TIMP-2 stimulates cell proliferation through c-Src activation, which influences a worse prognosis for pathological stage I lung adenocarcinoma

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Tissue inhibitors of metalloproteinases (TIMPs) have been known to be involved in tumorigenesis in both matrix metalloproteinase (MMP)-dependent and MMP-independent manner. This manuscript highlights key findings from our recent research describing the mechanism by which TIMP-2 stimulates lung adenocarcinoma cell proliferation. Our study showed for the first time that TIMP-2 induces lung adenocarcinoma cell proliferation through c-Src kinase activation, independent of MMP inhibition. c-Src kinase activity, induced by TIMP-2, concomitantly increased FAK, phosphoinositide 3-kinase (PI3-kinase)/AKT, and ERK1/2 activation. Furthermore, we showed from multiple cohorts that high TIMP-2 expression in lung adenocarcinomas is associated with a worse prognosis, especially for stage I lung adenocarcinoma. Through integrated analysis of The Cancer Genome Atlas data, Reverse Phase Protein Assay data showed that Src phosphorylation at Y418 significantly increased when TIMP-2 was highly expressed. TIMP-2 expression was significantly associated with the alteration of driving genes and activation of the PI3-kinase/AKT pathway. Taken together, our results suggest that TIMP-2 may play a key role in tumorigenesis of lung adenocarcinoma.

Keywords: TIMPs; tumorigenesis; c-Src; lung adenocarcinoma; PI3-kinase/AKT pathway

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In our recent work, published in Oncotarget, we have discovered that the growth-stimulatory activity of tissue inhibitors of metalloproteinase (TIMP)-2 is mediated through c-Src activation followed by the activation of FAK, phosphoinositide 3-kinase (PI3-kinase)/AKT, and ERK1/2, independent of matrix metalloproteinase (MMP) inhibition in lung adenocarcinoma cells [1]. TIMP-2 promotes cell growth of A549 lung adenocarcinoma cells by activating nuclear factor kappa B (NF-κB) in an insulin-independent manner. Overexpression of TIMP-2 often positively correlates with tumorigenesis in lung and breast cancer patients [4-7]. Specifically, TIMP-2 overexpression was detected in the lungs of mice bearing Lewis lung carcinoma cells and was positively correlated with tumor progression in non-small lung carcinoma (NSCLC) [7-8]. Previous findings from in and clinical studies support the idea that TIMP-2’s growth-stimulatory activity may play a key role in lung tumorigenesis. Thus, we examined the mechanism by which TIMP-2 stimulates cell proliferation in lung adenocarcinoma cells. Additionally, we evaluated the association of TIMP-2’s
growth-stimulatory activity with lung adenocarcinoma prognosis in multiple independent cohorts through analysis a genome-wide survey of gene-expression data.

The growth-stimulatory ability of various concentrations TIMP-2 was tested in several lung adenocarcinoma cell lines, including A549, NCI-H2009, SK-LU-1, HCC-827, and A427. To exclude the effect of MMP inhibition, a TIMP-2 C72S mutant that cannot inhibit MMP activity was used in all of experiments with TIMP-2. The highest levels of proliferation were achieved when the cells were treated with 250 pM of either TIMP-2 or TIMP-2 C72S. TIMP-2 had the greatest effect on A549 and NCI-H2009 cell proliferation. There are several mechanisms that are associated with the growth-stimulatory activity of TIMP-2 in various types of cancer cells. TIMP-2 mediates a mitogenic response by stimulating adenylate cyclase to produce cyclic AMP which in turn activates protein kinase A (PKA) in HT-1080 cells [9]. In MG-63 cells, TIMP-2 stimulates cell growth via the PKA-Ras-PI3-kinase signaling pathway [10]. In addition, TIMP-2 promotes cell growth by activating NF-κB in A549 cells and melanoma cells [2, 11]. To determine the signaling pathways involved in the growth-stimulatory activity of TIMP-2 in lung adenocarcinoma cells, we tested the effects of various signaling inhibitors. TIMP-2-induced cell proliferation was significantly inhibited by H89, PD98059, LY294002, and NF-κB treatment, but not inhibited by the adenylate cyclase inhibitor, SQ22536. This result suggested that TIMP-2’s growth-stimulatory activity in A549 cells involved the activation of ERKs, PI3-kinase, and NF-κB, which is consistent with previous results [2, 10]. However, reports have shown that TIMP-2 stimulates cell proliferation in HT-1080 and MG-63 human osteosarcoma cells via a cAMP/PKA-dependent mechanism [9,10]. In our study, H89, a PKA inhibitor, reduced TIMP-2-induced cell proliferation. However, inhibition of adenylate cyclase with SQ22536 or inhibition of PKA activity with siRNA against the PKA catalytic subunit did not block the growth-stimulatory effect of TIMP-2 in A549 cells. H89 inhibits other signaling molecules such as casein kinase I, MLCK, PKC, and ROCK-II. We found that inhibition of PKC, one of the effects of H89, considerably reduced TIMP-2-stimulated cell proliferation. Our results suggested that the growth-stimulatory activity of TIMP-2 may involve casein kinase I, MLCK, ROCK-II, and/or PKC via a cAMP/PKA-independent mechanism in A549 cells.

In previous reports, tyrosine kinase inhibitors, such as genistein, erbstatin, and herbimycin A, significantly blocked TIMP-2-mediated cell growth in MG-63 cells [12]. Because Src family of tyrosine kinase plays a key role in cell growth, we included PP2 in our assay. PP2 strongly inhibited TIMP-2-induced cell proliferation, indicating that the Src family of tyrosine kinases is involved in TIMP-2 growth-stimulatory activity. TIMP-2 and TIMP-2 C72S increased phosphorylation of Src at Y418 and c-Src kinase.
activity. Maximal Src kinase activation was approximately 2.5-fold higher than control. Our findings demonstrate that TIMP-2 significantly induces c-Src kinase activity in an MMP-independent manner. To further verify that c-Src is involved in the growth-stimulatory activity of TIMP-2, we performed experiments using c-Src-silenced A549 cells and A549 stable cell lines expressing kinase-dead Src (K297R). These experiments showed that TIMP-2 and TIMP-2 C72S did not stimulate cell proliferation in either the c-Src siRNA-expressing A549 cells or the kinase-dead Src cell To examine the involvement of FAK, MAP kinase, or PI3-kinase during TIMP-2-induced A549 cell proliferation, we evaluated the following: FAK phosphorylation on Y925 downstream of c-Src, AKT phosphorylation downstream of PI3-kinase, and ERK1/2 phosphorylation. TIMP-2 increased FAK phosphorylation on Y925, AKT phosphorylation, and ERK1/2 phosphorylation. Next, we evaluated signaling protein activation in KDSrc-a cells, the A549 stable cell line expressing kinase-dead Src (K297R). In KDSrc-a cells, TIMP-2 treatment did not induce c-Src, FAK, ERK1/2, or AKT phosphorylation when compared with control treatment. This result suggested that FAK, ERK1/2, PI3-kinase, and AKT are downstream of c-Src. To further analyze the order these downstream signaling proteins, we evaluated the effect of TIMP-2 treatment in the absence or presence of the signaling inhibitors. Our results showed that TIMP-2 growth-stimulatory activity was mediated by c-Src followed by the FAK, PI3-kinase/AKT, and ERK1/2 pathways in an MMP-independent manner in A549 cells (Figure 1). MT1-MMP and α3β1 integrin are known to be cell-surface receptors for TIMP-2 [13-14]. TIMP-2 binds to the surface of human microvascular endothelial cells via interaction with α3β1 integrin, and this interaction suppresses VEGF-A- or FGF-2- induced cell proliferation in vivo [13]. We showed that TIMP-2 significantly increased the phosphorylation of c-Src, FAK, AKT, and ERK1/2 in α3 integrin siRNA-treated A549 cells when compared with control siRNA-treated A549 cells, suggesting that there is another TIMP-2 receptor besides integrin and MT1-MMP. Future studies will be needed to identify the receptor that mediates the mitogenic activity of TIMP-2.

To evaluate the association between the growth-stimulatory activity of TIMP-2 and lung adenocarcinoma prognosis, we collected gene expression from the Gene Expression Omnibus and TCGA. We divided the exploration cohort (n=226, JNCC cohort, GSE31210) two groups based on the median value of TIMP-2 expression (i.e. high and low subgroups). Kaplan-Meier (KM) plots of overall survival (OS) and of recurrence-free survival (RFS) revealed that patients with high TIMP-2 expression had prognosis. Distinct separation of OS and RFS survival curves based on TIMP-2 expression was confined to patients with pathological stage (pstage) I lung adenocarcinoma. Patients with pstage I lung adenocarcinoma and high TIMP-2 expression had survival curves similar to those of patients with pstage II lung adenocarcinoma (Figure 2A-2B). When lung adenocarcinoma patients from another independent cohort (n=332; MGH + ACC + Nagoya, GSE13213 and GSE11969) and TCGA lung adenocarcinoma cohort (n=203) were divided into two groups according to TIMP-2 expression, KM plots showed significant difference in OS for only pstage I (p<0.001) (Figure 2C-2D). To investigate the correlation between high TIMP-2 expression and TIMP-2-induced signaling pathways as well as the alteration of driving genes, 93 patients with stage I lung from TCGA database were sorted according to TIMP-2 mRNA expression. Interestingly, Reverse Phase Protein Assay (RPPA) data showed that Src phosphorylation at Y418 significantly increased when TIMP-2 was highly expressed (Figure 2E). Also, the PIK3CA and AKT branch pathway is significantly activated in patients with high TIMP-2 expression, but Serine/Threonine Kinase 11 (STK11)-AMPK inactivation is related to low TIMP-2 expression. High TIMP-2 expression was associated with high rates of TP53 mutation, low rates of STK11 mutation, and EGFR and copy number alterations. These findings are consistent with our data suggesting that the growth-stimulatory activity of TIMP-2 is mediated through Src activation followed by the activation of PI3-kinase and AKT, and that patients with I lung adenocarcinoma and high TIMP-2 expression have a poor prognosis. Prior unsupervised analyses of lung adenocarcinoma gene expression have used varying nomenclature for transcriptional subtypes of the disease To coordinate naming of the transcriptional subtypes with the histopathological, anatomical, and mutational classifications of lung adenocarcinoma, TCGA Research Network proposed an updated nomenclature: the terminal
Using integrated analysis, we identified new treatment decisions based on the tumors’ molecular profiles. The activity may represent a tool that could help further refine our mechanistic investigation of TIMP-2 growth-stimulatory adjuvant chemotherapy in early stage lung adenocarcinoma, their ability to assess risk of recurrence and benefit from targeted therapy option for stage I patients with high TIMP-2 expression. These findings imply that the activation of TIMP-2 in certain subtypes of stage I lung adenocarcinoma may play a pivotal role in altering genes related to oncogenesis.

The American Joint Committee on Cancer staging system is currently used to guide treatment decisions and is the best predictor of prognosis for patients with NSCLC. While postoperative adjuvant chemotherapy is the standard care among patients with stage II to IIA NSCLC who have undergone complete resections, surgery remains as the only recommended guideline for treatment of stage I NSCLC. For patients with stage IA and IB NSCLC who are candidates for surgical resection, the 5-year OS is approximately 73% and 58%, respectively [21-22]. The recurrence rate in stage I patients who undergo surgical resection ranges from 13-41% [23-24]. Thus, postoperative recurrence is a major obstacle to prolonged survival in early stage NSCLC, and considerable differences exist among patients with the same pathological stage. This indicates that NSCLC is a very heterogeneous cancer even at the earliest stage, and this underlying heterogeneity is not well-reflected in the current staging system. A small fraction of NSCLC patients have underlying EGFR mutations or EML4-ALK fusions that are associated with relatively high response rates to targeted molecular therapies [25-27]. Src kinase inhibitor treatment could be a targeted therapy option for stage I patients with high TIMP-2 expression, high rates of TP53 mutation, low rates of STK11 protein kinase A; STK11: Serine/Threonine Kinase 11; TRU: terminal respiratory unit; PI: proximal-inflammatory; PP: proximal-proliferative.

Conclusions

Since current staging systems and biomarkers are limited in their ability to assess risk of recurrence and benefit from adjuvant chemotherapy in early stage lung adenocarcinoma, our mechanistic investigation of TIMP-2 growth-stimulatory activity may represent a tool that could help further refine treatment decisions based on the tumors’ molecular profiles. Using integrated analysis, we identified new pathway-dependent prognostic subgroups of lung adenocarcinoma that show significant differences in patient survival, especially in stage I lung adenocarcinoma. This study provides new knowledge by illuminating modes of genomic alteration, highlighting previously unappreciated TIMP-2-related genes, and enabling further refinement in sub-classification for improved personalization of treatment for this deadly disease. Thus, our results, if confirmed in prospective studies, may improve patient care by providing more practical guidance for treatment.

Conflicting interests

The authors have declared that no conflict of interests exist.

Author contributions

S. J. Lee handled everything of this manuscript.

Abbreviations


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