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Expression of Stat3 and Notch1 is associated with cisplatin resistance in head and neck squamous cell carcinoma

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Abstract. Cisplatin is the most important chemotherapeutic agent involved in treatment of head and neck squamous cell carcinoma (HNSCC), but cisplatin resistance in HNSCC is still a serious problem in clinic. The reasons why patients fail chemotherapy are unclear. We examined 25 HNSCC patients who were all tested for cisplatin sensitivity by CD-DST (collagen gel droplet embedded culture-drug sensitivity) method and expression of Stat3 and Notch1. We found that high expression levels of Stat3 and Notch1 were closely associated with cisplatin resistance respectively (P=0.014, P=0.000). In addition, cisplatin resistance of HNSCC was decreased after inhibition of Stat3 or Notch signaling *in vitro*. Our results provide first evidence that both high Stat3 and Notch1 expression are associated with cisplatin resistance in HNSCC patients, supporting the hypothesis that co-activation of Stat3 and Notch1 by their crosstalk induces the reprogrammed survival pathways in HNSCC responding to chemotherapy.

Introduction

Cisplatin was introduced for treatment of head and neck squamous carcinoma (HNSCC) since the early 1980s and 30 to 40% of complete response rates were reported (1,2). Although cisplatin, as the most important chemotherapy agent for HNSCC, there were still nearly 70 to 80% of patients treated for relapsed or recurrent disease showing no response (3,4). The reasons of patients failing chemotherapy are unclear. Cisplatin resistance in HNSCC may be mediated by a number of different mechanisms, including drug detoxification, up-regulation of DNA repair enzymes, gene amplification or the overexpression of gene products that provide a tumor cell with survival advance relative to normal cells (5).

Stat3 protein is a cytoplasmic transcription factor that translocates into the nucleus following cytokine activation (6-8), it has important roles in several biological responses such as proliferation, differentiation and apoptosis (9-11). Increasing studies have suggested that Stat3 oncogenic pathway is associated with intrinsic drug resistance. Activation of Stat3 has been shown to confer resistance to Fas-mediated apoptosis in multiple myeloma and liver cancer (12,13). Paclitaxel resistant ovarian cancer cells showed abnormal increase of Stat3 activity and the RNAi-mediated down-regulation of the transcription factor reduced paclitaxel resistance (14). In chronic myelogenous leukemia, imatinib mesylate-resistant cells became sensitive to the combination of flavopiridol and bortezomib, and were linked with the inactivation of Stat3 (15). It seems that Stat3 signaling pathway acts as a predictive marker of drug resistance (16).

According to Kamakura *et al* and our previous studies, there is cross-talk between Notch pathway and Stat3 signaling (17,18). Notch1 signaling related with chemoresistance has been reported in different kinds of tumor cells. Nefedova *et al* indicated that Notch1, but not Notch2 resulted in protection of myeloma and malignant lymphoid cells from melphalan- and mitoxantrone-induced apoptosis. This protection is associated with up-regulation of p21 and growth inhibition of cells (19). Inhibition of Notch1 signaling also prevented bone marrow-mediated drug resistance and sensitized myeloma cells to chemotherapy (20). Our previous study also showed that there was higher expression of Notch1 in cisplatin resistance HNSCC compared with cisplatin-sensitive cases (21). Few studies have reported both Stat3 and Notch1 expression related with HNSCC chemoresistance. The aim of this study was to characterize the association of Stat3, Notch1 expression with cisplatin resistance in HNSCC.

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In our present study, we chose 25 HNSCC patients who were all tested for cisplatin sensitivity by CD-DST (collagen gel droplet embedded culture-drug sensitivity) method. We found that the high expression levels of Stat3 and Notch1 were closely associated with cisplatin resistance. In addition, by inhibition of Stat3 and Notch1 signaling, respectively, in HNSCC cells, the cisplatin sensitivity was significantly elevated. Thus, our study suggests that Stat3 and Notch1 may be candidate molecules for potential therapeutic targets in HNSCC cisplatin resistance.

Materials and methods

Patients. Twenty-five HNSCC patients were selected from the outpatients of Department of Head and Neck Carcinoma, Tianjin Medical University Cancer Institute and Hospital during the period of January 2007 to May of 2008. None of the patients had received chemotherapy before hospitalization. The tumors originated from the oral cavity (n=10), hypopharynx (n=7) and larynx (n=8). Nineteen patients had stage 2 HNSCC and the remaining 6 patients had stage 3 or 4 HNSCC. Two of the patients were women and 23 were men. The median age was 50 years (range: 44-74). The specimens from the operations were divided into two pieces, one for histopathological and immunohistochemical examination and one for CD-DST analysis. Participation of the patients in the clinical part of the study was approved by the Ethics Committee, Tianjin Medical University Cancer Institute and Hospital.

Drug sensitivity test by CD-DST. CD-DST was performed as described previously by Kobayashi (22,23). Briefly, biopsied or surgically resected specimens were digested in dispersion collagenase enzyme and the dispersed cancer cells were incubated in a collagen coated flask. The viable cells alone adhering to the collagen gel layer were then collected and added to reconstructed Type I collagen solution (Cellmatrix type CD™; Niita Gelatin Inc., Yao, Japan). Three drops of these mixtures were placed in each well of a 6-well multiplate and cisplatin (0.2 μg/ml) were then added to each well, and the plate was incubated for 24 h. After removal of the medium containing cisplatin, each well was incubated with PCM-2 medium (Primaster, Niita gelatin Inc., Yao, Japan) for 7 days. Neural red was added to stain colonies in the collagen gel drops, which were finally fixed with formalin. The *in vitro* chemosensitivity effect of each chemoagent was expressed as a ratio of the surviving cells (T) of the total treated cells to that of the untreated cells (C). Originally, a sample with a ratio of T to C of ≤50%, >60%, and from 51 to 60% was defined as *in vitro* sensitive, resistant, and borderline, respectively, but in the present study, the cutoff ratio was regarded as 60%, thus, samples with a ratio of T/C of ≤60% were considered as *in vitro* sensitive.

Cell culture and antibodies. Human HNSCC cell line Tb was obtained from Shanghai Ninth People's Hospital affiliated to Shanghai JiaoTong University School of Medicine (Shanghai, China) and cells were cultured in a complete medium (RPMI-1640 supplement with 10% FCS). The antibodies toward Stat3, p-Stat3, Hes-1 and Notch1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Immunohistochemistry. Immunohistochemisty was performed using standard techniques. Antigen retrieval was performed by autoclaving. Incubation with 10% normal goat serum in phosphate-buffered saline was performed for 15 min to eliminate non-specific staining. Incubation with Stat3 and Notch1 antibody respectively was carried out. Finally, sections were lightly counterstained with 10% Mayer's hematoxylin, dehydrated, mounted and observed. Staining was evaluated by a pathologist and an investigator blinded to diagnosis. Sections were classified + (focal and weak immunoreactivity), ++ (diffuse and weak or focal and intense immunoreactivity), +++ (diffuse and intense immunoreactivity).

Inhibition of Stat3 and Notch signaling and statistic analysis. Inhibition of Stat3 or Notch signaling with AG490 (Sigma) or the γ-secretase inhibitor N-[N-(3, 5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester DAPT (Calbiochem), respectively, were described previously (24-29). In brief, AG490 was dissolved in DMSO to a stock concentration of 50 mM and was diluted to the final concentration of 60 μM with conventional culture medium just before use. DAPT was used at a final concentration of 1.0 μM diluted in DMSO. DMSO group was mock treated with conventional medium containing the same concentration of DMSO carrier only. Control group was mock-treated with conventional medium only.

Western blotting. Western blotting was performed as described previously (18). The cells were lysed by 1X SDS lysis buffer (Tris-HCl, pH 6.8, 62.5 mM, 2% SDS, 10% glycerol) followed by centrifugation at 10,000 rpm for 10 min at 4˚C. Equal amounts of cell lysates (20-40 μg total protein/lane) were loaded and separated by SDS-PAGE and proteins were transferred onto nitrocellulose membranes (Immobilon-P, Millipore, Billerica, MA, USA), probed with anti-Stat3 (1:1000), P-Stat3 (1:1000), Hes-1 (1:1000) and GAPDH (1:5000) followed by AP-conjugated secondary antibodies.

Statistics. The data were analyzed by SPSS 11.5 statistical package. Spearman rank correlation was used in Tables and Student's t-test was used in Fig. 3.

Results

Cisplatin resistance was associated with Stat3 and Notch1 expression. The specimens of 25 HNSCC patients were examined for cisplatin resistance by the CD-DST method. There were 8 patients sensitive to cisplatin and 17 showed resistances to it. Five of 8 patients (62.5%) had weak Stat3 expression in the sensitive cases, while only one case (5.88%) had weak Stat3 expression in the 17 cisplatin-resistant patients. Resistant patients showed 70.58% (12/17), and sensitive cases 25% (2/8) of moderate Stat3 expression, respectively. The statistical analysis showed that there was significant difference in expression level of Stat3 between the cisplatin-sensitive and -resistant groups (Table I, P=0.014).

Furthermore, we detected the expression level of Notch1 in the same specimens of the 25 HNSCC patients. Six of 8 patients (75%) had weak Notch1 expression in the sensitive

Table I. Correlation between cisplatin resistance and Stat3 protein expression in HNSCC.

Cisplatin sensitivity	Stat3 protein expression (cases)			
	+	++	+++	Total
Sensitivity	5	2	1	8
Resistance	1	12	4	17
Total	6	14	5	25

r_s=0.484, P=0.014 (Spearman rank correlation).

Table II. Correlation between cisplatin resistance and Notch1 protein expression in HNSCC.

Cisplatin sensitivity	Notch1 expression (cases)			
	+	++	+++	Total
Sensitivity	6	2	0	8
Resistance	1	5	11	17
Total	7	7	11	25

r_s=0.738, P=0.000 (Spearman rank correlation).

cases, while only one case (5.8%) had weak Notch1 expression in the 17 cisplatin-resistant patients. No patient showed intense Notch1 immunoreactivity in the sensitive group, however, there was 64.7% (11/17) of patients who had strong expression in the cisplatin-resistant group. The statistical analysis showed that there was a significant difference in the expression level of Notch1 between the cisplatin-sensitive and -resistant groups (Table II, P=0.000).

The statistical analysis showed that there was a significant correlation of Stat3 and Notch1 expression in the HNSCC patients (P=0.049). The expression of both proteins in each patient is summarized in Table III.

Table III. Correlation of Stat3 and Notch1 expression in HNSCC.

Notch1 expression	Stat3 expression			
	+	++	+++	Total
+	1	6	0	7
++	4	3	0	7
+++	1	5	5	11
Total	6	14	5	25

r_s=0.397, P=0.049 (Spearman rank correlation).

Pathological expression of Stat3 and Notch1 in HNSCC patients. Immunohistochemical staining of Stat3 in HNSCC patients are shown in Fig. 1, three levels of immunoreactivity was classified as described in methods. Normal squamous epithelial tissues have little Stat3 expression as shown in Fig. 1A. Focal and weak immunoreactivity in cisplatin sensitive HNSCC patient is shown in Fig. 1B and C showing focal and intense immunoreactivity, and Fig. 1D represent the diffuse and intense immunoreactivity, respectively, in

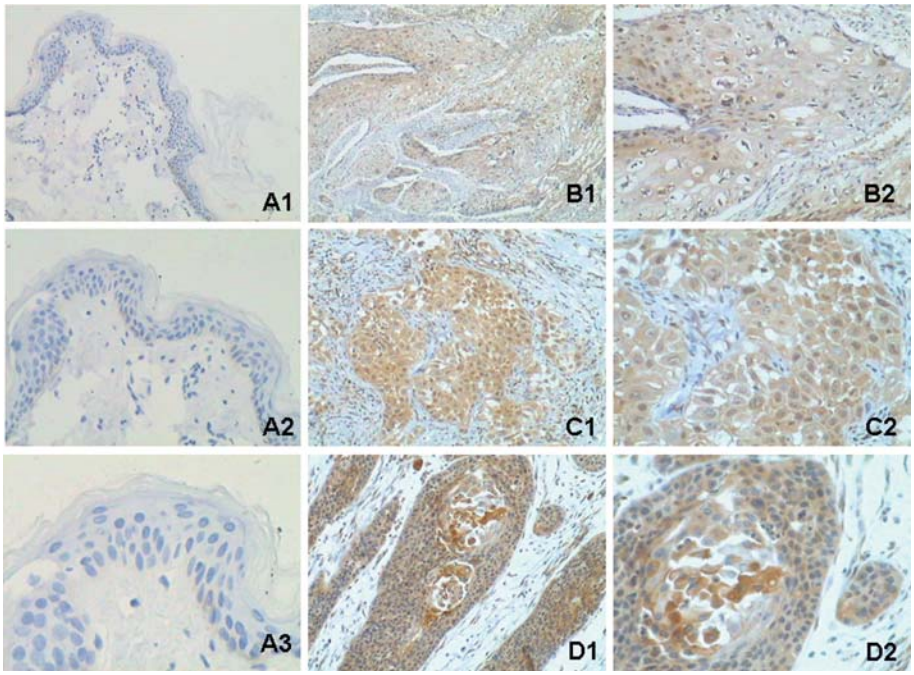

Figure 1. Expression of Stat3 protein in HNSCC patients. (A) Normal epithelial tissue. (B) Focal and weak expression of Stat3 protein in cisplatin-sensitive HNSCC patient. (C) Focal and intense Stat3 expression in cisplatin-resistant HNSCC patient. (D) Stat3 protein is strongly expressed in cisplatin-resistant HNSCC patient. Data show representative immunohistochemical staining. (x100: A1, B1, C1, D1; x200: A2, B2, C2, D2; x400: A3).

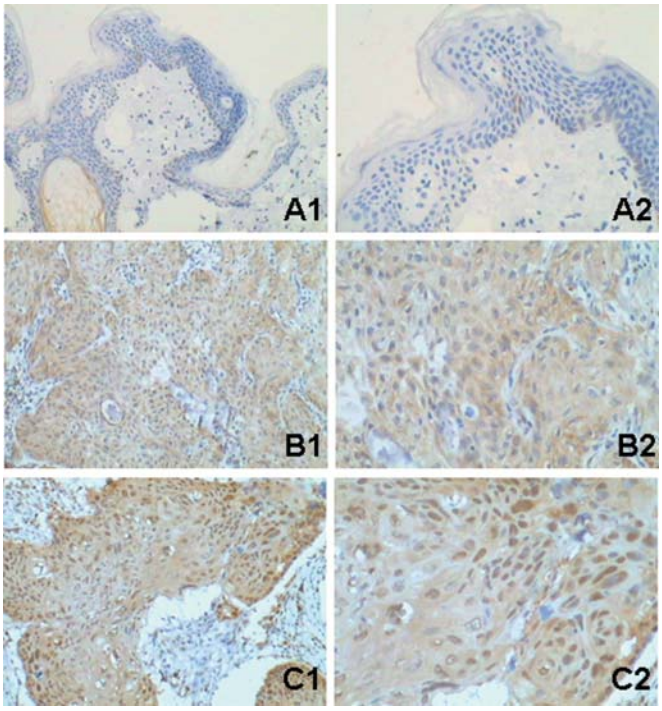

Figure 2. Expression of Notch1 protein in HNSCC patients. (A) Normal epithelial tissue. (B) Focal and weak expression of Stat3 protein in cisplatin-sensitive HNSCC patient. (C) Focal and intense Stat3 expression in cisplatin-resistant HNSCC patient. Data show representative immunohistochemical staining (x100: A1, B1, C1, D1; x200: A2, B2, C2, D2).

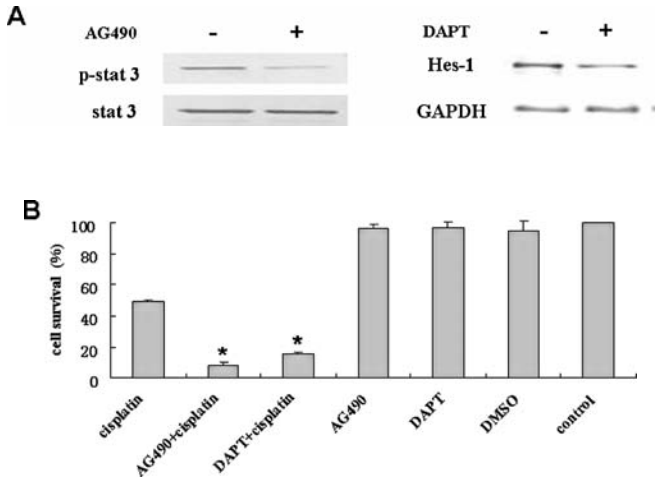

Figure 3. Inhibitor of Stat3 or Notch1 treatment increased Tb HNSCC cell sensitivity to cisplatin. (A) Tb cells were treated with 60 μM of AG490 and 1.0 μM of DAPT, respectively. Twenty-four hours later, cells were collected and the expression of phosphorylated Stat3 and Hes-1 were detected by Western blotting. Expression of Stat3 and GAPDH were used as control, respectively. (B) Tb cells were treated as indicated in the graph. After 24 h of treatment, cells were collected for the CD-DST test. Control, mock treatment with culture medium only. Data were from 3 independent experiments and analyzed by Student's t-test (*P<0.05).

cisplatin-resistant HNSCC patients. We also detected the Notch1 expression in the same HNSCC patients. Different from the Stat3 expression level, we did not find intense immunoreactivity of Notch1 in the 8 cisplatin-sensitive HNSCC patients. Normal squamous epithelial tissues have little Notch1 expression as shown in Fig. 2A. Weak immunoreactivity in cisplatin-sensitive HNSCC patient is shown in Fig. 2B and C showing focal and intense immunoreactivity of Notch1 in the cisplatin resistance HNSCC patients.

Inhibitor treatment of Stat3 or Notch1 increased cisplatin sensitivity. As described above, cisplatin sensitivity of HNSCC patients was highly associated with the Stat3 and Notch1 expression. In order to confirm that Stat3 and Notch1 are involved in the cisplatin sensitivity in HNSCC patients, we applied the selective inhibitor of Stat3 signaling AG490 and Notch inhibitor DAPT, respectively. AG490 is a JAK2-specific inhibitor and selectively inhibits Stat3 activity (26,27). First, we detected the inhibitor function in cultured HNSCC Tb cells. With the treatment of 60 μM of AG490 in Tb cells, phosphorylated Stat3 was significantly decreased compared with the absence of AG490 group without affecting expression level of Stat3 (Fig. 3A). A major transcriptional downstream regulator of Notch1 pathway is the helix-loop-helix (HLH) transcription factor Hairy/Enhancer of Split 1 (Hes-1). Application of 1.0 μM of DAPT (γ-secretase inhibitor) in Tb cells led to reduced expression level of Hes-1 (Fig. 3A). Cultured Tb cells were divided into 5 groups, which were treated with 0.2 μg/ml cisplatin, 60 μM AG490, combination of AG490 and cisplatin, DMSO and mock treatment, respectively, with culture medium was regarded as control. Cisplatin sensitivity detection by CD-DST method indicated that cell treatments with AG490 or DMSO were almost the same as mock control group. The ratio of combination group (AG490 and cisplatin) was ~8.1%, much lower than that of cisplatin group (48.6%) (Fig. 3B, P=0.02). Tb cells were also treated with 1.0 μM of DAPT for inhibiting Notch signaling to confirm whether Notch1 is involved in cisplatin sensitivity. The ratio of combination group (DAPT and cisplatin) was ~15.6%, significantly lower than that of cisplatin group (Fig. 3B, P=0.037). It suggested that inhibition of Stat3 signaling or Notch1 signaling caused the HNSCC cells to be more sensitive to cisplatin treatment.

Discussion

Stat3 protein as a transcriptional activator and activates its target genes to affect a variety of critical cellular processes (30-32). It has been reported that activation of Stat3 was associated with drug resistance in ovarian cancer, hepatocellular carcinoma and non-small cell lung cancer (33-35). In the present study, we provided first evidence that high expression of Stat3 was correlated with cisplatin resistance in HNSCC patients. The precise mechanisms of Stat3 involved in drug resistance remain unclear. It is already well known that Stat3 activates C-Myc expression and the up-regulation of C-Myc induces DNA damage and genomic instability (36), so one possible mechanism is that the Stat3/C-Myc pathway might induce genetic alterations of chromosomes linking to drug resistance and clinical outcome (16). Chemoresistance was initially correlated to a reduced concentration of the drug via efflux pumps, to detoxification enzymes or to

ced DNA repair activity. It is now clear that tumor escape cytotoxic treatments by reprogramming their survival pathways. It has been reported that Stat3 is phosphorylated following genotoxic treatments such as irradiation or topoisomerase inhibition (37), this leads to another hypothesis that the transcriptional activity of Stat3 might be reprogrammed not only during the initial step of tumorigenesis but also during chemotherapy (16).

Notch signaling could participate in chemoresistance by protecting the cells from apoptosis, possibly as it activates targets which are involved in cellular survival, such as PI3K/Akt, Bcl-xl and survivin (38-40). Accumulating studies indicated that overexpression of Notch1 increased the chemoresistance of T cells to etoposide, breast cancers to melphalan and mitoxantrone, cervical cancers to doxorubicin and lung cancers to cisplatin and paclitaxel (38,39,41,42). In this study, we provided evidence that high expression of Notch1 was correlated with cisplatin resistance in HNSCC patients.

Although both Notch and JAK/STAT signaling pathways fulfill overlapping roles in growth and differentiation regulation, a physiologically crucial crosstalk between them was first demonstrated in 2004. Stat3 is activated in the presence of activated Notch, Notch1 target gene Hes1 associates with JAK2 and Stat3 to facilitate complex formation between JAK2 and Stat3, thus promoting Stat3 phosphorylation and activation (17). In the present study, we provided evidence that both high Stat3 and Notch1 expression were associated with cisplatin resistance in HNSCC patients supporting the hypothesis that co-activation of Stat3 and Notch1 by their crosstalk induces the reprogrammed survival pathways in HNSCC responsing to chemotherapy.

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