Potential Prognostic Molecular Signatures in a Preclinical Model of Melanoma

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Abstract. Numerous studies have revealed a variety of pathways involved in the development of melanoma, however, the molecular and genetic divergence of underlying mechanisms remain vague. In a mouse model, we studied the expression pattern of insulin-like growth factor 2 mRNA-binding protein 1 (*Igf2bp1*) and target genes microphthalmia-associated transcription factor (*Mitf*), v-myc avian myelocytomatosis viral oncogene homolog (*Myc*), B-cell lymphoma 2 (*Bcl2*), prothymosin alpha (*Ptma*) and melan-A (*Mlana*) in relation to tumor-growth characteristics. The *in vivo* expression of the aforementioned genes was assessed by quantitative Real Time-Polymerase Chain Reaction (RT-PCR) in tumors established by B16-F1-derived clones. Gene expression was correlated with tumor growth characteristics. Simultaneous expression of elevated levels of *Myc*, *Igf2bp1*, *Ptma* and *Mitf* characterizes tumors with a more aggressive phenotype. Our findings introduce a tumor-specific molecular signature possibly associated with melanoma heterogeneity. The concomitant overexpression of key molecules such as *IGF2BP1*, *PTMA*, *MYC* and *MITF* could serve as prognostic or predictive marker.

Melanoma is a very heterogenic cancer with great *in vivo* plasticity (1). Although the diversity of melanomas is known, the underlying molecular and genetic differences remain obscure (2). Thus, identifying intra-tumoral molecular signatures might introduce novel biomarkers of prognostic significance. A number of studies have unveiled a variety of molecules and several distinct pathways involved in the onset of melanoma. Some focused on the multifaceted and complicated role of wingless-related integration site (Wnt)/ β -catenin pathway (3, 4). However, other studies have also

pinpointed microphthalmia-associated transcription factor (*MITF*) and insulin-like growth factor 2 mRNA-binding protein 1 (*IGF2BP1*) as drivers in melanoma initiation and progression.

MITF is a major transcription factor, dynamically transcribed by β -catenin complexes (5), active in normal melanocytes and malignant melanoma cells (6). Its deregulated expression has been described as a causal event in melanomas, resulting from gene amplification (7), as well as, from the ectopic expression of the RNA binding protein *IGF2BP1* (8). As an RNA-binding protein, *IGF2BP1* exerts its mode of action *via* regulating the intracellular levels of its targets. Among them, besides *MITF*, there are many notorious oncogenes such as v-myc avian myelocytomatosis viral oncogene homolog (*MYC*), glioma-associated oncogene family zinc finger 1 (*GLI1*), insulin-like growth factor 2 (*IGF2*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), multidrug resistance protein 1 (*MDR-1*), beta-transducin repeat containing E3 ubiquitin protein ligase pseudogene 1 (*BTRCP1*) and homing cell adhesion molecule (*CD44*) (9). It has been reported that overexpression of *IGF2BP1* results in up-regulation of one or more of these target genes, depending on the specific type of tissue (10). Although its expression is absent or scarce in normal adult tissues, it is detected in almost all types of cancers (9). It is common knowledge that heterogeneity from a single cell level to the whole solid tumor is mainly the outcome of the well-orchestrated differential expression of a multitude of genes, carved under the pressure of the tumor microenvironment.

Given the established role of *IGF2BP1* and *MITF* in melanoma, here we investigated the expression pattern of these genes, along with the associated molecules *Myc*, B-cell lymphoma 2 (*Bcl2*), prothymosin alpha (*Ptma*) and melan-A (*Mlana*), in relation to growth characteristics, in the context of melanoma heterogeneity. Although the Achilles' heel of all types of melanomas is very difficult to identify, novel combinations of gene expression patterns, often described separately to be involved in melanoma, might better serve as a more representative molecular signature for melanoma characterization and prediction.

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Key Words: Melanoma, *Igf2bp1*, *Ptma*, β -catenin/Wnt pathway, molecular signatures.

Table I. Sequences of primers for the gene expression analysis.

Gene	Protein	Forward primer 5'-3'	Reverse primer 5'-3'	Tm	Reference (no)
<i>Gapdh</i>	Glyceraldehyde 3-phosphate dehydrogenase	GTGTTCTACCCCAATGTG	GTCATTGAGAGCAATGCCAG	59	(26)
<i>Igf2bp1</i>	Insulin-like growth factor 2 mRNA-binding protein 1	GGCTGCTCCCTATAGCTCCT	CTGGTGGTGCAATCTTGATG	59	(26)
<i>Bcl2</i>	B-cell lymphoma 2	CTGTGGATGACTGAGTACCT	AGCCAGGAGAAATCAAACAG	60	M&M section
<i>Myc</i>	v-myc avian myelocytomatosis viral oncogene homolog	AGCGACTCTGAAGAAGAGCAA	GTTGTGCTGGTGAGTGGAGA	62	(26)
<i>Ptma</i>	Prothymosin alpha	TTGTGGAGGAGGCAGAGAAT	TTGGTGTCCACATCGTCATC	62	M&M section
<i>Mlana</i>	Melan-A	ATGTGAGAGCCCTGATCACC	TAAGGTGGCGGTGAAGAGAG	59	M&M section
<i>Mitf</i>	Microphthalmia-associated transcription factor	GTGCAGACCCACCTGGAAAAC	AGTTAAGAGTGAGCATAGCCATAG	60	(27)

Materials and Methods

Cell lines and generation of clones. B16-F1 melanoma murine cell line (American Type Culture Collection, Wesel, Germany) Cultures maintained in logarithmic phase of growth and sub-cultured using Trypsin 0.25%-ethylene-diaminetetraacetic acid solution (Life Technologies, Waltham, MA, USA). Clones were generated applying the limiting dilution assay, by seeding 1 cell/well in 96-well plates Dulbecco's Modified Eagle Medium (Life Technologies, Waltham, MA, USA). Media were replenished every two days. The total number of clones was 22, in compliance with the estimated numbers by the likelihood equations of this assay.

Proliferation assay. The proliferative rate of each cell line was estimated by thymidine incorporation. Briefly, cells were seeded at 5,000 cells/well in 96-well flat bottomed plates (Greiner Bio-One GmbH, Kremsmünster, Austria) and pulsed with 1 μ Ci/well [methyl-³H] thymidine (specific activity: 53.0 Ci/mmol, 1 mCi/ml; Amersham, GE Healthcare, Amersham, UK) for additional 18 h. Cells were harvested 48 h post seeding and counted on a liquid scintillation counter (Wallace Trilux, No. 1450 Microbeta; PerkinElmer Life Sciences, Waltham, MA, USA).

Animals. All protocols were in compliance with the Greek and European regulations on Animal Welfare and with Public Health Service recommendations. Female C57BL/6 mice, aged 6-8 weeks old (Hellenic Pasteur Institute, Athens, Greece) were randomly assigned (at least five per clone or line) to receive 5×10^5 cells in 100 μ l Phosphate Buffered Saline (Biochrom GmbH, Berlin, Germany), by subcutaneous injection in their flank (single flank/mouse). Tumor growth was monitored daily and growth measurement is presented as the orthogonal mean: $(d1+d2)/2$. All animals were sacrificed by CO₂ inhalation, on the 16th day post inoculation. Tumors were excised, weighed and dissociated mechanically. Single-cell suspensions were used to assess antigen expression by flow cytometric analysis and gene expression by quantitative real-time polymerase chain reaction (qRT-PCR). In order to facilitate our combinatorial analysis, all samples were divided for each parameter (growth and gene expression) into two groups based on the median value of this parameter in all tumors (irrespective of the clone of origin) namely high and low.

Flow cytometry. Cells were washed and stained as previously described (11). In detail, cells were stained with the monoclonal

antibodies anti-CD45 (leukocyte common antigen (LCA)) (clone 30-F11; eBioscience, Cheshire, UK) and anti-CD44 (clone IM7, eBioscience), fixed and analyzed on a BD FACS Canto II instrument (BD Biosciences, Milano, Italy) using DIVA 6.0 software (BD Biosciences, Milano, Italy).

RNA extraction and qRT-PCR. Total RNA extraction was performed using Nucleospin RNA II kit (Macherey-Nagel GmbH&Co, Düren, Germany) according to the manufacturer's instructions. Total RNA (1 μ g) was reverse transcribed using PrimeScript Reverse Transcriptase (TaKaRa, Bio.Inc, Shiga, Japan) and random hexamer oligonucleotides (Invitrogen, Life Technologies). qRT-PCR was performed on a Rotor-Gene 3000 instrument (Corbett Research, Life Technologies). cDNA solution (200 ng) KAPA SybrFast qPCR kit (KapaBiosystems, London, UK) and 200 nMol of specific primers (VBC Biotech Service, Wien, Austria) were used in a 20 μ l reaction. Sequences of primers are listed in Table I. Primers for *Ptma*, *Bcl2* and *Mlana* were designed via Primer3 software v.0.4.0 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>). In order to confirm amplification specificity, melting curve analysis was performed and the $\Delta\Delta$ Ct method was applied for gene expression quantification. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used for internal normalization and the B16-F1 cell line as reference sample.

Statistical analysis. Statistical analyses were performed by GraphPad Prism v.5.0 (GraphPad software, La Jolla, USA), using Spearman's correlation with 95% confidence interval, Fisher's exact test and Mann-Whitney *t*-test, accordingly. *p*-Values of less than 0.05 were considered statistically significant.

Results

Generation of a preclinical model reflecting melanoma heterogeneity and differential expression of *Igf2bp1* in vitro and in vivo. In order to create a preclinical melanoma model reflecting melanoma diversity, we used B16-F1-derived clones exhibiting different *in vitro* proliferation rates (Figure 1a), cell morphology, anchorage independence, melanin production and gene expression pattern to generate tumors in C57BL/6 mice (data not shown). The differential profile of the clones noted *in vitro*, still existed *in vivo*, as was confirmed by the tumor growth rate (Figure 1a) and gene expression analysis (data not shown).

Figure 1. *a*: Left: *In vitro* proliferation rate of B16-derived clones 13,15,17 and 22. Bars represent the fold difference of each clone in relation to the maternal cell line (B160-F1=1.0). Right: *In vivo* proliferation, where each line represents growth of an individual tumor. *b*: *In vitro* and *in vivo* expression of insulin-like growth factor 2 mRNA-binding protein 1 (*Igf2bp1*) mRNA (median value of AU) and homing cell adhesion molecule (CD44) antigen in clones 13 (50.21, range:23.49-72.20), 15 (22.76, range:12.07-111.7), 17 (18.95, range:0.92-27.93) and 22 (12.5, range:9.21-36), ($n=5/\text{group}$). AU: Arbitrary Units; MFI: mean Fluorescence Intensity.

CD44 and *Igf2bp1* levels of expression were assessed on the 16th day (Figure 1b). Considering the role of IGF2BP1 in melanoma, we chose clones 13 and 22, which exhibited the most significant difference in *in vivo* expression of *Igf2bp1* ($p=0.0317$), as well as of antigen CD44 (Figure 1b). Our group (10) and others have shown that high levels of IGF2BP1 and the presence of CD44 molecule are related to more aggressive melanoma phenotype (11), characterize mesenchymal cells and are linked post-transcriptionally (12). Tumors generated by these clones had similar growth (gr), since their median mass did not differ significantly ($p=0.3651$; Figure 2a). However, the diameter of clone 22 tumors was significantly greater than

Figure 2. Growth characteristics and gene expression in tumors derived from clones 13 and 22. *a*: Boxplots represent median values of mass, diameter, and mass to diameter ratio of tumors derived from clones 13 (0.94 gr, 1.3 cm, 0.74, respectively) and 22 (0.98 gr, 1.5 cm, 0.65, respectively) ($n=5/\text{group}$). *b*: Bars represent the frequency of tumors with high or low level of insulin-like growth factor 2 mRNA-binding protein 1 (*Igf2bp1*), prothymocin alpha (*Ptma*), microphthalmia-associated transcription factor (*Mitf*), v-myc avian myelocytomatosis viral oncogene homolog (*Myc*), melan-A (*Mlana*) and B-cell lymphoma 2 (*Bcl2*) expression ($n=5/\text{group}$).

that of clone 13 tumors ($p=0.0171$; Figure 2a), suggesting a different pattern of *in vivo* growth, possibly implying a preferential growth in depth (reflected by mass/diameter ratio) for clone 13.

Gene expression pattern differs between clones with different Igf2bp1 expression. Clones 13 and 22 were analyzed for their gene expression pattern. Figure 2b presents the percentages of tumors exhibiting high or low expression of each gene studied. Interestingly, *Igf2bp1*, *Ptma*, *Bcl2* and *Mlana* exhibited a common pattern of expression. The majority of clone 13-derived tumors showed high levels of expression of these genes compared to clone 22. All clone 13-derived tumors (100%) expressed high levels of *Igf2bp1* and *Ptma* but low levels of *Myc*. It is noteworthy that clone 22-derived tumors exhibited the opposite pattern of gene expression.

Figure 3. Correlations of gene expression. Graphs present correlations in gene expression (AU) of all genes studied. *Igf2bp1*: insulin-like growth factor 2 mRNA-binding protein 1; *Ptma*: prothymocin alpha; *Myc*: v-myc avian myelocytomatosis viral oncogene homolog; *Mlna*: melan-A; *Bcl2*: B-cell lymphoma 2; *Mitf*: microphthalmia-associated transcription factor.

Potential correlations in gene expression. Figure 3 depicts only the genes with positive correlations that were statistically significant. Correlations of *Myc* with *Ptma* ($p=0.0161$) and *Bcl2* ($p=0.0033$), and *Mitf* with *Mlna* ($p=0.0013$) and *Igf2bp1* ($p=0.0089$) were in line with previous studies regarding control of gene expression (13-15). However, strong positive correlations were also observed for *Ptma* with *Igf2bp1* ($p<0.0001$), *Mitf* ($p=0.0009$) and *Bcl2* ($p<0.0001$), as well as *Mlna* ($p=0.0025$). Moreover, *Mlna* weakly correlated with *Igf2bp1* ($p=0.0362$).

Combinatorial analysis of gene expression in relation to tumor growth reveals distinct molecular patterns. Analysis of expression of single genes in relation to growth characteristics showed no significant correlation (data not shown). Therefore, we sought to test whether the concomitant expression of two or more genes correlated with similar *in vivo* tumor growth characteristics. Tumors generated by all clones were divided in sub-categories, based on the level of simultaneous expression of two genes of interest each time. The group with high levels was compared to the group with low levels, regarding their mass (Figure 4a), diameter (Figure 4b) and mass/diameter (Figure 4c). Interestingly, the analysis revealed that high expression of *Igf2bp1* with *Ptma*, *Myc* with *Ptma* or *c-Myc* with

Figure 4. Tumor growth analyzed in relation to gene co-expression. Bars represent the frequency of tumors with high (H) or low (L) co-expression of both insulin-like growth factor 2 mRNA-binding protein 1 (*Igf2bp1*) and prothymocin alpha (*Ptma*) ($n=14$), v-myc avian myelocytomatosis viral oncogene homolog (*Myc*) and *Ptma* ($n=10$), and *Igf2bp1* and *Myc* ($n=10$) in relation to high or low mass (a), diameter (b) or mass to diameter ratio (c).

Igf2bp1 correlated with high tumor mass and mass/diameter. Given the correlation for gene expression described previously, we decided to investigate the concomitant expression of three or more genes. We observed that co-expression of high levels of *Myc*, *Igf2bp1* and *Ptma* had an even stronger correlation with high tumor mass (Figure 5a) and mass/diameter (Figure 5c), which was maintained after including *Mitf*.

The effect of the expression pattern of *Igf2bp1*, *Myc* and *Ptma* on tumor growth was verified following analysis of tumors derived from clones with opposite reverse growth characteristics (clone 15 and 17 data not shown). Hence, expression of *Igf2bp1*, *Myc* and *Ptma* might serve as a possible molecular signature underlying melanoma *in vivo* growth rate.

Discussion

The results presented herein strongly suggest that concomitant activation of *Igf2bp1*, *Myc*, *Ptma* and *Mitf* are associated with

Figure 5. Combinatorial analysis of gene expression. Bars represent the frequency of tumors with high (H) or low (L) co-expression of insulin-like growth factor 2 mRNA-binding protein 1 (*Igf2bp1*), prothymocin alpha (*Ptma*) and v-myc avian myelocytomatosis viral oncogene homolog (*Myc*), (n=9), and of *Igf2bp1*, *Ptma*, *Myc* and microphthalmia-associated transcription factor (*Mitf*), (n=6) in relation to tumor mass (a), diameter (b) and mass/diameter ratio (c).

a more aggressive melanoma phenotype in our preclinical model. More specifically, our findings indicate that augmented co-expression of these genes characterizes melanoma tumors with increased *in vivo* growth rate.

Given the fact that high levels of *Igf2bp1* and CD44 have been correlated with metastatic potential and aggressiveness in melanoma (11, 13), we decided to investigate the expression pattern of *Igf2bp1*, *Myc*, *Ptma*, *Mitf*, *Mlana* and *Bcl2* in tumors with high or low expression of these genes. Based on our data, high levels of *Igf2bp1*, *Ptma*, *Bcl2* along with *Melan-A*, an already established marker in melanoma characterization (16, 17), are associated with tumor of small diameter but high mass, implying melanoma invasiveness (18).

Previous reports have described the selected genes studied here as associated players in the Wnt/ β -catenin pathway, which is deregulated in most cases of melanoma (4). *Mitf* participates in a feed-back loop regulatory mechanism with β -catenin (19) and may alter its transcriptional activity (5). Additionally, *Igf2bp1* and *Ptma* are direct targets in the β -catenin pathway (20, 21). Moreover, *Igf2bp1* post-transcriptionally regulates *Mitf* and *Myc*, whose transcription is in turn controlled by β -catenin (22). These interactions dictate the high complexity of involvement of the Wnt/ β -catenin pathway in melanoma tumorigenesis. In line with this, we studied the above mentioned genes aiming to assign a specific expression pattern to differential melanoma growth rates.

Our analysis revealed significant correlations in gene expression, particularly for *Ptma* with *Bcl2*, *Myc* and *Igf2bp1*. Correlation of *Ptma* and *Bcl2* is anticipated given the fact that PTMA has been described as an anti-apoptotic molecule (23). A previous study found elevated PTMA correlated to increased *Myc* level in human colon carcinoma and was further associated with multiple established clinicopathological factors as well as the clinical outcome (24). Notably, the significant correlation of *Igf2bp1* and *Ptma*, reported here for the first time to our knowledge, could be explained either by the fact that both these genes are affected by β -catenin in a parallel way, or by the hypothesis that *Igf2bp1* may bind to *Ptma* mRNA positively regulating its pattern of expression. This observation should be further investigated by gene silencing or analyses to elucidate the possibility that *Ptma* is another target of *Igf2bp1*.

Given the limited number of reports regarding concomitant expression of *Igf2bp1* and *Ptma* in cancer, we studied their association with already described molecules in our effort to identify a prognostic signature in melanoma. In particular, high co-expression of *Igf2bp1*, *Ptma* and *Myc* characterizes tumors with high mass and high mass/diameter but not tumors with large diameter and low mass/diameter ratio. When *Mitf*, the major melanoma regulator, is added to the signature, this correlation persists, even though the remaining sample size is very small. Noteworthy, the results of the current study should be considered only as hypothesis generating, due to the large intra-group variation, possibly explained by melanoma plasticity (25), along with the limited number of animals analyzed.

Our findings suggest that the role of Wnt/ β -catenin pathway-related molecules in melanoma is not only qualitative but also quantitative. We propose that the concomitant overexpression of key molecules such as *Igf2bp1*, *Ptma*, *Myc* and *Mitf*, although perhaps not solely driven by the Wnt/ β -catenin pathway, may better reflect the aggressiveness of melanoma phenotype. Thus, this molecular signature could serve as prognostic or predictive marker for melanoma, adding more arrows to our quiver against this disease. Finally, extensive studies in primary human melanoma tissues, from patients with available follow-up data, are required in order to validate the prognostic significance of this signature.

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Received October 20, 2016

Revised November 15, 2016

Accepted November 22, 2016