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Development of novel parameter for monitoring of malignant melanoma progression

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ABSTRACT

Objective: Increasing HIFs in malignant melanoma, the highly aggressive skin tumour, results in the stimulation of invasiveness. Increased HIF-1α fallouts in inhibition of the activity of some mitochondrial enzymes and leads to preference of cytosol energetic metabolism. Increase of aerobic glycolysis is reflected in an increase of free NADH (Warburg effect) and develops the malignant melanoma.

Our goal was to find a link between hypoxia, or hypoxia mimicking factors and the stage of malignant melanoma. Furthermore, we focused on the finding of the experimental parameter which could monitor melanoma patients.

Patients and methods: We targeted HIF-1α gene expression and VDR rs2107301 gene polymorphism by PCR analysis. We detected the level of NADH in blood plasma by fluorescence spectroscopy (excitation and emission spectra).

Results: Analysis of the obtained data from patient samples has shown an increase in HIF-1α which correlates with the disease stage. Investigation VDR rs2107301 polymorphism of patient samples does not show any significant changes in single nucleotide polymorphism, and the low vitamin D level in blood is not a result of VDR mutation in mitochondria. NADH levels vary under hypoxic and pseudohypoxic conditions and refer to the cancer stage.

Conclusions: The apparent mismatch between HIF-1α expression and NADH fluorescence has become the basis for the design of an algorithm for monitoring malignant melanoma based on the sensing of NADH fluorescence and the determination of HIF-1α.

1. Introduction

Malignant melanoma (MM) is the seventh most common cancer in the Europe (the fifth most common cancer in women) [1]. Melanoma accounts for 4% of all dermatological tumours, but the percentage of mortality is highest, up to 95% [2,3]. The annual incidence of new cases of melanoma, depending on the population, is 10–42 new cases per 100,000 inhabitants and increases by 3–8% per year for Europeans [4]. Malignant melanoma affects predominantly young and middle-aged people; the median age of diagnosis is 57 years. A positive phenotype is observed for patients from 15 to 50 years old. About one half of the patients are between 35 and 65

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years old [5]. Melanoma is presented on the skin in 95% of cases while is also observed in the eye (uvea), oral and genital mucosa, gastrointestinal and genitourinary tract, leptomeningitis and lymph nodes [5].

Real or limited oxygen deficiency is one of the most important factors in the formation of the tumour microenvironment. A hypoxic environment leads to a change in the metabolic processes of the cells, which in turn leads to changes at the transcriptional and translational levels. Cancer cells prefer rapid energy production by glycolysis. Activation of the pseudohypoxic response of cells in a normoxic environment results in the accumulation of lactate and NADH, a process well-known as the Warburg effect [6].

Adaptation to hypoxia is regulated by hypoxia-induced factor 1 (HIF-1). This α/β heterodimeric protein is a transcription factor that plays a major role in cell response to hypoxia. HIF-1 transcriptionally affects the activity of genes modulating oxygen homeostasis in the cell [7]. In healthy melanocytes under physiological conditions (in normoxia), while HIF-1α is hydroxylated by the enzyme PHD (prolyl 4-hydroxylase), enabling the binding of VHL (Von Hippel-Lindau) and Rbx1 (Ring-Box 1). Prolyl-hydroxylated HIF-1α is then subsequently available for ubiquitination, and proteasome destruction of the 26S subunit occurs [8].

In melanoma cells, due to the E318K mutation, MITF (Microphthalmia-associated transcription factor) sumoylation worsens, leading to an increased expression of hypoxic transcription factors [9,10]. The increasing HIF signalling pathway, dominantly HIF-1α pathway [11], in cancer cells leads to increased invasiveness, angiogenesis, metastasis and the secretion of tumour factors, cytokines and growth factors, which increase the production of immunosuppressive cells [12].

Increased HIF-1α activity affects the expression of genes of various enzymes (glucose metabolism enzymes, mitochondrial enzymes) by reducing activity or inactivating the respiratory complexes, oxidative phosphorylation (OXPHOS) is disconnected from ATP production. Vitamin D, one of the main actors in activation of malignant transformation of cancer cells, is physiologically hydroxylated in mitochondria at position 1α and 25 (mitochondria of hepatocytes and kidney cells) [13]. Pigment cell in contrast to most of the tissues are independent in production of active vitamin D. However, in melanoma cells the disorder in activity of respiratory complexes results in impaired hydroxylation of vitamin D. Furthermore, the disconnection of respiratory complexes favours the cytosolic metabolism which leads to increased aerobic glycolysis and NADH production.

The ratio of free NADH and NAD^+ in the cell cytoplasm controls cellular redox homeostasis and can thus serve as a specific cell metabolic marker [14]. NADH and NAD^+, respectively, are cofactors for a number of enzymes involved in redox metabolic processes. The NADH/NAD^+ ratio in the cells is stable, and a its change indicates the presence of pathology. Elevated NADH is a characteristic of the developing tumour microenvironment [15]. NADH molecules arise not only in the process of glycolysis, which is referred to as the major metabolic pathway of the Warburg effect, but also by the metabolism of fatty acids in the mitochondria. Thus, NADH is one of the major metabolites that, together with lactate, characterises the tumour environment. Increasing glycolytic activity in the cytosol of cancer cells also leads to an adequate increase in NADH. NADH molecules are readily transferred to the matrix of mitochondria where they can be involved in the OXPHOS process. Recent research has shown that malignant cells do not use this energy source and accumulate free NADH [16,17]. NADH molecules belong to endogenous fluorophores and are fluorescently detectable in body fluids (blood, urine) in oncogenic-metabolic cell disorders in MM.

In light of previous studies in this field we have investigated the correlation between HIF-1α expression as well as blood plasma levels of NADH and stage of MM. Based on our findings we have attempted to design a novel parameter which could be used as a marker for MM progression.

2. Material and methods

2.1. Material

RNeasy Mini Kit (QIAGEN, Germany), Promega ReliaPrep Blood gDNA Miniprep System (Promega, USA), First strand synthesis reverse H Minus M-MLV kit (Thermofischer), SensiFast SYBR NO-ROX kit (Bioline), LightCycler FastStart DNA Master HybProbe kit, LC Green plus dye, 0.2 M Na_2HPO_4/KH_2PO_4 pH = 7.4 (Sigma-Aldrich, Austria).

2.2. Patients

Two groups of subjects were involved in the study. A control group (50 probands) without personal and a prior family history of cancer, mean age of the control group: 40 years old, 1:1 ratio of male and female, volunteers selected at the Transfusion Department of the University Hospital in Košice. Probands in control group were intake supplementary vitamin D to ensure that all controls will not have hypovitaminosis based on well-known situation that in population is highly spread hypovitaminosis D [18]. The group of patients with a malignant melanoma disease (126 probands), average age of the group: 63 years (from 19 to 87 years old), 51% female and 49% male, who have been ambulatory patients at the Clinic of Plastic, Reconstructive and Aesthetic Surgery of the Pavol Jozef Šafárik University in Košice, Faculty of Medicine). All participating probands agreed to the use of the provided biological material and personal data for scientific experiments in accordance with the Declaration of Helsinki Code of Ethics in 1975, with the last modification in 2013.

We also declare that the study was assessed and approved by the institutional ethics committee/institutional review board and that the letter of approval is available for examination. Permission from ethics committee was issued by Pavol Jozef Šafárik University in Košice, Faculty of Medicine (20N/2016).

2.3. Molecular analysis

RNA isolation was performed from a Paxgene RNA blood tube (Qiagene) using a commercial RNAeasy Mini kit (Qiagene). DNA

isolation was performed from blood collected in K_3EDTA Vacutainer tube using a commercial ReliaPrep Blood gDNA Miniprep System (Promega). The concentration and purity measurements of the isolated RNA and DNA samples were performed using the Nanodrop LC 3000 (Thermo Scientific).

Changes in the expression level of mRNA for specific HIF-1α (Hypoxia-inducible factor 1-alpha) and the housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as well as single-nucleotide polymorphisms (SNPs) of VDR rs2107301 in the whole blood of MM patients were detected. The sequences of used reverse and forward primers are shown in Table 1.

The acquired RNA was transcribed by reverse PCR into cDNA using specific reverse primers individually for HIF-1α using the First strand synthesis reverse H Minus M-MLV kit (Thermofischer). The cDNA samples were amplified using a commercial SensiFast SYBR NO-ROX kit (Bioline), 34 cycles (denaturation: 95 °C/5 min; cycle: 95 °C/15 s, 58–62 °C/20 s, and 72 °C/25 s) protocol using the corresponding specific primer sequences. Rotor-Gene Q-PCR Thermocycler (Qiagene) was used for RT-PCR analysis. Detection of genotype of VDR rs2107301 was performed by capillary qRT-PCR. DNA samples were amplified using a commercial LightCycler FastStart DNA Master HybProbe kit and LC Green plus dye, 70 cycles (denaturation: 95 °C/5 min; cycle: 95 °C/10 s, 60 °C/15 s, and 72 °C/15 s; melting: 95 °C/30 s, 40 °C/2 min, and 85 °C/1 s) protocol using the corresponding specific primer sequences. Roche LightCycler II Capillary Real Time PCR System was used for capillary RT-PCR analysis.Due to the biological variability of the biological material samples, the analysed samples were measured in triplicate for each gene of interest. Changes in mRNA expression for the genes of interest HIF-1α were evaluated by comparative quantification and Ct values using the Q Rotor Gene Software, respectively, GraphPad Prism. Changes in DNA gene polymorphism (SNP genotyping) of VDR rs2107301 were evaluated by LightCycler Software 4.0, respectively, GraphPad Prism.

2.4. Fluorescence analysis of biological material

The blood plasma sample was diluted 50-fold with 0.2 M Na_2HPO_4/KH_2PO_4 buffer (pH = 7.4) at room temperature and then analysed using the Horiba Dual FL spectrophotometer. The fluorescence value was read at the emission maximum (460 nm) of the fluorescence spectrum excited at λ_{ex} = 340 nm. Fluorescence was recorded in a 1 cm quartz cuvette, slit 5/5.

2.5. Statistical analysis

Relative quantification measures the relative change in mRNA expression. It determines the changes in calibrator mRNA levels of a gene (housekeeping gene) across multiple samples and expresses it relative to the levels of analysed mRNA levels (gene of interest). Calculation and comparison of relative quantification (ΔCt) is a result of Ct analysis for an analysed gene and a housekeeping gene.

The obtained data were analysed with GraphPad Prism version 5.04 (GraphPad Software), respectively, with the Q Rotor Gene Software (Qiagen) and LightCycler Software 4.0 (Roche). The presented data are mean ± standard error of mean (SEM) in 3 independent experimental repeats. The two-tailed Student's t-test was used to compare the data obtained in the two groups. When comparing values among multiple groups, the one-way ANOVA statistical method was used to evaluate statistical significance, more specifically Bartlett's test and Tukey's Multiple Comparison test. Statistically significant results were found to have a p-value below 0.05.

3. Results

The level of free NADH, transcriptional activity of the HIF-1α gene and SNP of VDR rs2107301 in blood, resp. Blood plasma samples of patients with histopathologically confirmed malignant melanoma were monitored. Demographic and clinical characteristics of the examined patients is shown in Table 2. Vitamin D levels in blood plasma as one of the commonly used parameters, were studied in all patient samples over the period of year. The Clinical stage, pT-stage and average level of vitamin D of examined patients is shown in Fig. 1-A, B and C.

3.1. Level of HIF-1α expression in blood

HIF-1α gene transcription activity was determined in MM patients with various stages of Clinical Stage I – IV melanoma (IABC – IVABC) and pT-stage melanoma 1–4 (1 ab – 4 ab). The monitored expression of mRNA of HIF-1α isoform increased with increasing stage of MM (Fig. 2-A, C). The correlation of HIF-1α expression between the control group (fold change = 0.0954) and the MM group (fold change = 0.622) was at $p < 0.0001$ as well as differences in the expression at respective disease stages are highly significant ($p < 0.0001$).

Table 1
Gene sequences.

gene	forward (sense) sequence	reverse (antisense) sequence
GAPDH	TGGGGCCAAAAGCATCATCTC	GCCGCCTGCTTCACCACCTTCTT
HIF-1α	CGTTCCTTCGATCAGTTGTC	TCAGTGGTGGCAGTGGTAGT
VDR rs2107301	AGAAGGCTCCGATGACCCC	AAGGTAAAAGACTGGTTGGAGCG
VDR rs2107301	**probe** – CCGCTCTCCATGCTGCCCCACC	

Table 2
Demographic and clinical characteristics of the examined patients (vitamin D levels in ng/mL; NM – nodular melanoma; SSM – superficial spreading melanoma; LM – lentigo maligna melanoma; C I – Clark stage I; C II – Clark stage II; C III – Clark stage III; C IV – Clark stage IV, C V – Clark stage V; pT-stage -; N0 – no melanoma cells in the nearby lymph nodes; N1 – melanoma cells in one lymph node or there are in-transit, satellite or microsatellite metastases; N2 – melanoma cells in 2 or 3 lymph nodes or there are melanoma cells in one lymph node and there are also in-transit, satellite or microsatellite metastases; N3 – melanoma cells in 4 or more lymph nodes or there are melanoma cells in 2 or 3 lymph nodes and there are in-transit, satellite or microsatellite metastases or there are melanoma cells in any number of lymph nodes and they have stuck to each other; NX – regional nodes not assessed; M0 – cancer has not spread to another part of the body; M1 – cancer has spread to another part of the body).

vitamin D	*winter-spring*						*summer-autumn*		
women patients	13.03						19.08		
men patients	15.16						18.11		
control	32.09						34.04		
types of MM	***NM***		***SSM***				***LM***		***other***
	51%		38%				5%		6%
vitamin D	13.14		19.16				10.23		15.02
Clark Stage	***I***		***II***	***III***		***IV***			***V***
	4%		20%	39%		35%			2%
vitamin D	17.96		20.17	18.33		15.43			15.36
Clinical Stage	***0***		***IABC***	***IIABC***		***IIIABC***			***IVABC***
	4%		26%	18%		44%			8%
vitamin D	18.08		16.38	14.91		15.24			24.20
pT-stage	***pT1a***	***pT2a***	***pT2b***		***pT3a***	***pT3b***		***pT4a***	***pT4b***
	27%	12%	4%		12%	9%		10%	26%
vitamin D	17.00	11.95	19.97		15.02	15.31		16.36	17.66
N-stage	***N0***		***N1***	***N2***		***N3***			***NX***
	70%		12%	6%		8%			4%
vitamin D	16.42		13.3	7.85		20.43			17.29
M-stage	***M0***						***M1***		
	94%						6%		
vitamin D	15.95						15.01		

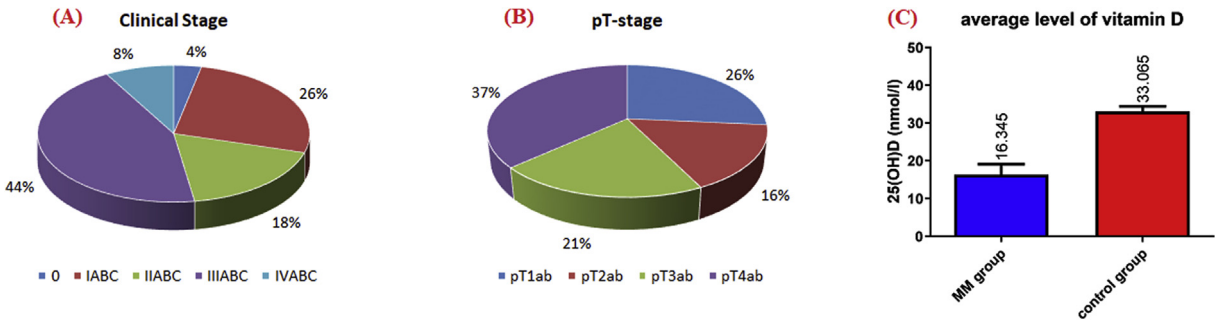

Fig. 1. A: Demographic description of patient group by Clinical Stage (study group consists of 4% of patients with clinical stage 0, 26% in clinical stage IABC, 18% in stage IIABC, 44% in clinical stage IIIABC, and 8% in clinical stage IVABC); B: Demographic description of patient group by TMN classification, pT-stage respectively (pT1ab had 26% of patents, pT2ab had 16% of patients, pT3ab had 21% of patients, and pT4ab had 37% of patients); C: The level of vitamin D in studied group measured in spring was 16.345 μg/mL in patient group and 33.065 μg/mL in control group (controls supplemented with vitamin D).

3.2. Analysis of VDR rs2107301 gene polymorphism in blood

The observed group of patients with MM was predominantly represented by the homozygous GG group in 53%, followed by the heterozygous AG group in 40% of cases and the least homozygous AA group in 7% of patients with MM. All controls were GG homozygous.

When patients with malignant melanoma were categorised according to the SNP of the VDR rs2107301, we found that HIF-1α expression increased approximately two times in the group of AA homozygotes as compared to GG homozygotes or AG heterozygotes (Fig. 3-C).

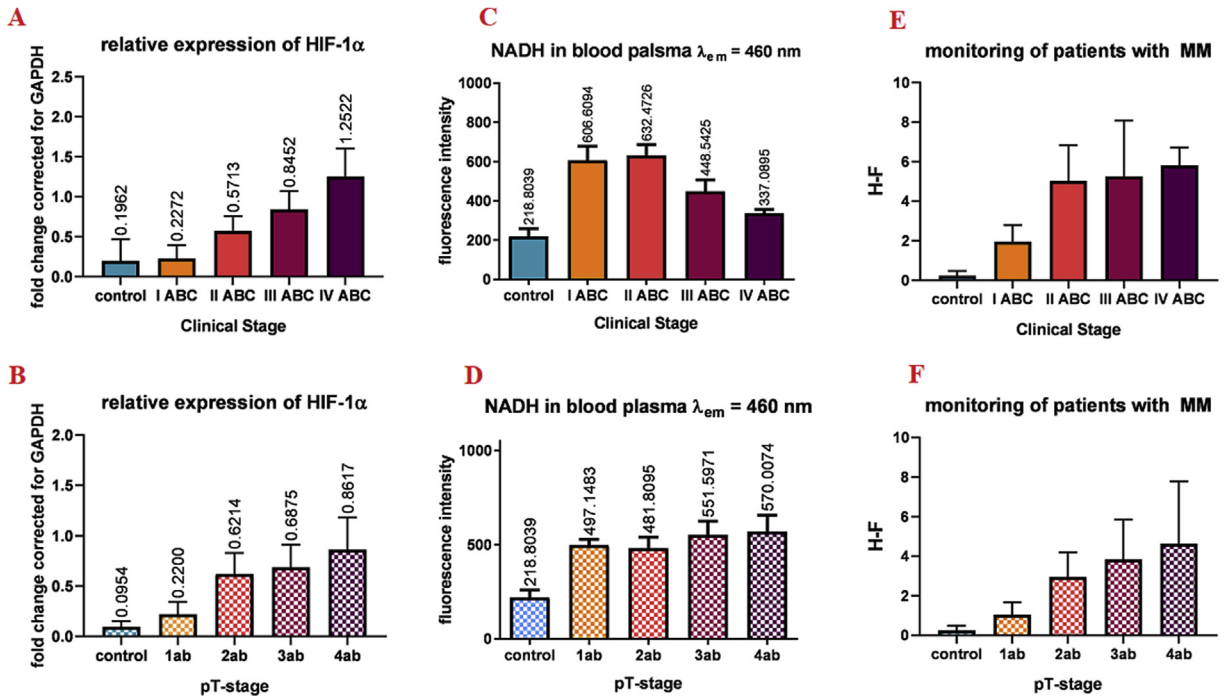

Fig. 2. Relative expression of HIF-1α by A: Clinical Stage; B: pT-stage, fluorescence intensity of NADH; C: Clinical Stage; D: pT-stage; H–F Parameter E: Clinical Stage; F: pT-stage.

3.3. Detection of NADH level in blood plasma of MM patients

Both NADH and NADPH display almost identical fluorescent behaviour in blood plasma; therefore, the characteristic fluorescence signal in emission spectra ($\lambda_{ex} = 340$ nm) found at 460 nm reflects the concentration of both cofactors. While the level of free NADPH is significantly lower and even further reduced in MM [19] in our study we have attributed the emission intensity only to the NADH [20, 21].

By comparing the average fluorescence values (F) in the controls and MM patients at different stages of melanoma, significant differences were found in NADH levels at the level $p < 0.01$.

Compared to the control group ($F_{CON} = 269$), the NADH level was approximately twice as high in patients with MM ($F_{MM} = 532$). We noted a significant, almost 2-fold increase in free NADH in the clinical stages of 1ABC and 2ABC, followed by a gradual decrease in blood NADH levels with increasing 3ABC and 4ABC clinical stages (Fig. 2-B). We have found a significant, almost 2-fold increase in free NADH in the primary tumour (pT) stages pT1ab and pT2ab, and almost 2.5-fold increase in free NADH in pT3ab and pT4ab (Fig. 2-D). pT-stages are part of TNM classification based on of evidence of tumour thickness (Breslow), mitoses, and ulceration [22].

4. Discussion

During the course of MM development, vitamin D cannot be hydroxylated and thus activated because the vitamin D receptors in the

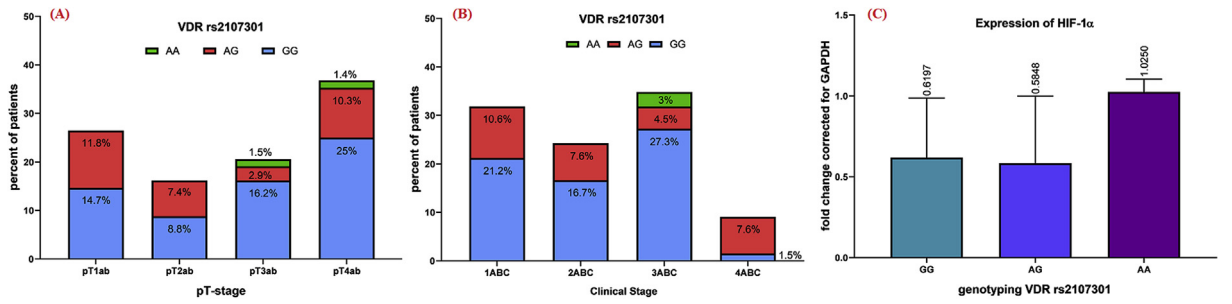

Fig. 3. Levels of VDR rs2107301 gene polymorphism by A: pT-stage; B: Clinical Stage; C: relative expression of HIF-1α linked with VDR rs2107301 polymorphism.

mitochondria are inhibited by malfunction of respiratory complexes or by detached OXPHOS. Therefore, vitamin D is a marker routinely used for monitoring of most skin diseases including MM [23,24]. Screening of vitamin D levels in blood plasma within our study revealed presence of hypovitaminosis in all patients, although no correlation between the stage of MM and vitamin D levels was found.

The obtained data of HIF-1α expression correspond to the previously published results of other research groups [11,25–27] which indicate that the overexpression of HIF-1α which promotes the survival of cells under adverse conditions often results in formation of tumour microenvironment. MM cells are able to produce active vitamin D which is a function of VDR presented in the cells and ability of VDR convert the non-active vitamin D into active vitamin D which is tightly connected with the type and stage of the disease [28]. The VDR polymorphism rs2107301 is characterised by a C < T change; which is in reverse PCR shown as a G < A change. A 9.1% homozygote AA was observed for Europeans as described in a study to detect the incidence of VDR rs2107301 polymormism in Europe, East and South Asia, Africa and America [29], approximately equal to the observations in the study group of patients. Patients studied in the homozygous AA group were in the 3ABC clinical stage or in the pT3ab and pT4ab groups (Fig. 3-A, B), respectively. Similar results were observed by the other research teams [30–32]. Patients with the GG genotype and patients with heterozygous AG mutation (AG mutation leads to an increased risk of MM) occurred in all analysed groups. The genotypic representation of VDR rs2107301 in the healthy control group was in all cases GG (GG genotype represent physiological VDR mutations unaffected by malignancy [33]). The change of HIF-1α in the group of AA homozygotes as compared to GG homozygotes or AG heterozygotes was not significant, but suggests that patients with AA homozygous polymorphism have a higher HIF-1α gene expression and therefore a higher cancer risk or stage of cancer.

The NADH molecule, which originates in the process of glycolysis, is the source of energy gains in tumour cells (the Warburg effect) and is the most prominent metabolite showing changes in the tumour cells. A change in respiratory efficiency in the pseudohypoxic tumour microenvironment affects the change in substrate preference from glucose to glutamine. Reduced utilization of NADH in the mitochondrial respiratory chain leads to reductive carboxylation of glutamine [34–36]. The reaction sequence results in production of citrate, from which acetyl-CoA and oxaloacetate are formed in the cytosol by reaction with CoA. Acetyl-CoA is involved in the synthesis of fatty acids, which are essential for the synthesis of proliferating cell membranes. On the other hand, oxaloacetate is a substrate for gluconeogenesis which leads to consumption of NADH [37,38]. Additionally, oxaloacetate can be converted via malate to pyruvate to generate NADPH, which is a “reducing energy” for the fatty acid synthesis [39].

The obtained results indicate that while the gradual increase in HIF-1α expression is linked with the increase of NADH in the early clinical stages, a decrease of NADH concentration is observed in the higher stages of MM. This paradox might be explained by a complete change in tumour-cell metabolism during hypoxia or mimic hypoxia in the course of disease progression [40]. It is worth noting, that a decrease in the NADPH level due to extensive fatty acid synthesis in proliferating cells associated with a decrease in its contribution to the fluorescence intensity might also contribute to the reduced signal found in clinical stage III and IV [19]. However, when the patients were classified according to the pT-stages a slight increase in NADH level was observed between the patient groups 1 ab – 4 ab. This finding suggests that the selected classification plays an important role in monitoring of NADH levels and possibly also other endogenous metabolic parameters during the MM progression.

In attempt to find a novel marker for screening of MM we have designed a H–F parameter, calculated as a product of HIF-1α expression (H) and NADH fluorescence intensity value (F). As can be seen in Fig. 2 (E, F) the H–F value correlates with both clinical stage and pT-stage classification and therefore appears to be useful for monitoring of MM progression.

5. Conclusions

Investigation of SNP has shown a significant increase in the VDR rs2107301 gene polymorphism in AA homozygotes which could be responsible for low level of activated vitamin D. However, in the vast majority of the analysed samples we detected the GG homozygotes and AG heterozygotes of VDR rs2107301 which exhibit vitamin D deficiency as well Thus, according to our study the low vitamin D levels are not directly related to the SNP of VDR rs2107301 gene.

Our further study of HIF-1α expression in patients has revealed that a significant increase of expression correlates with the stage of MM progression. Moreover, the blood plasma levels of NADH detected by fluorescence measurements in similar manner reflects the disease progression. Outcomes of our investigation has resulted in development of H–F parameter which encompasses both HIF-1α expression and blood plasma levels of NADH. Unlike these individual parameters the values of H–F show a correlation with clinical stage as well as newer pT-stage classification, therefore it seems a suitable candidate for monitoring of MM.

Even though a more thorough study of the H–F parameter on a larger patient group is needed in order to establish it as a marker for clinical praxis, we believe that our preliminary results are promising for the field of MM diagnosis and treatment.

All authors whose names appear on the submission

1) made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work;

2) drafted the work or revised it critically for important intellectual content;

3) approved the version to be published; and.

4) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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On behalf of all contributing authors.

Dr. Ivana Špaková, PhD.

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Declaration of competing interest

There is no conflict of interest of any of the authors with the results of this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2020.e00182.

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