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Atypical chemokine receptors predict lymph node metastasis and prognosis in patients with cervical squamous cell cancer ☆

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HIGHLIGHTS

- We described the characterization of ACR (DARC, D6, and CCX-CKR) expression with clinicopathological and immunohistochemical features.
- We found that ACR expression was correlated with lymph node status and prognosis of cervical squamous cell carcinoma.
- We found that the three ACRs could be used as prognostic markers in cervical cancer patients.

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ABSTRACT

Objective. Atypical chemokine receptors (ACRs), including CCX-CKR, DARC, and D6, have been reported to be involved in cancer invasion and metastasis. The objective of this study was to investigate the prognostic importance of ACRs in patients with cervical squamous cell carcinoma (CSCC).

Methods. The expression of three ACRs was investigated by immunohistochemical (IHC) examination in a total of 317 cervical specimens including 40 normal cervical tissues, 50 cases of carcinoma in situ of cervix (CIS), and 227 cases of CSCC by immunohistochemistry.

Results. The expression rate of DARC and CCX-CKR in CSCC, CIS, and normal cervix increased gradually ($p < 0.01$). D6 expression is decreased in CSCC compared to either in CIS or in normal cervix ($p < 0.05$). In addition, the expression of CCL2 and CCL19 was inversely associated with ACR expression ($p < 0.05$), while that of LCA was positively correlated with ACR expression ($p < 0.05$). Moreover, DARC expression, CCX-CKR expression, and ACR coexpression were negatively correlated with lymph node metastasis ($P < 0.01$). D6 expression and ACR coexpression were negatively related to tumor size ($p = 0.018$) and recurrence ($p = 0.028$). In multivariate Cox regression analysis, CCX-CKR expression was a positive indicator for overall survival ($p = 0.008$), and D6 expression was an independent predictor of both overall and recurrence-free survival ($p = 0.041$) in CSCC.

Conclusions. Our results suggest that the loss of ACRs may play important roles in the tumorigenesis and migration of cervical cancer. ACR expression may be considered as prognostic markers in patients with CSCC.

Introduction

Cervical cancer is the second most common malignancy of female reproductive system [1]. One of the most important prognostic parameter in patients with cervical cancer is regional lymph node metastasis [2]. About one-half of the cervical cancer patients with pelvic lymph node metastasis will have recurrence of disease, with most of them dying of uncontrolled disease [3]. The reported 5-year survival rate for patients who undergo relapse ranges from 3 to 13% [4,5], while there is the lack of predictive markers for lymph node metastasis in patients with cervical cancer.

Chemokines and their receptors, such as CXCL12/CXCR4, CCL19 (CCL21)/CCR7, and CXCL13/CXCR5, have been implicated mostly in cell migration [6]. Recently, increasing attention has been drawn to atypical chemokine receptors (ACRs), which comprise a group of 7 transmembrane domain proteins structurally similar to G protein-coupled receptors [7]. However, atypical chemokine receptors do not induce classical signaling via the typical G protein-mediated pathways [8]. This may be due to the lack of canonical DRYLAIV motif within the second intracellular loop, which normally enables G protein coupling and induces the G protein-mediated signaling [9]. The ACR family, including Duffy antigen receptor for chemokines (DARC), D6, and Chemocentryx chemokine receptor (CCX-CKR), has the potential to

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modify the bioavailability of chemokines [10]. DARC was initially discovered as the Duffy red blood group antigen, and it mediates the internalization of inflammatory chemokines of the CC (cysteines next to each other) and CXC (cysteines separated by a single amino acid) groups [11]. D6 binds multiple inflammatory CC chemokines, while it does not recognize homeostatic CC chemokines [12]. CCX-CKR was recently discovered and was found to localize predominantly in the epithelial cells of heart and lung [13]. Previous studies have shown that ACRs are involved in the process of tumor progression and metastasis [14,15]. As chemokines contribute to immune suppression, tumor infiltration, angiogenesis, and migration [16], ACRs inhibit tumor progression by competitively binding to chemokines and in turn decreasing their bioactivities [17]. However, there is no published report of whether the expression of ACRs correlates with progression or clinical outcomes of cervical cancer.

In this study, we report for the first time the characterization of DARC, D6, and CCX-CKR expression in human cervical squamous cell cancer. We found that the expression of DARC and CCX-CKR was conversely correlated with lymph node status. Multivariate analysis suggested that D6 and CCX-CKR expression were independent prognostic markers for CSCC patients. Our results strongly suggest that ACRs might play an anticancer role in cervical cancer development and might be valuable prognostic and metastatic markers for CSCC patients.

Material and methods

Patients and tissue specimens

A total of 317 paraffin-embedded samples were obtained from CSCC patients, who underwent radical hysterectomy at Sun Yat-Sen University Cancer Center between Jan 2001 and June 2006. The samples were composed of 40 normal cervical tissues, 50 CIS, and 227 CSCC specimens. For the use of these clinical materials for scientific purposes, prior patient's consent and approval from the Institute Research Ethics Committee were obtained. None of the patients had received chemotherapy or radiotherapy before surgery. After surgery, patients were treated with adjuvant radiotherapy or concurrent chemoradiation therapy, depending on the lymph node status, the stage of the disease, the parametrial status, and tumor differentiation, according to the national guidelines. Postoperative radiotherapy was given to 65 patients. Postoperative chemotherapy was administered to 110 patients. Patients with lymph node metastasis received both chemotherapy and radiotherapy postoperatively. The clinical stage of the patients was classified according to the International Federation of Gynecology and Obstetrics criteria as follows: 130 were allocated to stage IB1, 38 to stage IB2, 40 to stage IIA1, 13 to stage IIA2, and 8 to stage IIB. The median age of the patients was 42.7 years (range 25–68 years).

Immunohistochemistry

IHC analysis was performed to examine protein expression of DARC, D6, CCX-CKR, chemokine (C–C motif) ligand 2 (CCL2), chemokine (C–C motif) ligand 19 (CCL19), and leukocyte common antigen (LCA). Briefly, freshly cut 4 μm sections were deparaffinized and rehydrated in declining grades of ethanol. Antigen retrieval was performed by submerging the sections into a 10 μmol/L citrate buffer solution (pH 6.0) for 10 min in a microwave oven. The slides were then treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with 1% fish skin gelatin to block the nonspecific staining. Tissue sections were incubated overnight with antibodies against DARC (Abcam, 1:250), D6 (Abcam, 1:250), CCX-CKR (Abcam, 1:250), LCA (Abcam, 1:500), CCL2 (Abcam, 1:100), and CCL19 (Abcam, 1:100). After washing, the sections were incubated with prediluted secondary antibody (Abcam), followed by further incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB). Finally, the slides were counterstained with hematoxylin and mounted in an aqueous mounting medium.

All stained slides were separately evaluated by two pathologists. For DARC, D6, CCX-CKR, CCL2, and CCL19, the IHC score was defined by multiplying the percentage of cytoplasmic positive cells by the intensity. The intensity of stained cells was graded semi-quantitatively into four levels: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate staining = yellow brown) and 3 (strong staining = brown); and the percentage was scored as: 0, negative; 1, 10% or less; 2, 11% to 50%; 3, 51% to 80%; or 4, 80% or more positive cells. The scoring system for DARC, D6, and CCX-CKR was defined as negative for score 0 and as positive for scores of 1–12, whereas that for CCL2 and CCL19 was defined as negative for scores of 0–3, and as positive for scores of 4–12. Two or three coexpression of ACRs was regarded as the coexpression of ACRs. For LCA, scoring was undertaken using a Chalkley point array [18]. In brief, three hot spots with the highest density of positive cells were selected per tumor. A 25 cross hair grid was used to score each hot spot at a magnification of ×200. Positive immune cells that touched or overlapped with tumor epithelial compartments were counted as stained cells. A region was considered positive if there were more than five stained cells per unit area, and was considered negative if there were 0–5 stained cells per unit area. The cutoff values were chosen on the basis of a measure of heterogeneity with the log-rank test statistical analysis with respect to overall survival and recurrence-free survival.

Follow-up and statistical analysis

All statistical analyses were carried out using SPSS (version 16.0, Chicago, USA) statistical software. Follow-up was available for all patients with a median time of 60.4 months (range 0.5–131.6 months). The overall survival and recurrence-free survival were calculated as the time from the date of the primary surgery to the date of death or first recurrence. Survival of patients was estimated by Kaplan–Meier analysis and the differences were compared by the log rank test. Cox proportional hazards multivariate regression model was used to select independently significant prognostic factors for CSCC. The correlation between ACR expression and clinicopathologic features was assessed using the χ^2 test or Fisher's exact test, while the correlation between ACRs with CCL2, CCL19, and LCA staining was evaluated using the Spearman's rank. $P < 0.05$ in all cases was considered statistically significant.

Results

Expression of ACRs in CSCC, CIS, and normal cervical tissues

To investigate the potential roles of ACRs in the development and progression of cervical cancer, we determined the expression of DARC, D6, and CCX-CKR in 227 CSCC, 50 CIS, and 40 normal cervical tissues. The three ACR proteins were mainly located in the cytoplasm of tumor cells, but rarely in the nucleus. The representative immunostaining of DARC, D6, and CCX-CKR in normal cervical tissues (Fig. 1, A–C), CIS (Fig. 1, D–F), and CSCC (Fig. 1, G–L) was shown in Figure 1. Normal cervical tissue showed positive DARC in 39 (97.5%), D6 in 32 (80.0%), and CCX-CKR in 40 (100%) cases, CIS presents 42 (84.0%), 44 (88.0%), and 43 (86.0%), and CSCC positively stained 168 (74.0%), 162 (71.4%), and 179 (78.9%), respectively (Table 1).

Correlation of ACR expression with CCL2, CCL19, and LCA

Since chemokines play a crucial role in modulating immune response, we assessed the correlation of ACR expression with the expression of their chemokine ligands (CCL2 for DARC and D6, CCL19 for CCX-CKR). We also analyzed the correlation of ACR expression with lymphocyte infiltration by evaluating the expression of LCA, a marker of leukocytes. The