Association of insulin-like growth factor binding protein-3 expression with melanoma progression

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Abstract

Previous studies from our laboratory have identified several endothelial-associated marker genes implicated in human melanoma metastasis via tumor vasculogenic mimicry. In this study, we used dual model systems composed of melanoma cell lines and clinical melanoma samples to validate the importance of insulin-like growth factor binding protein-3 (IGFBP-3) as a marker involved in disease progression. Gene expression analysis was done using a microarray approach for both primary and metastatic melanoma samples. The expression of IGFBP-3 was decreased using a small interfering RNA (siRNA) knockdown approach and quantified with real-time quantitative reverse transcription-PCR analysis. The expression of insulin-like growth factor binding protein 3 (IGFBP-3) was up-regulated by nearly 20-fold in WM266-4 compared with WM35 cells. A subsequent parallel analysis using freshly isolated primary and metastatic melanoma cell samples and melanoma tissue array confirmed the previous findings. The functional significance of IGFBP-3 in melanoma invasion was further investigated using a siRNA gene knockdown approach, with the expression of IGFBP-3 markedly reduced. Additionally, siRNA knockdown resulted in a significant reduction in cell motility, migration, and invasive capacity of WM266-4 cells in vitro. These results strongly suggest that IGFBP-3 expression may be a vital cell motility, migration, and proliferation factor necessary for melanoma metastasis and is an important biomarker in human melanoma. [Mol Cancer Ther 2006;5(12):3078–84]

Introduction

The incidence of melanoma in the United States is rapidly increasing, resulting in an epidemic of newly diagnosed early and advanced patients with melanoma. Although the 5-year survival rate is >90% for early-stage melanoma, the rate decreases significantly with advanced disease. Recent efforts have focused on the discovery of genes that may play a central role in melanoma progression, by using technology such as high-throughput gene expression analysis (1–4). This approach has helped to identify numerous potential disease progression markers in both in vitro and in vivo melanoma model systems (1). Previously, we used an unbiased expression analysis approach to identify several endothelial-associated marker genes that were overexpressed in metastatic melanoma cells (1). Among these endothelial-associated genes, insulin-like growth factor binding protein-3 (IGFBP-3) was discovered to be a candidate gene owing to its key role in cell proliferation and metastasis (5). IGFBP-3 is overexpressed in lymph node–derived metastatic melanoma Hs.688(B) compared with primary Hs.688(A) cells. IGFBP-3 has also been shown to be expressed in several other human cancers, such as osteosarcoma, renal cell carcinoma, and breast and esophageal cancers (6–9). IGFBP-3 is one of the six members of the IGFBP family binding to the IGF-I and IGF-II proteins with higher affinity than the IGF receptor (10, 11). IGFBP-3, with a posttranslational molecular weight of 43 to 45 kDa, has also been shown to modulate IGF activity as its carrier in the cellular microenvironment (12).

Although IGFBP-3 has been shown to regulate cell proliferation through IGF-dependent and IGF-independent mechanisms, little is known about its expression and function in malignancy (5, 13–19). Depending on the cellular context of different experimental models, IGFBP-3 has been shown to have growth-stimulatory properties in certain tumors, whereas it has an antiproliferative effect in other cases (20–22).

In an attempt to validate some of the previously discovered endothelial-associated marker genes in human melanoma while gaining insight into how IGFBP-3 expression affects cell proliferation and metastatic potential, we did gene expression analysis experiments using early primary (WM35) and metastatic (WM266-4) melanoma cell lines. The WM35 line was established from a primary superficial spreading melanoma in radial growth phase, with the WM266-4 cell line derived from a patient with metastatic melanoma. We discovered that IGFBP-3 was highly overexpressed in metastatic WM266-4 cells compared with early primary WM35 cells. We also found that the down-regulation of IGFBP-3 using RNA interference approach significantly reduced the invasion potential of WM266-4 cells in vitro. This is the first report to show that IGFBP-3 may play a key role in modulating melanoma metastasis and may prove to be a potential prognostic marker for melanoma progression. It may also become a good candidate gene for targeted drug therapy for melanoma.
Materials and Methods

Cell Culture and Clinical Samples

WM35 and WM266-4 cell lines were purchased from Wistar Institute/American Type Culture Collection (Manassas, VA). WM35 and WM266-4 are human melanoma cell lines derived from early primary and metastatic melanoma. WM35 cells were maintained in 2% tumor medium containing a 4:1 mixture of MCD 153 medium with 1.5 g/L sodium bicarbonate and Leibovitz’s L-15 medium with 2 mmol/L l-glutamine supplemented with 0.005 mg/mL bovine insulin, 1.68 mmol/L CaCl₂, and 2% fetal bovine serum. WM266-4 cells were cultured at 37°C in a humidified atmosphere of 5% CO₂, in DMEM (Life Technologies, San Diego, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). For the IGFBP-3 expression analysis experiments, WM-35 cells were grown in either the cell maintenance medium or switched to the same medium condition as the WM266-4 cells.

Freshly procured melanoma samples were obtained at the time of definitive surgical resection by a single surgeon and a portion was macromdesectioned from the surrounding tissue and snap-frozen in liquid nitrogen. The sample set was comprised of primary cutaneous melanoma samples (n = 14) and metastatic melanoma samples (n = 30), all obtained with written patient consent from the H. Lee Moffitt Cancer Center and Research Institute with full approval by the institutional review board.

RNA Isolation and cDNA Synthesis

TRIzol reagent (Invitrogen, San Diego, CA) was used to isolate total RNA from cultured cells. RNA was treated with DNase I (Promega, Madison, WI). The integrity of total RNA was determined by 1% formaldehyde-agarose gel electrophoresis. cDNA synthesis was carried out with the cDNA synthesis kit (Applied Biosystems, Inc., Foster City, CA) by using 1 μg of total RNA as the template and random primers.

Quantitative Gene Expression Analysis via Real-time Quantitative Reverse Transcription-PCR

Real-time quantitative reverse transcription-PCR (qRT-PCR) analysis was done on the experimental mRNAs. The PCR primers and probes for insulin-like growth factor binding protein 3 (IGFBP-3), and internal control gene GAPDH were purchased from Applied Biosystems. qRT-PCR was done on an ABI 7500HT instrument under the following conditions: 25°C, 10 min and 37°C, 30 min of reverse transcription; 95°C, 10 min; 95°C, 15 s; 60°C, 1 min. The reaction was done for up to 40 cycles (n = 3).

Gene Expression Analysis via Microarray

Gene expression analysis based on both primary and metastatic melanoma patient samples were done by using the U133-plus Affymetrix GeneChip based on the manufacturer’s protocol. Gene expression analysis was carried out on GeneSpring software version 7.2, which allows multiplex comparisons of data from different experiments and resulting in global normalization, generation of restriction lists, and the functional classification of the differentially expressed genes. A further data filtration supervised analysis was used to exclude all genes with a <2-fold change. The expression profiles of the different groups were compared using one-way ANOVA (lower significance cutoff P < 0.01).

Small Interfering RNA Design and Knockdown

Small interfering RNA (siRNA) molecules were purchased from Dharmacon Research (Lafayette, CO), including IGFBP-3, positive control (Lamin A/C), and mismatch control. The sequences for siRNAs are as follows:

IGFBP-3 siRNA: 5'-GCACAGAUACCAGACUUUUAUU-3' and 5'-UUCGUGUCAUUGGUCUGAA-5'.

Mismatch siRNA: 5'-GCAAGAGACCCGAGAAUUUUU-3' and 3'-UUCGUUUCUGGCUCUUUA-5' (bold letters, mismatch).

Oligofectamine (Invitrogen)–mediated transfection of siRNA was carried out in six-well tissue culture plates according to the manufacturer’s instructions. Transfection mixtures containing 100, 200, or 400 nmol/L siRNA and 4 μL of Oligofectamine in 200 μL of Opti-MEM (Invitrogen) were directly added to 2 × 10⁵/mL of preincubated WM266-4 cells in 800 μL of Opti-MEM. Then, cells were incubated for 4 h and further cultured in DMEM supplemented with 10% fetal bovine serum (n = 3). After 48-h transfection, cells were harvested and subjected to the invasion assay, qRT-PCR, and Western immunoblot analysis.

Western Immunoblot Analysis

Western immunoblot analysis was used to characterize IGFBP-3 expression in WM266-4 cells after gene knockdown by RNA interference. Equal amounts (10 μg) of samples were resolved by SDS-PAGE on 10% gels by the method of Laemmli (23). Proteins were probed with mouse anti-IGFBP-3 monoclonal antibody (1:200 dilution; BD Biosciences, San Diego, CA), β-actin (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with a horseradish peroxidase–conjugated secondary antibody (1:1,000 dilution, Bio-Rad, Hercules, CA). Proteins were visualized with a chemiluminescence detection system using the Super Signal substrate (Pierce, Rockford, IL).

Tumor Invasion Assay

The in vitro invasion assay was done by using a cell invasion assay kit based on the manufacturer’s protocol (Chemicon, Billerica, MA) as previously described (24). WM266-4 cells at 2 × 10⁵/mL were treated with 100 nmol/L siRNA for 48 h (n = 3). Control and siRNA-treated cells were trypsinized and used for the invasion assay. In brief, the Chemicon Cell Invasion Assay was done in an invasion chamber, based on the Boyden chamber principle. Each kit contains 24 inserts; each insert contains an 8-μm-pore polycarbonate membrane coated with a thin layer of polymerized collagen. The collagen layer occludes the membrane pores, blocking noninvasive cells from migrating through. Invasive cells, on the other hand, migrate through the polymerized collagen layer and cling to the bottom of the polycarbonate membrane. Invaded cells on the bottom of the insert membrane were incubated with Cell Stain Solution, then subsequently extracted and detected on a standard microplate reader. Cells (2 × 10⁵) suspended in
serum-free DMEM were placed in the individual inserts. Lower chambers were filled with DMEM containing 10% fetal bovine serum. After incubation for 48 h at 37°C, cells that had penetrated the membrane were detached, lysed, and stained by provided buffers. The mixture was read by fluorescence plate reader using 480/520-nm filter set (Bio-Rad).

Scratch-Wound Assay

To evaluate the effect of IGFBP-3 on cell motility and migration, a scratch-wound assay (25) was done on WM266-4 cells transfected with IGFBP-3–specific siRNA, random control siRNA, or transfection vehicle control. The expression of IGFBP-3 was knocked down using IGFBP-3–specific siRNA as described above. Monolayer cells were scraped with a pipette tip to generate a scratch wound after cells reached confluence. The wounded surface was washed with 1× PBS and incubated in DMEM with 10% fetal bovine serum. A series of time frames (2, 4, 8, 12, and 24 h) of migration pattern at the wound edge were monitored by phase microscopy using an Axiovert 200M microscope with digital camera (Carl Zeiss, Thornwood, NY). The images were captured by AxioVision 4.0 imaging software (Carl Zeiss). The closure of the initial gap area was assessed by calculating the difference between the initial and the remaining wound area at each time point. Cell numbers from three separate image fields were counted within the gap area at 24 h. The average cell number from the control wells was used as the denominator to calculate the percentage inhibition in cell migration.

Immunohistochemistry

Formalin-fixed, paraffin-embedded melanoma tissue array (Biomax, Inc., Rockville, MD) was deparaffinized with xylene and gradually hydrated. The endogenous peroxidase was quenched by 3% hydrogen peroxide solution for 6 min. After careful washing with 1× PBS, slides were blocked with 1:50 goat serum for 30 min. Slides were incubated with primary antibody against IGFBP-3 (1:100; Santa Cruz Biotechnology) overnight at 4°C. After washing with 1× PBS thrice, the slides were incubated with biotin-conjugated secondary antibody for 30 min and streptavidin peroxidase for another 30 min. 3,3′-Diaminobenzidine substrate was added onto the slides for 10 min; the tissue staining images were acquired using Zeiss Axiovert 200M microscope and processed with AxioVision 4.0 imaging software (Carl Zeiss).

Statistical Analysis

Statistical studies were done using MedCalc for Windows, version 8.1.1.0 (MedCalc Software, Mariakerke, Belgium). The statistical differences of IGFBP-3 expression level between the primary and metastatic melanoma samples were calculated by Wilcoxon rank test with two-tailed probability. Values were considered significant if the P value was <0.05.

Results

Characterization of Endothelial Marker Genes by Using a Melanoma Cell Line Model

In this study, we chose two melanoma cell lines derived from patients with different stages of disease progression: early primary and metastasis as our model system. IGFBP-3 was selected based on our previous data (1) and was profiled and analyzed in WM35 and WM266-4 cells by using real-time qRT-PCR analysis. The results are shown in Fig. 1A, with the expression of IGFBP-3 up-regulated ~16-fold in metastatic WM266-4 cells. The expression of IGFBP-3 at the protein level was confirmed by Western immunoblot analysis and was found to be consistent with the qRT-PCR analysis (n = 3; Fig. 1B).

Expression analysis of IGFBP-3 using human whole-genome microarray. The average expression level of IGFBP-3 in metastatic melanoma patient samples (n = 30) was significantly higher (Wilcoxon test with two-tailed probability, P = 0.0005; 95% confidence interval for the median, 0.7592–2.5170) than primary melanoma patient samples (n = 14; 95% confidence interval for the median, 0.4005–0.9653).

Figure 1. A, qRT-PCR analysis was done in WM35 and WM266-4 cells for IGFBP-3. Data were analyzed by normalizing with the internal control gene, GAPDH. Columns, mean (n = 3); bars, SE. B, IGFBP-3 protein expression analysis using Western immunoblot analysis. β-Actin was used as a loading control.

Figure 2. Expression analysis of IGFBP-3 using human whole-genome microarray. The average expression level of IGFBP-3 in metastatic melanoma patient samples (n = 30) was significantly higher (Wilcoxon test with two-tailed probability, P = 0.0005; 95% confidence interval for the median, 0.7592–2.5170) than primary melanoma patient samples (n = 14; 95% confidence interval for the median, 0.4005–0.9653).
Overexpression of IGFBP-3 in Metastatic Melanoma

Gene expression analysis using high-throughput microarray profiling from primary and metastatic melanoma patient samples further confirmed the in vitro cell line results. The median expression level of IGFBP-3 in primary melanoma samples was 0.48 versus 1.38 in metastatic melanoma samples ($P = 0.0005$; Fig. 2).

Overexpression of IGFBP-3 by Using Human Melanoma Tissue Array

To further understand the significance of IGFBP-3 overexpression in melanoma, an immunohistochemical analysis was done using a human melanoma tissue array containing 80 primary and metastatic specimens. We selected 23 metastatic and 18 primary melanoma tissues for quantification of IGFBP-3 staining intensity. Negatively stained samples for IGFBP-3 were defined as having a low expression and samples with positive staining signals were treated as overexpressing IGFBP-3. Our results indicate that the IGFBP-3 expression was highly overexpressed in $>25\%$ (6 of 23) of the metastatic melanoma samples (Fig. 3B) compared with only $5\%$ (1 of 18) of the primary melanoma samples (Fig. 3C).

Decreasing IGFBP-3 Expression by Using siRNA

To understand the biological significance of IGFBP-3 overexpression in melanoma, we used a gene knockdown approach with siRNA created against IGFBP-3. We transfected cultured WM266-4 cells with siRNA molecules at three different concentrations (100, 200, and 400 nmol/L). Positive control siRNA against Lamin A/C was used to monitor knockdown efficiency (data not shown). The knockdown of IGFBP-3 expression was confirmed by real-time qRT-PCR (Fig. 4A) and Western immunoblot analysis (Fig. 4B). The protein level of IGFBP-3 became undetectable after siRNA treatment at concentrations of 100 nmol/L (lane 3), 200 nmol/L (lane 4), and 400 nmol/L (lane 5; Fig. 4B).

Down-Regulation of IGFBP-3 Results in Decreased Melanoma Tumor Invasion

To investigate the effect of IGFBP-3 on the capacity for WM266-4 cell invasion, we carried out the invasion assay in the presence and absence of siRNA knockdown for IGFBP-3. Our results show that decreasing IGFBP-3 gene expression significantly impaired the invasive capacity of WM266-4 cells in vitro by $>50\%$ compared with control samples (Fig. 5).
Down-Regulation of IGFBP-3 Results in Decreased Melanoma Cell Migration

To further evaluate the effect of IGFBP-3 on cell motility and migration ability, a scratch-wound migration assay was used in the presence and absence of siRNA knockdown for IGFBP-3. The total average number of cells within the gap area of the control wells was used as denominator to calculate the percentage inhibition on migratory capability. The average cell number (201 ± 8 cells) in the control sample was set as 100%. Mismatch controls (195 ± 10 cells) were found to be 94 ± 2% and the IGFBP-3 knockdown samples (10 ± 3 cells) were 4.5 ± 1.5% compared with controls. Thus, our results show that decreasing IGFBP-3 gene expression significantly impairs the motility and migration capacity of WM266-4 cells in vitro by ~95% compared with control samples (Fig. 6).

Discussion

It is hypothesized by many that the complex process of tumor cell invasion and metastasis is governed by a sequential, multistep process regulated by a key set of genes. In addition, it is the local tumor cell microenvironment that contributes an essential influence on this process of metastasis. Unbiased gene expression analysis has allowed us to better identify such marker genes by using different melanoma cell lines as a model in vitro cell system (1). IGFBP-3 was found to be overexpressed in the metastatic melanoma cell line Hs688(B) compared with the primary melanoma cell line Hs.688(A) (1). In this study, we further confirmed, by using an early primary WM35 and metastatic WM266-4 melanoma cell line model, that IGFBP-3 was deregulated. The functional significance of IGFBP-3 in melanoma progression was elucidated in detail by various in vitro studies. Based on qRT-PCR analysis, IGFBP-3 was elevated at both the mRNA transcript and protein levels in metastatic WM266-4 cells (Fig. 1A and B). We speculate that IGFBP-3 may play a central role in melanoma progression. We also previously showed that IGFBP-3 is overexpressed in esophageal cells and is regulated by epidermal growth factor receptor. IGFBP-3 has also been described as being up-regulated in pancreatic cancer in parallel with the oncogene c-Met; both are overexpressed in nonmetastatic pancreatic endocrine neoplasms. In addition, IGFBP-3 was overexpressed in metastatic sites, such as the lymph nodes (86%) and liver (100%; ref. 26).

Our findings seem to be consistent with other published reports on the described hypothetical role for IGFBP-3 overexpression in melanoma progression. The in vivo significance of IGFBP-3 overexpression was further confirmed by using both primary and metastatic melanoma patient samples, with the median expression level of IGFBP-3 in metastatic samples significantly higher compared with primary melanoma samples (P = 0.0005; Fig. 2). The immunohistochemical results of IGFBP-3 expression is consistent with the mRNA expression analysis using a melanoma tissue array (Fig. 3), with the intracellular distribution of IGFBP-3 suggesting that its expression may be through the endocrine pathway.

To further investigate the functional and biological significance of IGFBP-3 overexpression in melanoma, a siRNA gene knockdown approach was used. We found a marked down-regulation of IGFBP-3 gene expression in WM266-4 cells (Fig. 4A–B), with a concomitant reduction
of nearly 50% in tumor invasion capacity using the in vitro invasion assay (Fig. 5). In addition, decreasing IGFBP-3 expression reduced the motility and migration capacity of WM266-4 cells by 95% compared with control cells, further supporting the hypothetical role of IGFBP-3 in melanoma disease progression (Fig. 6).

There are several potential mechanisms by which IGFBP-3 may play a role in melanoma progression and metastatic behavior. One possibility is that IGFBP-3 is able to recruit IGF-I and IGF-II growth factors to the local tumor microenvironment, thereby promoting invasion and metastasis. Previously, others have shown that IGFBP-3 has the capacity to enhance IGF-I production through the interactions of fibrinogen and fibrin in wound healing (27). Enrichment of IGF-I to the wound site by IGFBP-3 was found to stimulate stromal cell function and proliferation. However, our results indicated that the expression of IGF-I and IGF receptor did not change in response to decreased IGFBP-3 expression (data not shown). Therefore, it is unlikely that the effect of IGFBP-3 in melanoma disease progression is directly related to IGF. However, it is reasonable to speculate that such an effect may be mediated through an IGF-independent pathway, such as a transforming growth factor-β-mediated pathway (28). It has been recently shown that in breast cancer, the expression of IGFBP-3 is closely associated with bony metastases derived from primary breast cancer via a transforming growth factor-β-mediated pathway (29).

In conclusion, we have provided evidence that IGFBP-3 may be an important biomarker in the early diagnosis of melanoma, further representing a possible choice for targeted drug therapy for patients with advanced melanoma. Additional studies are necessary to further elucidate the detailed signaling pathway of IGFBP-3 expression and how it is involved in the complex processes of neoplastic proliferation, migration, and invasion.

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References

Figure 6. In vitro cell motility and migration assay. A, transfection vehicle control. B, mismatch siRNA (100 nmol/L) control. C, IGFBP-3–specific siRNA (100 nmol/L) knockdown. Average cells from the defined gap area of the control wells were used as denominator to calculate the percentage inhibition in migration. The average cell number (201 ± 8) in control sample was set up as 100%. Mismatch control (195 ± 10 cells) was 94 ± 2% compared with control and the IGFBP-3 knockdown sample (10 ± 3 cells) was 4.5 ± 1.5% compared with control.


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