációt tekintve a tüdő, mellékvese, emésztőrendszer és a vese áttétekben szignifikánsnak bizonyult a BRAF mutáns klónok felszaporodása a primer tumorhoz képest. A BRAF mutáns esetek 31.1%-ában és az NRAS mutáns betegek 50%-ában egyezett a mutáns allélok megoszlása a primer tumorban és a metasztázisban, míg a többi esetben eltérést tapasztaltunk. Amikor egy betegből származó multiplex metasztázisok közti mutáns allél frekvenciát néztük, 12.5%-ban találtunk jelentős eltérést a primer tumorhoz képest.

A primer és áttéti, UV-indukálta és nem UV-indukálta (ALM) bőrre lokalizált melanomákat egyformán tartalmazó csoportban a KIT gén mutációs gyakorisága 15%-nak (N=227) adódott hazánkban, ami kissé magasabb a nemzetközi átlagnál, bár az irodalmat tekintve nagy a szórás. A mutációk a 11. exonban (44.7%), majd a 9. exonban (21.1%) voltak leggyakrabban fellelhetőek. Azonban a BRAF/NRAS vad bőr melanomák (N=79) esetén 43% volt a KIT mutációs gyakoriság, igaz, ebben a csoportban az ALM (non-UV) szignifikánsan nagyobb mértékben volt képviselve, mint az UV-indukálta (SSM, NM, LMM, NOS) melanoma (58.8% vs. 31.1%, p=0.014). Kontrollként megvizsgáltunk 17 nyálkahártya melanomát is, ahol a KIT mutációs ráta a dupla vad, bőr melanomák esetén látott eredménnyel jól összevethetőnek, 41.2%-nak adódott. Nyálkahártyáról kiinduló melanomák esetén azonban a 9. exon mutációi voltak a leggyakoribbak, ezt követte a 13. és a 17. exon. A leggyakoribb mutációkat az alábbi helyeken detektáltuk: 9. exon 482/491/492 kodon, 11. exon 559, 572, 570 kodon, 13. exon 642. kodon, 17. exon 822. kodon és a 18. exon 853. kodon.

Eredményeink nyomán következtetésképpen levonható, hogy a mutáns allél frekvencia előre nem megjósolható irányban változik tumorprogresszió során, ezért feltétlenül fontos az áttétek mutációs státuszának vizsgálata célzott terápia bevezetése előtt, illetőleg melanoma altípustól függetlenül a BRAF és NRAS vad eseteket legalább a KIT gén 11. és 13. exonjára vonatkozóan is informálódni kell a klinikusnak, hiszen KIT inhibitorokkal is beszámoltak sikeres kezelésekről.

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11 Bibliography of the candidate's publications

Publications related to the PhD thesis

(1)**Doma V**, Kárpáti S, Raso E, Barbai T, Timar J. Dynamic and unpredictable changes in mutant allele fractions of BRAF and NRAS during visceral progression of cutaneous malignant melanoma. BMC Cancer, 2019. 19(1): p. 786.

(2)**Doma V,** Barbai T, Beleaua MA, Kovalszky I, Raso E, Timar J. KIT mutation incidence and pattern of melanoma in Central Europe. Pathol Oncol Res, 2020. 26: 1 pp. 17-22.

Publications not related to the PhD thesis

(1)Kim Y, Gil J, Pla I, Sanchez A, Betancourt LH, Lee B, Appelqvist R, Ingvar C, Lundgren L, Olsson H, Baldetorp B, Kwon HJ, Oskolas H, Rezeli M, **Doma V**, Karpati S, Szasz AM, Nemeth IB, Malm J, Marko-Varga G. Protein Expression in Metastatic Melanoma and the Link to Disease Presentation in a Range of Tumor Phenotypes. Cancers (Basel), 2020. 12(3). doi: 10.3390/cancers12030767.

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