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Elevated *PDGFRB* gene copy number gain (CNG) is prognostic for improved survival outcomes in resected malignant pleural mesothelioma

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Abstract

Background—PDGF/PDGFR pathway has been implicated in malignant pleural mesothelioma (MPM) carcinogenesis and evidence suggests autocrine mechanisms of proliferation. We sought to evaluate the incidence of *PDGFRB* gene copy number gain (CNG) by fluorescence in situ hybridization (FISH) and PDGFR pathway protein expression by immunohistochemistry (IHC) and correlate it to patient clinical outcome.

Methods—88 archived tumor blocks from resected MPM with full clinical information were used to perform IHC biomarkers (PDGFR α , PDGFR β , p-PDGFR β) and FISH analysis of *PDGFRB* gene CNG. Spearman's rank correlation, Wilcoxon rank-sum test, Kruskal-Wallis test, BLiP plots, and Kaplan-Meier method were used to analyze the biomarkers and correlation to clinical outcome.

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Results—Several correlations between the IHC biomarkers were seen; however, none correlated to clinically relevant patient demographics or histology. In the CNG analysis, *PDGFRB* gene CNG in > 10% of tumor cells had lower cytoplasmic p-PDGFR β ($p=0.029$), while *PDGFRB* gene CNG in > 40% of tumor cells had a higher cytoplasmic PDGFR β ($p=0.04$). *PDGFRB* gene CNG status did not associate with patient demographics or tumor characteristics.

PDGFR pathway IHC biomarkers did not associate with survival outcomes. However, patients with *PDGFRB* CNG > 40% of tumor cells had improved relapse-free survival [HR 0.25 (95% CI 0.09, 0.72), $p=0.0096$] and improved overall survival [HR 0.32 (95% CI 0.11, 0.89), $p=0.029$].

Conclusions—*PDGFRB* CNG > 40% of MPM tumor cells is a potential prognostic biomarker for surgery and may identify a unique population of mesothelioma patients. Future validation of this biomarker in prospective trials is needed.

Keywords

PDGFRB; pleural mesothelioma; copy number gain; prognostic biomarker

Introduction

Malignant pleural mesothelioma (MPM) affects close to ~3000 new patients each year in the United States and carries a grim prognosis.¹ Standard of care therapies for earlier stage resectable patients still only provide ~15 – 17% overall survival rates^{2,3} with unresectable patients having a less than 1% rate for 5-year survival. The treatment for a resectable patient also carries significant morbidity and a 4-8% mortality rate for extrapleural pneumonectomies (EPP), the most aggressive surgical resection that can be done.^{3,4}

Currently, significant controversy exists as to whether MPM patients should receive surgery or which type of surgery.^{4,5} In a recent publication, Treasure et al.⁵ proposed that MPM patients are harmed by EPP surgical resection and a prior study by Flores et al.⁴ describes that pleurectomy-decortications (P/D) can provide improved survival benefits over EPP. In addition, the role of peri-operative chemotherapy in small non-randomized studies has been shown to add survival benefit to a subgroup of MPM patients.¹⁻³ The current practice in the United States is for the multidisciplinary team to make subjective decisions based on clinical characteristics^{6,7} on whether to operate, what type of operation, adjuvant radiation, and whether to administer neoadjuvant or adjuvant chemotherapy.

Identification of a prognostic biomarker that could determine which patients would benefit from aggressive surgical resection would be optimal. Unfortunately, there are currently no standardized molecular assays with proven benefit. Prior gene expression studies⁸ have suggested that gene expression ratios can provide prognostic information, yet this has not been adopted widely into practice due to limitations in real-time usage. Therefore identifying simple prognostic molecular biomarkers to recognize which MPM patients would benefit from aggressive intervention is crucial to optimize treatment.

The PDGF/PDGFR pathway has been implicated in mesothelioma carcinogenesis with both autocrine and paracrine mechanisms of proliferation in mesothelioma.⁹⁻¹¹ There are 2 receptor subtypes, PDGFR α which binds to 3 different ligand combinations PDGF (AA,

AB, and BB) and PDGFR β which binds to PDGF BB.¹⁰ Although the 2 receptors have a similar structure and only differ in their extracellular binding domain; their distinct regulatory roles in mesothelioma remains unknown. Mesothelioma tumor cell lines have been reported to secrete elevated mRNA levels of PDGF A-chain, PDGF-BB, and to express both receptors α and β .^{9,10,12} However, many of the early translational studies suggested that PDGF-BB and PDGFR β pathway were more relevant in malignant mesothelioma^{12,13}, whereas PDGFR α pathway was involved in normal mesothelial cell proliferation.^{14,15} Some reports indicate that upregulation of PDGFR β pathway leads to a worse prognosis in mesothelioma via TIE2/Tek¹⁶, whereas others¹⁷ suggest that PDGF could promote growth of the tumor associated connective tissue stroma via PDGFR β -integrin $\alpha 3 \beta 1$ interactions. It is presumed that inhibiting PDGF-BB expression can thus lead to diminished mesothelioma cell proliferation.⁹

Although there have been few publications in the literature on this topic, the studies were largely based on small numbers of tumor specimens. Several of the analyses provide conflicting information and there have been no prior studies that evaluated *PDGFRB* gene copy number gain (CNG) and its prognostic value in MPM. With the plethora of novel agents that now target PDGF/PDGFR pathway, and the potential for a biomarker that could identify a population of MPM patients that would benefit; we sought to determine the incidence and prognostic value, in a large set of archived resected mesothelioma tumor tissues, of *PDGFRB* gene CNG and protein expression by immunohistochemistry (IHC) in the mesothelioma tumor cell cytoplasm, membrane, nucleus, and stroma.

Materials and Methods

Tumor Tissue Specimens

Archived surgically resected pleural mesothelioma cases were selected from a review of the University of Texas M.D. Anderson Cancer Center Thoracic Tissue Bank from 1986-2006. Patients could have received an extrapleural pneumonectomy (EPP) or pleurectomy/decortication (P/D) or a debulking procedure if neither EPP nor P/D was achieved. Patient tumor specimens were selected for this analysis based on several factors: tumor tissue availability, signed informed consent to perform additional research on archived tumor specimens, adequate tumor tissue for immunohistochemistry biomarker analysis, and presence of existing clinical outcomes information. A total of 88 tumor block cases were identified that had adequate tissue to perform the analyses. Patient characteristics are listed in Table 1. The diagnosis of malignant mesothelioma was confirmed by an independent histopathology review and tissue immunohistochemistry (IHC) diagnostic markers. Immunohistochemical biomarkers for PDGFR α , PDGFR β and p-PDGFR β , and fluorescence in situ hybridization (FISH) were performed for analysis of *PDGFRB* gene copy number gain (CNG).

Immunohistochemistry

We performed standard IHC studies of 3 biomarkers and evaluated them in subcellular locations (cytoplasmic, stromal, membrane, and nuclear): PDGFR β , p-PDGFR β , PDGFR α . The histology sections were incubated with primary antibodies PDGFR- β (P-20, dilution

1:300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p-PDGFR β (Tyr 1021, dilution 1:400, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 65 min, and PDGFR α (dilution 1:100, Cell Signaling Technology, Danvers, MA) for 90 min, at room temperature. Tissue sections were then incubated with the secondary antibody (EnVision Dual Link+; DAKO, Carpinteria, CA) for 30 min, after which diaminobenzidine chromogen was applied for 5 min. Tumor malignant cells and stroma were evaluated using the same methodology for PDGFR β , p-PDGFR β , and PDGFR α protein expressions. Briefly, for each marker, two experienced thoracic pathologists (I.I.W. and J.F.) examined both the intensity and extent of immunostaining by light microscopy using a $\times 20$ magnification objective. Immunoreactivity of all antibodies was detected in the cytoplasm¹⁸ of malignant cell and stroma fibroblast, and p-PDGFR β was detected in both the cytoplasm and nucleus. Cytoplasmic or nuclear expressions were quantified using a four-value intensity score (0, none; 1+, weak; 2+, moderate; and 3+, strong) and the percentage (0-100%) of the extent of reactivity. A final expression score was obtained by multiplying the intensity and reactivity extension values (range, 0-300).¹⁸

Fluorescence in situ hybridization (FISH) for analysis of gene copy number gain (CNG)

In addition, *PDGFRB* gene copy CNG was evaluated in tumor tissue. We analyzed the gene copy number per cell using a dual-color FISH assay. The *PDGFRB* probe was prepared from the Vysis LSI CSF1R probe (5q33-34) SpectrumOrange (Abbott Molecular, Illinois). The Abbott Vysis probe encompasses *PDGFRB* (5q33.1) and *CSF1R* (5q32) genes. Although the manufacturer's manual indicates that these probes also covers the *SLC28A2* gene, we discovered that this is not correct since this gene is located in 5q15 region (<http://www.ncbi.nlm.nih.gov/gene/9153>). This mistaken information was recently discussed with the probe manufacturers who have stated that they will revise their manual. A similar probe Vysis LSI D5S23, D5S721 SpectrumGreen Probe (Abbott Molecular, Illinois) mapping to chromosome 5p was used as an internal control. The 4 μ m thick sections were incubated for two hours to overnight at 56°C, deparaffinized in Citri-Solv (Fisher, Waltham, MA) and washed in 100% ethanol. The slides were sequentially incubated in 2 $\times$ Saline-sodium Citrate Buffer (SSC) at 75°C for 18 to 23 min, digested in 0.5mg/ml proteinase K/2 $\times$ SSC at 45°C for 18 to 23 min, washed in 2 $\times$ SSC for 5 min, and dehydrated in ethanol. DNA denaturation was performed for 15 min at 85°C and hybridization was allowed to occur at 37°C for 36-48 hours. Chromatin was counterstained with 4',6-diamidino-2-phenylidole (0.3 μ g/ml in Vectashield mounting medium, Vector Laboratories). Gene copy number analysis was done in approximately 50 nuclei per tumor in at least four areas, and the selection of the area was guided by H&E-stained section. *PDGFRB* gene copy number was evaluated by three ways: a) gene amplification, defined as presence of loose or tight gene cluster or *PDGFRB* gene to centromeric probe 5 ratio ≥ 2 ; b) copy number gain (CNG) defined at 2 levels: ≥ 4 copies in $> 10\%$ cells and in $> 40\%$ of cells as previously published.^{19,20}

Statistical analyses—The correlation between IHC biomarkers was assessed using the Spearman's rank correlation. Wilcoxon rank-sum test or Kruskal-Wallis test is used to detect marker differences between groups.²¹ The distribution of the biomarkers is summarized by BLiP plots.²² The distributions of overall survival (OS) and recurrence free survival (RFS) are estimated by the Kaplan-Meier method.²³ Log-rank test²⁴ is performed to test the

difference in survival between groups. Regression analyses of survival data based on the Cox proportional hazards model²⁵ are conducted on OS defined as the time of surgery to the time of death or to the time of last contact. Recurrence free survival (RFS) is defined as the time of surgery to the time of relapse or death (all causes) or the time of last contact. Patients who had residual disease were considered to have a relapse at the surgery date. SAS version 9.1 and S-Plus version 8.0 are used to carry out the computations for all analyses.

Results

Immunohistochemistry (IHC) correlations

There were several specific associations of IHC biomarkers to one another. Higher activated cytoplasmic p-PDGFR β was associated with increased membrane p-PDGFR β ($p=0.0023$) but lower nuclear expression of p-PDGFR β ($p=0.0001$). Higher membrane p-PDGFR β correlated with a higher stromal expression of PDGFR β ($p=0.042$). Higher IHC expression of cytoplasmic PDGFR β was associated with higher levels of stromal PDGFR α ($p=0.014$). Higher stromal PDGFR α expression correlated to lower nuclear p-PDGFR β expression ($p=0.015$). Higher nuclear p-PDGFR β expression corresponded to higher expression levels of stromal PDGFR β ($p=0.031$). There was no association of cytoplasmic PDGFR α expression levels to any other IHC biomarker.

There was no association of IHC biomarkers to age, smoking status, smoking pack-years, histologic subtype, cause of death, or gender, although a trend towards higher expression of membrane p-PDGFR β in women was seen ($p=0.099$). Nuclear p-PDGFR β IHC expression was more highly expressed in non-Caucasian patients ($p=0.048$). And higher levels of cytoplasmic PDGFR α were found in patients who had no known exposure to asbestos ($p=0.029$) and left sided primary pleural tumors ($p=0.053$).

Patients who were able to receive EPP had tumors that expressed higher levels of cytoplasmic PDGFR β ($p=0.0029$) and nuclear p-PDGFR β ($p=0.053$). Patients that underwent pleurectomy-decortication had higher expression of membrane p-PDGFR β ($p=0.006$) and cytoplasmic p-PDGFR β ($p=0.02$). Patients with stage II disease had higher stromal PDGFR β than patients with stage III and IV disease ($p=0.041$). Patients with T3 primary tumors had a trend towards higher expression of cytoplasmic PDGFR β ($p=0.057$) than other T-stage tumors. There was no association of the IHC biomarkers to N-stage or M-stage. However, higher cytoplasmic PDGFR β ($p=0.004$) and lower nuclear p-PDGFR β ($p=0.07$) were associated with a lower risk of having residual disease after surgery. There was no association of the biomarkers to local or regional relapse rates. A trend towards higher cytoplasmic p-PDGFR β and decreased risk of distant metastasis at time of surgery was seen ($p=0.07$).

Gene copy number gain (CNG)

PDGFRB gene CNG was able to be successfully completed in 61 patients. Patients with *PDGFRB* gene CNG in $> 10\%$ of cells had lower median expression of cytoplasmic p-PDGFR β ($p=0.029$). Patients with *PDGFRB* gene CNG in $> 40\%$ of cells had a higher median expression of cytoplasmic PDGFR β ($p=0.04$). There were no other IHC differences

between tumors with and without *PDGFRB* gene CNG. There was no association with patient demographics or tumor characteristics or tumor histology with *PDGFRB* gene CNG. There were 5 epithelioid and 1 sarcomatoid patient who had *PDGFRB* gene CNG > 40%.

Survival associations

For relapse-free survival (RFS), the univariate analysis showed improved prognostic factors for younger age < 60 at surgery [HR 0.58 (95% CI 0.37, 0.89), $p=0.013$], female gender [HR 0.42 (95% CI 0.22, 0.81), $p=0.01$], no known asbestos exposure [HR 0.58 (95% CI 0.36, 0.94), $p=0.027$], stage II disease [HR 0.37 (95% CI 0.14, 0.95), $p=0.041$] and stage III [HR 0.56 (95% CI 0.36, 0.88), $p=0.011$] disease, N₀ nodal disease [HR 0.44 (95% CI 0.26, 0.75), $p=0.002$], and N₁ disease [HR 0.49 (95% CI 0.49, 0.25), $p=0.049$]. Receiving an EPP instead of P/D led to better outcomes [HR 0.14 (95% CI 0.08, 0.26), $p<0.0001$] and the addition of adjuvant chemotherapy improved RFS [HR 0.35 (95% CI 0.13, 0.99), $p=0.048$]. For patients who had *PDGFRB* CNG > 40% of cells, the RFS was improved compared to patients with *PDGFRB* < 40% CNG [HR 0.25 (95% CI 0.09, 0.72), $p=0.0096$] (see Figure 1A). In the multi-covariate analyses for RFS, there was no association with any IHC biomarker. After adjusting for gender, known asbestos exposure, EPP, and adjuvant chemotherapy, patients with *PDGFRB* CNG > 40% of cells had an improved median RFS [HR 0.27 (95% CI 0.09, 0.80), $p=0.018$]. In Figure 2, the patients ($n=2$) with *PDGFRB* CNG > 40% of cells who also received peri-operative chemotherapy appeared to have the best RFS ($p=0.017$).

For the OS analyses, univariate analysis of patient demographics demonstrated that the worse prognostic factors were male gender [HR 2.5 (95% CI 1.35, 4.46), $p=0.004$], African-American ethnicity compared to Caucasians [HR 3.8 (95% CI 1.2, 12.45), $p=0.026$], former smoking history [HR 2 (95% CI 1.27, 3.36), $p=0.004$], biphasic histology [HR 2.5 (95% CI 1.28, 4.73), $p=0.004$], and sarcomatoid histology [HR 3.4 (95% CI 1.47, 7.83), $p=0.004$]. In contrast, having *PDGFRB* gene CNG > 40% of cells was prognostic for an improved overall survival [HR 0.32 (95% CI 0.11, 0.89), $p=0.029$] with surgery (Figure 1B). In the multi-covariate biomarker analysis, only *PDGFRB* CNG > 40% of cells (HR 0.29, $p=0.02$) was associated with improved OS. Biphasic [HR 2.74 (95% CI 1.41, 5.33), $p=0.003$] and sarcomatoid [HR 2.83 (95% CI 1.19, 6.68), $p=0.018$] histologies had a lower median OS compared to epithelioid histology in the multi-covariate Cox model.

Discussion

The results of our translational study on archived resected MPM tissues demonstrate that *PDGFRB* CNG in > 40% of tumor cells appears to be a potential prognostic biomarker that identifies MPM patients with an improved relapse-free and overall survival with surgical resection. Other reports have noted the presence of increased *PDGFRB* copy number gain in mesothelioma; from the publically available data from the Progenetix database (www.progenetix.org), they report that 13 out of 20 (65%) mesotheliomas with copy number variations demonstrated gain of the *PDGFRB* gene region (5q33.1), as well as the *CSFRI* gene region (5q32). This is a higher incidence than what we identified in our archival specimens, but could be related to varying platforms for analysis. Future studies such as from The Cancer Genome Atlas (TCGA) Project will be crucial in determining the precise

incidence of *PDGFRB* copy number gain. This biomarker identifies a unique subgroup of MPM patients who may have different disease biology, possibly being less invasive/proliferative or less likely to spread distantly. This is the first analysis to report this finding.

The molecular explanation for why this occurs remains unclear. In our protein biomarker assessment, we had findings that were consistent with the literature demonstrating high expression of cytoplasmic PDGFR β in mesothelioma tumors. Patients who had *PDGFRB* CNG > 40% of tumors cells were more likely to have higher median IHC expression of cytoplasmic PDGFR β , suggesting increased protein production. However, cytoplasmic PDGFR β IHC by itself was not prognostic for either RFS or OS, indicating a more complex regulatory environment in MPM tumors.

One possible theory for the improved prognosis seen in our MPM patients relies on the ligand-receptor negative feedback loop. Studies²⁶ in other tumor types have shown that PDGF-BB overexpression increased endothelial cell pericyte coverage and lead to a subsequent decrease in tumor cell proliferation. This mechanism is supported by an analysis showing that inhibition of PDGFR tyrosine kinase activity via imatinib mesylate causes a contrary response by decreasing pericyte coverage and increasing tumor cell proliferation.²⁶ Also, studies have shown that chronic stimulation by ligand leads to down-regulation of PDGFR.^{11,27} Prior studies have reported higher baseline levels of PDGF in serum of MPM patients compared to NSCLC and healthy controls.²⁸ It is possible that MPM patients with *PDGFRB* CNG in over 40% of tumor cells have co-existing elevation of high ligand PDGF-BB levels via the autocrine loop mechanism; and that once the ligand values reach a key level, this subsequently causes a negative feedback switch and suppression of tumor cell growth.

In addition, the precise regulatory environment between the α and β receptors remains undefined, yet distinct differences have been reported. Prior preclinical studies²⁹ using knock-out PDGFR isoforms showed distinct gene expression profiles between PDGFR α and PDGFR β . The gene sets activated by PDGFR α included C21-steroid hormone biosynthesis and in the PDGFR β pathway contained angiogenic and epidermal growth factor receptor pathways.²⁹ PDGFR β activation has been associated with epidermal growth factor receptor (EGFR) activation, and EGFR overexpression has been reported in mesothelioma (pleural and peritoneal) tumor cells.^{30,31} It is possible that the subtypes of receptors interact in a regulatory fashion and may also impose a negative feedback inhibition. The complex regulatory environment and paracrine effects of the receptor subtypes remain unknown in mesothelioma but may be relevant in therapeutic interventions.

This study is limited by several factors. First, this is a retrospective analysis that spans 20 years and was a preliminary evaluation of several biomarkers in a heterogeneous group of patients with variable and differing treatments. The sample size for the patients was small and the tissue specimens were archived over an extended period of time. The subsequent therapies (including systemic treatment) were inconsistent and this could potentially have impacted the survival results, especially in the patients whose tissue specimens were procured after pemetrexed was FDA approved.

Thus, although definitive conclusions cannot be drawn from this retrospective archival study with limited numbers of patients; this study nevertheless points to the significance of PDGFR pathway in mesothelioma. The identification of PDGFRB CNG in > 40% of cells as a prognostic biomarker for surgical resection will need to be prospectively validated in clinical trials. Additional systemic therapy trials can also be constructed to assess whether *PDGFRB* gene CNG is predictive for an improved sensitivity to chemotherapy. Also, further assessment of the distinct roles of PDGFR α/β signaling and the potential regulatory effect on the other receptor isoforms may be clinically relevant.

Conclusions

This is the first study to report that *PDGFRB* CNG in > 40% of tumor cells is a potential prognostic biomarker in surgically resected MPM tumors. Future validation of this biomarker in prospective trials is needed.

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Figure 1. Improved relapse-free and overall survival outcomes in patients with PDGFRB gene CNG > 40% of tumor cells.

Figure 2. Improved relapse-free survival in patients (n=2) with *PDGFRB* CNG in >40% of tumor cells with the addition of chemotherapy.

Figure 3.

A. Representative microphotographs of malignant pleural mesotheliomas (MPMs) with epitheloid and sarcomatoid histology showing PDGFR-α, PDGFR-β and phosphorylated(p)-PDGFR-β expression (brown staining) by immunohistochemistry. In the epitheloid tumor, the malignant cells show strong cytoplasmic PDGFR-α expression, and strong nuclear PDGFR-α and p-PDGFR-β expressions. In the sarcomatoid tumor, the malignant cells show strong cytoplasmic PDGFR-β and p-PDGFR-β expressions, and strong nuclear p-PDGFR-β expression. (Original Magnification × 200.)

B. Representative microphotographs of two epitheloid pleural mesotheliomas (MPMs) examined by FISH showing gain of PDGFRB copies in the malignant cells. (Original magnification × 1,000.) Red signals represent PDGFRB gene copies and green signals

represent the chromosome 5 centromeric probe. (Cell Nuclei Stained With 4',6-diamidino-2-phenylidole.)

Table 1

Patient demographics.

Characteristic (n=88)	Percentage (%)
Age 60	50%
Male	84%
Ethnicity	
Caucasian	90%
African-American	3%
Hispanic	6%
Asian	1%
Known Asbestos Exposure	66%
Smoking Status	
Current	11%
Former	51%
Never	38%
Type of Surgery	
Extrapleural Pneumonectomy (EPP)	66%
Pleurectomy/Decortications (P/D)	10%
Debulking procedure less than P/D	24%
AJCC 6th edition Pathologic Stage	
Stage II	6%
Stage III	49%
Stage IV	44%
Pathologic T-stage	
T1	1%
T2	23%
T3	42%
T4	30%
Unknown	4%
Pathologic N-stage	
N0	41%
N1	13%
N2	42%
Nx	4%
Histology	
Biphasic	31%
Epithelioid	56%

Characteristic (n=88)	Percentage (%)
Sarcomatoid	13%
Neoadjuvant chemotherapy	22%
Adjuvant chemotherapy	6%