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Analysis of Malignant Melanoma Cell Lines Exposed to Hypoxia Reveals the Importance of *PFKFB4* Overexpression for Disease Progression

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Abstract. *Background/Aim: Most melanomas develop in hypoxic conditions. Since hypoxia via HIF-1 induces glycolysis, a process essential for malignant melanoma growth/survival, the goal of this study was to analyze the influence of hypoxia on the expression of HIF-1 target genes involved in glucose metabolism. Materials and Methods: The response of melanoma cell lines to hypoxic conditions was analyzed by RT-PCR and western blotting. A Kaplan–Meier survival analysis for patients with high and low expression level of PFKFB4 was performed. Further analysis of patients' data was performed using the R/Bioconductor environment. Results: Induction of PFKFB4 gene expression can be considered a crucial mechanism behind glycolysis enhancement in hypoxic melanoma cells. Analysis of a publicly available database revealed that high PFKFB4 expression contributes to poor prognosis of melanoma patients. Conclusion: Currently available anti-melanoma therapeutic strategies may significantly benefit from agents targeting PFKFB4 activity.*

The incidence of melanoma is relatively low, representing less than 5% of all skin cancers, nevertheless, its frequency has been increasing over the past decades. Moreover, advanced-stage melanoma is highly aggressive and invasive malignancy constitutes around 70% of skin cancer-related deaths (1-4).

Melanoma arises from skin pigment cells (melanocytes) located in the basal layer of epidermis that are responsible for melanin production. One of the major risk factors for melanoma is chronic sun exposure of the skin, leading to UV- induced melanocyte mutations, especially within *NRAS, BRAF* and *cKIT* protooncogenes (5). On the other hand, a considerable group of melanoma patients reports a family history of disease, pointing out the hereditary background of this highly lethal cancer, characterized predominantly by *BRAF* mutations (5, 6). In general, mutations in the genes involved in proliferation, apoptosis, metabolism and cell cycle constitute the main cause of malignant melanoma transformations (5).

Although the genetic alterations in melanocytic DNA are crucial for melanoma development, emerging data highlight the significant role of the skin microenvironment in melanoma initiation and progression. Skin microenvironment is a structural and functional constellation composed of normal skin cells such as keratinocytes, fibroblasts, endothelial cells, melanocytes and cells of the immune system subjected to mutual interactions. Disruption of this homeostasis may promote the development of melanoma (7). Importantly, the inherent part of skin microenvironment is low oxygen partial pressure, deepening during cancer progression and promoting its development (8-11).

Notably, literature data indicate that 50-60% of locally advanced tumors, including melanomas are characterized by areas of hypoxia or even anoxia in which oxygen concentration ranges from 0.5 to 1.5% O_2 (8, 9, 12). In melanoma, hypoxia was observed to accelerate malignant transformation (13-16) and tumor development/progression (12, 16-19). Of note, oxygen-deprived environment also contributes to treatment resistance (20-25) and phenotype switching of melanoma cells (26, 27). All the alterations observed in hypoxic cells result from hypoxia-mediated gene expression changes, initiated mainly by the hypoxia-inducible factor-1 (HIF-1) transcription factor (stabilized in hypoxic conditions, below 2% O_2) (28). Among the HIF-1 target genes are those coding for glycolytic enzymes and glucose transporters, that allow for sufficient ATP

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production in oxygen-deprived environment. Notably, enhanced glycolysis has been recently reported as a pathway associated with resistance to adoptive T cell therapy in melanoma mouse model (29). Moreover, it has been shown that the attenuation of glycolysis is crucial in the response to BRAF inhibitors (BRAF-i) as by in BRAF-i resistant melanomas a decrease of glucose breakdown induces cell death (30, 31). As most melanomas reside in a hypoxic environment, the glycolytic pathway seems to be constantly induced. In this paper, using melanoma cell lines as a model, the influence of hypoxia on the expression of glycolysis related genes was analyzed. Importantly, our study revealed that the *PFKFB4* gene product, 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 4 (PFKFB4), is the crucial enzyme enhancing the glycolysis in hypoxic melanomas, the overexpression of which is associated with the poor prognosis of melanoma patients.

Materials and Methods

Cell lines and cell culture. Two human melanoma cell lines representing different stages of tumor progression were studied: WM115 from vertical growth phase and its metastatic derivative WM266-4 line. Both cell lines were obtained from ESTDAB Melanoma Cell Bank (Tubingen, Germany) and were grown according to their recommendation. Reduced oxygen culture conditions (1% O_2 hypoxia) were obtained, as described previously (32).

RNA isolation and cDNA synthesis. Total amount of RNA was extracted from melanoma cells using RNeasy Plus Mini kit (Qiagen, Hilden, Germany). The concentration and purity of all isolated RNA samples were determined with the use of NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For each sample 125 ng of RNA was used for reverse transcription. cDNA synthesis was carried out using NG dART RT kit (EURx, Gdansk, Poland) with oligo (dT)20 primer and dART reverse transcriptase, as described in the manufacturer's protocol (EURx).

Reverse transcription polymerase chain reaction (RT-PCR). PCR reaction was carried out using Color OptiTaq PCR Master Mix (2x) (EURx) according to the manufacturer's protocol. The PCR mixture contained 0.6 μl of each 10 μM primers (forward and reverse), 5 μl of Color OptiTaq PCR Master Mix (2x) and 5 μl of nuclease free deionized water. 1 μL of cDNA was added to each PCR mixture. The cDNA was amplified using the MJ Research PTC-200 Thermal Cycler. PCR reaction started with initial denaturation for 5 min at 95˚C. Then the PCR conditions for 26 cycles were as followed: 30 sec at 95˚C, 30 sec at 65˚C (*PFKFB3*) or 58˚C (all other genes) and 30 sec at 72˚C. The extension reaction was for 10 min at 72˚C. The PCR products were analyzed on 1.5% w/v agarose gels. Bands were normalized using HPRT1 to correct for differences in loading of the cDNAs samples.

Western Blot. Western-blot analysis was performed as described previously (32).

Patients and statistical analysis. The gene expression and survival data of 214 melanoma patients (GSE65904) were downloaded from the NCBI Gene Expression Omnibus (GEO), a public repository of microarray data (33). The data were analyzed in the R environment (34). A number of libraries as GEOquery (35), Affy (36), Limma (37) were used to analyze gene expression. Based on the expression level of *PFKFB4* gene, the patients were divided in three groups G1, G2 and G3 using R/Bioconductor environment and segmented library (38) for fitting the regression models in case of broken-line relationships. Using limma, an R/Biocondictor software package significantly up-regulated genes in the G3 group (with the highest PFKFB4 expression) in comparison to the G1 group (with the lowest PFKFB4 expression) were found. All the selected genes were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID), and the Kyoto Encyclopedia of Genes and Genomes (KEGG). The survival curves were estimated by the Kaplan–Meier method and compared by Log-rank test in GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA, USA).

Results

The response of melanoma cells to low oxygen concentrations at the protein level. In the first stage, to validate our experimental setup, the influence of hypoxic stimulation on two melanoma cell lines, WM115 and WM266-4, was investigated. Hypoxia induced accumulation of HIF-1 alpha subunit protein (Figure 1A). In order to determine whether HIF-1 accumulation triggers a transcriptional response, RT-PCR assay was performed. The gene selected for this analysis, Carbonic Anhydrase IX (CAIX), has been shown to be a HIF-1 target (39, 40) and its HIF-1-dependent regulation has been reported in malignant melanoma (12). Interestingly, according to the literature, expression of CAIX significantly contributes to progression of melanoma as CAIX seems to be crucial in adaptation of melanoma cells to extracellular acidosis (41, 42), accompanying hypoxia. Expression of *CAIX* in normoxic conditions was negligible in both analyzed cell lines (Figure 1B). However, hypoxia induced expression of *CAIX* in WM115 and WM266-4 cells (Figure 1B).

Effect of low oxygen conditions on glycolytic gene expression in melanoma cell lines. Since glycolysis is the essential energy-yielding process accelerated in low oxygen environment and glycolysis enhancement was found to be associated with worse clinical outcome (43), semi-quantitative RT-PCR was used to examine the effect of reduced oxygen culture conditions on the expression of HIF-1 target genes involved in glucose breakdown in melanoma cells. The panel of selected genes comprised of *HK2* (Hexokinase 2), *PFKFB3* (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3), *PFKFB4* (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 4), *ALDO A* (Aldolase A), *ENO1* (Enolase 1), *PKM* (Pyruvate kinase, muscle), *LDH A* (Lactate Dehydrogenase A) and *SLC2A1* (Solute carrier family 2, facilitated glucose transporter member 1). All the genes were previously shown to be HIF-1 target genes (44) containing HRE (Hypoxia Response Elements) elements in their promoters (39, 45-52). As shown in Figure 2, in both cell lines the majority of analyzed genes had a high

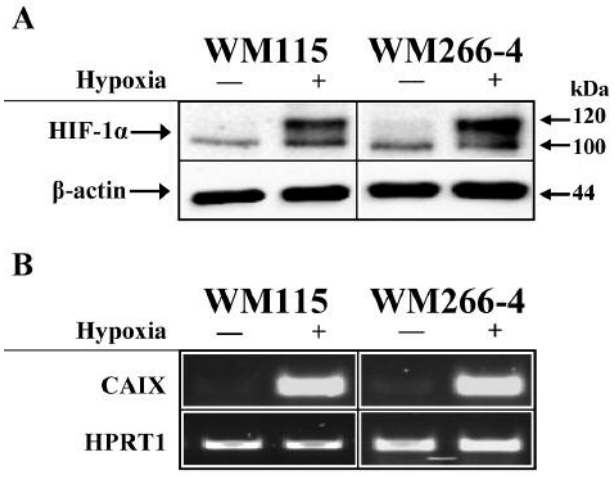

Figure 1. *Response of WM115 and WM2264 melanoma cell lines to hypoxic conditions A) HIF-1 alpha accumulation in WM115 and WM266-4 melanoma cells. WM115 and WM266-4 cells were cultured for 24 h in normoxic or hypoxic (24 h, 1% O_2) conditions. HIF-1 alpha accumulation was detected using western blot. β-actin is shown as an internal control for equal protein loading. B) Expression of HIF-1 target gene, CAIX, in WM115 and WM2264 cell lines. WM115 and WM266-4 cells were cultured for 24 h in normoxic or hypoxic (24 h, 1% O_2) conditions. CAIX expression was detected by RT-PCR. HPRT1 is shown as an internal control for equal amount of cDNA.*

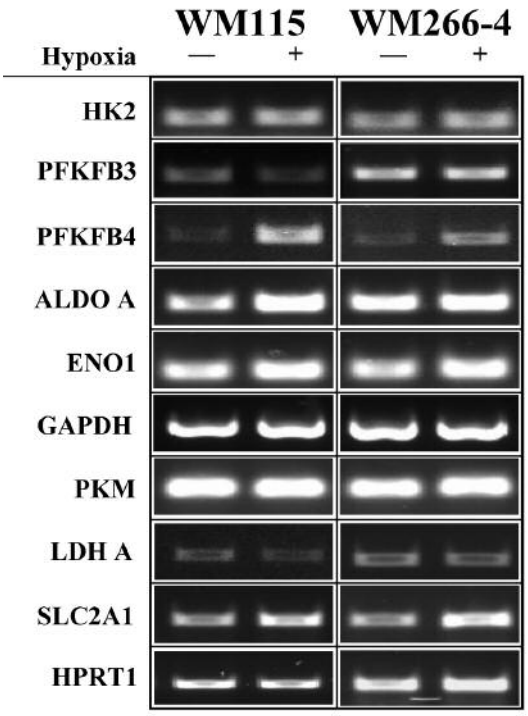

Figure 2. *Expression of HIF-1 glycolytic target genes under reduced oxygen conditions in WM115 and WM2264 melanoma cell lines. The expression of HIF-1 glycolytic target genes HK2, PFKFB3, PFKFB4, ALDOA, ENO1, PDK1, PKM, LDHA, SLC2A1 in cells cultured for 24 h in normoxic or hypoxic (24 h, 1% O_2) conditions was determined by RT-PCR. HPRT1 is shown as an internal control for equal amount of cDNA.*

expression in normoxic conditions indicating that HIF-1 is not the crucial transcription factor controlling their expression. The only gene, with low expression in normoxic conditions and clear induction in hypoxia was *PFKFB4*, the one coding the cancer specific isoenzyme of phosphofructokinase II (PFK-II). In conclusion, these results indicate that, in contrast to the majority of studied genes encoding glycolytic enzymes, HIF-1 induced strong expression of *PFKFB4*.

PFKFB4 expression in melanoma cell lines and patients. The observation that the *PFKFB4* gene can be crucial for the enhancement of glycolytic pathway in melanoma cells under hypoxic conditions, prompted us to investigate its basal expression in a panel of melanoma cell lines. As shown in Figure 3, in WM793, 1205Lu, A375P, WM239A WM115 and WM266-4 cell lines the expression of *PFKFB4* was comparable under normoxic conditions., However, in WM35 and WM3211the expression *PFKFB4* was as high as in WM266-4 hypoxia treated cells. To determine if high expression of PFKFB4 is the consequence activation of HIF-1 pathway in normoxic conditions, in the same panel of cell lines, the expression of CAIX, the hallmark of active HIF-1 signaling in melanoma cells, was analyzed. Based on CAIX expression (Figure 3), which seems to be controlled exclusively by HIF-1 transcription factor in melanoma cell lines, it can be

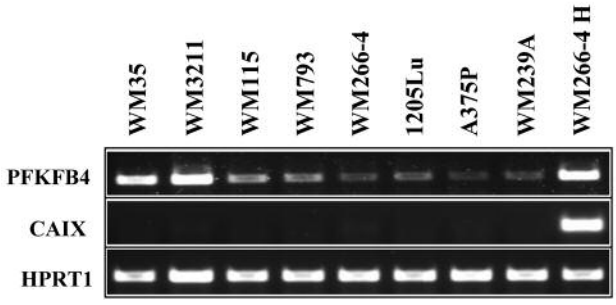

Figure 3. *Basal expression of PFKFB4 and CAIX in the panel of human melanoma cell lines determined by RT-PCR. The expression PFKFB4 and CAIX was determined by RT-PCR. cDNA isolated from hypoxic WM266-4 (WM266-4H) was used to compare the basal normoxic expression of PFKFB4 and CAIX in melanoma cell lines with the expression level observed in hypoxic environment. HPRT1 is shown as an internal control for equal amount of cDNA.*

concluded that there is a lack of HIF-1 activity in WM35 and WM3211. Thus, the high expression of PFKFB4 in WM35 and WM3211 is not a consequence of hypoxia-

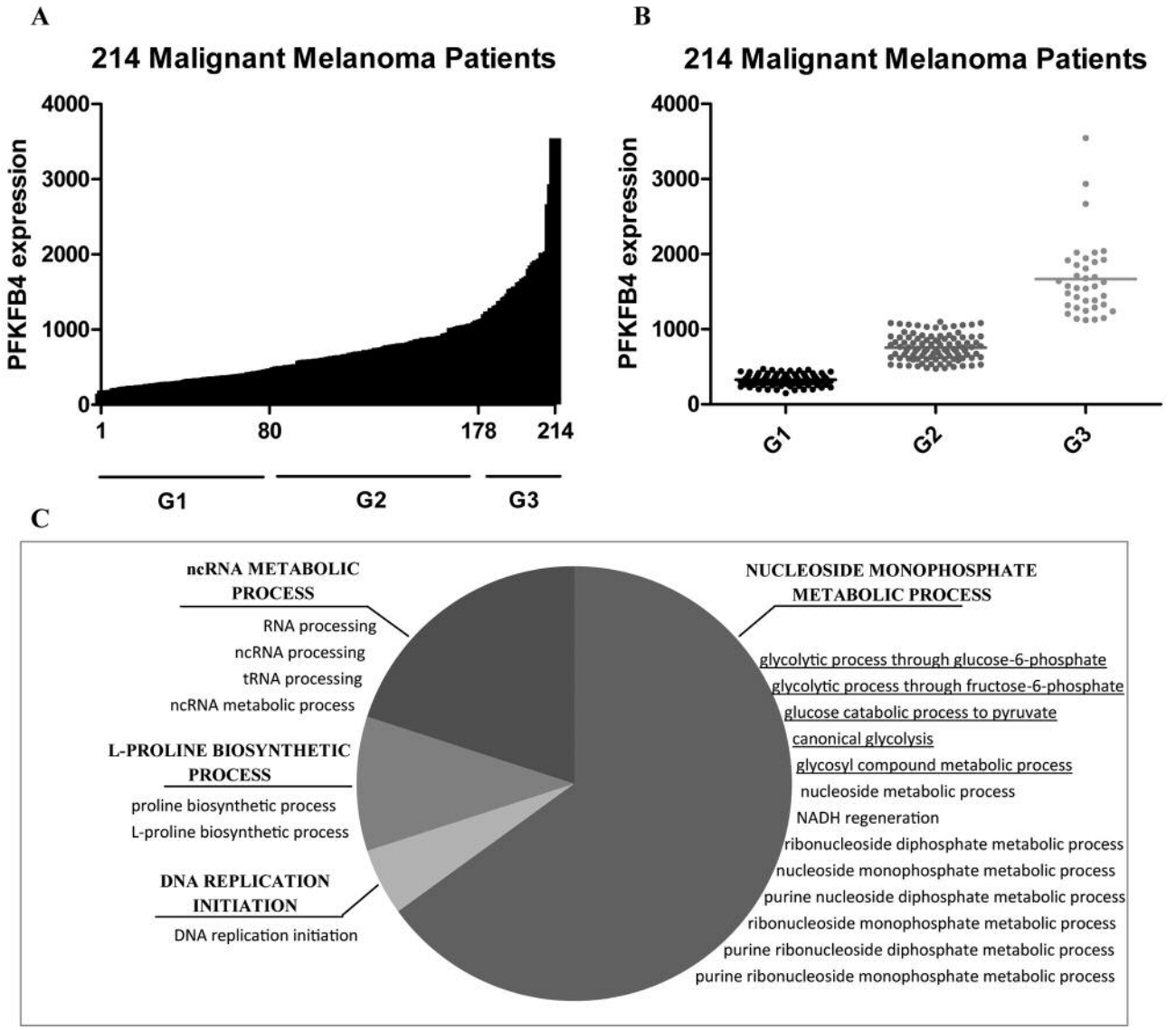

Figure 4. *Analysis of PFKFB4 mRNA expression in malignant melanoma patients using a publicly available data set GSE65904. A) 214 malignant melanoma patients were classified in accordance to PFKFB4 mRNA expression. The patients were divided into three expression groups (G1, G2, G3) using the segmented regression available in R package (34). B) The PFKFB4 mRNA expression in G1, G2 and G3 groups; for subsequent gene expression comparison the three patients with the highest PFKFB4 were excluded from the analysis. C) Gene ontology processes with significant over-representation among the genes overexpressed (fold change ≥2) in G3 in comparison to G1. Only the pathways having significant alterations ($p<0.05$) are presented. The glycolysis related processes are underlined.*

independent HIF-1 signaling in normoxic conditions, indicating that PFKFB4 can be expressed in melanoma cells also regardless of the presence of HIF-1 transcription factor. Next, the PFKFB4 mRNA expression was examined in melanoma patients using publicly available data set, GSE65904. As presented in Figure 4A, among malignant melanoma patients there was a group with high expression of PFKFB4 gene. Next, melanoma patients were divided, using segmented regression, in three groups (G1, G2, G3) based on the expression level of *PFKFB4* (Figure 4A and B) and subsequently significantly up-regulated genes in the G3 group (with the highest *PFKFB4* expression), in comparison to G1 group (with the lowest *PFKFB4* expression), were identified. Annotation analysis performed on up-regulated genes with DAVID and KEGG showed that a significant number of up-regulated genes was involved in

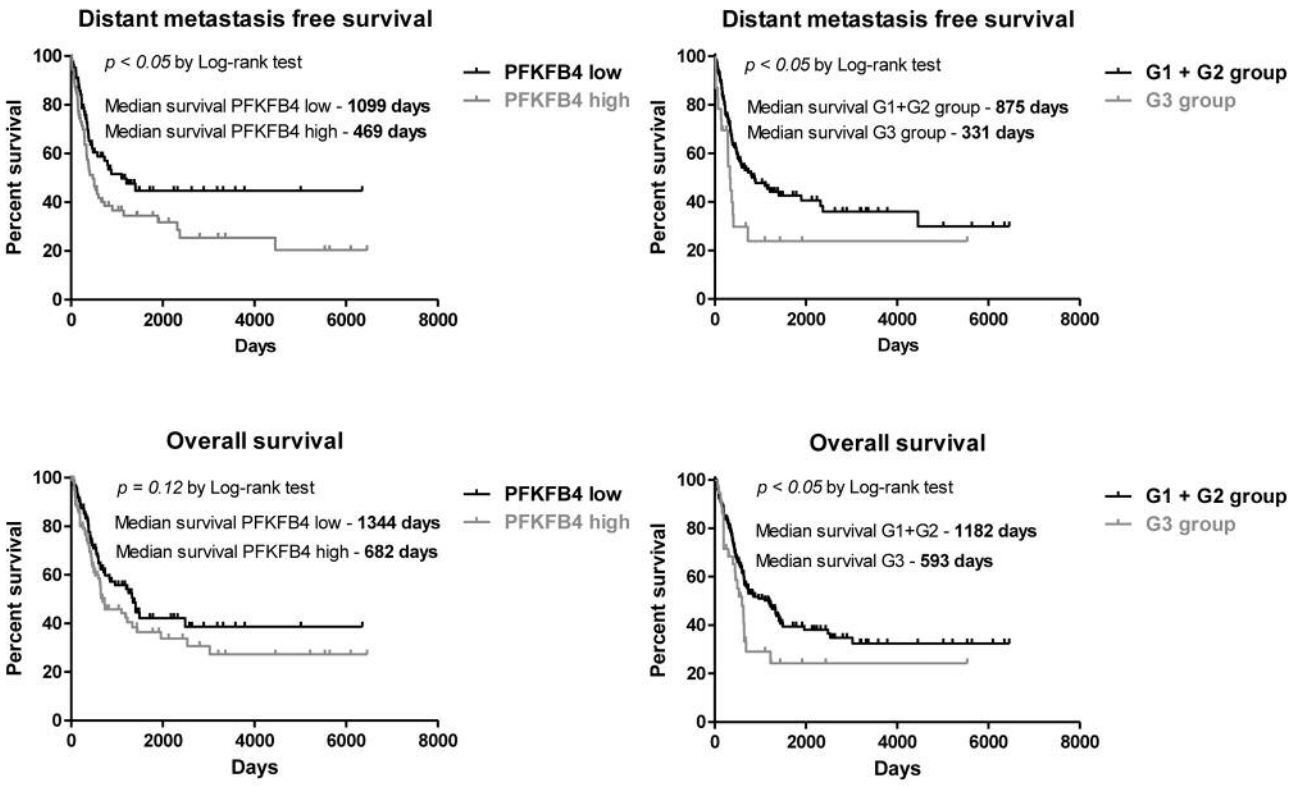

Figure 5. *High PFKFB4 expression is associated with poor prognosis of melanoma patients. The publicly available micro-array data set GSE65904 was used to correlate PFKFB4 mRNA expression (median lowest, median highest, and G3 versus G1+G2) to disease outcome. Kaplan–Meier curves are presented for distant metastasis free survival (upper panel) and overall survival (lower panel). The median survival for the compared groups is given in the graphs. High PFKFB4 expression is associated with worse distant metastasis free survival and overall survival, p<0.05, by Log-rank test.*

glucose breakdown (Figure 4C). These data clearly indicate that the group of patients with the highest PFKFB4 expression can be considered as the one with high rate of glucose breakdown. Taking into consideration the importance of glycolysis in the progression of melanoma, the melanoma patients were divided in groups based on the PFKFB4 expression (median lowest, median highest, and G3 *versus* G1+G2) and comparison of distant metastasis free and overall survival between groups was performed. As shown in Figure 5 (upper panel), the group with high *PFKFB4* expression selected based on the median and PFKFB4 high group, identified by segmented regression (G3 group), showed significantly worse distant metastasis free survival, clearly indicating that cancer specific isoenzyme of phosphofructokinase II, PFKFB4, can have an important impact on the progression of malignant melanoma. As for the overall survival (Figure 5, lower panel), in the PFKFB4 high group identified based on the median expression the trend for the worse overall survival was clearly visible, whereas the G3 group had significantly worse OS survival in comparison to G1+G2 group.

Discussion

In this study we focused on the influence of hypoxia on the expression of genes involved in glucose metabolism in melanoma cells. Hypoxia occurs/appears when oxygen supply fails to meet demands of the body tissue and is a common phenomenon in advanced solid tumors (53) including most melanomas. Tumor hypoxia and HIFs affect most of the cancer hallmarks including: cell survival, vascularization, metabolism reprogramming, immune response, invasion, treatment resistance, metastasis, apoptosis and genomic instability (54-56). Our study revealed that many of analyzed HIF-1 target genes, which code for proteins involved in glucose metabolism had high basal normoxic expression indicating high glycolytic profile of melanoma cells at normoxic conditions. On the other hand, expression of certain genes involved in glucose metabolism was not apparently induced in hypoxic conditions in the studied cell lines. Interestingly, however, in both studied cell lines, very strong induction of *PFKFB4* gene, which codes for cancer specific isoenzyme modulating glycolysis – phosphofructokinase II

(PFK-II)/fructose-2,6-bisphosphatase (FBPase-II), was observed. The product of its kinase domain, Fru-2,6-P_2, constitutes an essential activator of the glycolytic flux, activating glucose breakdown through allosteric modulation of the rate-limiting enzyme of glycolysis, phosphofructokinase I (PFK-I) (57, 58). As mentioned above, the protein coded by *PFKFB4* gene is not a *bona fide* glycolytic enzyme, but its activity is associated with carcinogenesis and significantly enhances the rate of glucose breakdown. The fact that its basal normoxic expression is rather low (at least in most studied melanoma cell lines) suggests that HIF-1 mediated PFKFB4 induction can be considered as a universal mechanism for enhancement of hypoxia-mediated glucose breakdown in malignant melanoma. Although our data stays in line with studies conducted by Buart *et al*. (59), who indicated *PFKFB4* gene as the one belonging to hypoxic signature in melanoma, our cell line analysis pointed that its expression is not restricted to hypoxic environment. The fact that there are melanoma cell lines with high PFKFB4 expression in normoxic conditions irrespective of active HIF-1 signaling suggests that in melanoma patients high basal PFKFB4 expression cannot be considered a direct hallmark of hypoxia. Important for deciphering the role of PFKFB4 in melanoma behavior/biology were the findings that came from the study of publicly available data set, revealing that its high expression significantly correlates with shorter overall survival of malignant melanoma patients.

Taking into consideration that melanoma cells mostly reside in hypoxic conditions and high glycolytic rate is involved in the resistance to BRAF inhibitors and adoptive T cell therapy (29), *PFKFB4* seems to be an important gene, the function/significance of which should be subjected to further detailed study, especially in the context of anti-melanoma treatment.

According to our knowledge, this is the first study showing the impact of *PFKFB4* overexpression in the progression of malignant melanoma. In addition, we found that in patients with high *PFKFB4* gene expression there was an up-regulation of genes involved in glucose metabolism. Altogether our data indicate that phosphofructokinase II isoenzyme 4, coded by *PFKFB4* gene, may become a novel target in anti-melanoma therapeutic strategies and inhibition of its activity may significantly improve the outcome of currently applied therapies against malignant melanoma.

Conflicts of Interest

The Authors have no conflicts of interest to disclose.

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References

1 Rastrelli M, Tropea S, Rossi CR and Alaibac M: Melanoma: Epidemiology, risk factors, pathogenesis, diagnosis and classification. In Vivo *28(6)*: 1005-1011, 2014.

2 MacKie RM, Hauschild A and Eggermont AM: Epidemiology of invasive cutaneous melanoma. Ann Oncol *20(Suppl 6)*: vi1-7, 2009.

3 Matta AS: Melanoma and hypoxia melanoma is an aggressive skin tumor. J Pigment Disord *2*: 9, 2015.

4 Kosmidis C, Baka S, Sapalidis K, Mixalopoulos N, Atmatzidis S, Koulouris H, Anthimidis G, Varsamis N, Zarogoulidis P, Man Y, Georgakoudi E, Mantalovas S, Koimtzis G, Tsakalidis A and Kesisoglou I: Melanoma from molecular pathways to clinical treatment: An up to date review. J Biomed *2*: 94-100, 2017.

5 Shain AH and Bastian BC: From melanocytes to melanomas. Nat Rev Cancer *16(6)*: 345-358, 2016.

6 Soura E, Eliades PJ, Shannon K, Stratigos AJ and Tsao H: Hereditary melanoma: Update on syndromes and management: Genetics of familial atypical multiple mole melanoma syndrome. J Am Acad Dermatol *74(3)*: 395-407; quiz 408-310, 2016.

7 Olbryt M: Role of tumor microenvironment in the formation and progression of skin melanoma. Postepy Hig Med Dosw (Online) *67*: 413-432, 2013.

8 Vaupel P, Kallinowski F and Okunieff P: Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: A review. Cancer Res *49(23)*: 6449-6465, 1989.

9 Lartigau E, Randrianarivelo H, Avril MF, Margulis A, Spatz A, Eschwege F and Guichard M: Intratumoral oxygen tension in metastatic melanoma. Melanoma Res *7(5)*: 400-406, 1997.

10 Evans SM, Schrlau AE, Chalian AA, Zhang P and Koch CJ: Oxygen levels in normal and previously irradiated human skin as assessed by ef5 binding. J Invest Dermatol *126(12)*: 2596-2606, 2006.

11 Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C and Kieda C: Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. J Cell Mol Med *15(6)*: 1239-1253, 2011.

12 Bedogni B and Powell MB: Hypoxia, melanocytes and melanoma – survival and tumor development in the permissive microenvironment of the skin. Pigment Cell Melanoma Res *22(2)*: 166-174, 2009.

13 Bedogni B, Welford SM, Cassarino DS, Nickoloff BJ, Giaccia AJ and Powell MB: The hypoxic microenvironment of the skin contributes to akt-mediated melanocyte transformation. Cancer Cell *8(6)*: 443-454, 2005.

14 Monsel G, Ortonne N, Bagot M, Bensussan A and Dumaz N: C-kit mutants require hypoxia-inducible factor 1alpha to transform melanocytes. Oncogene *29(2)*: 227-236, 2010.

15 Hanna SC, Krishnan B, Bailey ST, Moschos SJ, Kuan PF, Shimamura T, Osborne LD, Siegel MB, Duncan LM, O'Brien ET, 3rd, Superfine R, Miller CR, Simon MC, Wong KK and Kim WY: Hif1alpha and hif2alpha independently activate src to promote melanoma metastases. J Clin Invest *123(5)*: 2078-2093, 2013.

16 Huber R, Meier B, Otsuka A, Fenini G, Satoh T, Gehrke S, Widmer D, Levesque MP, Mangana J, Kerl K, Gebhardt C, Fujii H, Nakashima C, Nonomura Y, Kabashima K, Dummer R, Contassot E and French LE: Tumour hypoxia promotes melanoma growth and metastasis *via* high mobility group box-1 and m2-like macrophages. Sci Rep *6*: 29914, 2016.

17 Homsi J, Cubitt CL, Zhang S, Munster PN, Yu H, Sullivan DM, Jove R, Messina JL and Daud AI: Src activation in melanoma and src inhibitors as therapeutic agents in melanoma. Melanoma Res *19(3)*: 167-175, 2009.
18 Asnaghi L, Lin MH, Lim KS, Lim KJ, Tripathy A, Wendeborn M, Merbs SL, Handa JT, Sodhi A, Bar EE and Eberhart CG: Hypoxia promotes uveal melanoma invasion through enhanced notch and mapk activation. PLoS One *9(8)*: e105372, 2014.
19 Craig EA, Weber JD and Spiegelman VS: Involvement of the mrna binding protein crd-bp in the regulation of metastatic melanoma cell proliferation and invasion by hypoxia. J Cell Sci *125(Pt 24)*: 5950-5954, 2012.
20 Lenggenhager D, Curioni-Fontecedro A, Storz M, Shakhova O, Sommer L, Widmer DS, Seifert B, Moch H, Dummer R and Mihic-Probst D: An aggressive hypoxia related subpopulation of melanoma cells is trp-2 negative. Transl Oncol *7(2)*: 206-212, 2014.
21 Warfel NA and El-Deiry WS: Hif-1 signaling in drug resistance to chemotherapy. Curr Med Chem *21(26)*: 3021-3028, 2014.
22 Elcheva I, Tarapore RS, Bhatia N and Spiegelman VS: Overexpression of mrna-binding protein crd-bp in malignant melanomas. Oncogene *27(37)*: 5069-5074, 2008.
23 Qin Y, Roszik J, Chattopadhyay C, Hashimoto Y, Liu C, Cooper ZA, Wargo JA, Hwu P, Ekmekcioglu S and Grimm EA: Hypoxia-driven mechanism of vemurafenib resistance in melanoma. Mol Cancer Ther *15(10)*: 2442-2454, 2016.
24 Pucciarelli D, Lengger N, Takacova M, Csaderova L, Bartosova M, Breiteneder H, Pastorekova S and Hafner C: Hypoxia increases the heterogeneity of melanoma cell populations and affects the response to vemurafenib. Mol Med Rep *13(4)*: 3281-3288, 2016.
25 Li H, Chen J, Wang X, He M, Zhang Z and Cen Y: Nodal induced by hypoxia exposure contributes to dacarbazine resistance and the maintenance of stemness in melanoma cancer stemlike cells. Oncol Rep *39(6)*: 2855-2864, 2018.
26 O'Connell MP and Weeraratna AT: Change is in the air: The hypoxic induction of phenotype switching in melanoma. J Invest Dermatol *133(10)*: 2316-2317, 2013.
27 Widmer DS, Hoek KS, Cheng PF, Eichhoff OM, Biedermann T, Raaijmakers MIG, Hemmi S, Dummer R and Levesque MP: Hypoxia contributes to melanoma heterogeneity by triggering hif1alpha-dependent phenotype switching. J Invest Dermatol *133(10)*: 2436-2443, 2013.
28 Bracken CP, Fedele AO, Linke S, Balrak W, Lisy K, Whitelaw ML and Peet DJ: Cell-specific regulation of hypoxia-inducible factor (hif)-1alpha and hif-2alpha stabilization and transactivation in a graded oxygen environment. J Biol Chem *281(32)*: 22575-22585, 2006.
29 Cascone T, McKenzie JA, Mbofung RM, Punt S, Wang Z, Xu C, Williams LJ, Bristow CA, Carugo A, Peoples MD, Li L, Karpinets T, Huang L, Malu S, Creasy C, Leahey SE, Chen J, Chen Y, Pelicano H, Bernatchez C, Gopal YNV, Heffernan TP, Hu J, Wang J, Amaria RN, Garraway LA, Huang P, Yang P, Wistuba, II, Woodman SE, Roszik J, Davis RE, Davies MA, Heymach JV, Hwu P and Peng W: Increased tumor glycolysis characterizes immune resistance to adoptive t cell therapy. Cell Metab *27(5)*: 977-987 e974, 2018.
30 Parmenter TJ, Kleinschmidt M, Kinross KM, Bond ST, Li J, Kaadige MR, Rao A, Sheppard KE, Hugo W, Pupo GM, Pearson RB, McGee SL, Long GV, Scolyer RA, Rizos H, Lo RS, Cullinane C, Ayer DE, Ribas A, Johnstone RW, Hicks RJ and McArthur GA: Response of braf-mutant melanoma to braf inhibition is mediated by a network of transcriptional regulators of glycolysis. Cancer Discov *4(4)*: 423-433, 2014.
31 Paluncic J, Kovacevic Z, Jansson PJ, Kalinowski D, Merlot AM, Huang ML, Lok HC, Sahni S, Lane DJ and Richardson DR: Roads to melanoma: Key pathways and emerging players in melanoma progression and oncogenic signaling. Biochim Biophys Acta *1863(4)*: 770-784, 2016.
32 Kocemba-Pilarczyk KA, Ostrowska B, Trojan S, Aslan E, Kusior D, Lasota M, Lenouvel C and Dulinska-Litewka J: Targeting the hypoxia pathway in malignant plasma cells by using 17-allylamino-17-demethoxygeldanamycin. Acta Biochim Pol *65(1)*: 101-109, 2018.
33 Edgar R, Domrachev M and Lash AE: Gene expression omnibus: Ncbi gene expression and hybridization array data repository. Nucleic Acids Res *30(1)*: 207-210, 2002.
34 R Core Team: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2016.
35 Davis S and Meltzer PS: Geoquery: A bridge between the gene expression omnibus (geo) and bioconductor. Bioinformatics *23(14)*: 1846-1847, 2007.
36 Gautier L, Cope L, Bolstad BM and Irizarry RA: Affy--analysis of affymetrix genechip data at the probe level. Bioinformatics *20(3)*: 307-315, 2004.
37 Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W and Smyth GK: Limma powers differential expression analyses for rna-sequencing and microarray studies. Nucleic Acids Res *43(7)*: e47, 2015.
38 Muggeo VMR: Segmented: An r package to fit regress models with broken-line relationships. R News *8(1)*: 20-25, 2008.
39 Potter C and Harris AL: Hypoxia inducible carbonic anhydrase ix, marker of tumour hypoxia, survival pathway and therapy target. Cell Cycle *3(2)*: 164-167, 2004.
40 Kaluz S, Kaluzova M, Liao SY, Lerman M and Stanbridge EJ: Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: A one transcription factor (hif-1) show? Biochim Biophys Acta *1795(2)*: 162-172, 2009.
41 Andreucci E, Peppicelli S, Carta F, Brisotto G, Biscontin E, Ruzzolini J, Bianchini F, Biagioni A, Supuran CT and Calorini L: Carbonic anhydrase ix inhibition affects viability of cancer cells adapted to extracellular acidosis. J Mol Med (Berl) *95(12)*: 1341-1353, 2017.
42 Federici C, Lugini L, Marino ML, Carta F, Iessi E, Azzarito T, Supuran CT and Fais S: Lansoprazole and carbonic anhydrase ix inhibitors sinergize against human melanoma cells. J Enzyme Inhib Med Chem *31(sup1)*: 119-125, 2016.
43 Falkenius J, Lundeberg J, Johansson H, Tuominen R, Frostvik-Stolt M, Hansson J and Egyhazi Brage S: High expression of glycolytic and pigment proteins is associated with worse clinical outcome in stage iii melanoma. Melanoma Res *23(6)*: 452-460, 2013.
44 Golinska M, Troy H, Chung YL, McSheehy PM, Mayr M, Yin X, Ly L, Williams KJ, Airley RE, Harris AL, Latigo J, Perumal M, Aboagye EO, Perrett D, Stubbs M and Griffiths JR: Adaptation to hif-1 deficiency by upregulation of the amp/atp ratio and phosphofructokinase activation in hepatomas. BMC Cancer *11*: 198, 2011.
45 Luo W, Hu H, Chang R, Zhong J, Knabel M, O'Meally R, Cole RN, Pandey A and Semenza GL: Pyruvate kinase m2 is a phd3-stimulated coactivator for hypoxia-inducible factor 1. Cell *145(5)*: 732-744, 2011.

46 Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P and Giallongo A: Hypoxia response elements in the aldolase a, enolase 1, and lactate dehydrogenase a gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem *271(51)*: 32529-32537, 1996.

47 Minchenko O, Opentanova I, Minchenko D, Ogura T and Esumi H: Hypoxia induces transcription of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 gene *via* hypoxia-inducible factor-1alpha activation. FEBS Lett *576(1-2)*: 14-20, 2004.

48 Obach M, Navarro-Sabate A, Caro J, Kong X, Duran J, Gomez M, Perales JC, Ventura F, Rosa JL and Bartrons R: 6-phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. J Biol Chem *279(51)*: 53562-53570, 2004.

49 Lee HG, Kim H, Son T, Jeong Y, Kim SU, Dong SM, Park YN, Lee JD, Lee JM and Park JH: Regulation of hk2 expression through alterations in cpg methylation of the hk2 promoter during progression of hepatocellular carcinoma. Oncotarget *7(27)*: 41798-41810, 2016.

50 Hayashi M, Sakata M, Takeda T, Yamamoto T, Okamoto Y, Sawada K, Kimura A, Minekawa R, Tahara M, Tasaka K and Murata Y: Induction of glucose transporter 1 expression through hypoxia-inducible factor 1alpha under hypoxic conditions in trophoblast-derived cells. J Endocrinol *183(1)*: 145-154, 2004.

51 Lu S, Gu X, Hoestje S and Epner DE: Identification of an additional hypoxia responsive element in the glyceraldehyde-3-phosphate dehydrogenase gene promoter. Biochim Biophys Acta *1574(2)*: 152-156, 2002.

52 Graven KK, Yu Q, Pan D, Roncarati JS and Farber HW: Identification of an oxygen responsive enhancer element in the glyceraldehyde-3-phosphate dehydrogenase gene. Biochim Biophys Acta *1447(2-3)*: 208-218, 1999.

53 Muz B, de la Puente P, Azab F and Azab AK: The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia (Auckl) *3*: 83-92, 2015.

54 Wigerup C, Pahlman S and Bexell D: Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. Pharmacol Ther *164*: 152-169, 2016.

55 Sormendi S and Wielockx B: Hypoxia pathway proteins as central mediators of metabolism in the tumor cells and their microenvironment. Front Immunol *9*: 40, 2018.

56 Arends J: Metabolism in cancer patients. Anticancer Res *30(5)*: 1863-1868, 2010.

57 Van Schaftingen E: Fructose 2,6-bisphosphate. Adv Enzymol Relat Areas Mol Biol *59*: 315-395, 1987.

58 Hue L and Rider MH: Role of fructose 2,6-bisphosphate in the control of glycolysis in mammalian tissues. Biochem J *245(2)*: 313-324, 1987.

59 Buart S, Terry S, Noman MZ, Lanoy E, Boutros C, Fogel P, Dessen P, Meurice G, Gaston-Mathe Y, Vielh P, Roy S, Routier E, Marty V, Ferlicot S, Legres L, Bouchtaoui ME, Kamsu-Kom N, Muret J, Deutsch E, Eggermont A, Soria JC, Robert C and Chouaib S: Transcriptional response to hypoxic stress in melanoma and prognostic potential of gbe1 and bnip3. Oncotarget *8(65)*: 108786-108801, 2017.

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