ONCOGENIC DRIVERS OF CUTANEOUS MALIGNANT MELANOMA AND THEIR ROLE IN PROGRESSION

PhD thesis

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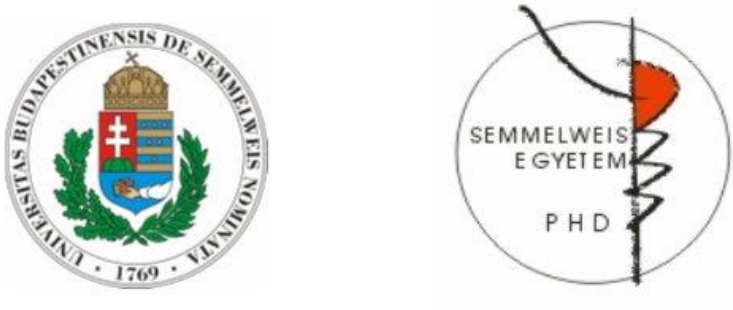

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Introduction

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. Today cardiovascular diseases, malignancies, respiratory diseases and diabetes account for more than 70 percent of mortality worldwide. 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. One of the well-known risk factors of skin cancer is ultraviolet (UV) radiation, 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. It plays a key role in damaging the skin's cellular DNA: excessive UV radiation produces genetic mutations that can lead to skin cancer. 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. 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. 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, which in 2018 was the second most common cancer type in both sexes, in all age groups.

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.

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. 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 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. 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. 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.

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.

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.

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%). Therefore, our aims in this thesis were the following:

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. 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.

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. According to certain studies, the high percentage of BRAF MAF may predict better response to RAF/MEK inhibition.

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).

Patient selection

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.

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).

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. 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.

DNA extraction

Prior to DNA isolation from FFPE blocks, all sections were stained with hematoxylin and eosin to evaluate T/N ratio by counting nuclei at three visual fields using 40x objectives. T/N ratio 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).

PCR

Concentration of the primers was 1 μM for each reaction. 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).

RFLP of BRAF exon 15 PCR products

PCR amplification of exon 15 with BRAF specific primers 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.

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. 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%.

Pyrosequencing

Primary melanomas and corresponding metastases carrying different genotype via Sanger sequencing were reinvestigated using a higher sensitivity (>2%) CE IVD pyrosequencing technology. 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.

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). Based on the T/N ratio, the adjusted MAF values were defined.

It is of note that the obtained PyroMark MAF value was corrected for T/N 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.

Statistical analysis

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

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.

KIT molecular epidemiology study

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

Results

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.

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. 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).

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.

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%). 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.

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. 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.

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.

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). 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.

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, whereas another patient showed codon 12 mutation, namely G12C.

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.

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 tumor to normal cell ratio. Extremely low and high T/N cases were found to have low MAF rates or vice versa. The MAF values were also corrected for T/N.

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.

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, while no significant alterations were detected in metastases of NRAS mutant melanomas as compared to the primary tumors.

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).

(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).
(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). 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.

Changes in MAF levels during metastatic progression

For better presentation of the above patterns, the individual samples were artificially grouped into low (MAF < 15%), medium (MAF between 15-40%) and high (MAF > 40%) 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. 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, as well as in 9 out of 12 NRAS mutant cases shown. Heterogeneous metastatic cases - where the MAF values are different between the corresponding multiplex metastases – are observable in 9 out of the 32 BRAF mutant cases and 3 out of the 12 NRAS mutant cases. 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: 6 out of 32 (18.75%) in BRAF-mutant homogeneous cases, 4 out of 32 (12.5%) in BRAF-mutant heterogeneous cases and 2 out of 12 (16.7%) in NRAS-mutant cases. 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%).

Conclusions

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, the NRAS mutation rate was 20%, which is also in accordance with other geographical regions. Reading the melanoma literature, one might have the impression that KIT mutations in cutaneous melanoma are rare. 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. 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. For example, according to data coming from the neighbouring Slovenia, the KIT mutation frequency showed an extremely low value of 1.3%, 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). 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 analyzed, 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. 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). 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.

As regards mutational hotspots, in case of GIST codons 502/503 in exon 9 were described, 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, however, the neighbouring codon 559 occurred in melanoma cases as well. Regarding exon 13, GIST 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. Another similarity between GIST 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. Another malignancy with KIT mutation is AML, where the response to KIT inhibitor therapy is not known for exon 17 codon 816 mutations. 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.

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

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 T/N ratio, since these factors were compensated for. Our findings are actually in line with previous studies found in the literature.

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. 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 analyze 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. 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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