BRAF MUTATION AND THE POSSIBILITY OF IDENTIFYING PROGNOSTIC MARKERS FOR METASTASIS OF UVEAL MELANOMA

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SUMMARY

Aim: The aim is to assess the BRAF gene mutations in patients with posterior uveal melanoma.

Material and methods: Retrospective analysis of the group of patients with malignant melanoma of the uvea, who were indicated to enucleation between 1.1 2015 to 1.3.2016. We analyzed stage of uveal melanoma, volume, cell type and BRAF gene mutations.

Results: In clinical study of 20 patients after enucleation due to uveal melanoma at the Department of Ophthalmology in Bratislava, patient age was ranged from 22 to 89 years with a median of 62 years. In 14 patients (70 %) enucleation was the primary treatment and in 6 patients (30 %) enucleation was after irradiation (brachytherapy, Leksell gama knife, linear accelerator). In 17 cases (85 %) the mutation of the BRAF gene was negative and in 3 cases the sample was not assessable for the BRAF mutation.

Conclusion: BRAF gene mutation is confirmed by several studies found in malignant melanoma of the skin. The histopathology findings in our group did not confirmed our theory, that since the uveal melanoma itself has the similar origin as skin melanoma, should also contain a BRAF mutation.

Key words: malignant melanoma of the uvea, mutation of the BRAF gene, chromosomal abnormalities as a prognostic factor

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INTRODUCTION

In the case of intraocular tumours, the most frequent in terms of its incidence is malignant melanoma of the uvea (MMU). It appears most often in the choroidea (78-85%), followed by the corpus ciliare (9-16%) and the iris (6-9%). This has also been confirmed by more extensive studies (12).

From the perspective of histopathology it is possible to divide MMU into the following types: fusocellular type A, in the case of which cells have a deficiency of cytoplasma, a dense core with absent nucleolus and a uniform shape and size, the second type is fusocellular type B, in the case of which cells have a fusiform shape with a visible nucleolus, the third type is the epithelioid type, which is formed by polyhedral cells of various size and visible nucleoli, and the fourth type is referred to as the mixed type, in which there is a synthesis of the epithelioid and fusocellular type (12, 16, 19, 46).

In the case of treatment of MMU in more advanced stages this concerns an endeavour to remove the tumour as a whole and prevent the spread of metastases into the surrounding structures, chiefly into the orbit but also into the entire organism. In the past the treatment of MMU proceeded exclusively to enucleation of the bulb or exenteration of the orbit (11). At present other methods are also now used in treatment (ionising radiation – application of brachytherapy, Leksell gamma knife, linear accelerator, proton radiation) (13, 14, 15, 17, 18).

On the basis of clinical trials and histopathological images, the prognosis for survival is more favourable in women aged up to 40 years, in patients with small tumours without scleral infiltration and also in the case of the incidence of fusocellular types (A or B) of tumours. From a prognostic perspective it is possible to indicate as unfavourable the epithelioid type of MMU, high mitotic activity, as well as a certain type of vascularisation. In the past vascularisation was considered a potential prognostic factor, but at present the prognostic significance of this factor is far lower in comparison with others. The prognostic significance of indicators is summarised in the current TNM classification (dimensions of tumour, presence or absence of extrascleral progression etc.) and in the morphological classification ensuing from Callender's classification (17, 18).

Molecular aspects of uveal melanoma

The development of tumorous pathology is frequently linked with genome instability and the acquisition of genome

heterogeneity, the generation of both populations of cancer cells, both clonal and non-clonal (2, 29, 49). Certain mutations in the cellular cycle may lead to aneuploidy, for example during mitosis (2, 35). Here there is a very fine line in the balance between the possible benefit from an accumulation of genetic and epigenetic changes on one hand, and lethal genetically unstable cells on the other (32). Polyploidy is known in tumorous proliferation and has a tendency to occur in tumours with an aggressive phenotype (4, 28).

Current research is focusing on seeking the paths involved in carcinogenic potential, and is thereby attempting to understand the origin, the development of the tumour in the early stage, and its transition up to the stage of a metastatic pathology.

Chromosomal anomalies as a prognostic factor

Certain chromosomal anomalies such as deletion of chromosome 1p, monosomy of chromosome 3 or acquisition of chromosome 8q correlate very strongly with reduced survival of patients with uveal melanoma. The chromosomal aberration monosomy of chromosome 3 is found most frequently in uveal melanoma and is located primarily in metastasising tumours (41). In a univariate analysis, monosomy of 3 was the most significant predictor ($p < 0.0001$) of a poor prognosis for uveal melanoma, followed by localisation of the tumour and diameter (2, 34). This is considered to be of primary significance from the perspective of the fact that they occur in combination with other chromosomal aberrations in uveal melanomas, such as loss of chromosome 1p, amplification of 6p and acquisition of 8Q (35). In rare cases melanomas have been described with partial aberrations on chromosome 3 or translocation, so it is very difficult to map the expected tumour suppressor genes (36, 37).

Simultaneous loss of chromosomes 1p and 3 has a stronger correlation with metastatic pathology than the loss of one of them separately (30). The ordinarily deleted area on chromosome 1 is within the range of 1p34.3 to 36.2 (1, 21, 25). Association with chromosome 8q was somewhat less significant than in the case of monosomy of chromosome 3, but a strong inverse correlation ($p < 0.0001$) of other copies of 8q to survival was observed (26). The acquisition of chromosome 8 or the procurement of isochromosome 8q is a secondary event in uveal melanoma, because a variable number of copies of 8Q may be present in one tumour (24, 39). This often occurs in tumours that have lost one copy of chromosome 3, and is an independent prognostic factor of a progressive pathology (36, 38, 43, 46).

In the group of posterior uveal melanomas, the presence of chromosome 6p is predictive of a more favourable result (27, 28). These tumours with acquisition of chromosome 6p represent a specific group of uveal melanomas with an alternative genetic pathway in carcinogenesis, because acquisition of 6p occurs frequently in tumours with disomy 3 (10, 23, 29, 31, 32).

Genes involved in uveal melanoma

Deregulation of RAS-RAF-MEK-EKR (Rat sarcoma – Rapidly Accelerated Fibrosarcoma – Mitogen-activated protein kinase extracellular-signal-related kinases) or mitogen-activated protein kinase (MAPK) is common in several human malignant tumours (27). Mutations of specific parts of these key molecular signal pathways were incorporated into the tumorigenesis of skin melanoma. Many of these frequently known oncogenes (e.g. BRAF) or suppressor genes of the tumour (PTEN – Phosphatase and tensin homologue, CDKN2A – cyclin-dependent kinase Inhibitor 2A) were also analysed in the case of uveal melanoma, and it was determined that mutations of these genes occasionally occur. Although to date no correlation has been found with the development of metastatic pathology, the presence of somatic mutations in these genes may provide a starting point for the timely detection of metastatic cells in blood. More promising are the recent results of two frequently mutated genes of the MAP-kinase pathway, GNAQ (G protein subunit alpha q) and GNA11 (G protein subunit alpha 11), and the gene BAP1 (BRCA1 associated protein 1) (20).

BRAF

The BRAF gene occurring on chromosome 7q34 is indicated as the proto-oncogene BRAF and v-RAF murine sarcoma viral oncogene homologue B1. It consists of 18 exons and its length of transcribed mRNA is 2478 bp. BRAF can be indicated as an oncogene which acts in several types of tumours, whereby it has various forms in which changes in the BRAF protein occur. These can cause simplified growth and also widespread incidence of tumour cells.

An analysis of BRAF identified at least 30 mutations with a change in the sense of "missence mutation". Various forms of mutation of the BRAF gene can be identified in kinase domains, which are localised on exons 11 to 15. A substantial proportion – up to 89% - of BRAF mutations are localised in codons 595-600 of exon 15. The most typical mutation occurring in exon 15 occurs as a consequence of a point mutation of the DNA of nucleotide 1799, which is linked with a substitution of the nitrogenous base of thymine (T) for adenine (A), which is indicated as T1799A. This mutation has the effect of a replacement of amino acids and subsequent activation of BRAF kinase. A substitution of the amino acid valine (V) with glutamic acid (E) takes place in codon 600 (7). The mutation is indicated as BRAFV600E, or Val600Glu. The substitution of V for E causes a constitutive activation of BRAF.

The BRAF gene codes a protein with the same designation, which belongs to the family of RAF kinases. BRAFV600E destabilises the kinases and contributes to tumorogenesis on the basis of activation of the MAPK pathway (47). The BRAF protein, by regulating the MAPK/ERK signal cascade, influences cell division, differentiation and secretion. The dephosphorylated standard BRAF protein persists in an inactive state as a consequence of hydrophobic interactions between the activation point and the ATP-binding point. V600E mutation influences the spread of these interactions, as a consequence of which phosphorylation takes place, which in the activation segment regulates the transcription activity of BRAF by means of incorporation of BRAF into the membrane activated by RAS. Phosphorylation enables the attainment of maximum kinase activity, and also contributes to basal activity, which is higher in the case of BRAF than the residual RAF isomers.

Uveal melanoma is the most frequently occurring type of tumour in ophthalmology affecting adults. The annual incidence is 6 to 7 cases per one million, in 50% of cases leading to death within 5 years due to subsequent metastases, which are associated also with the histological and demographic prognostic factors (cellular type, dimensions of tumour, localisation, chromosome aberrations, age and also sex).

Uveal to skin melanomas originated from identical precursor cells, from melanocytes, which migrate from the neural band to the relevant location during the period of embryonic development. The similar genetic background and certain common histological characteristics point to the fact that the pathogenesis of uveal and skin melanoma could be very similar. However, risk factors such as pale complexion and blue coloured iris may be pathognomonic, UV radiation and solar effects appear to be significant mainly in the pathogenesis of skin melanomas. In addition to this, uveal melanoma metastasises the blood pathway, especially to the liver, whereas skin melanoma has a tendency to metastasise and pass through the lymphatic system, usually regionally affecting lymph nodes. Despite this, however, there are still few observations on the molecular pathogenesis of uveal melanoma. The loss of function of the gene as a consequence of deletion or inactivating mutations is a characteristic tumour cells.

The importance of oncogenetic mutations in the RAS/F/RAF/MEK/ERK pathway has been well documented. More than 15% of tumours conceal point mutations of RAS. Constant activation of this pathway provides a powerful mitogenic force which leads to an abnormal proliferation and differentiation in several types of tumours. The association between RAS and mutation and uveal melanoma has been examined in several studies. However, it is still unknown as to whether other genes play a role in the RAS/RAF/MEK/ERK pathway in the origin of uveal melanoma.

The mutation BRAF V600 has been identified in pre-cancerous lesions, which indicates participation in the transformation of melanocytes. Certain naevi which contain this mutation may have the consequence of transformation into melanoma, but it is necessary to note that not all naevi with this mutation are pre-cancerous. In addition to this, whilst BRAF mutation V600 contributes to the development of melanoma, studies have determined that mutations in other cellular proteins may also play a role in this type of tumorous pathology (5).

Studies indicate a significant and essential role of RAS-RAF signalling in the origin of melanoma. The presence of activating BRAF mutations has been determined in up to 82% of benign naevi. Certain experts are of the opinion that BRAF mutations in benign naevi may serve as markers of malignant melanoma upon sensitivity of the individual.

RAF signalling cascade

Enzymes catalysing the binding of phosphate to lateral chains of the amino acids serine, threonine or tyrosine are indicated as protein kinases. They play a role in the quantity of the aspects of cellular biology. Phosphorylation brings changes to the properties of enzymes such as for example regulation of their metabolism, catalytic activity, transcription and apoptosis. RAF mutations manifest incidence in approximately one third of all detected types of human tumours (44).

Structure of RAF proteins

RAF refers to the family of protein-serine/threonine kinases. RAF proteins may be designated as essential connectors between the RAS and MEK-ERK signal pathway. In the case of mammals we can distinguish three different RAF isoforms: ARAF (v-raf murine sarcoma 3611 viral oncogene homologue 1), BRAF amd CRAF (v-raf-1 murine leukaemia viral oncogene homologue 1). From the perspective of functionality, RAF protein-kinase domains are typical N-terminal small and large C – terminal loops. N-terminal loops contain an ATP-phosphate binding loop which is referred to as a P-loop. C-terminal loops are usually alpha-helical, binding MEK 1/2. In the case of all three RAS isoforms we find a common structure, which consists of three conservative regions that differ in their functions, designated CR1 (Conserved region 1), CR2 (Conserved region 2) and CR3 (Conserved region 3) (44). CR1 consists of a RAS binding domain which is essential as a consequence of interaction with the RAS and membrane phospholipids, and also with a cysteine rich domain which serves as a secondary RAS – binding location. In CR2 phosphorylation of CRAF takes place. CR3 occurs in the region of the C end, contains the kinase domain and also an activation segment, the phosphorylation of which is essential for the kinase activity of BRAF (26).

RAS-RAF-MEK-ERK signal pathway

In the case of all three isoforms of the RAF family, a proportion there of is present in the so-called RAS-RAF-MEK-ERK signal transduction cascade. It is designated as a mitogen-activated protein kinase cascade (50). It takes part in a wide spectrum of processes, including apoptosis, progression of the cellular cycle, differentiation, proliferation and transformation into cancerous state. The skeleton of the RAF pathway is widely branched, consisting of a three-level kinase cascade which enables extension of regulation by crossing signalling pathways and also an increase in sensitivity to incoming signals (3). The signal pathway is activated by RAS protein. Inactive RAS – GDP, which binds to the membrane, is transformed under the influence of phosphorylation into active membrane RAS – GDP, which subsequently activates further elements of the pathway. Binding of RAS – GDP with RBD, which is located on the N-terminal regulating section of kinase triggers activation. A subsequent analysis of the RAS family demonstrated that only H-RAS, N-RAS and K-RAS are capable of activating BRAF (43).

Inactive RAF kinase proteins are located in cytosol. All RAF proteins share MEK 1/2 kinase as a substrate. Despite the ability of all members of the RAF family to bind and phosphorylate MAK in vitro, activity of the directions to MEK differs widely. The strongest with regard to activity to MEK is BRAF. In the case of ARAF and CRAF, activity is on the boundary of detectability. Activated RAF kinases phosphorylate both MEK isoforms in the activation loop. Phosphorylation of RAF proteins increases the activity of MEK, which is then capable of binding, phosphorylating and activating ERK. MEK 1/2 are dually specific protein kinases which mediate the activation

of tyrosine and serine in ERK1 or ERK2 with the influence of their phosphorylation. The ERK pathway is characterised by subsequent regulation of a large quantity of proteins, which mediate cross-talk with the residual signal pathways, whilst ERK regulates various biological functions such as cellular proliferation, differentiation, migration or apoptosis. Post -translation modification is necessary in order to sustain RAS – signalling, as a result of which it is possible to bind RAS proteins on to the internal cell membrane – farnesylation and prenylation (33).

MATERIAL AND METHOD

The cohort comprises 20 patients with malignant melanoma of the uvea, for whom enucleation of the afflicted bulb was indicated in the period from 1 January 2015 to 1 March 2016. Basic clinical data was gathered about the patients' tumours. Tissue samples were routinely processed by the formol-paraffin method. The histopathological finding was supplemented with a histological typing of the tumours and an evaluation of their propagation. We conducted an analysis of the characteristics of the uveal melanoma (stage, volume, cellular type) and level of BRAF.

RESULTS

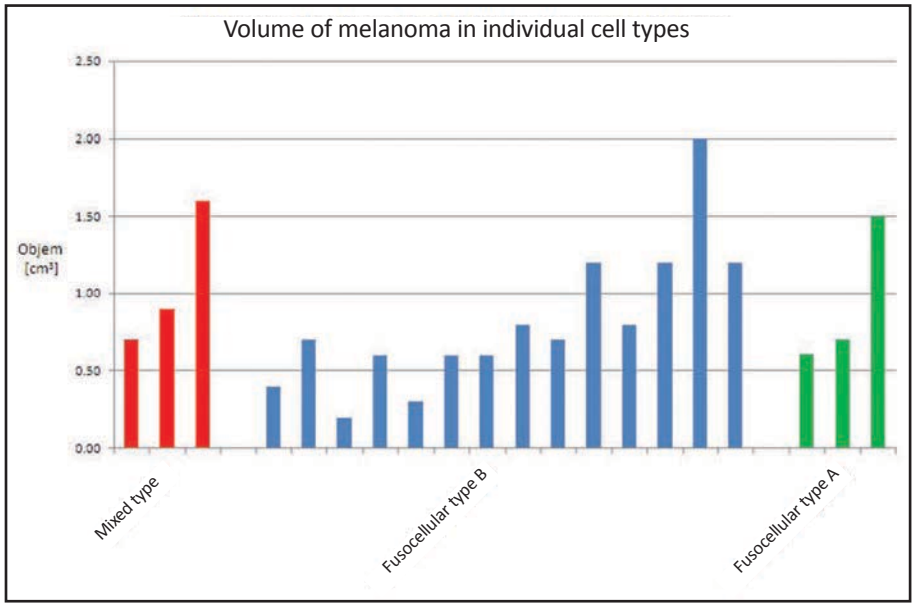
We indicated 20 enucleations at the Department of Ophthalmology of the Faculty of Medicine, Comenius University and University Hospital in Bratislava in the period from 1 January 2015 to 1 March 2016 due to malignant melanoma of the uvea. The average age of the patients was 62 years (from 22 to 89 years). The average age of the men was 62 years and the average age of the women was 59 years). Men predominated in the cohort. Enucleation in 14 cases (70%) was indicated as primary enucleation for melanoma, and in 6 cases (30%) this concerned enucleation following prior treatment with radiation (brachytherapy, Leksell gamma knife, linear accelerator). In our cohort the left eyeball was afflicted by MMU more frequently than the right eyeball. No metastases were determined at the time of indicated enucleation in any of the patients. In 17 cases (85%) mutation of the BRAF gene was negative, and in three cases it was not possible to examine the sampled material for mutation of the BRAF gene (table 1).

In our cohort fusocellular type B was the most frequently

Table 1 Overview of cohort of patients following enucleation for malignant melanoma at the Department of Ophthalmology, Faculty of Medicine, Comenius University and University Hospital Bratislava in the period from 1 January 2015 to 1 March 2016

Patient	Sex	Age	Localisation	Side	Histopathological type/stage	Volume (cm3)	Prior treatment	BRAF mutation	Metastases
1	M	77	MMCH	R	Fusocellular type B / IIb	0.7	No	No	Not determined
2	M	56	MMCH	L	Fusocellular type B / II	0.4	No	No	Not determined
3	M	75	MMCH	L	Fusocellular type B / II	0.6	No	No	Not determined
4	M	20	MM corp. cil.	L	Fusocellular type A / IV	1.5	Yes (Leksell gamma knife)	No	Not determined
5	F	47	MMCH	R	Fusocellular type B / II	0.3	Yes (SRCH)	Not examined	Not determined
6	F	78	MM corp. cil. et. choroidae	L	Fusocellular type B / IV	2.0	No	Not examined	Not determined
7	F	67	MMCH	L	Mixed type / III	0.9	Yes (SRCH)	Not examined	Not determined
8	F	75	MMCH	R	Fusocellular type B / IIb	0.8	No	No	Not determined
9	M	67	MMCH	L	Fusocellular type B / IIi	1.2	No	No	Not determined
10	M	86	MMCH	L	Fusocellular type A / II	0.6	No	No	Not determined
11	M	72	MMCH	R	Fusocellular type B / II	0.6	Yes (SRCH)	No	Not determined
12	F	53	MMCH	L	Fusocellular type A / II	0.7	No	No	Not determined
13	F	63	MM corp. cil. with infiltr.	R	Mixed type / II	0.7	No	No	Not determined
14	F	35	MMCH	L	Fusocellular type B / III	0.8	No	No	Not determined
15	M	89	MMCH	L	Fusocellular type B / IIb	0.7	No	No	Not determined
16	M	42	MMCH	R	Fusocellular type B / IIIa	1.2	Yes (SRCH)	No	Not determined
17	M	41	MMCH	L	Mixed type / IIIb	1.6	No	No	Not determined
18	M	56	MMCH	R	Fusocellular type B / III	1.2	No	No	Not determined
19	M	64	MMCH	L	Fusocellular type B / Ib	0.2	Yes (brachytherapy)	No	Not determined
20	F	57	MMCH	R	Fusocellular type B / IIa	0.6	No	No	Not determined

(MM – malignant melanoma, MMCH – malignant melanoma of the choroidea, SRCH – stereotactic radiosurgery)



Graph 1 Cohort of patients after enucleation for malignant melanoma at the Department of Ophthalmology, Faculty of Medicine, Comenius University and University Hospital Bratislava

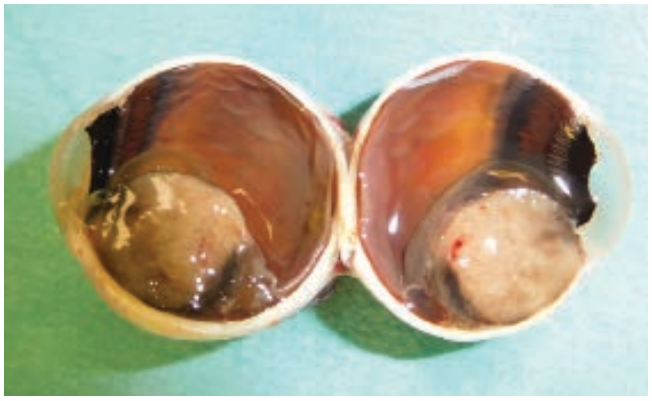


Fig. 1 Patient no. 13 – macro photo of enucleated eyeball following enucleation for MM of the corpus ciliare with infiltration into the iridocorneal angle; histopathologically this concerns a mixed type which penetrates into the surface of the bulb without propagation into the optic nerve (photo – A. Furdová)

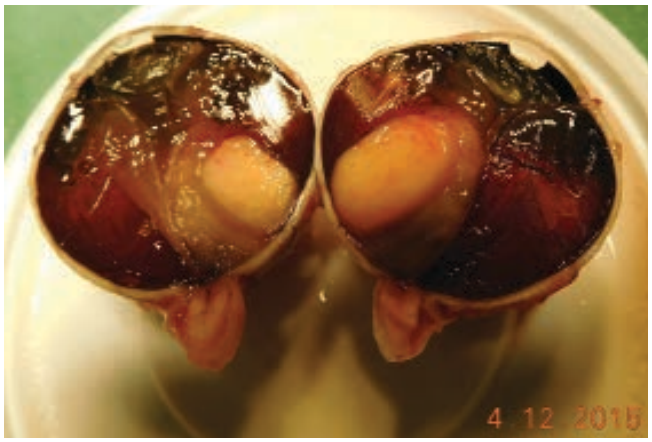


Fig. 2 Patient no. 20 – macro photo of enucleated eyeball following enucleation for MMCH; histopathologically this concerns fusocellular type B, which grows into the surface of the wall of the bulb without propagation into the optic nerve (photo – A. Furdová)

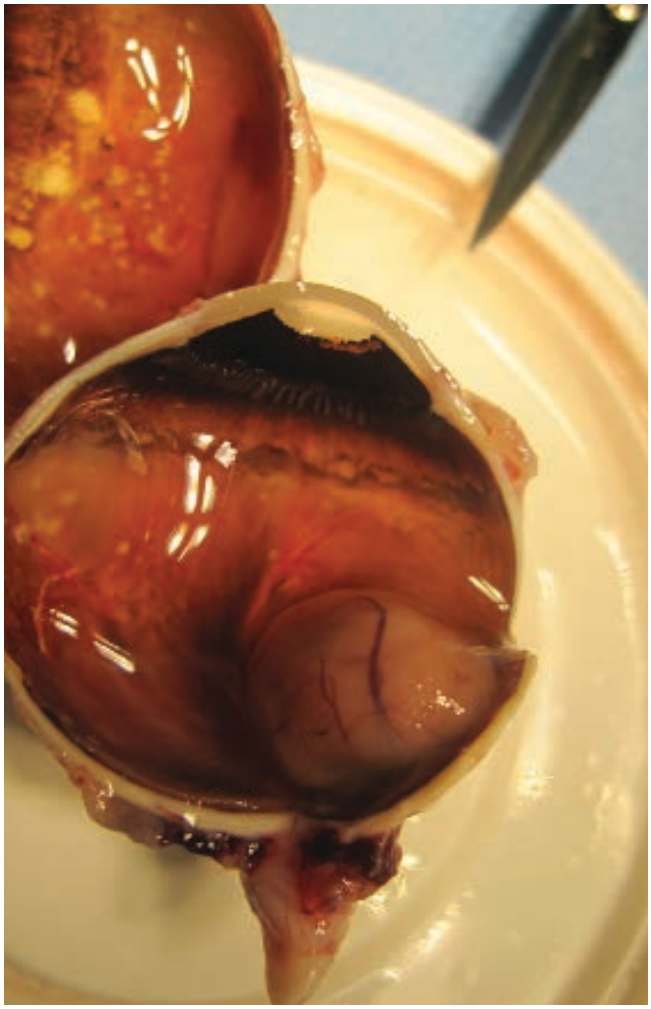


Fig. 3 Patient no. 1 – macro photo of enucleated eyeball following enucleation for MMCH; histopathologically this concerns fusocellular type B, which grows into the surface of the wall of the bulb without propagation into the optic nerve (photo – A. Furdová)

represented (14 times), with fusocellular type A 3 times and mixed type 3 times (fig. 1, 2, 3). The average volume for fusocellular type B was 0.81 cm3, for fusocellular type A 0.93 cm3 and for mixed type 1.07 cm3 (graph 1).

DISCUSSION

We do not know the influence on overall survival for patients with a BRAF mutation. Within a general context, endeavours are now under way to somehow combine the benefits of the "immunity" and "molecular" levels. This is being projected into clinical practice with the launch of new, either combined regimes or sequences in the form of clinical trials. In the metastatic stage of the disease we therefore have at least two new treatments on the horizon, namely biological therapy and immunological therapy, in addition to which we have a large number of questions. Recently several key molecular pathways have been detected in the pathogenesis of melanoma, anticipating an impact on clinical practice in the sense of division into individual subgroups. Depending on the "chief" oncogenic pathway we would then be able to develop and apply targeted biological therapy. We would consider MAPK (Mitogen-Activated Protein Kinase) and the c-kit kinase pathway to be the most important pathways which influence the differentiation, proliferation, survival and migration of melanocytes. The prevalence of BRAF mutations is different for various oncological disorders, but in the majority of cases they appear on codon 600, with the result of substitution of glutamate with valine. In the case of malignant melanomas it represents approx. 50-60% (8).

It appears that the location of incidence of the primary deposit is linked with genome expression. Melanomas localised in places with low exposure to solar radiation such as acral and mucosal melanomas, or in places with chronic damage to the skin by solar radiation, have a low incidence of BRAF mutations. A further "activation pathway" in melanoma appears to be the c-kit signal cascade. Earlier studies indicated that the growth and invasion of a melanoma may be linked with a loss of c-kit expression (9). The last studies select the significance of this pathway, and thereby also select melanomas as such. Within the framework of the juxtamembrane domain of c-kit there is a presence of activating mutations, e.g. in up to 21% of mucosal melanomas, 11% of acral melanomas, 17% of melanomas originating the skin following solar radiation and 15% of anal melanomas (10, 11).

The thus defined subgroup of melanomas with c-kit aberrations in general shall probably be a candidate for targeted therapy with tyrosine kinase inhibitors (TKi) especially in the case of c-kit mutated form. At present phase III clinical trials are under way with a number of preparations, though in addition to the relatively small number of patients, there is a problem with recruitment into the trial, in which the subjects must have an appropriate mutation (22).

In the case of several tumorous pathologies, biological therapy has effectively succeeded in extending the time before the progression of the disease, as well as of overall survival. Until recently MMU was an "evil disease", considered to be chemo and radio resistant, as a result of which its prognosis in the fourth clinical stage was highly unfavourable. Intensive research in the field of the intracellular pathways which detect the "chief" cascades of oncogenesis in MM has brought hope of effective treatment using inhibitors of the BRAF-MEK-ERK pathway. However, to date this therapy is only for a selected subgroup of patients with BRAF mutation (40, 45).

Immunotherapy is the second platform for the development of treatment, without an unequivocal predictor. It appears that multiple control points exist in antitumour immunity, which can be influenced by effective targeted immunomodulation therapy in such a manner that it shall be possible to achieve long-term remission. In the following period it will be important, naturally in addition to the entry of further new molecules, to be able to time the treatment correctly and select a group of patients who will gain the maximum benefit from this type of therapy with minimal side effects. At present neither adjuvant therapy with interferon nor any other currently tested adjuvant therapy for uveal melanoma has any documented efficacy, and in Slovakia, even upon the potential conflagration of the disease, no "biological" therapy but chemotherapy with DTIC is indicated.

CONCLUSION

Mutation of the BRAF gene is confirmed by several studies, located especially in malignant melanoma of the skin. In 2003 Rimoldi and Cruz confirmed that mutation of the BRAF gene does not occur in the case of uveal melanoma. In a histological examination this mutation was not confirmed in our study, even though uveal melanoma and skin melanoma have the same histological basis. This may be caused also by the very low incidence of malignant melanoma of the uvea (6, 42).

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