Dysregulated activation of c-Src in gestational trophoblastic disease contributes to its aggressive progression

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ABSTRACT

Introduction: Gestational trophoblastic disease (GTD) is a heterogeneous group of pregnancy-related disorders. Hydatidiform mole (HM) is the most common type of GTD, whereas gestational choriocarcinoma is the most aggressive. Non-receptor tyrosine kinase c-Src contributes to the transformation to a malignant phenotype in various cancers. However, the role of c-Src in the pathogenesis of GTD remains largely unknown.

Methods: The expression level of phosphorylated c-Src was determined by immunohistochemistry and Western blotting assay. JAR and JEG-3 cells were treated with hCG, specific c-Src inhibitor saracatinib and PP2, and PKA specific inhibitor, PKI. Cell growth rate and cell migration/invasion ability was determined by cell proliferation and transwell assays respectively.

Results: c-Src was highly activated in HM tissues and choriocarcinoma cells (JAR and JEG-3). c-Src was activated by hCG in a time and concentration-dependent manner, which was abrogated by specific c-Src and PKA inhibitors. Inhibition of c-Src activity in JAR and JEG-3 cells by saracatinib leaded to a decrease in the rate of cell growth and cell migration/invasion ability. Furthermore, inhibition of c-Src phosphorylation induced cell cycle arrest and reduced expressions of cyclin A2, cyclin B1, cyclin E1, FOXD3 and NANOG. Moreover, inhibition of c-Src activity resulted in decreased p-FAK Tyr397 phosphorylation.

Discussion and conclusion: Our findings indicate an important role of c-Src in the pathogenesis of GTD, and we propose that c-Src inhibitors are potential adjuvant chemotherapeutic drugs for the treatment of GTD.

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1. Introduction

Gestational trophoblastic disease (GTD) refers to a spectrum of pregnancy-related disorders, ranging from the pre-malignant forms of partial and complete hydatidiform mole (HM), to the malignant forms of invasive mole, choriocarcinoma, placental-site trophoblastic tumor (PSTT) and epithelioid trophoblastic tumor (ETT). The malignant forms of GTD are termed gestational trophoblastic neoplasia (GTN) [1,2]. HM is the most common type of GTD. It is characterized by overgrowth of trophoblasts and stromal swelling of placental villi companied by stunted or absent embryonic development [3]. Most women with HM can be cured after suction evacuation and curettage, however, postmolar GTN developed in approximately 15–20% with HM [4,5]. The prophylactic chemotherapy after evacuation of high risk HMs by drugs (such as methotrexate or actinomycin D) reduced the incidence of postmolar GTN down to 3–8% [5–7]. However, potential drug resistance, possibly delayed treatment of GTN and toxic side effects make the use of prophylactic chemotherapy limited. Thus, to better understand the pathogenesis of GTD and develop safer and more effective medical treatment is need.

c-Src is a non-receptor tyrosine kinase and plays essential roles in cell proliferation, survival, adhesion, morphology and motility [8,9]. The expression and kinase activity of c-Src are elevated in a large variety of human cancers including liver, colon, pancreas, lung, breast and ovary cancers [10]. Moreover, studies demonstrate that c-Src activated signaling pathways are involved in cell motility and invasion, which contributes to the transformation of metastatic phenotype [11,12]. The enzyme activation of c-Src is determined by phosphorylation at either Tyr416 (p-SrcTyr416) or Tyr527 (p-
Src Tyr527 residues. p-Src Tyr416 allows c-Src access to the substrate and catalytically active whereas p-Src Tyr527 renders the enzyme inactive. The N-terminal of c-Src contains a myristoyl group responses for its membrane localization [13]. Highly selective c-Src kinase inhibitors, such as dasatinib [14], saracatinib [15,16], bosutinib [17] and PH006 [18] have anti-tumor activity in vitro and in vivo without obvious intolerable toxicity even in high dosage: In phase I/II clinical studies, saracatinib alone or in combination with paclitaxel or gemcitabine inhibited c-Src activity effectively and showed acceptable toxicity [16,19,20].

The role of c-Src in the pathogenesis of GTD is not established. In the current study, we investigated the role of activated c-Src in the pathogenesis of GTD and explored the potential therapeutic effect of c-Src inhibitors in GTD treatment.

2. Materials and methods

2.1. Reagents and antibodies

Leupeptin, aprotinin and phenylmethylsulfonyl fluoride were purchased from Sigma–Aldrich Inc (St. Louis, MO), PP2 and saracatinib (AZD0530) were from Selleck Chemicals (Houston, TX). Antibodies against c-Src and phosho-tyrosine, p-Src Tyr416 were purchased from Cell Signaling Technology (Danvers, MA), and antibodies against FAK and phospho-FAK (Tyr-397) were from Epitomics Inc. (Burlingame, CA), Anti β-actin antibody was from Abmart Inc. (Shanghai, China), HRP-conjugated antimouse/rabbit IgG secondary antibody were from Jackson Immunoresearch (West Grove, PA). Other reagents were commercially available in China.

2.2. Clinical samples and cell lines

A total of 120 trophoblastic tissues (26 normal first trimester placentas and 94 HM tissues) were retrieved from the archives of Departments of Pathology and Bio-bank, the International Peace Maternity and Child Health Hospital (IPMCH), Shanghai Jiao Tong University (SJTU). HMs were diagnosed according to the commonly accepted morphological and clinical criteria [21]. Ethical approval for the use of tissues in this study has been obtained from institutional ethical review board of IPMCH, SJTU.

JAR and JEG-3 choriocarcinoma cell lines were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin. Routinely, the purity of trophoblast cells was tested by immunofluorescence with antibodies against cytokeratin 7 and vimentin (eBioscience) as described elsewhere [24]. Positive cytokeratin 7 and negative vimentin staining was taken as successful isolation.

2.3. Isolation of primary human trophoblasts

Primary human trophoblasts were isolated from first trimester placentas (6–9 gestational weeks) by 30 min digestion with 1 U/ml Dispase II (Roche) and 0.2% collagenase I (Sigma) at 37 °C. Collagenase I and phospho-tyrosine, Src Tyr416 were purchased from Cell Signaling Technology (Danvers, MA), and antibodies against FAK and phospho-FAK (Tyr-397) were from Epitomics Inc. (Burlingame, CA), Anti β-actin antibody was from Abmart Inc. (Shanghai, China), HRP-conjugated antimouse/rabbit IgG secondary antibody were from Jackson Immunoresearch (West Grove, PA). Other reagents were commercially available in China.

2.4. Total RNA isolation and quantitative RT-PCR assay

Total RNA was extracted from JAR cells using TriZol reagent (Invitrogen, Carlsbad, CA). The 2 µg RNA was treated with DNase I, subsequently cDNA was obtained using reverse transcriptase kit from Takara Biotech (Tokyo, Japan) according to the manufacturer’s instructions. Quantitative real time RT-PCR (qRT-PCR) analysis of Cyclin A2, Cyclin E1, Cyclin B1, P18, CDK4, FOXD3 and NANOG 4 gene expression was performed with SYBR Green premix (Takara Biotech) on an ABI StepOnePlus system (Applied Biosystems, Foster City, CA). The primers for qRT-PCR assay were listed in Supplemental Table 1. The relative mRNA levels of target genes were normalized to the amount of 18S rRNA in an identical cDNA sample using the 2 −ΔΔCt method.

2.5. Western blotting assay

Western blotting assays were performed as described previously [25,26]. Briefly, 50–100 mg trophoblastic tissues were homogenized in 1 ml RIPA buffer (Beyotime, Nanjing, China) and 106 cells were suspended in 100 µl SDS lysis buffer (Beyotime, Nanjing, China). Lysates were separated by electrophoresis in an 8% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). After blocking with 5% bovine serum albumin (BSA), the membrane was incubated with primary antibodies against c-Src, p-Src Tyr527, GAPDH, p-FAK Tyr992 and FAK (Supplemental Table 2) for 2 h at room temperature and with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h. The membranes were then washed 3 times in TBS containing 0.1% Tween-20. Protein band was developed using the enhanced chemiluminescence (ECL) assay kit (Tiangen Biotech, Beijing, China). GAPDH was used as the endogenous loading control.

2.6. Immunohistochemistry staining

Paraffin-embedded tissues were cut at 5 µm thick. The sections were deparaffinized, rehydrated, incubated with 3% H2O2 in methanol for 10 min to quench the activity of endogenous peroxidase and subjected to antigen retrieval by heating the slides at 100 °C for 15 min in 1 mM Ethylene Diamine Tetraacetic Acid (EDTA) buffer (pH 8.0). Immunohistochemistry was performed using the SuperPictureTM 3rd Gen IHC Detection Kit Horseshadish Peroxidase Polymer (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. A monoclonal rabbit anti-human p-Src Tyr416 antibody was applied in 1:50 dilution and incubated overnight at 4 °C. Subsequently, HRP-labeled secondary antibody was incubated for 30 min at 37 °C. The sections were then developed in 0.05% diamobenzidine containing 0.01% hydrogen peroxide for 5 min at room temperature and counterstained with hematoxylin. Negative controls were prepared by replacing the primary antibody with normal Rabbit IgG. Staining intensity was evaluated as 0-negative; 1-weak; 2-moderate; 3-intense according to a previous report [27]. Images were captured at magnification of 200× under a Leica microscope.

2.7. Cell proliferation assay

Cell proliferation assay was performed using the Cell Counting Kit-8 (CCK-8) from Dojindo (Kanamishiki-gun Kumanomito, Japan) according to the manufacturer’s instructions. The method is based on tetrazolium salt reduction assay. Briefly, JAR and JEG-3 cells were seeded on 96-well plates at a density of 5000 cells/well and treated with DM50 or 10 µM saracatinib for 1–3 days. At each time point, cells were incubated with CCK-8 reagent for 1 h at 37 °C, followed by measuring the absorbance at 450 nm with the Universal Microplate Reader (BIO-TEK Instruments, Minneapolis, MN). All experiments were carried out in triplicate.

2.8. Cell cycle analysis

About 1 × 106 JAR or JEG-3 cells were collected, washed twice in PBS, and fixed in ice-cold 70% ethanol overnight. The fixed cells were washed and stained with propidium iodide/RNAse staining solution (BD Biosciences, Rockville, MD) for 30 min at 37 °C. The DNA content was determined by flow cytometry with standard optics of Beckman Coulter flow cytometer (Beckman Coulter Inc., Miami, FL). The percentage of cells in each phase of cell cycle was calculated using the Multicycle Program (Beckman Coulter Inc., Miami, FL).

2.9. Transwell cell migration and invasion assay

Cell migration assay was performed as described previously using Transwell chambers (tissue culture treated, 6.5 mm diameter, 0.4 µm pores from Costar Corp, Cambridge, MA) [26,28]. For invasion assay, the upper membranes were coated with Matrigel (BD Biosciences, Rockville, MD). Briefly, serum starved JAR and JEG-3 cells were suspended and counted. Then 105 cells in 100 µl serum-free medium were added to the upper chamber and 500 µl DMEM medium with 10% FBS was added to the lower chamber. Cells were incubated for 24 h at 37 °C. Non-migratory cells on the upper membrane surface were removed and the migratory cells on the undersurface were fixed, stained with 0.1% crystal violet for 20 min at room temperature and counted on a Leica microscope.

2.10. Statistics

Data are shown as mean ± SEM. Statistical analysis was performed using the Statistical Package for Social Science (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA). The Student’s t-test, two-way ANOVA and chi-squared test were used to evaluate the experimental data. Differences were considered statistically significant at p < 0.05.

3. Results

3.1. c-Src was highly activated in HM tissues and choriocarcinoma cells

To explore the role of c-Src in GTD, we firstly examined the activation status of c-Src in normal first trimester placentas and HM tissues. Western blotting assay on freshly collected samples was applied for the expressions of c-Src and p-Src Tyr416. As shown in Fig. 1A, the expression of activated form p-Src Tyr416 was about 3-fold higher in HM tissues than that in first trimester placentas (n = 7, p < 0.001, student’s t-test), whereas no significant differences of total c-Src protein level were observed. By immunohistochemistry (IHC), we found p-Src Tyr416 staining signal was...
predominantly located on the plasma membrane of the syncytiotrophoblasts (ST), cytotrophoblasts (CT) and villous intermediate trophoblasts (VIT) (Fig. 1B). Signal intensity was showed as 0-negative; 1-weak; 2-moderate; 3-intense for IHC score. In accord with the Western blotting findings, significantly higher p-SrcTyr416 immunostaining signal was observed in the HM tissues (99% samples positive staining and about 50% samples displayed strong intensity) compared with normal first trimester samples (65% samples positive staining and only about 7% samples showed strong intensity) (p < 0.001, chi-squared test, Fig. 1C). Furthermore, we demonstrated that p-SrcTyr416 was upregulated in choriocarcinoma cell lines, JAR and JEG-3 when compared with primary human trophoblasts (PHT, Fig. 1D). Together, these findings suggest that c-Src is highly activated in HM tissues and choriocarcinoma cells.

3.2. c-Src was activated by hCG in trophoblastic cells

To dissect molecular mechanism underlying the enhanced activation of c-Src in GTD, we evaluated the potential induction effect by hCG in choriocarcinoma cells. JAR cells were serum starved overnight, and treated with 0, 5, 12.5, 25, 50 or 100 IU/ml hCG for 30 min. hCG showed dose-dependent stimulatory effects on p-SrcTyr416 level (Fig. 2A). Furthermore, serum starved JAR cells were treated with 50 IU/ml hCG for 15, 30, 45, 60 or 120 min p-SrcTyr416 level was up-regulated at 15 min and peaked at 60 min (Fig. 2B). To further ascertain whether the regulation exists between endogenous hCG and c-Src, we treated JAR cells with 10 μg/ml neutralization antibody against β-hCG. As expected, p-SrcTyr416 level was dramatically reduced upon hCG signal blocking (Fig. 2C). In addition, the inductive role of hCG for activation of c-Src in choriocarcinoma cells was abrogated by co-administration of specific inhibitors of c-Src kinase, saracatinib and PP2 (Fig. 2D). These data demonstrated the activation of c-Src was hCG dependent in trophoblastic cells.

3.3. PKA inhibition abolished the effect of hCG induced c-Src activation

Previous reports suggested that PKA might be involved in c-Src activation as a downstream kinase in G-protein coupled receptor pathway [29,30]. To ascertain whether PKA was required for p-SrcTyr416 activation downstream of hCG/hCG receptor, we treated serum starved JAR cells with 50 IU/ml hCG together with/without 10 μM PKI (a PKA specific inhibitor). As shown in Fig. 3, PKA inhibition almost abolished the effect of hCG induced p-SrcTyr416 elevation, which suggested that activation of c-Src by hCG might be in a PKA dependent manner.
3.4. Inhibition of c-Src signaling significantly attenuated cell proliferation and cell cycle progression

To better understand the role of c-Src in the pathogenesis of GTD, we examined the cell growth rates by cell proliferation assay. JAR and JEG-3 cells were treated with DMSO or saracatinib (1 μM) for 1–3 days. As shown in Fig. 4A, the administration of saracatinib significantly inhibited the growth of JAR and JEG-3 cells (n = 8, p < 0.001, two-way ANOVA). On the basis of previous studies [31,32] and our findings, we supposed that the impact of c-Src on cell growth might be associated with cell cycle regulation. To verify this, the cell cycle distributions of JAR and JEG-3 cells treated by saracatinib for 24 h were detected with flow cytometer. Compared to the DMSO group, JAR and JEG-3 cells under saracatinib treatment were arrested at G0/G1 phase (Fig. 4B; p < 0.01 for JAR, p < 0.05 for JEG-3, student’s t-test). To further investigate the effect of saracatinib on cell cycle, we examined the mRNA expressions of cell cycle-associated genes. Administration of saracatinib for 48 h attenuated expressions of cell cycle promoting genes (Cyclin A2, Cyclin E1, Cyclin B1 and CDK4), while enhanced expression of cell cycle progression inhibiting gene (P18). In addition, trophoblastic and embryonic stem cell transcriptional factors (FOXD3 and NANOG), which promote trophoblastic cell growth and cell cycle progression, were also reduced under saracatinib treatment (Fig. 4C). These data indicated that saracatinib induced choriocarcinoma cells growth inhibition through cell cycle attenuation.

3.5. Inhibition of c-Src signaling attenuated the migration and invasion of choriocarcinoma cells

We further assessed whether c-Src activation modulated the migratory and invasive ability of choriocarcinoma cells. JAR and JEG-3 cells were treated with DMSO or 1 μM saracatinib in transwell chambers for 24 h. We found that the motility of choriocarcinoma cells were substantially impeded shown representatively in Fig. 5A. Inhibition of c-Src activity by saracatinib attenuated the migration (about 52% inhibition for JAR and 67% inhibition for JEG-3) and invasion (about 60% inhibition for JAR and 74% inhibition for JEG-3) of choriocarcinoma cells compared to DMSO treated cells (Fig. 5B, p < 0.001, p < 0.001). Furthermore, the activity of FAK, a key regulator of cell migration and invasion [33], was also down-regulated with saracatinib treatment for 24 h, as shown by markedly decreased p-FAK\textsubscript{Y397} levels in JAR and JEG-3 cells (Fig. 5C). These results indicated that activation of c-Src induced FAK activation promoted migration and invasion of choriocarcinoma cells in vitro.

4. Discussion

c-Src tyrosine kinase plays an important role in various cellular functions, including cell survival, cell cycle progression, growth and migration [8,9]. In this study, we revealed that c-Src is highly activated in GTD tissues when compared with normal placentas. We also found, by immunohistochemistry, that activated c-Src predominantly localized in the plasma membrane of the trophoblasts. The membrane association of c-Src depends on its N-terminal residues containing an N-myristoylation motif. A mutation eliminating myristoylation blocks cell transformation by activated c-Src, the membrane association is thus very critical for c-Src signaling [34–36].
As a key regulator in signal transduction, c-Src can be activated by a variety of extracellular signals. Previous studies have highlighted c-Src as an important activator in MAPK/ERK signaling downstream of EGF and estrogen [25]. Activated EGFR family receptor tyrosine kinases (RTKs) recruit and activate c-Src, and c-Src in turn further activates RTKs [37,38]. c-Src, which is activated by ligand-bound estrogen receptor (ER), rapidly and transiently stimulates activation of downstream signaling [39]. Here we showed that c-Src expressed in trophoblastic cells was activated by hCG in a time and concentration-dependent manner, which required recruitments of GPCR and PKA cascade. Previous studies in villous trophoblast and BeWo cells identified PKA as a mediator for the biological effects of cAMP on cell differentiation and hormonogenesis during the syncytialization process. In line with this notion, we showed PKA played an essential role in hCG induced c-Src activation. Whether c-Src is involved in trophoblast differentiation and hCG secretion needs further study.

The role of c-Src in cell proliferation and cell cycle regulation has been established [31,32]. Marcotte et al. showed that c-Src deficiency resulted in arrested proliferation in PyVmT tumor cells,
as loss of c-Src leaded to reduced expression of Cyclin A, Cyclin B and Cyclin E, increased expression P27KIP1 and P16INK4A, and inefficient progression beyond the G1-S transition [31]. Another study demonstrated that c-Src phosphorylated P27KIP1 impaired its CDK2 inhibitory action and reduced the binding between P27KIP1 and Cyclin E-CDK2 complex, leading to P27KIP1 proteolysis and cell cycle progression [40].

Our in vitro experiments demonstrated that inhibition of c-Src signaling attenuated cell proliferation by inhibiting the expressions of Cyclin A2, Cyclin E1, Cyclin B1 and CDK4, and arresting cell cycle in choriocarcinoma cells. NANOG and FOXD3 are key regulatory transcription factors involved in the expansion development of embryonic and trophoblastic cells. One previous study demonstrated that NANOG overexpressed in HMs and choriocarcinoma cells, was involved in the pathogenesis and clinical progress of GTD [41]. In another study, we also revealed that FoxD3 was overexpressed in GTN and dysregulated the functions of choriocarcinoma cells (unpublished data). Here we showed that inhibition of c-Src kinase reduced expressions of NANOG and FOXD3, which provides extra-mechanism for the involvement of c-Src in pathogenesis of GTD.

The cell migration is an integrated process that requires the coordination with extracellular factors, kinases, phosphatases, scaffold proteins and cell skeleton proteins [42]. c-Src/FAK dual complex kinases play key roles in the process, which phosphorylize paxillin and p130Crk-associated substrate and recruit key molecules to adhesion sites and actin cytoskeleton [43,44]. In this respect we have now demonstrated that hCG induced FAK and c-Src activation, as well as cell migration and invasion, via c-Src kinase activity in choriocarcinoma cells. These data extend our current understanding of the role of hCG in trophoblast regulation.

Although most women with HM can be cured after suction evacuation and curettage, some women are still at the risk of developing into malignant GTN [4]. It was reported that administering prophylactic chemotherapy after evacuation of HM reduced the incidence of postmolar GTN from 15 to 20% down to 3–8% [6,7]. However, giving prophylactic chemotherapy to prevent malignant transformation of HM remains controversial, as the potential risks are unclear. In this study, we showed that saracatinib, a specific inhibitor of c-Src with low toxicity, could attenuate choriocarcinoma cells proliferation, migration and invasion. Thus, we proposed a possible adjuvant therapeutic value for specific inhibitors of c-Src in treating patients with GTN, which warrants further investigation.

In summary, our findings defined a possible mechanism by which hCG induced c-Src activation and promoted gestational trophoblastic disease progression. c-Src signaling was induced by hCG/CGR/PKA cascade in choriocarcinoma cells. c-Src promoted malignant choriocarcinoma cells proliferation by upregulating transcription of cell cycle-promoting genes and trophoblastic stem cell transcriptional factors. In addition, c-Src augments choriocarcinoma cells migration and invasion through induction of FAK phosphorylation. Herein, our data identified a critical role of c-Src in pathogenesis of GTD with its effects on cell proliferation and migration, and proposed a potential treatment targeting c-Src activity in GTD.
Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.placenta.2014.07.012.

References


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