Deregulation of Glyceraldehyde-3-Phosphate Dehydrogenase Expression During Tumor Progression of Human Cutaneous Melanoma

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Abstract. *Background/Aim: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a highly abundant housekeeping gene. GAPDH overexpression has been reported in diverse types of human cancers including cutaneous melanoma. Our goal was to quantify GAPDH mRNA and protein expression in the whole spectrum of primary and metastatic melanomas in the search for a specific role for this ubiquitous molecule during tumor progression. Materials and Methods: Intratumoral GAPDH mRNA expression was quantified by real-time PCR in 71 cases, including 29 primary melanomas and 42 metastatic cases. Relative expression levels in thin (≤1 mm) and thick (>1 mm) primary tumors and 'in-transit', lymph node and distant metastases were compared. Similarly, protein expression was investigated by means of immunohistochemistry. Specific exons of GAPDH were analyzed by DNA sequencing. Results: GAPDH mRNA expression was significantly up-regulated in thick melanomas when compared to primary thin melanomas. Similar differences were also encountered between metastatic melanomas when compared to lymph-node metastatic melanomas. Interestingly, GAPDH protein immunoexpression was higher in thick melanomas and distant metastases than in thin tumors and lymph node metastases, respectively. However, no specific point-mutations in GAPDH-specific exons were found in any patient. Conclusion: Deregulation of GAPDH during melanoma progression was demonstrated in our series by mRNA and protein expression studies.*

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a highly abundant molecule in humans commonly used as an endogenous reference gene in quantitative gene expression studies (1-3). Extensive data obtained in the last decade strongly support the concept that GAPDH is a multi-functional protein which, in addition to being an integral factor in glycolysis, plays an outstanding role in apoptosis and a large variety of cellular functions within membrane, cytoplasmic and nuclear compartments (4-9). Accordingly, GAPDH expression may vary significantly in different cellular settings, and, particularly, in transformed tumor cells (2, 3, 10, 11). Indeed, Otto Warburg first described high levels of GAPDH in tumor cells in comparison to normal tissues (12, 13). Consequently, GAPDH cannot be currently recommended as an endogenous reference gene in quantitative studies of gene expression in cancer research (2, 3, 11, 14).

In comparison to normal tissues, GAPDH has been demonstrated to be overexpressed in breast, lung, kidney, pancreatic, cervical, and skin carcinomas (15-18). In contrast, in a series of cutaneous melanocytic lesions, Seykora *et al.* found GAPDH expression to be slightly diminished in nodular primary melanomas and melanoma metastases in comparison to melanocytic nevi (19), whereas other studies described a remarkable up-regulation of melanoma tissues compared with normal samples (14). In addition, specific point-mutations in the *GAPDH* gene have recently been identified as a new melanoma tumor antigen recognized by tumor-infiltrating T-lymphocytes from one patient with metastatic melanoma (20).

In an attempt to clarify the specific role of this molecule during progression of human melanoma tumor, we carried out a quantitative study of *GAPDH* mRNA expression, as well as a semi-quantitative protein expression analysis in the whole spectrum of patients with melanoma, from primary (thin and thick) to metastatic ('in-transit', lymph node and systemic) cutaneous melanomas.

Materials and Methods

Human tissues. Seventy-one consecutive specimens of cutaneous melanoma were included in the study. Primary melanomas (n=29) were sub-divided according to the current American Joint Committee on Cancer (AJCC) staging system [21] into thin (Breslow index ≤1 mm) (n=14), and thick (Breslow index >1 mm) (n=15) tumors. Metastatic melanomas (n=42) included 'in-transit' metastases (n=16), regional lymph node metastases (n=13), and distant metastasis (n=13; four in brain, two in liver, two in bone, one in pleura, two in skin, and two subcutaneous). Likewise, four cases of normal skin specimens (including non-tumor melanocytes, keratinocytes and normal appendages) were also included as a non-malignant control group. All tumor specimens were received at the Department of Anatomic Pathology, Valencia University Clinic Hospital, from November 2002 to April 2005.

Two contiguous slices were obtained from macrodissected tumor areas from each specimen. One slice was immediately frozen in liquid nitrogen and stored at −80˚C until molecular analysis. The remaining fresh tumor tissue from each case was fixed in formalin and embedded in paraffin for routine diagnosis and, subsequently, for the construction of tissue microarrays (TMA) platforms in order to facilitate homogeneous morphological and immunohistochemical studies (MTA1-Manual Tissue Arrayer; Beecher Instruments Inc; Sun Prairie, Wisconsin, USA). This protocol was approved by the Ethics Committee (H1413973212956) of the Valencia University Clinic Hospital/University of Valencia. In each case, informed consent was obtained from the patient.

Immunohistochemistry. Protein expression was determined by means of immunohistochemistry. Five TMA blocks were constructed and subsequently, each TMA was arranged by grouping cases into different prognostic groups of patients with melanoma in triplicate. GAPDH (clone 6C5; 1:200 dilution; Chemicon Merck Millipore, Darmstadt, Germany) was utilized as the primary antibody and Giemsa counterstaining was used so as to be able to differentiate true immunoreaction from melanin pigment. Citrate-buffered solution was utilized for antigen retrieval (pH=6), and the reaction was visualized with EnvisionFlex revealing system (DAKO Denmark, Glostrup, Denmark). Finally, protein expression levels were semiquantitatively evaluated by three different pathologists separately, and discordant judgments were resolved in consensus. Both cytoplasmic and nuclear reactions were recorded. Nuclear staining was scored as a total percentage of positively stained tumor cells, whereas the intensity of cytoplasmic immunoreactivity was stratified according to intensity as: 0: absent, 1: weak, 2: moderate and 3: strong immunostaining; and the percentage of positively stained tumor cells: 0: none; 1: 1-25%; 2: 26-50%, and 3: more than 50%. Consequently, the final score ranged from 0 to 6.

Cardiac muscle fibers were utilized as external positive control with the maximum staining level and utilized then as the reference in comparison of expression levels of GAPDH in our cases.

Real-time Polymerase Chain Reaction (PCR). Total RNA extraction was performed according to the manufacturer's instructions using 1 ml TRIzol Reagent (Gibco BRL, Gaithersburg, MD, USA) per 50 mg of tissue. RNA was quantified by spectrophotometry at 260 nm, and the integrity of the *28S* and *18S* ribosomal bands was checked by 1% ultraPURE agarose (Life Technologies, Paisley, Scotland, UK) gel electrophoresis and ethidium-bromide staining.

Relative quantification of messenger RNA (mRNA) of GAPDH was performed by real-time quantitative reverse transcriptase PCR (qRT-PCR). Briefly, 1 μg of total RNA was converted to single-stranded cDNA using the High-Capacity cDNA Archive Kit (Perkin-Elmer, Applied Biosystems Inc., Foster City, CA, USA). Then 25-μl PCR reactions were performed using TaqMan Universal PCR Master Mix (Perkin-Elmer, Applied Biosystems Inc.) and Assays-on-Demand gene expression products (Perkin-Elmer, Applied Biosystems Inc., Foster City, CA, USA), consisting of a mix of unlabeled PCR primers and the TaqMan MGB probes (dye-labeled) for the target gene *GAPDH* (Hs99999905_m1), and endogenous reference gene *18S* rRNA (Hs99999901_s1) (2). All reactions were performed in triplicate, and the results were automatically analyzed by an ABI-PRISM 7700 Sequence Detection System and software (Perkin-Elmer, Applied Biosystems, Inc.). For the relative quantification of gene expression, the comparative Ct method was used. The final amount of the target gene, normalized to an endogenous reference gene (ΔCt=Ct target gene – Ct reference gene) was given by the formula: $2^{-\Delta\Delta Ct}$ (22), which allowed for the comparison of the different samples of our study. Additionally, a standard curve with six serial dilutions of one of the cases was designed for both target and reference genes in each sample. The slope ('m' values) of the standard curve ranged from −3.1 to −3.5 with a correlation coefficient of 0.98 or more.

DNA sequencing. To screen for mutations in the *GAPDH* gene (codon 175 of exon 7), complementary cDNA was amplified by PCR using the following primer pair "*GAPDH*.ex7-F" (ATG TTC GTC ATG GGT GTG AA); "GAPDH.ex7-R" (ATC ACT GCC ACC CAG AAG AC).

The PCR products were checked on standard agarose gels and purified before they were sequenced. The sequence reactions were run on an Applied Biosystems 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA, USA). Data were collected and analyzed using Applied Biosystems sequencing analysis software, according to the manufacturer's protocols.

Statistical analysis. Statistical analysis was performed using the SPSS software package "version 12.0" (Statistical Analysis Department, University of Valencia, Valencia. Spain). Differences between the relative expression of "median values" of *GAPDH* in the different tumor groups (primary tumors with a Breslow index ≤1 mm, primary tumors with a Breslow index >1 mm, in-transit metastatic melanomas, lymph node metastases, and distant metastases) were analyzed by two-tailed univariate statistical non-parametric tests. The association of *GAPDH* mRNA expression in primary tumors with clinical and histopathological prognostic variables was also analyzed. Mann–Whitney *U* and Kruskal–Wallis tests were utilized for the statistical comparison between two or more groups of patients, respectively.

A *p*-value of less than 0.05 was considered statistically significant.

Results

Relative *GAPDH* mRNA expression demonstrated a strong correlation with GAPDH protein expression by means of immunohistochemistry on TMA paraffin blocks ($p<0.001$; correlation coefficient: 0.408), particularly when the cytoplasmic distribution of the protein was taken into

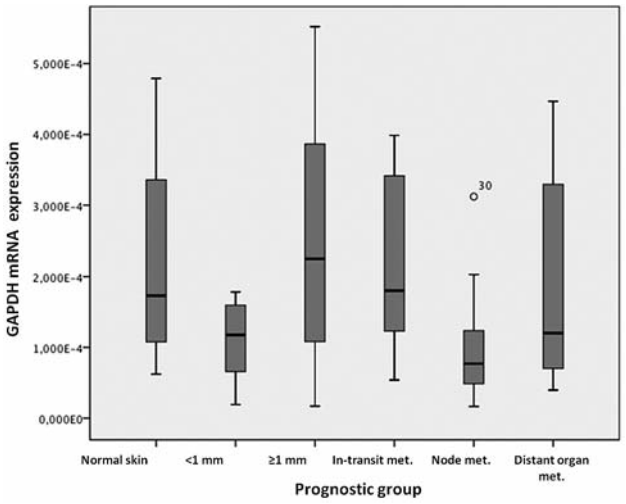

Figure 1. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels of median values in the different groups of primary and metastatic melanoma patients: Normal skin, non-tumoral; <1 mm, thin primary melanomas; ≥1 mm, thick melanomas; in-transit met., in-transit metastatic cases; node met., lymph node metastatic cases; distant organ met., melanomas metastatic to distant organs. Box plots with the median and range of the Ct values, the 25 and 75% percentiles, and outliers.*

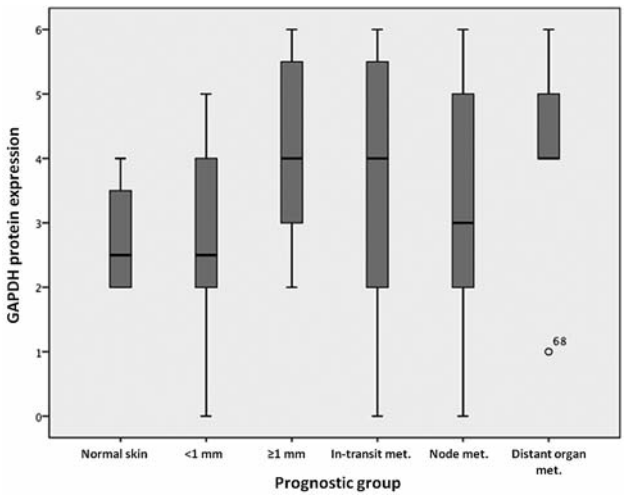

Figure 2. *Cytoplasmic immunoexpression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein in the different groups of patients with primary and metastatic melanoma: Normal skin, non-tumoral; <1 mm, thin primary melanomas; ≥1 mm, thick melanomas; in-transit met., in-transit metastatic cases; node met., lymph node metastatic cases; distant organ met., melanomas metastatic to distant organs. Box plots and the median and range of values of the immunohistochemical protein expression ranging between 0 and 6 are shown.*

account. On the contrary, in our study, nuclear protein immunoexpression did not show any correlation with mRNA gene expression. The differences found between qRT-PCR and immunohistochemistry could be related to the presence of a variable percentage of contaminating non-tumor cells in tumor samples, especially with lymphocytes in lymph-node metastatic melanomas.

GAPDH mRNA levels showed some differences among the different predictive groups of patients with melanoma (primary tumors with Breslow index ≤1 mm; primary tumors with Breslow index >1 mm; in transit metastases; lymph node metastases, and distant organ metastases) as shown in Figure 1. Interestingly, the strongest differences were found between thick primary melanomas *vs*. lymph node metastastic cases (p=0.041); thick primary melanomas plus in-transit metastases, when compared simultaneously to lymph node melanoma metastases (p=0.011); and, finally, in-transit *vs*. lymph node metastases (p=0.017).

GAPDH immunoexpression demonstrated that many cases presented with moderate or intense staining within each predictive group of patients with melanoma, although the vast majority of cases with a GAPDH immunohistochemical score of between 4 and 6 (high protein expression level) were recorded in the groups of patients with thick primary melanomas, in-transit metastases, and distant organ metastases. The strongest differences were observed among the following prognostic groups: thin *vs*. thick primary melanomas (p=0.005); thin melanomas *vs*. in-transit metastases (p=0.003); thin melanomas *vs*. distant metastases (p=0.009); thick melanomas *vs*. lymph node metastases (p=0.023); in-transit *vs*. lymph node metastases (p=0.019); and, finally, between lymph node *vs*. distant metastases (p=0.037), as shown in detail in Figures 2 and 3.

No point-mutations were encountered in any of the 45 cases analyzed by means of sequencing analysis of the specific exon 7 within the *GAPDH* gene.

Discussion

Understanding the molecular mechanisms that facilitate neoplastic dissemination in the carcinogenetic process is an essential step towards predicting and therapeutically-preventing the metastatic process, which is responsible for the high mortality rates associated with cutaneous melanoma (21, 23).

GAPDH constitutes an extremely abundant and multi-functional glycolytic enzyme that is also considered to be a major intracellular messenger mediating apoptotic cell death (5-7). Knockdown of *GAPDH* using antisense strategies demonstrated its role in apoptotic processes (7). Recent investigations indicated that GAPDH mediates a novel cell death cascade, namely cytotoxic stimuli, *via* nitric oxide generation, leading to the binding of GAPDH to the protein SIAH1 (Seven in Absentia Homolog protein 1), translocation of GAPDH–SIAH1 to the nucleus, and ultimately cell death

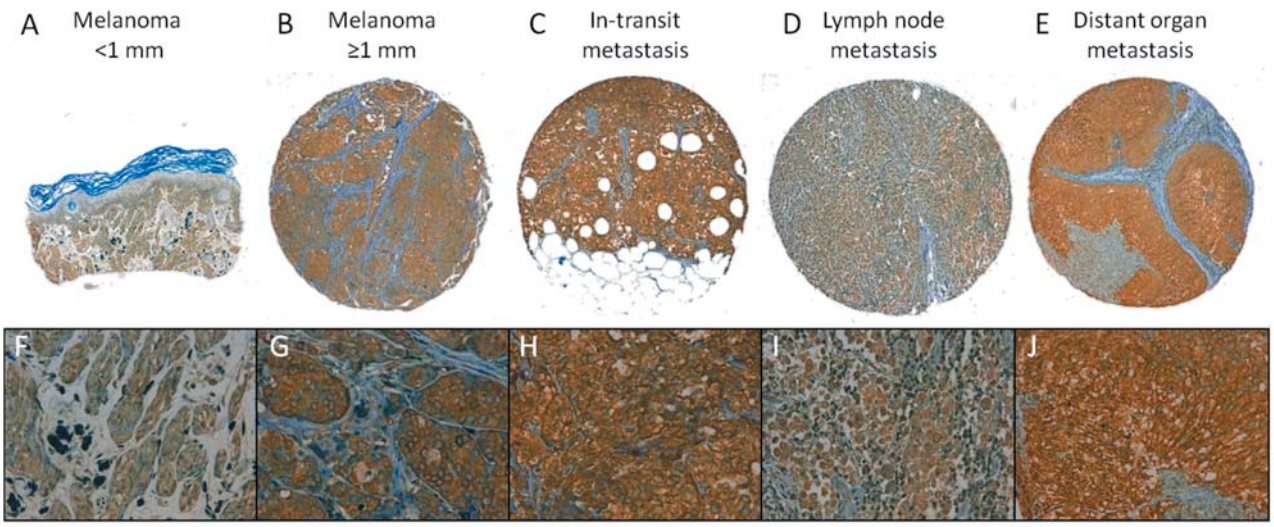

Figure 3. *Tissue microarrays of selected cases illustrating immunoexpression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each stage of melanoma progression. A-E: ×100; F-J: ×400.*

(6). Our cases frequently demonstrated high levels of GAPDH mRNA expression through all predictive groups of melanoma (primary and metastatic), together with the demonstration of positive cytoplasmic and nuclear GAPDH protein expression at the immunohistochemical level. As mentioned above, GAPDH plays a significant role in multiple membrane, cytoplasmic and nuclear pathways such as endocytosis, mRNA regulation, tRNA export, transcriptional control of histone expression, nuclear membrane fusion, recognition of fraudulently incorporated nucleotides in DNA, and the maintenance of telomere structure (4, 5). Interestingly, the interaction of GAPDH with disease-related proteins and drugs suggests that it could be considered a molecular target for drug development (7).

In diverse tumor models, glycolytic activity in the tumor cell population has been postulated to be dramatically enhanced in comparison with normal tissues. In this regard, Otto Warburg first described that some tumors possess an abnormally high metabolic rate of aerobic metabolism when compared to non-tumor tissues, so determining the so-called 'Warburg phenotype' of cancer cells (24), as well as its close relationship with the proliferative rate of tumor cells (25). Likewise, Ganapathy-Kanniappan *et al*. showed an increase of GAPDH expression in progression of murine hepatocarcinoma, so that this molecule could be considered as a probable target for future target therapies (25). Our results revealed a significant deregulation of GAPDH protein and mRNA in the progression of melanoma in patients through primary and metastatic disease; this fact also seems to support the hypothesis of an enhancement or loss of aerobic glycolytic pathways could predict the biological aggressiveness of the disease, especially when comparing thin with thick primary cutaneous melanomas.

In the literature, GAPDH was found overexpressed in breast, lung, pancreatic, and kidney carcinomas in comparison to normal tissue (15-18). In breast cancer, GAPDH levels were found to inversely correlate with patient age, oestrogen/progesterone receptor status, overall survival, and relapse-free survival, and was positively correlated with the histo-prognostic grading, cancer cell proliferation and tumor aggressiveness (15). Conversely, cutaneous melanomas appear to behave differently from carcinomas. In fact, according to a DNA microarray study, which included primary nodular cutaneous melanomas (two cases), melanoma metastases (two cases), and melanocytic nevi (four cases), GAPDH expression was slightly lower in melanomas when compared to melanocytic nevi (19). The latter study, which to our knowledge is one of the few reports focused specifically on GAPDH expression in human malignant melanoma, did not include thin primary melanomas and the number of cases was very limited. The unique study of Falkenius *et al*. indicates that higher mRNA and protein GAPDH expression levels correlate with worse survival in patients with stage III melanomas (26). In the current study, up-regulation of GAPDH protein and mRNA expression in melanoma metastases in comparison to thin primary melanomas suggests that low GAPDH levels (down-regulation of glycolytic activity in tumor cells) found in thin melanomas and melanocytic nevi might be a protective factor against metastatic dissemination of melanocytic tumor cells. Conversely, high levels of GAPDH activity probably mean an increase in the provision of energy and higher risk for tumor dissemination.

Distinct mechanisms by which GAPDH enhances the metastatic ability of melanoma tumor cells have been also described. Firstly, GAPDH plays a significant role in the

apoptotic cascade, and, therefore, high levels of this molecule may be responsible for a high cell death rate, which might then restrict tumor growth (8-9), although our results do not support this point. Secondly, GAPDH binds to Nm23-H1/Nucleoside Diphosphate Kinase A (27, 28), the first putative metastasis-suppressor gene, which was originally identified in murine melanoma cells (29) and is highly expressed in non-metastatic tumor cells in breast and ovarian carcinoma and melanoma (29, 30). In this regard, GAPDH binding to NM23-H1 activates histidine kinase function of NM23-H1 (28) and underlies its metastasis-suppressive effect (31, 32). In a large series of human melanomas, McDermott *et al*. found that strong immunoreactivity for NM23-H1 correlated positively with survival and inversely with indicators of poor prognosis (33). Other authors found reduced or lack of NM23-H1 immunostaining in thick primary melanomas and melanoma metastases (34). Moreover, such immunoreactivity was a predictor for cerebral metastasis in patients with primary melanomas (35). Therefore, high GAPDH levels may be associated with high histidine kinase activity of NM23-H1 and may suppress or reduce the metastatic potential of melanoma cells in thin primary tumors. However, our results indicate that up-regulation of *GAPDH* mRNA and, in particular, protein overexpression of cytoplasmic GAPDH, may serve as an indicator of potential aggressiveness in primary and metastatic melanoma due to the activation of glycolytic enzymes in tumor cells required for cancer cell growth and high proliferative rates in the progression of the disease. In contrast, nuclear translocation of GAPDH demonstrated by means of immunohistochemistry did not show any statistical correlation with any of the predictive groups analyzed in our series, which probably indicates a minor role of GAPDH in apoptotic deregulation or other nuclear functions.

Aerts *et al*. demonstrated that *GAPDH* was the housekeeping gene with the highest variability in mRNA expression techniques in diverse tumor models (36). Accordingly, the demonstration of remarkable overexpression of GAPDH mRNA in our patients as the disease progresses does not recommend the use of this molecule as a control gene in methodologies based on real-time PCR methods.

Intriguingly, mutated *GAPDH* (G- to-A substitution in codon 175) was identified as a human melanoma tumor antigen since it was recognized in intratumoral lymphocytes in a patient with metastatic melanoma (20). The mutated GAPDH epitope stimulates the release of high levels of interferon-γ from tumor-infiltrating 2098 T-cells (20). Zhou *et al*. suggested that the GAPDH molecule may be involved in tumor development and T-cell recognition (20). Unfortunately, no point-mutations were found in our cases of primary and metastatic melanomas in exon 7 of *GAPDH*. The frequency, clinical significance, and diagnostic value of *GAPDH* mutations in cutaneous malignant melanomas require further investigation in larger series.

In conclusion, our results appear to invalidate GAPDH as an appropriate housekeeping gene for mRNA expression studies in cutaneous malignant melanoma and suggest that in patients with melanoma, this ubiquitous molecule may facilitate tumor progression and metastatic dissemination of tumor cells by increasing glycolytic metabolism. Moreover, up-regulation of GAPDH protein and mRNA expression in thick primary and metastatic cases when compared to thin primary melanomas, together with the differences encountered between thick and thin melanomas, supports the concept that low GAPDH levels could be a protective or limiting factor against metastatic dissemination of skin melanoma (except for lymph node metastases; in which contamination with lymphocytes could explain mRNA down-regulation). Therefore, GAPDH might be considered a useful prognostic marker and could be taken into account in future strategies as a potential therapeutic target in patients with cutaneous melanoma. However, these results should be validated in a larger series of patients and other functional studies.

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Conflicts of Interest

None declared.

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References

1 Thellin O, Zorzi W, Lakaye B. De Borman B, Coumans B, Hennen G, Grisar T, Igout A and Heinen E: Housekeeping genes as internal standards: use and limits. J Biotechnol *75(2-3)*: 291-295, 1999.

2 Goidin D, Mamessier A, Staquet MJ, Schmitt D and Berthier-Vergnes O: Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and β-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem *295(1)*: 17-21, 2001.

3 Bas A, Forsberg G, Hammarstrom S and Hammarstrom ML: Utility of the housekeeping genes 18S rRNA, β-actin and glyceraldehyde-3-phosphate dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. Scand J Immunol *59(6)*: 566-573, 2004.

4 Sirover MA: Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in normal cell function and cell pathology. J Cell Biochem *66(2)*: 133-140, 1997.

5 Sirover MA: New nuclear functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells. J Cell Biochem *95(1)*: 45-52, 2005.
6 Sedlak TW and Snyder SH: Messenger molecules and cell death: therapeutic implications. JAMA *295(1)*: 81-89, 2006.
7 Chuang DM, Hough C and Senatorov VV: Glyceraldehyde-3-phosphate dehydrogenase, apoptosis, and neurodegenerative diseases. Ann Rev Pharmacol Toxicol *45*: 269-290, 2005.
8 Colell A, Green DR and Ricci JE: Novel roles for GAPDH in cell death and carcinogenesis. Cell Death Differ *16(12)*: 1573-1581, 2009.
9 Tristan C, Shahani N, Sedlak TW and Sawa A: The diverse functions of GAPDH: views from different subcellular compartments. Cell Signal *23(2)*: 317-323, 2011.
10 Goldsworthy SM, Goldsworthy TL, Sprankle CS and Butterworth BE: Variation in expression of genes used for normalization of northern blots after induction of cell proliferation. Cell Prolif *26(6)*: 511-518, 1993.
11 Bustin SA: Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. J Mol Endocrinol *29(1)*: 23-39, 2002.
12 Warburg O: On the origin of cancer cells. Science *23(3191)*: 309-314, 1956.
13 Warburg O: On respiratory impairment in cancer cells. Science *124(3215)*: 269-270, 1956.
14 Giricz O, Lauer-Fields JL and Fields GB: The normalization of gene expression data in melanoma: investigating the use of glyceraldahyde 3-phosphate dehydrogenase and 18S ribosomal RNA as internal reference genes for quantitative real-time PCR. Anal Biochem *380(1)*: 137-139, 2008.
15 Revillion F, Pawlowski V and Hornez L: Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. Eur J Cancer *36(8)*: 1038-1042, 2000.
16 Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K and Sakiyama S: Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res *47(21)*: 5616-5619, 1987.
17 Schek N, Hall BL and Finn OJ: Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in human pancreatic adenocarcinoma. Cancer Res *48(22)*: 6354-6359, 1988.
18 Vila MR, Nicolas A, Morote J and Meseguer A: Increased glyceraldehyde-3-phosphate dehydrogenase expression in renal cell carcinoma identified by RNA-based, arbitrarily primed polymerase chain reaction. Cancer *89(1)*: 152-164, 2000.
19 Seykora JT, Jih D, Elenitsas R, Horng WH and Elder DE: Gene expression profiling of melanocytic lesions. Am J Dermatopathol *25(1)*: 6-11, 2003.
20 Zhou J, Dudley ME, Rosenberg SA and Robbins PF: Persistence of multiple tumor-specific T-cell clones is associated with complete tumor regression in a melanoma patient receiving adoptive cell transfer therapy. J Immunother *28(1)*: 53-62, 2005.
21 Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG, Fleming ID, Gershenwald JE, Houghton A Jr, Kirkwood JM, McMasters KM, Mihm MF, Morton DL, Reintgen DS, Ross MI, Sober A, Thompson JA and Thompson JF: Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. J Clin Oncol *19(16)*: 3635-3648, 2001.
21 Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, Buzaid AC, Cochran AJ, Coit DG, Ding S, Eggermont AM, Flaherty KT, Gimotty PA, Kirkwood JM, McMasters KM, Mihm MC Jr., Morton DL, Ross MI, Sober AJ and Sondak VK: Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol *27(36)*: 6199-6206, 2009.
22 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods *25(4)*: 402-408, 2001.
23 Thompson JF, Scolier RA and Kefford RF. Cutaneous melanoma. Lancet *365(9460)*: 687-701, 2005.
24 Warburg O, Wind F and Negelein E: The metabolism of tumors in the body. J Gen Physiol *8(6)*: 519-530, 1927.
25 Vander Heiden MG, Cantley LC and Thompson CB: Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science *324(5930)*: 1029-1030, 2009.
26 Falkenius J, Lundberg J, Johansson H, Frostvik-Stolt, J. Lundeberg, Hansson J and Egyhazi S: A gene expression profile associated with clinical outcome in stage III melanoma. J Clin Oncol *29(suppl; abstr)*: 8551, 2011.
27 Engel M, Seifert M, Theisinger B, Seyfert U and Welter C: Glyceraldehyde-3-phosphate dehydrogenase and Nm23-H1/nucleoside diphosphate kinase A. Two old enzymes combine for the novel Nm23 protein phosphotransferase function. J Biol Chem *273(32)*: 20058-20065, 1998.
28 Engel M, Mazurek S, Eigenbrodt E and Welter C: Phosphoglycerate mutase-derived polypeptide inhibits glycolytic flux and induces cell growth arrest in tumor cell lines. J Biol Chem *279(34)*: 35803-35812, 2004.
29 Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA and Sobel ME: Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst *80(3)*: 200-204, 1998.
30 Hartsough MT and Steeg PS: Nm23/nucleoside diphosphate kinase in human cancers. J Bioenerg Biomembr *32(3)*: 301-308, 2000.
31 Hartsough MT, Morrison DK, Salerno M, Palmieri D, Ouatas T, Mair M, Patrick J and Steeg PS: Nm23-H1 metastasis suppressor phosphorylation of kinase suppressor of Ras *via* a histidine protein kinase pathway. J Biol Chem *277(35)*: 32389-32399, 2002.
32 Ouatas T, Salerno M, Palmieri D and Steeg PS: Basic and translational advances in cancer metastasis: Nm23. J Bioenerg Biomembr *35(1)*: 73-79, 2003.
33 McDermott NC, Milburn C, Curran B, Kay EW, Barry Walsh C and Leader MB: Immunohistochemical expression of NM23 in primary invasive malignant melanoma is predictive of survival outcome. J Pathol *190(2)*: 157-162, 2000.
34 Lee CS, Pirdas A and Lee MW: Immunohistochemical demonstration of the NM23-H1 gene product in human malignant melanoma and Spitz nevi. Pathology *28(3)*: 220-224, 1996.
35 Sarris M, Scolyer RA, Konopka M, Thompson JF, Harper CG and Lee CS: Cytoplasmic expression of NM23 predicts the potential for cerebral metastasis in patients with primary cutaneous melanoma. Melanoma Res *14(1)*: 23-27, 2004.
36 Aerts JL, Gonzales MI, Topalian SL, Chinnasamy N, Morgan RA and Topalian SL: Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR. Biotechniques *36(1)*: 84-91, 2004.

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