

Low PIAS3 Expression in Malignant Mesothelioma Is Associated with Increased STAT3 Activation and Poor Patient Survival

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Abstract

Purpose: Deregulation of STAT3 activation is a hallmark of many cancer cells, and the underlying mechanisms are subject to intense investigation. We examined the extent of PIAS3 expression in mesothelioma cells and human tumor samples and determined the functional effects of PIAS3 expression on STAT3 signaling.

Experimental design: We evaluated the expression of PIAS3 in mesothelioma tumors from patients and correlated the expression levels with the course of the disease. We also measured the effects of enhanced PIAS3 activity on STAT3 signaling, cellular growth, and viability in cultured mesothelioma cells.

Results: Gene expression databases revealed that mesotheliomas have the lowest levels of PIAS3 transcripts among solid tumors. PIAS3 expression in human mesothelioma tumors is significantly correlated with overall survival intervals ($P = 0.058$). The high expression of PIAS3 is predictive of a favorable prognosis and decreases the probability of death within one year after diagnosis by 44%. PIAS3 expression is functionally linked to STAT3 activation in mesothelioma cell lines. STAT3 downregulation with siRNA or enhanced expression of PIAS3 both inhibited mesothelioma cell growth and induced apoptosis. Mesothelioma cells are sensitive to curcumin and respond by the induction of PIAS3. Corroborative evidence has been obtained from STAT3 inhibition experiments. Exposure of the cells to a peptide derived from the PIAS3 protein that interferes with STAT3 function resulted in apoptosis induction and the inhibition of cell growth.

Conclusion: These results suggest that PIAS3 protein expression impacts survival in patients with mesothelioma and that PIAS3 activation could become a therapeutic strategy. *Clin Cancer Res*; 20(19); 5124–32. ©2014 AACR.

Introduction

The JAK–STAT signaling pathway provides an essential cellular communication route from the plasma membrane to the nucleus. It is initiated by the interaction of extracellular ligands with cellular surface receptors and results in alterations of transcription patterns. Cytokines and growth factors act as ligands and play central roles in the regulation of cell growth, differentiation, and metabolism in many different cell types and organs (1). STAT proteins, especially

STAT3, are not only important in the development and function of normal cells, but can assume oncogenic properties when the extent and duration of their activation are disturbed (2). Inappropriate STAT3 activation results in the prevention of apoptosis and the promotion of tumor cell proliferation (3, 4). STAT3 is constitutively phosphorylated and thereby activated in many forms of cancer. The inhibition of STAT3 activity often results in apoptosis and cell death (5, 6). STAT3 serves as a central integration point for multiple oncogenic signaling pathways to regulate genes involved in cell-cycle control, apoptosis, angiogenesis, tumor invasion, and metastasis. This makes it an attractive target for cancer therapies.

PIAS3 is part of a small gene family with four members (7, 8). PIAS3 was initially found to interact specifically with phosphorylated STAT3, and not STAT1, in IL6-activated murine myeloblast M1 cells and to decrease STAT3 DNA-binding capacity and transcriptional activity (9). We (10, 11) and others (12) have demonstrated that overexpression of PIAS3 can inhibit STAT3 transcriptional activity and promote growth inhibition *in vitro*. If PIAS3 counteracts transactivation by STAT3, it would be conceivable that

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Translational Relevance

The biologic principle of negative feedback regulation is being exploited by tumor cells. Mesothelioma cells suppress PIAS3, a negative regulator of STAT3 activity. Strategies for the reactivation of PIAS3 could result in STAT3 inhibition and the growth arrest of tumor cells.

PIAS3 expression could be decreased in malignant tissues with high STAT3 activation. This hypothesis has been confirmed. PIAS3 expression is absent in glioblastoma multi-forme tumor tissue when compared with the adjacent normal brain tissue (13). The expression of PIAS3 is inversely proportional to that of activated, phospho-STAT3 (pSTAT3) expression. We have examined expression of PIAS3 in 44 resected non-small cell lung cancer (NSCLC) specimens and found that 89% of adenocarcinomas stained positive for PIAS3 and only 38% of squamous cell carcinomas showed evidence of staining (14). In squamous cell

carcinomas in which PIAS3 could be detected, only low expression levels were found. Intense PIAS3 expression in adenocarcinomas was associated with the absence of pSTAT3.

These studies suggest that PIAS3 may represent a useful target to devise therapeutic strategies against cancer cells harboring activated STAT3. Here, we show that mesothelioma cell lines exhibit high STAT3 activity and use three experimental approaches to manipulate PIAS3 levels to demonstrate the principle. PIAS3 is able to inhibit STAT3 activity and cell growth in mesothelioma cells.

Materials and Methods

Cell culture and transient transfection

Human pulmonary epithelial cell line A549 and mesothelioma cell lines H2052, H2452, 211H, and H28 were purchased from ATCC and maintained in DMEM/Ham's F-12 medium supplemented with 10% (v/v) FBS (Hyclone, ThermoFisher Scientific) in a 5% CO₂ humidified incubator at 37°C. Cells were transfected with either pCMV5 (empty vector) or pCMV5-mouse *Pias3* using

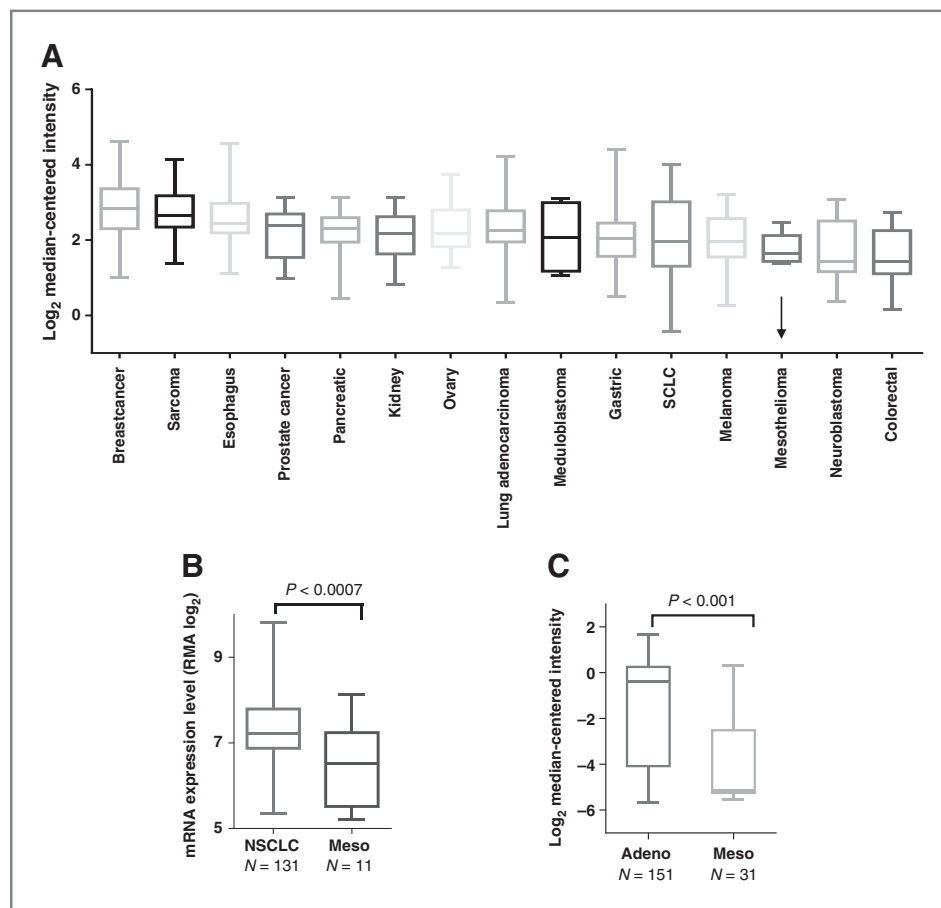


Figure 1. Mesothelioma shows low *PIAS3* expression in public datasets. **A**, global gene expression pattern for *PIAS3* across all types of cancer cell lines in the Cancer Cell Line Encyclopedia (CCLE) database. **B**, *PIAS3* expression in NSCLC and mesothelioma cells, showing a significant difference in *PIAS3* mRNA expression in the CCLE database. **C**, Whisker plots showing the Log₂ median-centered intensity of *PIAS3* expression in Gordon lung. Compared with adenocarcinoma, *PIAS3* expression in mesothelioma tissue samples is significantly lower.

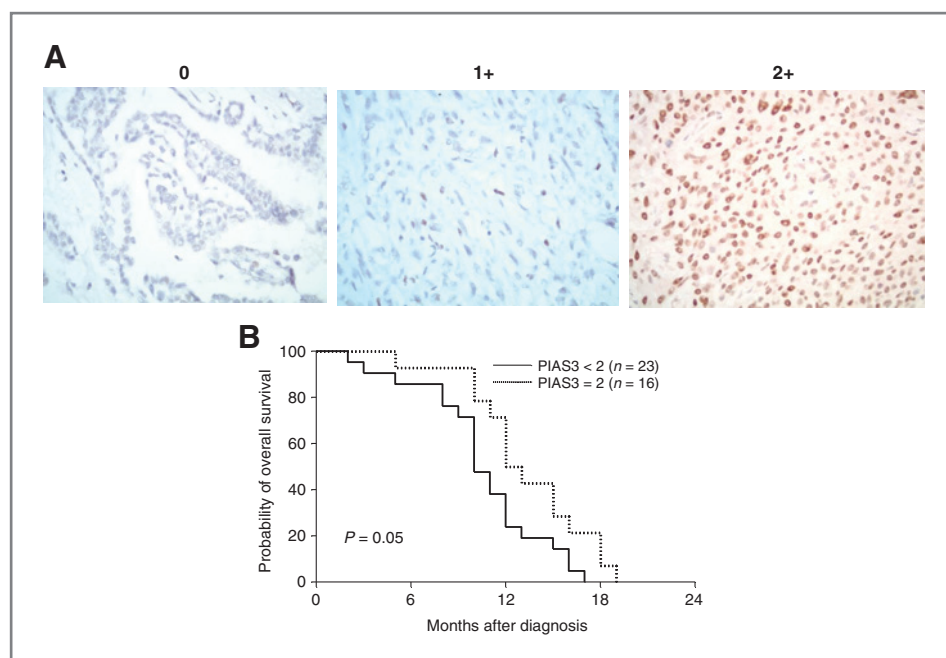


Figure 2. Mesothelioma tumors demonstrate reduced PIAS3 expression. A, IHC of mesothelioma tissue microarray: Representative pictures of PIAS3 staining and scoring. B, Kaplan-Meier estimation of OS by PIAS3 level (epithelioid histology only, from Cox model).

TransIT2020 (Mirus Bio LLC). After 5 hours, media were replaced with DMEM/F12 media containing FBS (10%). After 24 hours of incubation, cells were collected for further analysis.

STAT3 siRNA transfection

Downregulation of STAT3 by siRNA was achieved as described previously by Dabir and colleagues (15).

Immunoblotting and antibodies

Whole-cell lysates were prepared in RIPA buffer, as described previously (16). Antibodies used in Western blots were obtained from either Cell Signaling Technology (PIAS3 #4641, pSTAT3 #9145, Caspase 3 #9961, PARP #9542), or Sigma (β -actin #A5441).

Cell proliferation analysis

Cell growth and viability were assessed in manually counted cells by either Trypan blue dye exclusion or the MTS assay, as described previously (10).

Purification and cell treatment of peptide rPP-C8

Protein purification of the peptide rPP-C8 and cell treatment was performed as described previously (17). Briefly, 2,000 cells were seeded in 96-well plates, and the next day, medium was removed, 0.5 or 2.0 μ mol/L of the peptide (or PBS as solvent control) was diluted in 100 μ L medium and added to the cells.

Immunohistochemistry

The tissue microarray (TMA) slides were obtained from the National Mesothelioma Virtual Bank (NMVB; <http://mesotissue.org/>). The slides were deparaffinized with xylene rinses and then transferred through two changes

of 100% ethanol. Endogenous peroxidase activity was blocked by 30-minute incubation in a 2.5% hydrogen peroxide/methanol buffer. Antigen retrieval was performed by boiling the slides in a pressure cooker filled with a sodium citrate buffer (pH 6.0). After antigen retrieval, the slides were blocked using Background Sniper (Biocare #BS966M) for 20 minutes. The tissues were incubated with 1:400 dilution of rabbit anti-human PIAS3 antibody (Cell Signaling Technology) overnight at 4°C. Bound antibody was detected using an anti-rabbit MACH4 horseradish peroxidase-labeled polymer secondary antibody from Biocare (#MRH534L) for 30 minutes. The slides were rinsed in a TBS series and visualized with a 10-minute incubation of liquid 3,3'-diaminobenzidine in buffered substrate in the dark. Finally, the slides were counterstained with hematoxylin for 30 minutes and mounted with Biomount. The intensity of immunostaining for PIAS3 was scored visually and stratified into 3 staining groups: 0 = no nuclear staining, 1+ = minimal staining, and 2+ = moderate-to-strong staining in >50% of tumor nuclei. At least two different cores were analyzed by immunohistochemistry (IHC) for approximately 70% of the tumors, approximately 50% had ≥ 3 cores analyzed per tumor. The multiple scores from cores of a single tumor specimen were averaged to yield the final score.

Statistical analysis

Overall patient survival (OS) was measured from the date of diagnosis to the date of death. The difference of OS between groups was examined by log-rank test. The effect of PIAS3 as a continuous measurement was further evaluated using the Cox model (18) after controlling for tumor grade (low, intermediate, or high). Histologic type

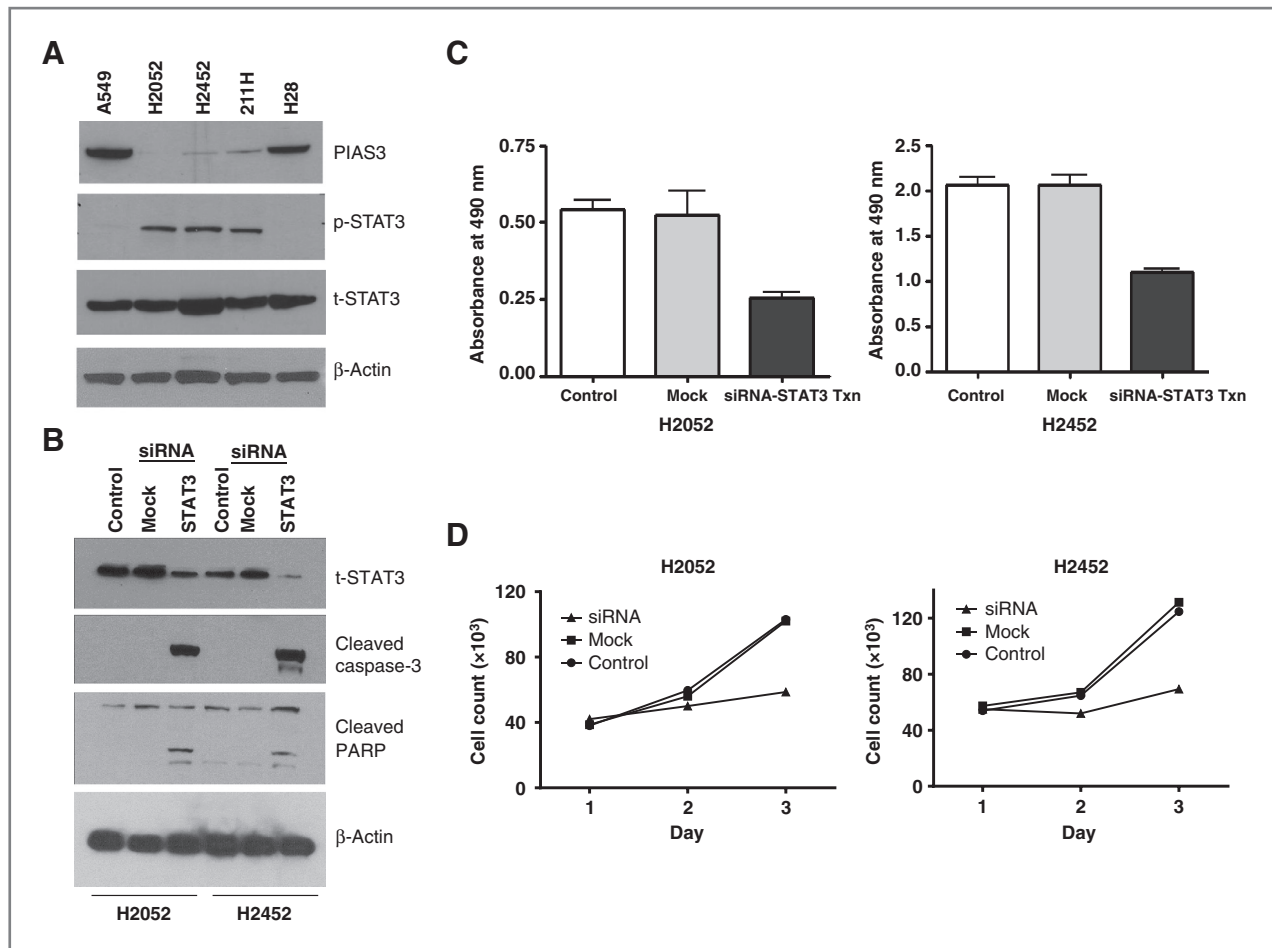


Figure 3. Mesothelioma cells are dependent on high STAT3 activity for growth. A, A549, H2052, H2452, 211H, and H28 cells were grown under basal conditions and protein lysates immunoblotted for PIAS3, p-STAT3 (active), total STAT3, and β -actin. B, H2052 and H2452 cells were treated with STAT3 siRNA, mismatch siRNA (mock), or untreated (control). Protein lysates were immunoblotted for total STAT3, cleaved caspase-3, PARP, and β -actin. C, knockdown of STAT3 in mesothelioma cells promotes apoptosis (MTS assay) and (D) cell growth inhibition.

(epithelioid, sarcomatoid, biphasic) was not included in the Cox analysis due to a lack of some histotypes. The large majority of patients had advanced stage disease (stages III/IV) and therefore staging was also not included in the Cox model used. The difference of *PIAS3* expression between two groups was examined using a *t* test. All tests are two-sided and a $P \leq 0.05$ was considered statistically significant.

Results

PIAS3 mRNA expression is low in cancer tissues

We assessed global gene expression patterns for *PIAS3* expression across all types of cancer cell lines in the Cancer Cell Line Encyclopedia (CCLE) database (<http://www.broadinstitute.org/ccle/home>; ref. 19). When cancer cell lines derived from solid tumors were compared, very low levels of *PIAS3* were found in mesotheliomas (Fig. 1A). When we specifically compared *PIAS3* expression between NSCLC and mesothelioma cells, we found a significant difference in *PIAS3* mRNA expression (Fig. 1B;

$P < 0.0007$). On the basis of these observations, we further searched the OncoPrint database (<https://www.oncoPrint.org/resource/login.html>) for analysis of *PIAS3* expression in tumor tissues. The study by Gordon and colleagues (20) compared *PIAS3* expression in thoracic cancers. The results demonstrated that mesothelioma showed significantly lower expression of *PIAS3* ($P < 0.001$) compared with adenocarcinoma (Fig. 1C).

PIAS3 expression predicts survival intervals of patients with mesothelioma

We also investigated *PIAS3* expression at the protein level. TMAs were obtained from the NMVB and *PIAS3* expression analysis was performed by IHC. Representative pictures of *PIAS3* staining and scoring for epithelioid cores are shown in Fig. 2A. *PIAS3* protein expression observed in the mesothelioma TMA showed mostly nuclear localization. The mean score for *PIAS3* expression was 1.46 ± 0.65 ; and the median was 1.71. Thus, about half of the tissue cores demonstrated low or negative *PIAS3* staining. These results

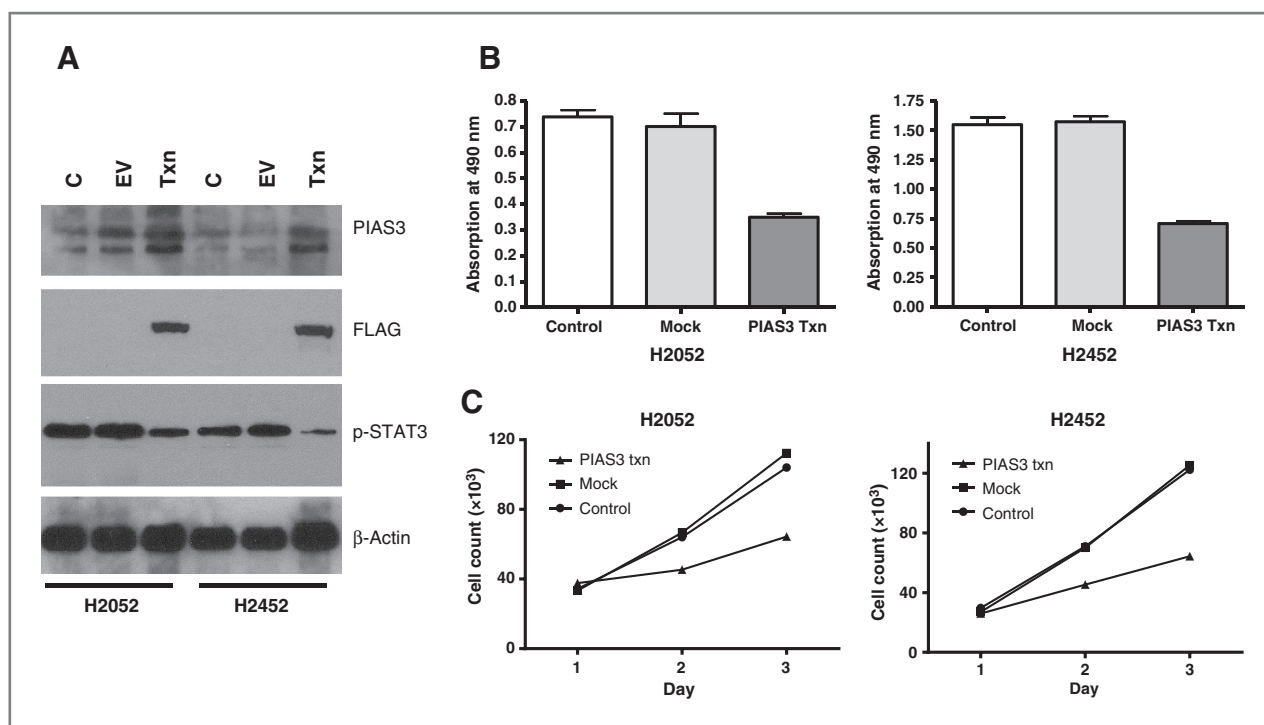


Figure 4. PIAS3 overexpression inhibits STAT3 activation and cell growth in mesothelioma cells. A, H2052 and H2452 cells were transfected with FLAG-*Pias3* (TXN), empty vector (EV), or untreated (C); 48 hours later, whole-cell extracts were prepared and immunoblotted for PIAS3, FLAG, p-STAT3 (active), and β -actin. B, PIAS3 overexpression results in apoptosis (MTS assay) and cell growth inhibition (C).

demonstrated that PIAS3 expression in a subset of mesothelioma tumors was consistent with the mRNA expression results.

To determine whether PIAS3 expression levels are correlated with the survival intervals of patients upon diagnosis of the disease, we analyzed PIAS3 scoring of 39 epithelioid tumors with available patient survival information. We did not analyze other patients with mesothelioma with biphasic, sarcomatoid, and mixed histologies due to their low frequency. Kaplan–Meier survival analysis (21) showed that after controlling for the effects of tumor grade, PIAS3 expression significantly predicted OS intervals ($P = 0.058$). Mortality in patients with high PIAS3 expression was about 44% lower 12 months after diagnosis (Fig. 2B).

STAT3 is activated in mesothelioma cell lines

PIAS3 protein expression was also measured in four mesothelioma cell lines (H2052, H2452, 211H, and H28) by Western blotting and compared with the expression levels in A549 lung cancer cells (Fig. 3A), a representative NSCLC cell line (10, 11, 14, 15). Because PIAS3 is thought to be an endogenous inhibitor of STAT3 activity, we examined whether any decreased PIAS3 expression in mesothelioma cells was accompanied by increased p-STAT3 expression. The results in Fig. 3A demonstrate an inverse correlation between PIAS3 and p-STAT3 expression; the three mesothelioma cells with high p-STAT3 levels (H2052, H2452, 211H) all have little detectable PIAS3 expression. The mesothelioma cell with high PIAS3 expres-

sion, H28, demonstrated no detectable p-STAT3. PIAS3 expression did not correlate with total STAT3 expression. Reprobing the blot with anti- β -actin shows that variability in protein loading could not account for the observed differences in p-STAT3 expression.

STAT3 siRNA induces apoptosis in mesothelioma cells

We established the functional significance of STAT3 activation in mesothelioma cells by measuring their survival and proliferation as a function of STAT3 transactivation. Downregulation experiments with STAT3 siRNA were carried out in H2052 and H2452 cell lines. Specific reduction in total STAT3 was demonstrated after STAT3 siRNA treatment, mismatch siRNA (mock) served as a negative control in H2052 and H2452 cells (Fig. 3B). The reduction in STAT3 levels was accompanied by the induction of apoptosis, as shown by the appearance of cleaved caspase-3 and PARP. Further experiments demonstrated that knockdown of STAT3 with siRNA resulted in substantial growth inhibition in both mesothelioma cell lines, as measured by the MTS (Fig. 3C) or by cell viability assays (Fig. 3D). Taken together, these results demonstrate that STAT3 activity is required for mesothelioma cell growth and survival.

PIAS3 overexpression inhibits cell growth and viability in mesothelioma cells

Most mesothelioma cells appear to be STAT3-dependent and we hypothesized that overexpression of PIAS3 would inhibit their growth. Indeed, transient overexpression of

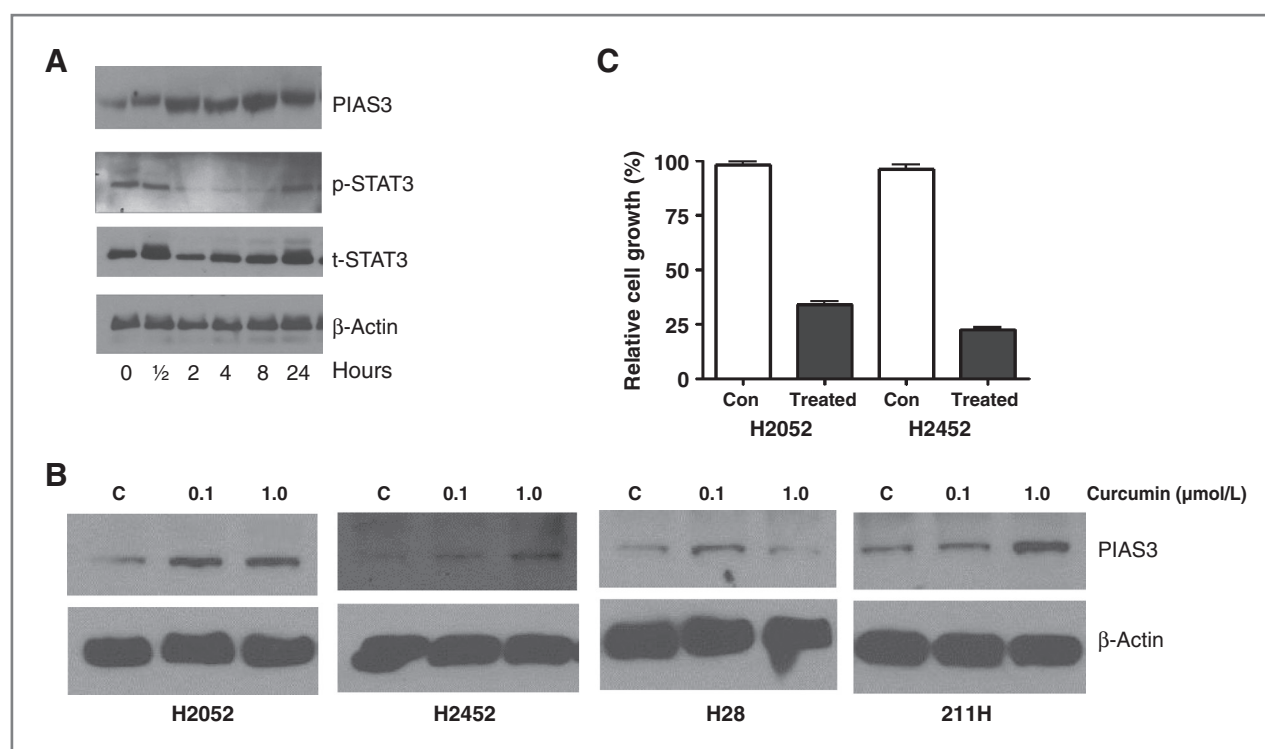


Figure 5. Curcumin increases endogenous PIAS3 expression in mesothelioma cells. A, H2052 cells were incubated with 1 $\mu\text{mol/L}$ curcumin for up to 24 hours and protein lysates immunoblotted for PIAS3, pSTAT3 (active), total STAT3, and β -actin. B, curcumin treatment at 0.1 or 1.0 $\mu\text{mol/L}$ for 24 hours shows an increase in PIAS3 protein expression level in most of the mesothelioma cell lines. C, curcumin inhibits cell proliferation. H2052 and H2452 cells were incubated for 24 hours in the absence or presence of 10 $\mu\text{mol/L}$ of curcumin and cell growth determined by DNA assay.

FLAG-tagged PIAS3 in H2052 and H2452 cells decreased p-STAT3 levels (Fig. 4A). Empty vector (EV)-transfected cells and untransfected cells served as controls (Fig. 4C). The decrease in activated STAT3 levels by PIAS3 overexpression also led to a decrease in cell growth, as measured by MTS (Fig. 4B) or cell viability assays (Fig. 4C). These results support our hypothesis that endogenous PIAS3 expression is downregulated in most mesothelioma cells and may thus contribute to the increased STAT3 activity that drives cell proliferation.

Curcumin induces PIAS3 expression in mesothelioma cell lines

Enhancement of PIAS3 expression represents a potential therapeutic strategy for cancers like mesothelioma which depend on activated STAT3 for sustained growth. Thus, the discovery of a small molecule that increases endogenous PIAS3 levels in STAT3-dependent cancer cells could potentially become beneficial. Curcumin may be such a molecule, as it is a well-known inhibitor of STAT3 activity (22–24). Indeed, there is a time-dependent increase in PIAS3 levels in H2052 mesothelioma cells exposed to 1.0 $\mu\text{mol/L}$ curcumin (Fig. 5A). This increase in PIAS3 was associated with a parallel decrease in p-STAT3 levels. No change in total STAT3 expression was observed, similar to β -actin. A dose-dependent increase in PIAS3 protein expression was observed upon curcumin treatment in all four mesotheli-

oma cell lines (Fig. 5B). Our experiments also demonstrated that curcumin treatment decreased mesothelioma cell growth (Fig. 5C). These results suggest that curcumin may produce growth inhibition in mesothelioma cells through increased PIAS3 expression.

A recombinant PIAS3 peptide induces apoptosis and inhibits growth in mesothelioma cells

It has recently been shown that rPP-C8, a C-terminal recombinant peptide derived from PIAS3, has the ability to inhibit STAT3 activity and cell growth in glioblastoma cells (17). The peptide is able to penetrate the plasma membrane upon addition to the growth medium. We incubated this peptide with mesothelioma cells to determine its effect on STAT3 activity. Western blotting experiments showed a decrease in p-STAT3 levels in H2052 and 211H cells after a 4-hour peptide treatment (Fig. 6A). There was no effect on total STAT3 or PIAS3 expression levels. Furthermore, addition of rPP-C8 to the medium of mesothelioma cells induced apoptosis, as shown by increased PARP cleavage (Fig. 6B). We next measured the effect of rPP-C8 on mesothelioma cell proliferation. H2052 and 211H cells were treated with 0.2 or 2.0 $\mu\text{mol/L}$ rPP-C8 peptide for 3 days. The mesothelioma cells demonstrated a dose-dependent decrease in cell viability upon addition of the peptide, compared with PBS-treated cells (Fig. 6C). This was confirmed by cell counting (Fig. 6D). Thus, the rPP-C8 peptide

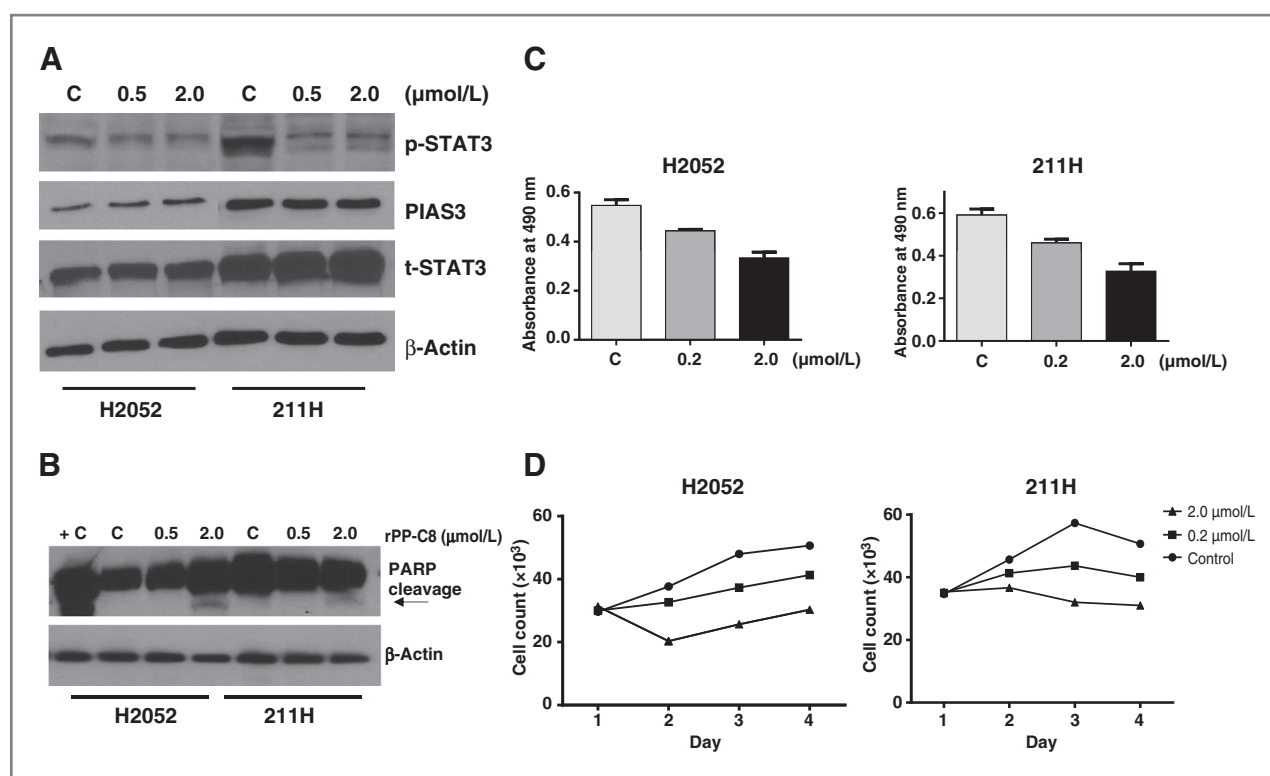


Figure 6. A recombinant PIAS3 peptide inhibits STAT3 activation and cell growth in mesothelioma cells. A, protein lysates were prepared after 4-hour exposure of H2052 and 211H cell lines to rPP-C8 peptide (0.5 and 2.0 μmol/L). Western blotting shows decrease in pSTAT3 (Tyr 705) without affecting total STAT3 or PIAS3 expression. β-Actin is a loading control. B, H2052 and 211H cells exposed to 0.5 or 2.0 μmol/L rPP-C8 peptide for 24 hours results in PARP cleavage at the higher concentration. +C is a positive control lysate. C, addition of the peptide to mesothelioma cells induces apoptosis (MTS assay) and cell growth inhibition (D).

appears to be efficiently taken up by mesothelioma cells and acts as a potent STAT3 inhibitor in mesothelioma cell lines.

Discussion

Our study addressed the question of whether the PIAS3 protein can be exploited as a potential drug target. We demonstrated elevated levels of STAT3 activation in mesothelioma and that mesothelioma may be "addicted" to STAT3 activity using siRNA. A recent report also showed that the active forms of STAT3 are highly expressed in most cases of mesothelioma (25). As the PIAS3 protein is being downregulated by cancer cells, a therapeutic strategy could be based upon the enhancement of PIAS3 expression because of its role in inhibiting activated STAT3 and thereby inducing apoptosis. The number of cases of mesothelioma is increasing and current chemotherapy, radiotherapy, and surgery treatments are mainly palliative (26). Clinical studies using molecular-targeted approaches have been promising for NSCLC, like EGFR inhibition and angiogenic blockade, but have failed to improve patient outcome in patients with mesothelioma (27, 28). Thus, new therapeutic strategies targeting PIAS3 could become valuable for cancers with persistent STAT3 activity, including mesotheliomas.

Many current cancer therapies target cell signal initiation at the level of membrane receptors, such as VEGFR and EGFR. However, multiple receptor tyrosine kinases are often activated in cancer, including mesothelioma (29, 30), making kinase-specific inhibitors less effective or subject to the development of resistance. For example, two recent phase II studies have shown that EGFR is highly expressed in 75% to 97% of mesothelioma tumor samples yet the disease was refractive to the EGFR tyrosine kinase inhibitors gefitinib (31) or erlotinib (32). These results indicate that downstream effectors of tyrosine kinase receptor signaling such as STATs may represent a more attractive choice for drug targeting because they represent an integration point for multiple oncogenic signaling pathways. Unfortunately, specific and effective STAT3 inhibitors are still only in early development, although promising lead compounds have recently been reported (33).

Here, we report for the first time the clinical significance of PIAS3 expression on overall patient survival. In this regard, our study has provided preliminary data that PIAS3 expression may be useful as a prognostic factor in mesothelioma. Previous reports have used immunohistochemistry to suggest an important role for PIAS3 expression in subsets of gastrointestinal carcinomas, glioblastoma cell tumors, and colorectal carcinoma (13, 34, 35). This further

emphasizes the potential role of PIAS3-targeted therapy in malignancies beyond mesothelioma.

Curcumin is regarded as the most active constituent derived from the spice turmeric (22). Turmeric has a long history in ancient Asian medicine and curcumin has been extensively studied in modern science as an anticancer agent both *in vitro* and *in vivo*. Here, we show that curcumin treatment can increase PIAS3 levels and thereby decrease STAT3 phosphorylation and cell viability in mesothelioma cells. In support of our findings, curcumin has been shown to increase PIAS3 levels and decrease STAT3 phosphorylation and cell viability in ovarian and endometrial cancer cells *in vitro* (24). These results suggest that curcumin may represent a lead compound to explore the potential of increasing endogenous PIAS3 levels as a therapeutic strategy against STAT3-dependent cancers. Although limited bioavailability of curcumin has hindered its clinical use (23), recent efforts to produce more pathway-specific analogs of curcumin, coupled with methods to increase its bioavailability, make this avenue more attractive for the treatment of mesothelioma (36, 37).

STAT3 inhibition can also be affected by peptides (5, 17, 38). Our study indicates that the uptake of a STAT3-inhibiting peptide into mesothelioma cells is effective and induces cell growth inhibition and apoptosis. There is an increase in the number of peptide drugs in preclinical and clinical development. Peptide degradation by proteolysis can be prevented by chemical modifications such as incorporation of D-amino acids or cyclization (39). Because of the tremendous advancement in the large-scale synthesis of peptides it will be possible to make peptide-based anticancer drugs more affordable to patients (40). Thus, peptides are poised to make a huge impact in the near future in the area of cancer treatment and diagnosis.

In conclusion, here we present PIAS3 as a potential prognostic marker that would be beneficial in the management of mesothelioma due to the variable biologic behavior of these tumors despite similar tumor stage, grade, and clinical presentations. Moreover, we present evidence that

downregulation of STAT3 or induction of PIAS3 protein expression by curcumin or using a recombinant PIAS3 peptide *in vitro* inhibit cell growth and induce apoptosis. Because the three-dimensional crystal structure of PIAS3 has recently been solved (<http://www.ebi.ac.uk/pdbe-srv/view/entry/4mvt/summary.html>), this should hasten work on developing effective PIAS3-directed therapeutics using small-molecule drug screens or finding structural analogs of the PIAS3 protein.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Dabir, G. Wildey, A. Dowlati

Development of methodology: S. Dabir, A. Dowlati

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Dabir, A. Kluge, A.M. Kresak, M. Yang, B. Groner, A. Dowlati

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Dabir, P. Fu, G. Wildey, A. Dowlati

Writing, review, and/or revision of the manuscript: S. Dabir, A.M. Kresak, B. Groner, G. Wildey, A. Dowlati

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Dabir, A.M. Kresak, G. Wildey, A. Dowlati

Study supervision: S. Dabir, G. Wildey, A. Dowlati

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