Activated Stat3 expression in gestational trophoblastic disease: correlation with clinicopathological parameters and apoptotic indices

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Aims: To assess the expression profile of the activated form of signal transducer and activator of transcription (Stat)3 in gestational trophoblastic disease (GTD) and correlate the findings with clinicopathological parameters.

Methods and results: By immunohistochemistry, both cytoplasmic and nuclear expression of p-Stat3-Ser727 was demonstrated in 88 trophoblastic tissues, including placentas and GTD. Nuclear immunoreactivity of p-Stat3-Ser727 was significantly higher in hydatidiform mole (HM) (P < 0.001) and choriocarcinoma (P = 0.009) when compared with normal placentas. Placental site trophoblastic tumours (PSTT) and epithelioid trophoblastic tumours (ETT) also demonstrated higher nuclear p-Stat3-Ser727 expression than their normal trophoblast counterparts. Higher p-Stat3-Ser727 expression was confirmed in choriocarcinoma cell lines, JEG-3 and JAR, than in a normal trophoblast cell line, with both nuclear and cytoplasmic fractions demonstrated by immunoblotting. Spontaneously regressed HM showed significantly increased nuclear and cytoplasmic p-Stat3-Ser727 immunoreactivity over those that developed gestational trophoblastic neoplasia (GTN) (P = 0.013, P = 0.039). There was a significant positive and inverse correlation between nuclear p-Stat3-Ser727 immunoreactivity and apoptotic indices [terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labelling and M30 CytoDeath antibody] (P = 0.001, P < 0.001, Spearman’s ρ test) and Bcl-2 expression (P = 0.034), respectively.

Conclusions: p-Stat3-Ser727 plays a role in the pathogenesis of GTD, probably through the regulation of apoptosis. p-Stat3-Ser727 immunoreactivity is a potential marker in predicting GTN in HM.

Keywords: gestational trophoblastic disease, Stat3

Abbreviations: CCA, choriocarcinoma; CM, complete mole; CT, cytotrophoblast; ETT, epithelioid trophoblastic tumour; GTD, gestational trophoblastic disease; GTN, gestational trophoblastic neoplasia; HM, hydatidiform mole; HRP, horseradish peroxidase; IT, intermediate trophoblast; LIF, leukaemia inhibitory factor; PM, partial mole; PSTT, placental site trophoblastic tumour; ST, syncytiotrophoblast; Stat, signal transducer and activator of transcription; TBS, tris-buffered saline; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labelling.

Introduction

Gestational trophoblastic disease (GTD) is a family of diseases derived from abnormal growth of trophoblast. It comprises hydatidiform mole (HM), choriocarcinoma (CCA), placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT). Whereas the latter three are evidently malignant tumours, HMs can...
be considered as an abnormal conceptus with potential for malignant transformation. Molar pregnancies are further divided into complete (CM) and partial mole (PM) depending on their genotype and pathological features. After treatment by suction evacuation, most HM will spontaneously regress. Although about 8–30% will develop into gestational trophoblastic neoplasia (GTN) requiring chemotherapy.1–4 Due to the unpredictability of the progression of HM, close follow-up with serial assay of human chorionic gonadotrophin levels is routinely carried out.

Signal transducer and activator of transcription (Stat), as its name implies, carries signals from the cytoplasm to the nucleus, where it serves as a transcriptional factor.5,6 Upon activation by cytokines such as leukaemia inhibitory factor (LIF), cytokine receptor dimerization and subsequent phosphorylation of Stat occurs. The activated Stat forms a dimer and translocates into the nucleus to initiate gene transcription. There are altogether seven mammalian members of the family, namely Stat1, Stat2, Stat3, Stat4, Stat5A, Stat5B and Stat6.7 Stat3 has been shown to be involved in a wide variety of biological responses.8 In cultured embryonic stem cells, LIF-mediated Stat3 activation is required to sustain self-renewal.9

Aberrant Stat activation has been found in a variety of human malignancies and their cell lines.10–15 Constitutively activated Stat3 is amongst the most common members involved in neoplastic transformation, invasion and metastasis of various tumours.16 In vitro studies involving p-Stat3-Tyr705 and choriocarcinoma cell lines have suggested the effect of Stat3 activity on regulation of proliferation and invasiveness.17–19

Phosphorylation at Ser727 and Tyr705 is essential for the enhanced transcriptional activities of Stat3.20,21 Ser727 is the equally important but less studied phosphorylation site on Stat3. The role of activated Stat3 in GTD has not been fully explored. This study aimed to assess the expression profiles of p-Stat3-Ser727 in GTD by immunohistochemistry and Western blotting in association with apoptotic activities and clinical outcome.

Materials and methods

Clinical samples and related data

For immunohistochemistry, 88 trophoblastic tissues, including 16 first trimester placentas, 10 term placentas, nine PM, 32 CM, 10 CCA, eight PSTT and three ETT, were retrieved from the archives of the Department of Pathology, Queen Mary Hospital, the University of Hong Kong. The tissues were collected with the approval of the Institutional Review Board.

The tissue sections were histologically reviewed by using generally agreed and accepted diagnostic criteria.1,2 Most HM cases had been confirmed by fluorescent microsatellite genotyping after microdissection and chromosome in situ hybridization for ploidy analysis.22,23 The clinical information of the patients was also retrieved. Among the 41 HM cases, nine developed GTN requiring chemotherapy. These cases have also been involved in previous studies on apoptosis and their regulatory genes.24,25

Immunohistochemical study

Paraffin sections (5 μm) were cut and deparaffinized. Antigen retrieval was carried out at 95 °C for 10 min in 10 mM sodium citrate buffer at pH 6.0.26,27 Immunohistochemistry was performed using the UltraVision LP Value Detection System Horseradish Peroxidase (HRP) Polymer (LabVision, Fremont, CA, USA). A rabbit anti-p-Stat3-Ser727 antibody (Cell Signaling Technology, Beverly, MA, USA) was applied at a dilution of 1 : 50 and incubated overnight at room temperature. 3-diaminobenzidine-hydrogen peroxide was used as chromogen and sections were counterstained with haematoxylin. A negative control was prepared by replacing the primary antibody with Tris-buffered saline (TBS). A case of HM with extensive immunoreactivity for p-stat3-Ser727 was used as a positive control.

Assessment of immunoreactivity

Immunoreactivity was assessed by two individual pathologists (H. J. Z. and A. N. Y. C.) as described previously.26,27 The percentage of immunopositive cells was scored according to the following criteria: 0, no positivity; 1, 0.1–25.0% of cells immunopositive; 2, 25.1–50.0% immunopositive; 3, 50.1–75.0% immunopositive; 4, 75.1–100% of cells immunopositive. A signal intensity score was assigned according to the following criteria: 0, immunonegative; 1, weak positivity; 2, moderate positivity; 3, strong positivity; 4, very strong positivity. An immunohistochemical score was then established by adding up the percentage of positive scores and the signal intensity scores, giving a range of 0, 2–8.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science 10.1 for Windows (SPSS Inc., Chicago, IL, USA). The significance of differences
in immunoreactivity scores between categories was analysed with the non-parametric Mann–Whitney test. The overall significance of differences among all the categories was analysed with the Kruskal–Wallis rank test. Correlation between p-Stat3-Ser\textsuperscript{727} scores and apoptotic indices was analyzed using Spearman’s ρ test. Results with $P < 0.05$ were considered to be significant.

**CELL CULTURE, TOTAL AND SUBCELLULAR PROTEIN EXTRACTION**

Two choriocarcinoma cell lines (JEG-3 and JAR) (American Type Culture Collection, Manassas, VA, USA) and one normal extravillous trophoblast cell line (TEV-1)\textsuperscript{28} were cultured in Minimum Essential Eagle’s Medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), and 100 U/ml penicillin and streptomycin (Invitrogen, San Diego, CA, USA). Total protein lysate from JEG-3, JAR and TEV-1 was extracted with lysis buffer [0.125 M Tris, pH 6.8 at 22°C containing 1% NP-40 (v/v), 2 mM ethylenediamine tetraacetic acid, 2 mM N-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 0.1 mM sodium okadate]. Cytoplasmic and nuclear extracts from JEG-3 were isolated using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA).

**WESTERN BLOT ANALYSIS ON TOTAL AND SUBCELLULAR PROTEIN EXTRACTS FROM CELL LINES**

Protein concentration was measured using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Thirty micrograms of total protein lysate/25 μg subcellular protein extracts was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions and electroblotted onto a nitrocellulose membrane.\textsuperscript{29} After blocking using 5% non-fat dry milk in TBS containing 0.1% Tween 20 for 1 h at room temperature, the nitrocellulose membrane was incubated with a rabbit anti-p-Stat3-Ser\textsuperscript{727} antibody (Cell Signaling Technology) at 1 : 1000 dilution overnight. HRP-labelled goat antirabbit immunoglobulin was then applied for 1 h. The signals were visualized using a chemiluminescent detection kit (ECL Plus Western blotting Detection Reagents; Amersham Biosciences, Little Chalfont, UK) and autoradiography. The same membrane was stripped and incubated with a rabbit anti-actin antibody (1 : 1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-α tubulin antibody (1 : 200; Santa Cruz Biotechnology, Inc.) and with a mouse anti-histone H1 antibody (1 : 200; Santa Cruz Biotechnology, Inc.).

**Results**

**IMMUNOHISTOCHEMICAL STUDIES**

**Immunoreactivity pattern of p-Stat3-Ser\textsuperscript{727} in normal placenta, HM and CCA**

Both cytoplasmic and nuclear immunoreactivity of p-Stat3-Ser\textsuperscript{727} was detected in syncytiotrophoblast (ST), cytotrophoblast (CT) and villous intermediate trophoblast (IT) in first-trimester placentas and GTD samples (Figure 1).

p-Stat3-Ser\textsuperscript{727} expression decreased in relation to gestational age. In first-trimester placenta, cytoplasmic p-Stat3-Ser\textsuperscript{727} expression was found more extensively in the CT and villous IT (80%) than ST (30%), whereas focal nuclear immunoreactivity of p-Stat3-Ser\textsuperscript{727} was also present (Figure 1A). In term placentas, cytoplasmic and nuclear expression of p-Stat3 Ser\textsuperscript{727} was almost undetectable and was significantly lower than that in first-trimester placenta ($P < 0.001$, Mann–Whitney test) (Figure 1B) (Table 1).

There was a significant difference in cytoplasmic immunoreactivity for p-Stat3-Ser\textsuperscript{727} among placentas, PM, CM and CCA ($P < 0.001$, Kruskal–Wallis test) (Table 1, Figures 1 and 2). Significantly higher nuclear immunoreactivity for p-Stat3-Ser\textsuperscript{727} was also found in PM, CM and CCA when compared with normal placenta, ($P < 0.001$, Kruskal–Wallis test) (Table 1, Figures 1 and 3).

More importantly, among the samples of HM, significantly higher nuclear ($P = 0.013$, Mann–Whitney test) and cytoplasmic ($P = 0.039$, Mann–Whitney test) immunoreactivity of p-Stat3-Ser\textsuperscript{727} was found in the nine HM that subsequently developed GTN (Figure 1C) than in the 32 HM that spontaneously regressed (Figure 1D) (Table 2).

**Expression profiles of p-Stat3-Ser\textsuperscript{727} in PSTT and ETT with their respective normal counterparts**

Trophoblastic tumours other than CCA also demonstrated stronger p-Stat3-Ser\textsuperscript{727} immunoreactivity when compared with their respective normal trophoblast counterparts. When compared with extravillous implantation site IT (Figure 1G), significantly higher cytoplasmic p-Stat3-Ser\textsuperscript{727} immunoreactivity was detected in PSTT (Figure 1F) ($P = 0.012$). Furthermore, weak nuclear p-Stat3-Ser\textsuperscript{727} was detected in PSTT, whereas extravillous implantation site IT was negative for nuclear p-Stat3-Ser\textsuperscript{727} immunoreactivity.
Similarly, ETT (Figure 1H) also showed moderate cytoplasmic and nuclear immunoreactivity, whereas chorionic-type extravillous IT (Figure 1I) displayed only weak focal cytoplasmic expression and no nuclear immunoreactivity. Owing to the limited number of ETT samples, statistical significance could not be achieved.

**Correlation with apoptotic indices**

Nuclear expression of p-Stat3-Ser727 correlated directly with apoptotic activity as assessed by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) and M30 CytoDeath antibody ($P = 0.001$, $P < 0.001$, Spearman’s $\rho$ test). An inverse correlation was found between nuclear immunoreactivity of p-Stat3-Ser727 and expression of Bcl-2, an anti-apoptotic gene ($P = 0.034$, Spearman’s $\rho$ test) (Table 3). On the other hand, there was no significant correlation between p-Stat3-Ser727 cytoplasmic immunoreactivity and these apoptotic indices ($P > 0.05$, Spearman’s $\rho$ test) (Table 3).
Expression of p-Stat3-Ser727 was found to be higher in choriocarcinoma cell lines (JEG-3 and JAR) than the normal trophoblast cell line (TEV-1) (Figure 4A). At subcellular level, p-Stat3-Ser727 expression was detected in both cytoplasmic and nuclear fractions of JEG-3 (Figure 4B).

**Discussion**

Stat3 has been documented to be constitutively expressed and phosphorylated in many tumours. Its role in malignant transformation, regulation of proliferation, differentiation, apoptosis and invasive behaviour has been suggested in various studies. Apart from the most widely discussed tyrosine phosphorylation site at Tyr705, Stat possesses another phosphorylation site, which is a serine phosphorylation site. However, there are few data regarding the functional significance of p-Stat3-Ser727 in trophoblast in vivo.

There was significantly higher nuclear localization of p-Stat3-Ser727 in HM and the frankly malignant tumour, CCA, when compared with first-trimester placenta. A similar trend was also confirmed by Western blot analysis using choriocarcinoma cell lines (JEG-3 and JAR) and the normal trophoblast cell line (TEV-1).
Elevated levels of p-Stat3-Ser 727 were detected in choriocarcinoma cell lines. PSTT and ETT, gestational trophoblastic tumours derived from extravillous implantation site IT and chorionic type IT, also displayed similarly stronger immuno-reactivity than their respective normal counterparts. The overexpression of p-Stat3-Ser 727 in GTD suggests that it may play an important role in the pathogenesis of trophoblastic neoplasia. The oncogenic effect of activated Stat3 has been demonstrated in various cancers, including prostatic cancer, ovarian cancer, gastric cancer, and cutaneous squamous cell cancer.

Stat3 (the total form) and p-Stat3-Tyr 705 (the activated form) have been found to be linked to the invasive behaviour of trophoblast. In the present study, the higher p-Stat3-Ser 727 expression in HM and frankly malignant trophoblastic tumours compared with normal placenta implies that p-Stat3-Ser 727 enhances trophoblast invasiveness.

The abundance of p-Stat3-Ser 727 nuclear and cytoplasmic expression was found to be correlated with the clinical outcome of molar pregnancy. Higher levels of p-Stat3-Ser 727 expression favoured spontaneous regression of molar pregnancy, whereas relatively lower levels of p-Stat3-Ser 727 expression were associated with progression to GTN. It is possible that in HM, besides exerting an oncogenic effect on trophoblasts, p-Stat3-Ser 727 may also play a self-limiting role in restricting the aggressive behaviour of HM. This safeguarding mechanism can be possibly explained by the inhibitory effect exerted by serine phosphorylation on tyrosine phosphorylation.

Furthermore, the higher nuclear p-Stat3-Ser 727 immunoreactivity in HM that spontaneously regressed may also be related to its effect on apoptosis. Previous studies from our group and other investigators have indicated that apoptotic activity is important in determining whether a HM will regress spontaneously or progress to GTN requiring chemotherapy.

In the current study, apoptotic indices evaluated by both the TUNEL technique and the M30 CytoDeath antibody were positively correlated with nuclear p-Stat3-Ser 727 expression. In contrast, Bcl-2, which is an anti-apoptotic gene, was inversely correlated with p-Stat3-Ser 727. Such findings concur with the hypothesis that p-Stat3-Ser 727 is important in determining outcome of HM through regulation of its apoptotic activity. The absence of correlation between p-Stat3-Ser 727 cytoplasmic expression and these apoptotic indices tends to support the role of Stat3 as a transcriptional regulator, since the effect of apoptosis is more prominent when the activated protein is located in the nucleus.

Figure 4. Western blot analysis of p-Stat3-Ser 727 expression in cell lines. A. Expression of p-Stat3-Ser 727 in choriocarcinoma cell lines, JEG-3 and JAR, was higher than that in the normal extravillous trophoblast cell line, TEV-1. B. Expression of p-Stat3-Ser 727 was demonstrated in both cytoplasmic and nuclear subcellular protein fractions (T, total cell lysate; C, cytoplasmic fraction; N, nuclear fraction) of JEG-3.

### Table 3. Correlation between p-Stat3-Ser 727 immunoreactivity score and apoptotic indices and apoptosis related gene

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<tr>
<td>Nuclear</td>
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interactions with apoptotic pathways. p-Stat3-Ser727 may be a potential marker predicting the progression of HM.

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