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Original article

Hypoxic Gene Signature of Primary and Metastatic Melanoma Cell Lines: Focusing on HIF1-Beta and NDRG-1

Ercin et al. HIF-1 Beta and NDRG1 are Hypoxia Related Key Players in Malignant Melanoma

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Background: Hypoxia is an important microenvironmental factor significantly affecting tumor proliferation and progression. The importance of hypoxia is not well known in oncogenesis of malignant melanoma.

Aims: To evaluate the difference of hypoxia related gene expressions signature in primary and metastatic melanoma cell lines and to find the expression changes of hypoxia related genes in primary melanoma cell lines at experimental hypoxia conditions.

Study Design: Experimental study

Methods: The mRNA expression levels of hypoxic genes in primary and metastatic melanoma cell lines and in primary cell line at experimental hypoxia conditions were evaluated by using real-time PCR. Depend on experimental data, we focused on two gene/protein, Hypoxia-inducible Factor-1 Beta (HIF-1 β) and N-Myc Downstream Regulated 1 (NDRG1). The protein expression levels of two proteins were investigated by immunohistochemistry methods, in 16 primary and metastatic melanomas, 10 intradermal nevi, and commercial tissue array comprised of 208 cores, including 192 primary and metastatic malignant melanomas.

Results: The real-time PCR study showed that hypoxic gene expression signature was different between metastatic and primary cell lines. Hypoxic experimental conditions significantly affect hypoxic gene expression signature. In immunohistochemical study, NDRG-1 expression was found to be lower in primary cutaneous melanoma compared to intradermal nevi (p=0.001). In contrast, the cytoplasmic expression of HIF-1 β was higher in primary cutaneous melanoma than in intradermal nevi (p=0.001). We also detected medium/strong significant correlations between studied two proteins in the study groups.

Conclusion: This study may show that hypoxic response consists of closely related proteins in more complex pathways. These findings will shed light to hypoxic processes in melanoma and unlock “Pandora’s box” for development of new therapeutic strategies.

Keywords: HIF-1 beta, hypoxia, melanoma, NDRG1

Melanomas are life-threatening malignant neoplasms originated from the pigment-producing cells derived from the pluripotent neural-crest tissue (1, 2). Cutaneous melanoma is the most common type of primary melanoma in the clinical practice. However, mucosal and ocular melanomas are relatively rare (3-5).

There is an enormous effort to establish the molecular mechanisms involved in melanoma progression due to the significant increase in its incidence of worldwide and the low chance of success in treatment of advanced stages

(6, 7). Molecular pathogenesis of melanoma is very complicated and thus, is intensely investigated. Besides the cellular and molecular mechanisms, it has been well documented that tumor microenvironment has a crucial role in oncogenesis and progression of melanoma (8, 9).

Hypoxia is one of the well-defined and important microenvironmental factors affecting tumor proliferation, progression and metastasis. Decreasing the physiological oxygen levels due to the rapid proliferation and high metabolic demands of the tumor is one of the characteristic features of solid tumors, and is called as tumor hypoxia (10-13). There has been strong support that oncogenic pathways are activated by hypoxia in melanoma progression (14).

Major conductor of the hypoxia signaling orchestra is hypoxia-inducible factor (HIF) which is composed of HIF-1 alpha (HIF1 α) and beta/ARNT (HIF1 β ; Aryl hydrocarbon receptor nuclear translocator) subunits (15, 16). Under normoxic conditions, HIF-1 α is degraded by the ubiquitin-dependent processes. However, in severe hypoxia conditions HIF-1 α accumulates in the cytoplasm and then translocates into the nucleus to heterodimerize with the β subunit (17, 18). This, in turn, causes an increase in the expression of the hypoxia-regulated target genes (19).

In a metastatic environment, tumor cells may exhibit distinct gene expression profiles as compared to primary tumors. This might be related to the different microenvironmental conditions or the differences of the metastatic clones from the primary tumors. Metastatic tumor cells are commonly subjected to hypoxic conditions more than the primary site and, thus, need further genetic, epigenetic and post-translational preventive measures (20, 21).

In this study, we aimed to evaluate the difference of hypoxia related gene expressions signature in primary and metastatic melanoma cell lines. We selected WM-115 and WM-266-4 cell lines are, respectively, primary (PMCL) and metastatic cell lines (MMCL), originated from same patient. We also targeted to find the expression changes of hypoxia related genes in PMCL at experimental hypoxia conditions. Depending on the current literature, the observed differences in the gene expression patterns, and pathway analysis, we focused two genes *HIF-1 Beta (HIF1 β)* and *N-Myc Downstream Regulated 1 (NDRG1)*. As a last step, we studied immunohistochemical expression levels of two selected proteins in nevus, primary and metastatic human melanoma tissues.

Material and Methods

Study Design

This study was designed as three steps. As a first step, we evaluated the differences of hypoxia related gene expression patterns in PMCL (WM-115) and MMCL (WM-266-4). Then, we investigated the differences in mRNA expression levels of these genes of PMCL in experimental hypoxia conditions. As a last step, we focused on two protein (HIF1 β and NDRG1) which was significantly changed in experimental studies. We established the distribution and differential expression pattern of two protein, in nevus, primary and metastatic melanoma.

Study Groups

Archival materials

Thirteen primary malignant melanomas (7M/6F) including two mucosal and one uveal melanoma, three metastatic melanomas (1M/2F) and 10 intradermal nevi (1M/9F) were included in this study. Mean age was 61.3 for the melanoma group, and 34.3 for the intradermal nevi group. Clinical and histopathological features of archival materials were demonstrated in Supplementary Table 1. Archive pathology specimens were placed in formalin immediately after procurement, so cold ischemia time kept in minimum.

Tissue array

Commercial tissue array ME2082 (US Biomax, USA) was comprised of 208 formalin-fixed, paraffin-embedded human tissues, including 128 primary malignant melanoma, 64 metastatic malignant melanoma, and sixteen normal skin tissues. 65 of the 128 primary malignant cores were cutaneous melanomas, 13 were ocular, 39 were mucosal, and 11 were primary melanomas not otherwise specified localized in soft tissues (Melanoma, NOS). Clinical and histopathological features of tissue arrays were listed in Supplementary Table 2.

Cell Culture

Both cell lines were procured from the American Type Culture Collection (ATCC). Cell lines were cultured in Eagle's minimum essential medium (Lonza Verviers, Belgium) supplemented with heat inactivated 10% FBS and 1% penicillin-streptomycin (v/v) in a humidified incubator at 34 °C (for WM-115) or 37 °C (for WM-266-4) in 5% CO₂ environment.

Hypoxia Experiment

Hypoxic environment was achieved using BD GasPak EZ Gas Generating Container Systems and GasPak EZ Anaerobe Container System Sachets (Becton Dickinson and Co., Cockeysville, MD., USA). This system produces an anaerobic atmosphere within 2.5 h with less than 1.0% oxygen. Primary malignant melanoma cell lines were harvested after being exposed to 1, 4, and 8 hours of hypoxia and pellets of these cells were prepared for qRT-PCR studies and immunohistochemical analyses.

RNA Purification and cDNA Synthesis

RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract total cellular RNA from primary and metastatic melanoma cell lines. cDNA was synthesized from 2 µg of total RNA using RT² First Strand Kit (Qiagen, Hilden, Germany).

Real Time-PCR

PCR studies were performed with the RT² profiler PCR array (PAHS-032Z-Human Hypoxia Signaling Pathway PCR Array, (Qiagen, Hilden, Germany) with RT² Real Time SYBR Green PCR Master Mix in Rotorgene thermocycler (Qiagen, Hilden, Germany) (Fig 1). qPCR experiments were done duplicated.

Gene expression analysis

Expression levels of a spectrum of 84 hypoxia related genes were determined via qRT-PCR and were analyzed using the Web-Based PCR Array Data Analysis software available in SABiosciences website. Expression values of all samples were normalized to their relative expression levels of five housekeeping genes (ACTB, B2M, GAPDH, HPRT1, and RPLP0). A p value <0.05 was assumed statistically significant and the cut-off Ct values was selected to be 33 cycles. The differentially expressed genes were also analyzed by web-based gene analyses tool kit. (WebGestalt- <http://www.webgestalt.org/>)

Immunohistochemistry Staining

Immunohistochemical studies of archival cases and tissue microarrays were performed automatically in the Bond Max equipment (Leica Microsystems Inc., Wetzlar, Germany). Antigen retrieval steps were performed in Bond-Epitope Retrieval Solution 1 (AR9961) for HIF1β antibody (Abcam, 1:200) and in Bond-Epitope Retrieval Solution 2 (AR9640) for NDRG1 antibody (Santa Cruz Biotech, 1:300), at 100°C. Detection was carried out with Bond Polymer Refine Red Detection kit (DS9390). Stained slides were dehydrated and covered with mounting medium (DAKO; s3023) and cover-slips.

Immunohistochemical Scoring

Stained tissue microarrays and histological slides were digitally imaged under 100x magnification using the Nikon Eclipse Ni-U and Nikon's NIS-Elements D Microscope Imaging Software Version 4.0 (Tokyo, Japan). Digital images were evaluated using the H-score method with minor modifications. (22) A simple MS Excel macro file was generated to calculate the H-scores (range 0-300) using the following equation. $H\text{-score} = \sum (P_i \times i)$, where P_i coefficient is the percentage of stained cells and i is the intensity of staining (3+ (strong), 2+ (moderate), 1+ (weak), and 0 (absent) intensity).

Statistical Analysis

Statistical analysis was performed using PASW statistics v. 17.0 (Chicago, IL, USA). Data were subjected to normality analysis of distribution using Shapiro-Wilk test. The differences between the HSCOREs of the groups were studied with the non-parametric Kruskal–Wallis one-way analysis test and then Mann-Whitney U test. The “Bonferroni correction” was also applied for reducing the type I errors. The correlation between the parameters was investigated by Spearman's correlation.

Ethics Statement

This study was ratified by the Ethics Committee of University (26.04.2012 Decision No: 12/175).

Results

Real Time PCR:

In this study, we aimed to show the expression differences of hypoxia related genes in primary and metastatic melanoma cell lines. As a result, 37 genes were altered more than 2-fold: 10 genes were upregulated while 27 genes were downregulated in the metastatic melanoma cell line (Table 1). *WebGestalt PathwayCommons* analysis showed that differently expressed genes involved in several important pathways demonstrated in Table 2. Relying on the qRT-PCR results, pathway analysis and the available literature, we selected *HIF-1beta* and *NDRG-1* genes for further investigation using immunohistochemical studies. *HIF-1 beta* which is the second component of HIF complex showed 7,8-fold upregulation in metastatic melanoma cell line. *NDRG-1* is an interesting protein which has numerous functions including metastasis suppressor and hypoxia responsive properties. *NDRG1* showed 8,7-fold downregulation in metastatic cell line. The both genes were also a part of all related pathways.

Hypoxia Experiments:

We analyzed the gene expression differences in PMCL after exposure to 1, 4 and 8 hours of hypoxia (23). Seventy-four of the studied 84 genes were significantly changed after being exposed to 4 hours of hypoxia. However only 31 of the genes will be still affected at 8 hours (Table 3). The gene profile of the genes at 8. hours were analyzed by *WebGestalt PathwayCommons*. The related pathways are demonstrated in Table 4. mRNA expression of our candidate genes; *HIF-1 Beta* and *NDRG-1* were upregulated after 4 hours of hypoxia. However, *NDRG-1* genes were then significantly downregulated after 8 hours. Immunohistochemistry staining showed that and *NDRG-1_{nuc}* were decreased while *HIF-1 Beta_{cyt-nuc}* and *NDRG-1_{cyt}* were increased in the primary melanoma cell line after 1, 4 and 8 hours of hypoxia.

Immunohistochemical Staining Results:

Two markers showed both cytoplasmic (-_{cyt}) and nuclear (-_{nuc}) positivity. HIF1β showed weak staining in intra-dermal nevus. However, in melanoma groups, significant positivity was detected (Fig 2). In intra-dermal nevus

NDRG1 staining was both strong and homogeneous. Although staining was weaker and more heterogeneous in melanoma, some primary melanomas and ocular melanomas show more intense positivity, though not strong and diffuse as in intradermal nevus (Fig 3). Quantitative immunohistochemical staining results (HSCORE) of the study groups were demonstrated in boxplots in Fig 3.

In cell lines, *NDRG-1_{nuc}* staining was decreased in the metastatic melanoma cell line but *HIF-1 Beta_{cyt-nuc}* and *NDRG-1_{cyt}* staining were increased.

Statistical Results:

NDRG-1_{cyt-nuc} was found to be significantly lower in primary cutaneous melanoma tissues compared to intradermal nevi ($p=0.001$). In contrast, *HIF-1 Beta_{cyt}* was significantly higher in primary cutaneous melanoma than in intradermal nevi ($p=0.001$). There was no statistically significant H-score difference in cytoplasmic-nuclear staining between primary and metastatic melanomas for two proteins.

A positive correlation was observed between *NDRG-1_{nuc}* and *HIF-1 Beta_{nuc}* in intradermal nevi group ($r=0.758$; $p=0.011$).

Discussion

During carcinogenesis, neoplastic cells face different challenging conditions including hypoxia. However, neoplastic cells usually have the capacity to adapt to hypoxic microenvironment thanks to various intracellular mechanisms. HIF-1 pathway is probably at the center of this adaptive hypoxic response in neoplastic cells (24, 25). Activation of this pathway results in overexpression of several other important proteins having crucial roles in carcinogenesis and metastasis (11).

In this study, two established cell lines derived from the primary and metastatic melanomas of the same patient were used to reveal the differences in hypoxic gene expression. qRT-PCR analyses of the PCR array, which includes 84 hypoxia-related genes, showed that the expression levels of 10 genes were upregulated while 27 genes were downregulated in the MMCL. The expression profile of the studied genes were related to several important pathways. Koh *et al.* reported that 576 genes' expression levels were significantly different between primary and metastatic melanoma, and of these, 402 genes associated with cell cycle regulation, cell adhesion, protease inhibitor activity, and keratinocyte related functions were down-regulated in metastatic melanoma (26, 27). In our study, the pathway analysis showed that besides expected HIF1 α pathways, important cancer progression, angiogenesis and metastasis related pathways were changed in MMCL. Our data show that MMCL gained different gene signature than PMCL basically on angiogenesis and migratory properties. It is well known that the affected pathways in our study; PDGFR, VEGF-VEGFR, integrin family, focal adhesion kinase and EGFR related pathways are closely related to invasion and metastasis in cancer. VEGF-VEGFR, PDGF and EGFR pathways also have important roles of tumor angiogenesis (28, 29). IGF1 and PI3K signaling are other well-known pathways which are significant contributors of carcinogenesis. (30, 31) Glypican 1, an important protein at the center of the GP1 pathway, has significant roles in tumor growth, angiogenesis, and metastasis.(32, 33)

Similarly to our study, Bertucci *et al.* observed that angiogenesis, invasion and apoptosis-related genes were upregulated and tumor suppressor genes were down-regulated in metastatic melanoma.(34) HIF1 α related hypoxic pathways are also changed in MMCL, these data may show these pathways are also important in metastasis. Bertucci *et al.* also concluded that hypoxia was especially important in melanoma progression and hypoxia-related genes were significantly upregulated in melanoma (34, 35). It has also been emphasized that, hypoxia response pathways are required for metastasis (36). Our findings support the previous studies and we speculate that hypoxia related gene expression profiles may be significantly important in metastatic melanoma clones.

We also investigated the hypoxia responses of 84 hypoxia related genes in PMCL under an experimental hypoxic condition. Seventy four out of 84 genes were changed after 4 hours of hypoxia followed by a significant reduction to 31 genes in 8-hour hypoxia. At 8. hours, pathway analysis showed that affected pathways, similar to MMCL, PMCL showed differentially expressed genes are basically involved in progression, invasion and metastasis pathways. Furthermore HIF1 α , IGF1 and Glypican 1 pathways were also affected in hypoxia. The other affected pathways at 8. hour hypoxia including CDC42 signaling, $\alpha9\beta1$ integrin signaling and ILK signaling have also significant roles in invasion and metastasis. (37, 38) mTOR signaling which is one of the well-known pathway in melanoma, controls cell growth, proliferation and survival. Recent studies have shown that mTOR plays a critical role in the regulation of tumor cell motility, invasion and cancer metastasis.(39, 40)

In this study, one of our selected genes/proteins HIF-1 β showed higher expression levels in metastatic tumor cells and in hypoxic experimental conditions. HIF-1 β has a critical role in hypoxic response (41). Contrary to initial findings, recent studies suggest that HIF-1 β levels are not constant. Instead, its levels fluctuate in response to hypoxic conditions, akin to its heterodimeric partner HIF-1 α (42). Similar to our results, Mandl M. *et al.* showed hypoxic responses of ARNT in melanoma cell lines; 518A2, and A375 (17, 43). However, it was thought that this response was cell type dependent. In tissue studies, we found HIF-1 β was significantly

higher in primary cutaneous melanoma than in intradermal nevi. The information about its role in carcinogenesis is not well-known. However there is some clues involved in cell proliferation and survival of cancer cells. (44)

Similar to HIF-1 β , we found NDRG-1 levels to be upregulated in hypoxic conditions. There have been strong clues that NDRG1 protein shows hypoxic responses. Upregulation of NDRG1 in hypoxic conditions can be HIF-1 dependent but was also postulated to be related to other mechanisms (45). Our studies showed that NDRG1 mRNA expression was initially upregulated, and then, was downregulated in experimental hypoxic conditions in melanoma cells. Immunohistochemistry staining results showed that NDRG1 cytoplasmic expression, but not nuclear expression, was upregulated in primary cutaneous melanoma cell lines after 1, 4- and 8-hour hypoxia. In the light of our data, it can be concluded that NDRG1 is one of the possible genes that respond to hypoxia.

NDRG1 mRNA levels were also down-regulated in metastatic carcinoma cell lines compared to primary melanoma cell lines. However, at protein levels, only NDRG1_{nuc} staining was decreased in metastatic carcinoma cell line, whereas the cytoplasmic H-scores were slightly increased. Although NDRG1 was downregulated in melanoma compared to nevus, we could not detect any difference between H-scores of primary and metastatic melanomas in tissue samples. It has been shown that NDRG1 was downregulated in majority of cancers and acts as a metastasis suppressor protein in at least a group of human carcinomas (46-48). The importance of NDRG1 in melanoma is not well known, yet. Cangul H. showed strong NDRG1 staining in melanoma samples compared to nevus. The discrepancy in these results and ours may be related to the differences in selected antibodies and cases, as well as the performed techniques (49).

In conclusion, we demonstrated that MMCL gain distinct gene expression signature compared to PMCL. Pathway analysis showed this signature is involved in pathways related to invasion and metastasis besides hypoxic response. Interestingly, PMCL cells in hypoxic condition showed similar changes. We also demonstrated that HIF1B and NDRG1 expression significantly different in nevus than melanoma in paraffin embedded tissue study. However, we cannot find any difference of two proteins between primary and metastatic melanomas. Our study in the light of previous literature showed that hypoxia is an important phenomenon and may contribute melanomagenesis and metastasis of melanoma.

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Uncorrected Proof

Table 1. Differentially expressed genes of MMCL compared to PMCL.		
Gene Sym	Name	Fold Change
<i>NAMPT</i>	Nicotinamide phosphoribosyltransferase	14,6
<i>ANGPTL4</i>	Angiopoietin-like 4	9,4
<i>CTSA</i>	Cathepsin A	9,4
<i>ANXA2</i>	Annexin A2	8,1
<i>ARNT</i>	Aryl hydrocarbon receptor nuclear translocator	7,8
<i>BLM</i>	Bloom syndrome, RecQ helicase-like	3,0
<i>COPS5</i>	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)	3,0
<i>EGLN2</i>	Egl nine homolog 2 (C. elegans)	2,2
<i>PER1</i>	Period homolog 1 (Drosophila)	3,5
<i>VDAC1</i>	Voltage-dependent anion channel 1	2,1
<i>IGFBP3</i>	Insulin-like growth factor binding protein 3	46,4
<i>NOS3</i>	Nitric oxide synthase 3 (endothelial cell)	-45,3
<i>MAP3K1</i>	Mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase	-28,9
<i>JMJD6</i>	Jumonji domain containing 6	-18,4
<i>HNF4A</i>	Hepatocyte nuclear factor 4, alpha	-16,6
<i>IER3</i>	Immediate early response 3	-12,6
<i>CA9</i>	Carbonic anhydrase IX	-10,1
<i>NDRG1</i>	N-myc downstream regulated 1	-8,7
<i>EGLN1</i>	Egl nine homolog 1 (C. elegans)	-8,6
<i>P4HB</i>	Prolyl 4-hydroxylase, beta polypeptide	-7,8
<i>NFKB1</i>	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-6,4
<i>BTG1</i>	B-cell translocation gene 1, anti-proliferative	-5,2
<i>MIF</i>	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	-5,2
<i>SLC16A3</i>	Solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	-4,9
<i>ADORA2B</i>	Adenosine A2b receptor	-4,1
<i>EPO</i>	Erythropoietin	-3,1
<i>PGK1</i>	Phosphoglycerate kinase 1	-2,9
<i>PGF</i>	Placental growth factor	-2,8
<i>PDK1</i>	Pyruvate dehydrogenase kinase, isozyme 1	-2,8
<i>PFKFB3</i>	6-phosphofructo-2-kinase/fructose 2,6-biphosphatase 3	-2,7
<i>LDHA</i>	Lactate dehydrogenase A	-2,6
<i>ODC1</i>	Ornithine decarboxylase 1	-2,4
<i>PFKP</i>	Phosphofructokinase, platelet	-2,3
<i>MET</i>	Met proto-oncogene (hepatocyte growth factor receptor)	-2,2
<i>ALDOA</i>	Aldolase A, fructose-bisphosphate	-2,1
<i>F10</i>	Coagulation factor X	-2,1
<i>TFRC</i>	Transferrin receptor (p90, CD71)	-2,1

Table 2. Pathways analysis of differentially expressed genes between MMCL and PMCL.		
Pathway	Gene Number	Gene Symbols
Hypoxic and oxygen homeostasis regulation of HIF-1-alpha	13	<i>EGLN2, PGK1, COPS5, NDRG1, ALDOA, LDHA, PFKFB3, EPO, HNF4A, ARNT, TFRC, EGLN1, CA9</i>
HIF-1-alpha transcription factor network	12	<i>PGK1, COPS5, NDRG1, ALDOA, LDHA, PFKFB3, EPO, HNF4A, ARNT, TFRC, EGLN1, CA9</i>
VEGF and VEGFR signaling network	19	<i>NDRG1, LDHA, PFKFB3, ODC1, TFRC, PGF, PGK1, COPS5, NFKB1, ALDOA, EPO, MET, ARNT, HNF4A, MAP3K1, IGFBP3, EGLN1, NOS3, CA9</i>
Integrin family cell surface interactions	19	<i>NDRG1, LDHA, PFKFB3, ODC1, TFRC, F10, PGK1, COPS5, NFKB1, ALDOA, EPO, MET, ARNT, HNF4A, MAP3K1, IGFBP3, EGLN1, NOS3, CA9</i>
EGF receptor (ErbB1) signaling pathway	18	<i>NDRG1, LDHA, PFKFB3, ODC1, TFRC, PGK1, COPS5, NFKB1, ALDOA, EPO, MET, ARNT, HNF4A, MAP3K1, IGFBP3, NOS3, CA9, EGLN1</i>
IGF1 pathway	18	<i>NDRG1, LDHA, PFKFB3, ODC1, TFRC, PGK1, COPS5, NFKB1, ALDOA, EPO, MET, ARNT, HNF4A, MAP3K1, IGFBP3, NOS3, CA9, EGLN1</i>
Signaling events mediated by focal adhesion kinase	18	<i>NDRG1, LDHA, PFKFB3, ODC1, TFRC, PGK1, COPS5, NFKB1, ALDOA, EPO, MET, ARNT, HNF4A, MAP3K1, IGFBP3, NOS3, CA9, EGLN1</i>
Class I PI3K signaling events	18	<i>NDRG1, LDHA, PFKFB3, ODC1, TFRC, PGK1, COPS5, NFKB1, ALDOA, EPO, MET, ARNT, HNF4A, MAP3K1, IGFBP3, NOS3, CA9, EGLN1</i>
PDGFR-beta signaling pathway	18	<i>NDRG1, LDHA, PFKFB3, ODC1, TFRC, PGK1, COPS5, NFKB1, ALDOA, EPO, MET, ARNT, HNF4A, MAP3K1, IGFBP3, NOS3, CA9, EGLN1</i>
Glypican 1 network	18	<i>NDRG1, LDHA, PFKFB3, ODC1, TFRC, PGK1, COPS5, NFKB1, ALDOA, EPO, MET, ARNT, HNF4A, MAP3K1, IGFBP3, NOS3, CA9, EGLN1</i>

Hypoxia 1 Hour		Hypoxia 4 hours				Hypoxia 8 hours	
Gene	Fold regulation*	Gene	Fold regulation*	Gene	Fold regulation*	Gene	*Fold regulation
<i>GBE1</i>	14,2	<i>GBE1</i>	85,8	<i>MIF</i>	4,0	<i>ARNT</i>	11,2
<i>CCNG2</i>	3,2	<i>IGFBP3</i>	33,2	<i>APEX1</i>	3,9	<i>HIF1AN</i>	8,4
<i>TPII</i>	2,7	<i>ANXA2</i>	17,2	<i>SLC2A3</i>	3,9	<i>GBE1</i>	6,6
<i>P4HB</i>	-199,5	<i>HIF1AN</i>	16,6	<i>F10</i>	3,8	<i>ENO1</i>	5,8
<i>MCT4</i>	-42,4	<i>ARNT</i>	12,9	<i>VDAC1</i>	3,6	<i>ADM</i>	4,9
<i>NAMPT</i>	-17,3	<i>HIF1A</i>	9,8	<i>HNF4A</i>	3,5	<i>IGFBP3</i>	4,6
<i>ADORA2</i>	-5,6	<i>PER1</i>	9,8	<i>EGR1</i>	3,5	<i>HIF1A</i>	3,2
<i>MXI1</i>	-5,6	<i>TPII</i>	9,8	<i>SLC16A3</i>	3,4	<i>VEGFA</i>	3,2
<i>PDK1</i>	-5,5	<i>P4HA1</i>	9,7	<i>BTG1</i>	3,4	<i>PER1</i>	2,5
<i>MIF</i>	-4,3	<i>BLM</i>	9,5	<i>LOX</i>	3,3	<i>ANGPTL4</i>	2,9
<i>SLC2A1</i>	-3,3	<i>LDHA</i>	9,1	<i>FOS</i>	3,2	<i>ANXA2</i>	2,3
<i>ENO1</i>	-2,9	<i>MMP9</i>	8,6	<i>HIF3A</i>	3,2	<i>LDHA</i>	2,1
<i>PLANH1</i>	-2,8	<i>BNIP3</i>	8,2	<i>PLANH1</i>	3,2	<i>PFKFB4</i>	2,0
<i>PGK1</i>	-2,5	<i>PFKFB4</i>	8,0	<i>PFKFB3</i>	3,1	<i>PGK1</i>	2,0
<i>ERO1L</i>	-2,1	<i>COPS5</i>	7,0	<i>PGK1</i>	3,1	<i>P4HB[‡]</i>	>-100
<i>HNF4A</i>	-2,2	<i>VEGFA</i>	6,8	<i>EGLN1</i>	2,9	<i>NDRG1</i>	-39,6
<i>NFKB1</i>	-2,2	<i>TFRC</i>	6,7	<i>EGLN2</i>	2,8	<i>PLAU</i>	-8,0
<i>P4HA1</i>	-2,0	<i>PGAM1</i>	6,3	<i>JMJD6</i>	2,7	<i>JMJD6</i>	-7,3
		<i>CCNG2</i>	6,1	<i>RUVBL2</i>	2,6	<i>PFKP</i>	-7,2
		<i>HMOX1</i>	6,0	<i>NFKB1</i>	2,6	<i>ADORA2B</i>	-6,5
		<i>F3</i>	5,5	<i>EDN1</i>	2,5	<i>USF2</i>	-5,6
		<i>RBPJ</i>	5,4	<i>PIM1</i>	2,5	<i>CTSA</i>	-3,5
		<i>ADM</i>	5,4	<i>CTSA</i>	2,4	<i>IER3</i>	-3,4
		<i>STRA13</i>	5,2	<i>PKM2</i>	2,4	<i>ERO1L</i>	-3,4
		<i>BNIP3L</i>	5,2	<i>CA9</i>	2,3	<i>NOS3</i>	-3,3
		<i>CSP</i>	5,1	<i>EPO</i>	2,3	<i>MXI1</i>	-3,2
		<i>BP-1</i>	5,1	<i>ERO1L</i>	2,1	<i>NCOA1</i>	-2,9
		<i>PFKL</i>	4,9	<i>NDRG1</i>	2,0	<i>HNF4A</i>	-2,3
		<i>TP53</i>	4,9	<i>P4HB[‡]</i>	>-100	<i>NAMPT</i>	-2,5
		<i>MET</i>	4,9	<i>PFKP</i>	-18,6	<i>SLC2A1</i>	-2,2
		<i>DDIT4</i>	4,8	<i>ADORA2</i>	-10,8	<i>CA9</i>	-2,0
		<i>GYS1</i>	4,6	<i>NAMPT</i>	-5,7		
		<i>LGALS3</i>	4,6	<i>PLAU</i>	-5,3		
		<i>ODC1</i>	4,6	<i>MXI1</i>	-2,9		
		<i>PGF</i>	4,4				
		<i>LPR2BP</i>	4,4				
		<i>GPI</i>	4,3				
		<i>ALDOA</i>	4,2				
		<i>PGAR</i>	4,2				
		<i>PDK1</i>	4,0				

‡ For this gene the downregulation is very significant. Since $\Delta Ct=29$ in qPCR experiment, fold regulation may not be exact. *Compared to primary cell line. ** Fold: Fold regulation.

Table 4. Pathways analysis of differentially expressed genes of PMCL at 8 Hours hypoxia.		
Pathways	Gene Number	Gene Symbols
Hypoxic and oxygen homeostasis regulation of HIF-1-alpha	13	<i>PGK1, HIF1A, NDRG1, SLC2A1, LDHA, HIF1AN, NCOA1, VEGFA, ENO1, HNF4A, ARNT, ADM, CA9</i>
HIF-1-alpha transcription factor network	12	<i>PGK1, HIF1A, NDRG1, SLC2A1, LDHA, NCOA1, VEGFA, ENO1, HNF4A, ARNT, ADM, CA9</i>
AP-1 transcription factor network	15	<i>PLAU, PGK1, HIF1A, NDRG1, SLC2A1, LDHA, NCOA1, VEGFA, ENO1, USF2, HNF4A, ARNT, ADM, CA9, NOS3</i>
Integrin-linked kinase signaling	15	<i>PLAU, PGK1, HIF1A, NDRG1, SLC2A1, LDHA, NCOA1, VEGFA, ENO1, USF2, HNF4A, ARNT, ADM, CA9, NOS3</i>
CDC42 signaling events	15	<i>PLAU, PGK1, HIF1A, NDRG1, SLC2A1, LDHA, NCOA1, VEGFA, ENO1, USF2, HNF4A, ARNT, ADM, CA9, NOS3</i>
Regulation of CDC42 activity	15	<i>PLAU, PGK1, HIF1A, NDRG1, SLC2A1, LDHA, NCOA1, VEGFA, ENO1, USF2, HNF4A, ARNT, ADM, CA9, NOS3</i>
Glypican 1 network	16	<i>NDRG1, SLC2A1, LDHA, VEGFA, ADM, PLAU, HIF1A, PGK1, NCOA1, USF2, ENO1, ARNT, HNF4A, NOS3, IGFBP3, CA9</i>
Alpha9 beta1 integrin signaling events	16	<i>NDRG1, SLC2A1, LDHA, VEGFA, ADM, PLAU, HIF1A, PGK1, NCOA1, USF2, ENO1, ARNT, HNF4A, NOS3, IGFBP3, CA9</i>
IGF1 pathway	16	<i>NDRG1, SLC2A1, LDHA, VEGFA, ADM, PLAU, HIF1A, PGK1, NCOA1, USF2, ENO1, ARNT, HNF4A, NOS3, IGFBP3, CA9</i>
mTOR signaling pathway	16	<i>NDRG1, SLC2A1, LDHA, VEGFA, ADM, PLAU, HIF1A, PGK1, NCOA1, USF2, ENO1, ARNT, HNF4A, NOS3, IGFBP3, CA9</i>

Brief Protocol Overview

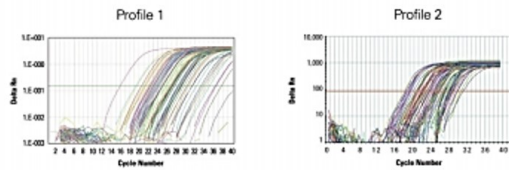
1. Convert Total RNA to cDNA.



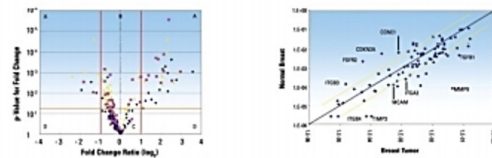
2. Add cDNA to RT² qPCR Master Mix & Aliquot Mixture Across PCR Array.



3. Run in Your Real-Time PCR Instrument.



4. Data Analysis.



cDNA Synthesis

- Genomic DNA Removal Step (5 min.)
- Reverse Transcription Step (20 min.)

Load Plates

- 1 Sample per PCR Array
- 2 minutes with multi-channel pipet

Run 40 cycle qPCR Program

- Standard cycling conditions for all Real Time PCR Instruments
- 2 hours

Upload and Analyze Data

- 15 minutes from Raw Ct to Fold Change Data

Figure 1. RT² Profiler PCR Array Brief Protocol Overview

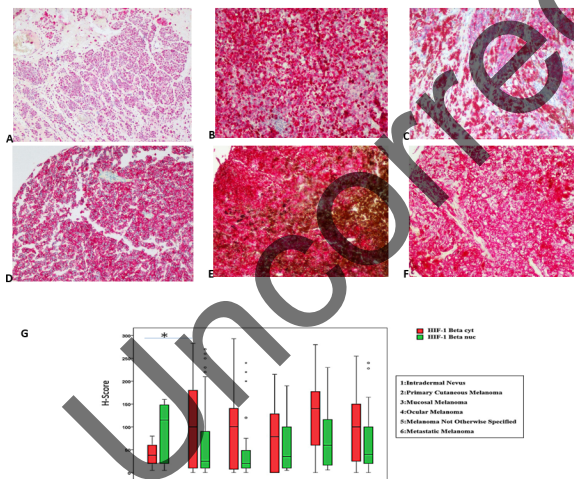


Figure 2. HIF1 β immunostaining in study groups except for low intense positivity in intradermal nevus (a), significant positivity was detected in primary cutaneous (b; c), rectal (d), ocular (e) and metastatic melanoma (f). Original magnifications A, x40; B; C; D; E; F, x100. Box-plot graphs for HIF1 β immunostaining H-scores of study groups (g).

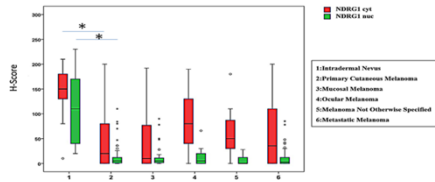
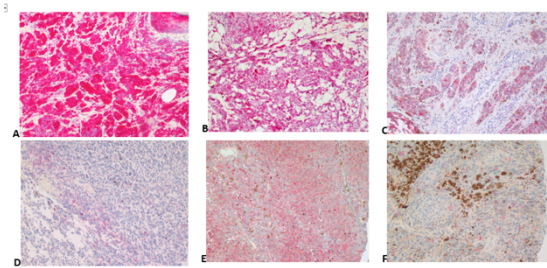


Figure 3. NDRG1 immunostaining in study groups. Strong positivity is easily detected in intradermal nevus (A), Weak and heterogenous positivity is detected in primary cutaneous (C), rectal (D), and metastatic melanoma (F). However, some primary melanomas (B) and ocular melanomas (E) show more intense positivities, though not strong as in intradermal nevus. Nuclear and cytoplasmic scores of NDRG1 in nevus are higher than melanomas (G) Original magnifications A, B, C x200, D; E; F, x100. Box-plot graphs for NDRG1 immunostaining H-scores of study groups (g).

Uncorrected Proof