Background and Objective: Notch1 belongs to the Notch family of transmembrane receptors and plays an important role in tumor cell proliferation and apoptosis. Notch1 affects chemosensitivity of tumors. However, its correlation to cisplatin (DDP)-sensitivity of head and neck squamous cell carcinoma is unclear. This study was to identify the expression of Notch1 in head and neck squamous cell carcinoma, and investigate its influence on the DDP-sensitivity. Methods: Twenty-five fresh specimens of head and neck squamous cell carcinoma were subjected to primary cell culture. DDP-sensitivity of tumor cells was detected using collagen gel droplet embedded culture-drug sensitivity test (CD-DST). The expression of Notch1 in head and neck squamous cell carcinoma, normal squamous epithelium, and tongue squamous cell carcinoma Tb3.1 cells was detected by immunohistochemistry or immunochemistry. Tb3.1 cells were divided into four groups, and received treatment of DMSO, DAPT, DMSO plus DDP, DAPT plus DDP, respectively. The absorbance of the four groups was detected by CD-DST to evaluate DDP-sensitivity. Results: The positive rate of Notch1 was significantly higher in head and neck squamous cell carcinoma than in normal squamous epithelium (100% vs. 35%, p < 0.001), and it was negatively correlated to DDP-sensitivity (r = -0.705, p < 0.01). There was no difference in absorbance between DMSO group and DAPT group (155.4 ± 2.3 vs. 154.7 ± 1.2, p > 0.05), while the absorbance was significantly higher in DMSO plus DDP group than in DAPT plus DDP (33.9 ± 1.3 vs. 26.6 ± 1.1, p < 0.05). Conclusions: Notch1 expression is negatively correlated to DDP-sensitivity of head and neck squamous cell carcinoma, and could be used to predict DDP-sensitivity. DAPT can enhance DDP-sensitivity of Tb3.1 cells via blocking Notch1 signaling.

Cisplatin (DDP)-based chemotherapy regimen is most widely used in patients with head and neck squamous cell carcinoma (abbreviated as HNSCC). The resistance of tumor cells to DDP remains to be a major cause of treatment failure. Notch1, as a member of a family of single-pass transmembrane receptors, plays an important role in the differentiation, proliferation and apoptosis of tumor cells. Some studies have indicated that the Notch1 signaling pathway is involved in the mediation of chemoresistance of multiple myeloma and a variety of tumor cell lines, and blocking Notch1 signaling pathway can reverse chemoresistance. However, the relationship of Notch1 with DDP-sensitivity of HNSCC remains to be clarified. In the present study, we analyzed the correlation of Notch1 expression level to DDP-sensitivity of HNSCC, and preliminarily explored the significance of Notch1 expression for predicting DDP-sensitivity, reversing DDP-resistance and developing targeted therapy in HNSCC.

Materials and Methods

Materials. A total of 25 fresh surgical specimens and archived paraffin blocks, obtained from HNSCC patients who were diagnosed and treated from January 2007 to May 2008 at the Tianjin Medical University Cancer Institute and Hospital, were collected. All specimens were pathologically confirmed as squamous cell carcinoma. Of the 25 cases, ten were well differentiated, 11 were moderately differentiated and four were poorly differentiated; ten were oral squamous cell carcinoma, seven were pharyngeal carcinoma, six were laryngeal carcinoma, one was upper esophageal carcinoma and one was maxillary sinus carcinoma. Moreover, 25 specimens of normal squamous epithelium were collected and embedded into paraffin blocks to prepare 4-μm slices.

The Primaster™ kit for CD-DST was purchased from Nitta Gelatin Co. (Japan); DMSO, DMEM/F12 medium, Hank’s solution and fetal calf serum (FBS) were purchased from Gibco Co.; six-well plates were purchased from Flacon Co.; DDP was purchased from Shandong Qilu Pharmaceutical Co., Ltd.; rabbit anti-human Notch1 polyclonal antibody was purchased from Aviva Antibody Co., Ltd. Anti-rabbit IgG antibodies were purchased from Biogenex Co. and used for immunostaining. The Primaster™ kit for CD-DST was purchased from Nitta Gelatin Co. (Japan); DMSO, DMEM/F12 medium, Hank’s solution and fetal calf serum (FBS) were purchased from Gibco Co.; six-well plates were purchased from Flacon Co.; DDP was purchased from Shandong Qilu Pharmaceutical Co., Ltd.; rabbit anti-human Notch1 polyclonal antibody was purchased from Aviva Antibody Co., Ltd. Anti-rabbit IgG antibodies were purchased from Biogenex Co. and used for immunostaining.
Collagen gel droplet embedded culture-drug sensitivity test (CD-DST). CD-DST was developed and authorized by Nitta Gelatin Co. The operating procedures were as follows. Tumor specimens were taken by an experienced doctor, rinsed three times with normal saline solution containing 100 μg/mL penicillin and 100 μg/mL streptomycin, finely cut into small pieces under aseptic conditions, incubated with collagenase in a shaker at 37°C for 1–2 h, and filtered to collect dissociated cells. Cells were suspended in pre-culture medium PCM-1 to prepare single cell suspension and seeded in collagen gel-coated culture flasks. When growing to a certain density (5 × 10⁴/flask), cells were washed with Hank’s solution, digested with collagenase and pelleted by centrifugation. Collagen gel solution was then prepared by mixing Solutions A, B and C at a ratio of 8:1:1 in an ice bath and used to resuspend cell pellet by pipetting. Subsequently, the resuspended cells were seeded in six-well plates (90 μL/well) and incubated at 37°C for 1 h to allow gelation. After DMEM/F-12 medium with 10% FBS was added (3 mL/well), cells were incubated for 24 h, treated with DDP (2 μg/mL) for 24 h (a blank control was also run) and washed three times with Hank’s solution. Serum-free PCM-2 medium was then added (3 mL/well), followed by a further culture for 7 d. Cells were stained with neutral red solution (30 μL/well) for 3 h. After fixation with 10% neutral formalin for 40 min, cells were washed and air-dried. The Scion Image software was used for photography and image analysis. Absorbance values were used to calculate cell survival of blank control group (C) and DDP group (T). The values of T/C×100% were used to evaluate DDP-sensitivity. Lower T/C value indicated stronger DDP-sensitivity. A T/C value of ≤ 50% was considered sensitive to DDP and that > 50% was considered insensitive to DDP.

Immunohistochemistry. The labeled streptavidin biotin (LSAB) method was used to detect the expression of Notch1 in HNSCC and normal squamous epithelium. Paraffin-embedded sections were dewaxed, hydrated and washed with PBS. After microwave antigen retrieval, slides were incubated with primary antibody (1:100) at 37°C for 90 min. The remaining steps were performed according to SP kit instructions. Positive control slides were provided by the manufacturer of the primary antibody, while negative controls were run by replacing the primary antibody with PBS. Signals were visualized by diaminobenzidine (DAB) coloration. Using the semi-quantitative scoring method reported by Carcangiu et al., as a reference and considering that the percentages of positive cells in 22 specimens were above 85%, Notch1 expression was evaluated according to staining intensity: colorless was defined as negative (-), light brownish yellow as mild positive (+), brownish yellow as moderate positive (++) and dark brown as intense positive (+++) staining. The expression of Notch1 in Tb3.1 cells was detected by immunocytochemistry using primary antibody (1:100) and DAB coloration.

Impact of DAPT-mediated blockade of Notch1 signaling pathway on DDP-sensitivity of Tb3.1 cells. After thawing, Tb3.1 cells were cultured in DMEM/F-12 medium containing 10% FBS at 37°C in humidified air containing 5% CO₂, inoculated into two culture flasks (5 × 10⁴ cells/flask) and cultured. Cells at logarithmic phase were treated for 24 h with 100 μmol/mL DAPT or DMSO, respectively. After being washed with Hank’s solution, cells were harvested, divided into DMSO group, DAPT group, DMSO plus DDP group and DAPT plus DDP group, then embedded in collagen gel and cultured. Cells in DMSO plus DDP group and DAPT plus DDP group were treated with DDP (2 μg/mL) for 24 h, while those in DMSO group and DAPT group were treated with normal saline solution. The remaining steps were performed according to the operating procedures for CD-DST. The absorbance values were then measured. This experiment was repeated five times.

Impact of DAPT-mediated blockade of Notch1 signaling pathway on DDP-sensitivity was analyzed using paired t-test. The level of significance was set at α = 0.05.

Results

CD-DST results. The success rate of CD-DST was as high as 88.0% (22/25). Pathologic examination revealed that three specimens failed the CD-DST due to too little tumor tissue contained. CD-DST results revealed individual differences in DDP-sensitivity among HNSCC patients. Of the 22 cases, three had T/C value of 0–25%, four had T/C value of 26–50%, eight had T/C value of 51%–75% and seven had T/C value of 76–100%. The response rate to DDP was 31.8% (7/22).

Expression of Notch1 in HNSCC. Immunohistochemistry showed that Notch1 was lowly or not expressed in normal squamous epithelium. In contrast, Notch1 was expressed as brown granules in cytoplasm of HNSCC cells. The positive rate of Notch1 was significantly higher in HNSCC than in normal squamous epithelium (100% vs. 35%, p < 0.001). Notch1 expression level was negatively correlated to DDP-sensitivity of HNSCC (r = -0.705, p < 0.01) (Table 1).

Impact of DAPT-mediated blockade of Notch1 signaling pathway on DDP-sensitivity of Tb3.1 cells. Notch1 was expressed as brown granules in the cytoplasm of Tb3.1 cells (Fig. 1). Tb3.1 cells were sensitive to DDP with a T/C value of (22.0 ± 1.4%). The absorbance values were similar in DMSO group and DAPT group (155.4 ± 2.3 vs. 154.7 ± 1.2, p > 0.05), while significantly higher in DMSO plus DDP group than in DAPT plus DDP group (33.9 ± 1.3 vs. 26.6 ± 1.1, p < 0.05), suggesting that DAPT may enhance DDP-sensitivity of Tb3.1 cells through blocking Notch1 signaling pathway.

Discussion

Chemotherapy plays an important role in reducing the risk of distant metastasis, improving survival rate and protecting organs in HNSCC patients. DDP is one of the most commonly used chemotherapeutic drugs. The resistance of tumor cells to DDP remains a major cause of treatment failure. Therefore, seeking molecular markers for prediction of DDP-sensitivity and analyzing factors...
Correlation of Notch1 expression and activation to cisplatin-sensitivity of head and neck squamous cell carcinoma

CD-DST can effectively imitate the in vivo micro-environment of cell growth and in vivo acting conditions for chemotherapeutic drugs through using type I collagen to create a three-dimensional culture system, thus being able to accurately evaluate drug sensitivity. Some clinical studies on lung cancer and breast cancer have proved that the response rate tested by CD-DST is accordant to that clinically reported. Although no large sample study is available to reveal such correlation in HNSCC, some case reports have showed that CD-DST can accurately predict the sensitivity of HNSCC to chemotherapeutic drugs. In the present study, the success rate of CD-DST was 88.0%, and pathologic examination revealed that the three cases of failure were due to too little tumor tissue contained, indicating that accurate and sufficient sampling is the key for the success of CD-DST. Since CD-DST revealed individual differences in DDP-sensitivity among HNSCC patients, conducting individualized chemotherapy in HNSCC patients is essential for improving the response rate to chemotherapy, reducing the side effects of chemotherapy and relieving the economic burden on patients. CD-DST can be used to predict DDP-sensitivity and guide individualized chemotherapy in HNSCC patients.

Notch1 is a kind of single-pass transmembrane receptor encoded by the Notch gene. The binding of the extracellular domain of Notch1 to its ligand (DSL family) induces the proteolytic release of Notch intracellular domain (NICD) mediated by tumor necrosis factor-α-converting enzyme and γ-secretase. Free NICD translocates to the nucleus and forms a complex with CSL family members. The complex binds to particular DNA sequences and activates the classical CSL-dependent Notch signaling pathway, and is thus involved in the differentiation, proliferation and apoptosis of tumor cells. Although studies show that the Notch1 signaling pathway is involved in the apoptosis of tumor cells, its relationship with chemotherapy sensitivity has not been fully clarified. Some studies have indicated that Notch1 mediates the resistance of multiple myeloma to melphalan and mitoxantrone which can be reversed by γ-secretase inhibitors through blocking the Notch1 signaling pathway. Similar conclusions have also been drawn in a variety of other tumor cell lines. Although no studies on the correlation of Notch1 expression to chemosensitivity of HNSCC are currently available, Duan et al. found that Notch1 is involved in the differentiation, proliferation and apoptosis of human tongue squamous cell carcinoma. In this study, immuno-histochemistry results proved that Notch1 was expressed in HNSCC with a positive rate of 100%, which was significantly higher than that in normal squamous epithelium. Furthermore, individual differences in Notch1 expression were noted. Notch1 expression level was negatively correlated to DDP-sensitivity of HNSCC (r = -0.705, p < 0.01), suggesting that Notch1 can be used to predict the DDP-sensitivity of HNSCC. DAPT is a γ-secretase inhibitor, while the activation of Notch1 signaling pathway depends on γ-secretase-mediated proteolysis. Shih et al. found that DAPT can effectively block Notch1 signaling pathway. Therefore, we used DAPT to block Notch1 signaling pathway in Tb3.1 cells to investigate the role of Notch1 signaling pathway in DDP-sensitivity. We found that after 24-hour treatment of DAPT followed by exposure to DDP, the absorbance value of Tb3.1 cells was significantly reduced as compared with that of control cells (p < 0.05), indicating that DAPT-mediated blockade of Notch1 signaling pathway can significantly enhance DDP-sensitivity.

In summary, this study shows: Notch1 expression is negatively correlated to DDP-sensitivity of HNSCC and can be used as a marker for prediction of DDP-sensitivity of HNSCC; blocking Notch1 signaling pathway can enhance DDP-sensitivity of HNSCC; Notch1 signaling pathway may mediate the resistance

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**Table 1** Correlation of Notch1 expression to cisplatin (DDP)-sensitivity of head and neck squamous cell carcinoma

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<thead>
<tr>
<th>DDP-sensitivity</th>
<th>Notch1 expression (cases)</th>
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<td>+</td>
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<tr>
<td>Sensitive</td>
<td>5</td>
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<td>Insensitive</td>
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r = -0.705, p < 0.01.
of HNSCC to DDP, therefore, it may serve as a target for reversing DDP-resistance and developing targeted therapy. However, since Tb3.1 cell line is not a DDP-resistant cell line, the correlation of Notch1 to DDP-resistance of HNSCC remains to be further investigated.

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References