Glycoprotein non-metastatic melanoma protein B promotes glioma motility and angiogenesis through the Wnt/ β -catenin signaling pathway

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Abstract

Glioma is a common tumor with high mortality and poor overall survival. However, the regulatory mechanisms of glioma tumorigenesis and glioma cell motility are completely unknown. Here, we investigated the role of glycoprotein non-metastatic melanoma protein B in glioma. The expression of glycoprotein non-metastatic melanoma protein B is observed to be aberrantly regulated in glioma tissues and cells, and high levels of glycoprotein non-metastatic melanoma protein B present an inverse correlation with the survival of glioma patients. Compared with the control, glycoprotein non-metastatic melanoma protein B inhibition significantly retarded the proliferation and migration of human glioma cells. The tube formation ability of HBMECs induced by glioma cells was also remarkably reduced by glycoprotein non-metastatic melanoma protein B silencing. Increased levels of VEGF-C and TEM7 were down-regulated by the suppression of glycoprotein non-metastatic melanoma protein B in glioma cells. Additionally, the activity of MMP-2/3/9 was assessed in glioma cells using Western blotting and gelatin zymography assay; their activities were strongly decreased following the suppression of glycoprotein non-metastatic melanoma protein B. Further studies suggested that canonical Wnt/ β -catenin pathway was activated, but was inactivated by glycoprotein non-metastatic melanoma protein B suppression in glioma cells. In conclusion, we demonstrate that glycoprotein non-metastatic melanoma protein B might be an inducer for glioma and could enhance matrix metalloproteinase activity through Wnt/ β -catenin pathway to contribute to glioma tumorigenesis. This may represent a new understanding for malignant glioma.

Keywords: Glioma, glycoprotein non-metastatic melanoma protein B, tumorigenesis, angiogenesis, Wnt/ β -catenin pathway, matrix metalloproteinases

Experimental Biology and Medicine 2016; 241: 1968–1976. DOI: 10.1177/1535370216654224

Introduction

Glioma is a common tumor, representing 80% of brain tumors. Glioblastoma (GBM) cells have high migratory potential and are capable of invading the surrounding normal brain tissue along blood vessels, resulting in very poor efficiency of completely removing the tumor by surgery, radiotherapy, and other therapeutic strategies.¹ Despite significant improvements in the neurosurgery and radiotherapy/chemotherapy approaches, the prognosis of malignant glioma patients, especially for highly malignant grade IV gliomas, is very poor with high mortality and overall survival of 12 to 15 months.² Hence, the knowledge about mechanisms related to glioma cells motility in the brain is necessary to improve the therapy for human diffuse gliomas.

As a transmembrane glycoprotein, glycoprotein non-metastatic melanoma protein B (GPNMB) is expressed in

bone, the hematopoietic system and various other normal tissues, and is involved in inflammation, cell differentiation, invasion, metastasis of malignant tumors, and other biological processes.^{3,4} Furthermore, GPNMB overexpression has been observed in many cancers, such as metastatic prostate cancer, glioma, and hepatocellular carcinoma.^{5,6} In terms of glioma, GPNMB was recently identified to be aberrantly overexpressed in human glioma tissue.³ As a potential molecular therapeutic target, GPNMB was selected to perform molecular targeting therapy for GPNMB-expressing gliomas by antibody-mediated delivery of cytotoxic agents.³ However, the role and regulatory mechanisms of GPNMB in human glioma tumorigenesis and glioma cell motility are completely unknown.

Angiogenesis is an important physiological process during physical development.⁷ In tumors tumorigenesis,

angiogenesis is implicated in cancer cell growth and metabolism by delivering necessary oxygen and is associated with high rates of proliferation and invasion of cancer cells. Recently, anti-angiogenic agents have been applied for the treatment of cancer.⁸ In GBM pathobiology, angiogenesis also has a significant effect on the highly malignant and metastatic state of glioma. Tumor angiogenesis is regulated by various pro-/anti-angiogenic factors secreted by the tumor cells themselves. Previous studies have indicated that VEGF-C plays key role as an angiogenic factor in GBM vascularization. Additionally, tumor endothelial marker 7 (TEM7) expression has an inverse relationship between microvessel density and patient survival.⁹

Besides angiogenic factors, the signaling pathway is reported to be involved in tumor angiogenesis.^{10,11} In GBM, the function of Wnt/ β -catenin pathway has been described.¹² In this paper, we determined the expression of GPNMB in glioma. Furthermore, we addressed whether and how GPNMB affects tumorigenesis and angiogenesis of gliomas.

Materials and methods

Glioma tissues and cells cultures

Frozen brain tissue was obtained from the First Affiliated Hospital of Xi'an Jiaotong University using a previously described protocol.² The gliomas were classified as grade II, grade III, and grade IV according to WHO criteria. The study was approved by Ethics Committee of First Affiliated Hospital of Xi'an Jiaotong University. All patients have signed the informed consents.

The glioma cell lines U-251 MG, U-87 MG, M059J, U-373 MG were obtained from ATCC. Human brain microvascular endothelial cells (HBMECs) were obtained from Lonza (Allendale, NJ). Cells were seeded in DMEM containing FBS (10%, Invitrogen), penicillin/streptomycin (1%, Gibco/Invitrogen) at 37°C.

Quantitative RT-PCR

The expression of GPNMB, VEGF-C, and TEM7 was measured using qPCR kit (Takara, Dalian, China). All primers were used as follows: GPNMB: (F) 5'-AGAGTCAAGCC CTGACTGGC-3'/(R) 5'-GAAGAGTGGGTTCCTCAGTCA-3'; VEGF-C: (F) 5'-GGCTGGCAACATAACAGAGA-3'/(R) 5'-GTGGCATGCATTGAGTCTTT-3'; TEM7: (F) 5'-GGAGT GGATGGACTATGGCT-3'/(R) 5'-AGGGAGGAGGAGGT AGTGGT-3'; GAPDH: (F) 5'-ACCACAGTCCATGCCATC AC-3'/(R) 5'-ACCACAGTCCATGCCATCAC-3'.

Western blotting

Total proteins and nuclear proteins were isolated from cell lysates, respectively. After separation via SDS-PAGE, equal amounts of proteins were transferred to PVDF. Blots were reacted with primary antibodies to GPNMB (1:1000, Abcam), VEGF-C (1:2000, R&D Systems China), TEM7 (1:1000, Biorbyt), MMP-2, MMP-3, MMP-9 (1:500, ProSci Inc.), β -catenin (1:500, Santa Cruz), β -actin and LaminB1 (1:3000, ABclonal) overnight at 4°C, followed by incubation

with secondary antibodies for 1 h at RT. Blots were measured by ECL (Amersham Pharmacia, NJ).

Cell transfection

Lipofectamine 2000 reagent (Invitrogen) was applied to cell transfection. Cells were transfected with synthesized GPNMB siRNA (5'-AUAUAACAUUUGCGGUGAA-3', Takara, Dalian, China) or negative siRNA for 48 h, followed by subsequent experiments.

Cell proliferation assay

A CCK-8/WST-8 Kit (Bioroot, Shanghai, China) was employed to analyze cell proliferation according to the direction.

Cell migration assay

Cells migration assay was performed with Transwell Boyden chamber (Costar). Briefly, after transfection and culturing for two days, cells were mixed in DMEM medium (growth factor-free), and were plated into the upper chamber (5×10^5 cells/ml). Additionally, 600 μ l DMEM (10% FBS) was filled into the lower chamber. Cells were then cultured in chamber for 4 h, fixed, and stained with ethanol and crystal violet for 15 min, respectively. A cotton swab was used to gently scrape off the non-transmigrated cells. Migrated cells were counted in five fields per chamber.

Cell apoptosis assay

Flow cytometric was performed to determine cell apoptosis. Briefly, transfected cells were seeded in DMEM medium (serum-free) for 16 h. After harvesting with ice-cold PBS, cells were re-suspended with binding buffer. PI (0.6 μ g/ml) and Annexin V-FITC (0.5 μ g/ml) were incubated with 250 μ l cells (5×10^6) for 15 min in the dark, followed by analysis using FACS CaliburTM (Becton Dickinson).

Preparation of CM (conditioned medium)

After 48 h of transfection, cells were re-suspended with fresh medium and cultured in the 96-well plates for 24 h at concentration: 5×10^5 cells/well in a volume of 1 ml. Then the CM was collected and frozen at -80°C for use.

Matrigel assay

Tube formation ability was determined using Matrigel (Chemicon, Temecula, CA). In brief, HBMECs were seeded into Matrigel-coated plates in DMEM without supplements. The conditioned medium was then incubated with HBMECs cells for 12 h, followed by imaging using microscopy. The quantification of tubes formed by cells per field (20 $\times$) was evaluated using four fields and five sample repeats.

Gelatin zymography

The assay was performed as previously described.^{13,14} In brief, equal amounts of proteins from the conditioned media were separated by SDS-PAGE (0.1% gelatin).

After washing with Triton X-100 (2.5%), gels were incubated with substrate buffer (50 mM Tris-HCl [pH 7.5] and 10 mM CaCl_2) at 37°C, followed by staining/destaining and analysis.

Statistical analysis

The results were showed as mean $\pm$ SEM. GraphPad Prism 5 was applied to compare the differences between groups via one way ANOVA. $P < 0.05$ indicates statistically significance. All experiments were performed at least three times.

Results

GNPMB is aberrantly expressed in glioma tissue and cells

To determine whether GPNMB has regulatory role in glioma tumorigenesis, we first determined the expression profile of GPNMB in glioma tissue from patients. As seen in Figure 1(a), GPNMB was maintained at low levels in normal brain tissue ($\Delta\text{Ct} = 15.69 \pm 0.32$) while it was aberrantly up-regulated in glioma tissue samples with a gradual increase in metastatic ability from grade II to IV GBM ($\Delta\text{Ct} = 9.18 \pm 0.39$; $\Delta\text{Ct} = 6.16 \pm 0.26$; $\Delta\text{Ct} = 3.25 \pm 0.15$). Notably, high levels of GPNMB presented a positive relationship with poor prognosis and short survival time in glioma patients, as shown in Figure 1(b).

Furthermore, we measured GPNMB levels in glioma cells. The GPNMB (approximately 100 kDa) was highly expressed in glioma cells in comparison with normal human astrocytes (ScienCell, San Diego, CA) (Figure 1c, d). Additionally, we detected two weakly immunoreactive bands of 55 and 75 kDa in glioma cell lysates (Supplementary Figure S1), which are consistent with those reported previously and might imply the different glycosylation forms of GPNMB in glioma cells.¹³ These results indicate that GPNMB might play positive role in the progression of human GBM.

GNPMB silencing inhibits glioma tumorigenesis *in vitro*

We then employed GPNMB siRNA knockdown to measure its effect on glioma cells. Transfection with GPNMB siRNA down-regulated GPNMB expression approximately 50–70% in glioma cells compared to negative control siRNA (Figure 2(a)), and the inhibition of endogenous GPNMB significantly reduced the proliferation capacity of glioma cells (Figure 2(b)). Consistently, when GPNMB was silenced, the migration of glioma cells was remarkably inhibited (Figure 2(c)), indicating the regulation of GPNMB to glioma cells proliferation and migration.

We also investigated the effect of GPNMB siRNA on the apoptosis capacity in glioma cells. Flow cytometric analysis showed that the apoptosis index of glioma cells exhibited

Figure 1 Expression of GPNMB in human glioma tissue and glioblastoma cell lines. (a) RT-PCR was used to measure the expression levels of GPNMB in normal brain tissue (normal) and human glioma tissues with grade II to IV glioblastoma (G II, G III and G IV) (** $P < 0.01$, * $P < 0.05$ vs. normal). (b) The expression levels of GPNMB and the survival rate in human glioma patients were assessed by the Kaplan-Meier test. GPNMB high represents high GPNMB mRNA expression (>3 -fold higher than in the normal brain sample) and GPNMB low represents low GPNMB mRNA expression (<3 -fold higher). (c) and (d) The levels of GPNMB in the human glioma cell lines U-87 MG, U-373 MG, U-251 MG and M059J as well as normal human astrocytes (NHA) were examined using RT-PCR and Western blotting. (** $P < 0.01$, * $P < 0.05$ vs. NHA)

Figure 2 GPNMB silencing inhibits glioma tumorigenesis. U-87 MG and U-373 MG cells were cultured in basal medium (control), followed by transfection with specific GPNMB siRNA or non-targeting control siRNA (s-Mock). (a) The protein levels of GPNMB were determined after 48 h by Western blotting to confirm the efficiency of GPNMB silencing. (b) Proliferation of cells transfected by GPNMB siRNA was tested by CCK-8 assays at 12, 24, 48, and 72 h of culture. (c) The migration of cells was examined using Transwell Boyden chambers after three days of transfection. (d) Flow cytometric analysis was performed to assess apoptosis. The apoptosis rate was quantified and is shown in (e). (* $P < 0.05$ vs. control)

no significant change in the GPNMB silenced group (Figure 2(d)). These data imply that high levels of endogenous GPNMB have a positive effect on glioma tumorigenesis via facilitating glioma cell proliferation and migration without an apparent effect on apoptosis.

Suppression of GPNMB decreases glioma angiogenesis

We next determined whether endogenous GPNMB is implicated in promoting angiogenesis during glioma progression. In the conditioned medium from glioma cells, a GPNMB band of approximately 100 kDa was tested with

significantly decreased levels in GPNMB silencing groups compared to control groups (Supplementary Figure S2). Conditioned medium from U-87 MG cells transfected with GPNMB siRNA was collected and added to HBMECs cells to assess tube formation ability. Compared to the control group, inhibition of GPNMB in U-87 MG cells remarkably reduced the tube formation in HBMECs (Figure 3(a) and (b)).

VEGF-C and TEM7 have been shown to be functionally linked to GBM angiogenesis.⁹ We further determined the change of VEGF-C and TEM7 levels in U-373 MG and U-87 MG cells. Results showed that VEGF-C and TEM7

Figure 3 Suppression of GPNMB decreases glioma angiogenesis. U-87 MG and U-373 MG cells were cultured in basal medium (Control), followed by transfection with specific GPNMB siRNA or non-targeting control siRNA (s-Mock). (a) The Matrigel assay was performed to determine the tube formation capacity of U-87 MG cells. The formation of branching points of U-87 MG cells was quantified and shown in (b) (* $P < 0.05$ vs. control). (c) Western blotting and (d) RT-PCR were used to detect the expression levels of VEGF-C and TEM7 in U-87 MG and U-373 MG cells as well as normal human astrocytes (NHA). (** $P < 0.05$, * $P < 0.05$ vs. NHA; # $P < 0.05$ vs. control)

were aberrantly up-regulated in glioma cells compared with normal human astrocytes (Figure 3(c) and (d)). However, the increased levels of VEGF-C and TEM7 were dramatically down-regulated by the suppression of endogenous GPNMB induced by specific siRNA knock-down, implying that the inhibitory effect of GPNMB silencing was on the pro-angiogenic ability of glioma. These data indicate that GPNMB might be a significant contributor to glioma angiogenesis.

GPNMB induces MMP-2, MMP-3, and MMP-9 expression

Previous reports revealed that matrix metalloproteinases (MMPs) play crucial roles in tumor metastasis and angiogenesis.^{7,15,16} To investigate the potential mechanism of GPNMB-mediated glioma tumorigenesis and angiogenesis, we analyzed the levels of MMP-2, MMP-3, and MMP-9 in glioma cells. As shown in Figure 4(a), MMP-2, MMP-3, and MMP-9 were expressed at low levels in normal human astrocytes but aberrantly regulated in both glioma cells. When GPNMB siRNA was transfected, the MMPs levels in both cell lines were greatly decreased (approximately MMP-2: -67.5%; MMP-3: -60%; MMP-9: -46.4%).

Furthermore, we employed gelatin zymography assay to determine the effect of GPNMB silencing on MMPs activity. Compared to the control group, MMP-2 and MMP-9 levels were significantly down-regulated in the conditioned media of glioma cells transfected by GPNMB siRNA

(Figure 4(b)). These data suggest that GPNMB specifically regulates MMPs secretion in glioma cells, which contribute to glioma progression.

GPNMB induces MMPs activity through Wnt/ β -catenin pathway

It is reported that the Wnt pathway plays regulatory roles in the progression of many cancers, including GBM.^{11,12} We therefore investigated the potential role of the Wnt pathway in GPNMB-mediated glioma progression *in vitro*. As seen in Figure 5(a), the levels of β -catenin were significantly up-regulated in glioma cells compared with normal human astrocytes, and this increase was attenuated by GPNMB suppression induced by siRNA, thus suggesting that Wnt/ β -catenin pathway is activated by high level of GPNMB in glioma cells.

Additionally, we employed a stimulator of Wnt/ β -catenin signaling (BIO) and an inhibitor (Dkk-1) to further elucidate the above results.^{17,18} BIO (1 nM, StemRD, Burlingame CA) treatment enhanced Wnt/ β -catenin signaling, while Dkk-1 (100 ng/ml, StemRD) abolished the activation of Wnt/ β -catenin (Figure 5(b)). And overexpression of MMP-2, MMP-3, and MMP-9 in glioma cells was blocked by treatment with Dkk-1, but increased by BIO (Figure 5(c)). Taken together, these results indicate that GPNMB might induce MMP activity through the activation of Wnt/ β -catenin signaling further to contribute to glioma motility.

Figure 4 Influence of GPNMB on the expression of MMP-2, MMP-3 and MMP-9. The levels of MMP-2, MMP-3 and MMP-9 were measured by Western blotting (a) in U-87 MG and U-373 MG cells as well as NHA (* $P < 0.05$ vs. NHA; # $P < 0.05$ vs. control). (b) Gelatin zymography was employed to evaluate MMP-2 and MMP-9 activity in conditioned media from U-87 MG and U-373 MG cells transfected by GPNMB siRNA or non-targeting control siRNA (s-Mock)

Discussion

GPNMB is a transmembrane glycoprotein and involved in inflammation, cell differentiation, invasion, and other biological processes.⁴ Previous studies have shown that GPNMB is up-regulated in many cancers, including metastatic prostate cancer, hepatocellular carcinoma, and glioma.^{13,16} Recently, GPNMB was observed to be aberrantly overexpressed in the brain tissue of GBM patients and was identified as a glioma-specific marker.³ In this study, we also showed that GPNMB is aberrantly up-regulated in GBM patients with high-grade glioma tumors and glioma cells. Additionally, high levels of GPNMB presented a positive relationship with the poor prognosis and short survival of glioma patients.

The pathology involved in GBM progression is associated with GBM cell motility.^{1,19} Cellular proliferation, migration, and invasion represent the main events in glioma tumorigenesis. Previous studies revealed that GPNMB is involved in the motility of various cancer cells. In human metastatic prostate cancer, GPNMB can increase the invasiveness of cancer cells.¹³ GPNMB also induces mammary tumor growth and metastasis by cooperating with neuropilin-1 (NRP-1) in human breast cancer.²⁰

In pancreatic cancer, GPNMB protein could regulate the apoptosis and proliferation capacity of cancer cells.²¹ Herein, we showed that GPNMB silencing in glioma cells significantly decreased cellular proliferation and migration without affecting apoptosis *in vitro*, indicating the crucial role of high levels of endogenous GPNMB in glioma tumorigenesis.

Angiogenesis is an important physiological process during embryonic development and the progression of various tumors.²² In grade IV GBM, the vascularity has been shown to be correlated with the poor prognosis of glioma.⁷ It is reported that GPNMB promotes the motility of human umbilical vein endothelial cells to induce angiogenesis in the bone regeneration by activating FGFR-1 signaling.²³ Herein, we determined the effect of endogenous GPNMB on the pro-angiogenic ability of glioma cells. Conditioned medium from the glioma cell U-87 MG strongly induced tube formation activity by HBMECs cells, and this capability was prevented by GPNMB suppression.

Various pro-angiogenesis and anti-angiogenesis factors secreted by tumor cells play important regulatory roles in tumor neovascularization.⁸ VEGF-C and TEM7 are two

Figure 5 GPNMB activates the canonical Wnt/β-catenin pathway. (a) Western blotting was used to measure the levels of nuclear β-catenin in NHA cells as well as U-87 MG and U-373 MG cells with GPNMB siRNA transfection or not (control). (b) Cells were treated with the stimulator BIO (1 nM) or the inhibitor Dkk-1 (100 ng/ml) for 48 h, followed by Western blotting to analyze the levels of nuclear β-catenin. (c) Cells were then prepared for Western blotting assay using specific antibodies for MMP-2, MMP-3 and MMP-9 (* $P < 0.05$ vs. NHA; # $P < 0.05$ vs. control)

such angiogenic factors. VEGF-C has crucial role in angiogenesis during physiological or pathological processes by binding to its specific receptor, VEGFR3. TEM7 is a transmembrane protein that is expressed intracellularly as well. Previous studies showed that TEM7 is implicated in cell migration and capillary morphogenesis by interacting with extracellular matrix components. VEGF-C and TEM7 are functionally linked to GBM vascularity. And high levels of VEGF-C and TEM7 are associated with worse survival in GBM patients and might represent an indicator of the degree of vascularity in GBM.⁹ In this report, we observed that VEGF-C and TEM7 were aberrantly up-regulated in glioma cells compared to normal human astrocytes, and this increase was attenuated by GPNMB suppression, further suggesting the inhibitory effect of GPNMB silencing on the pro-angiogenic ability of glioma.

The MMPs are able to degrade basement membranes type IV collagen and have been correlated with cell motility. In the invasive stage of carcinomas, it has been found that MMP-2 can promote the activation of the gelatinase MMP-9, and the overexpression of these MMPs has crucial roles in the metastasis of tumor cells.²⁴ Rocca *et al.*¹⁴ reported correlations between the activity of MMP-2/9 in serum and higher metastatic potential of breast cancer by gelatin zymography. A positive correlation between MMP-2/9 activation with angiogenesis was also demonstrated in colorectal adenocarcinoma.⁷ In terms of glioma, MMP-2 and MMP-9 could be regulated by microRNA-21 as well as specificity protein 1 (Sp1) in human glioma, thus

contributing to glioma cell invasiveness.^{25,26} Recently, GPNMB was reported to be capable of increasing the invasion capacity of metastatic prostate cancer cells by regulating the MMP-2/9 activation.¹³ In the present study, we demonstrated that MMP-2 and MMP-9 were aberrantly up-regulated in glioma cell lines, while the levels and activity were significantly down-regulated by GPNMB silencing in both cell lysates and conditioned medium. This implies that the expression and secretion of MMP-2/9 might be involved in the regulation of GPNMB during glioma progression.

Notably, MMP-3, another metalloproteinase, has also been identified to have roles in the invasiveness of malignant gliomas.¹⁵ Our present data indicate that significant down-regulation of MMP-3 was induced by GPNMB silencing in glioma cells. This is consistent with a previous study in which GPNMB was found to remarkably up-regulate the level of MMP-3 in activated microglia cells. This may provide evidence that GPNMB specifically regulates the activity of MMP-2, MMP-9, and MMP-3 in glioma cells and thus contributes to tumorigenesis and angiogenesis in glioma.

In addition to angiogenic factors, the Wnt pathway is also implicated in tumor tumorigenesis and angiogenesis in humans.²⁷⁻²⁹ In GBM, Augustin *et al.* reported that the Wnt protein Evi/Gpr177 regulates the migration and survival of glioma cells and is implicated in the pathogenesis of brain tumors by mediating Wnt signaling.¹⁹ By Wnt/β-catenin pathway, glucose-regulated protein 94 (GRP94) positively and miR-122 negatively regulate the aggressiveness and tumorigenicity of glioma cells.^{30,31} Additionally,

Wnt signaling is associated with the activity of MMPs, which may co-regulate the progression of glioma.^{10,32} Zhang *et al.*³³ reported that intracellular Wnt/ β -Catenin could induce MMP-9 expression in endometrial stromal cells by binding interaction between β -catenin/TCF3/LEF1 complex with MMP-9 promoter. Additionally, the Wnt/ β -catenin signaling is correlated with MMP-1 and MMP-7 levels in other tumor cells.^{34,35} We demonstrated that Wnt/ β -catenin pathway was activated in glioma cells, but inactivated by GPNMB suppression, and the activity of MMPs in glioma cells was mediated by Wnt/ β -catenin pathway.

In conclusion, we determined that GPNMB might be a promotor for glioma and could induce MMP activity through the Wnt/ β -catenin pathway, contributing to glioma motility and angiogenesis. This may represent a new understanding for malignant glioma.

Authors' contribution: GB and NW designed the experiment; NW, RL and G X conducted the experiment; PL and BH conducted statistical analysis; and GB wrote the manuscript.

ACKNOWLEDGMENTS

This study received no specific grant from any funding agency.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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(Received January 11, 2016, Accepted May 11, 2016)