CD44 affects the expression level of FOS-like antigen 1 in cervical cancer tissues

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Abstract. Cervical carcinoma is the second most prevalent type of malignancy in females worldwide. The crucial etiological factors involved in the development of cervical carcinoma include infection with the papillomavirus, and the structural or functional mutation of oncogenes and tumor suppressor genes. CD44 refers to a multifunctional family of type I transmembrane proteins. These proteins have been implicated in numerous biological processes, including cell adhesion, cell migration and metastasis. The present study examined the differences in the expression levels of ATP-binding cassette sub-family G member 2, CD24, CD44, CD133, cytokeratin (CK) 14 and CK19 between cervical cancer tissues and corresponding normal non-tumor tissues by flow cytometry. Then, the CD44+ or CD44-cells from cervical cancer tissues were sorted for identification and confirmation of differential expression by flow cytometry. The results demonstrated that the expression level of CD44 in cervical cancer tissues was higher than in the corresponding non-tumor normal tissues (t=3.12; P=0.0102). Compared with the CD44+ cells, the FOS-like antigen 1 (Fra-1), nestin, nuclear receptor subfamily 4, group A, member 2, OCT4 and p63 genes were highly expressed in CD44+ cells. The fold changes were 3.55, 3.55, 2.46, 2.87 and 2.56, respectively (P<0.05). However, BM11 polycomb ring finger oncogene, ck5, tumor protein p53 and lactotransferrin genes exhibited low expression levels in CD44+ cells.

It was verified by western blot analysis and flow cytometry that Fra-1 was highly expressed in CD44+ cells. Fra-1 was a potential target of miR-19a and miR-19b. The expression of miR-19a and miR-19b was downregulated by ~50% in CD44+ cells compared with CD44+ cells. These findings suggested that CD44 dysregulated the activation of the Fra-1 gene. The interaction of Fra-1 and CD44 may therefore be important in cervical carcinoma.

Introduction

Cervical carcinoma is the second most prevalent type of malignancy and the fifth most common cause of cancer-related mortality in females worldwide. Invasion and metastasis are major causes of cancer-associated mortality (1). Persistent infection with high-risk types of the human papillomavirus (HPV) is known to cause cervical cancer, however, additional genetic and epigenetic alterations are required for progression from precancerous disease to invasive cancer. DNA methylation is an early and frequent molecular alteration in cervical carcinogenesis. Dysregulated activation of numerous genes, including CD44 and SOX9, has been implicated in cervical cancer; however, the mechanism of regulation in human cervical cancer cells remains to be elucidated (2-4). It has been demonstrated that the inactivation of tumor suppressor genes and activation of oncogenes caused by genetic and epigenetic alterations is important in carcinogenesis. miRNAs are closely associated with the occurrence and regulation of cervical cancer (5). Emerging studies have evaluated the association of miRNA single nucleotide polymorphisms with cancer risk; however, the results remain inconclusive (6). In addition, the etiology of cervical carcinoma remains poorly understood.

CD44 refers to a multifunctional family of type I transmembrane proteins. The CD44 gene contains at least 21 exons, 11 of which can be variably spliced and produce a variety of heavily glycosylated cell surface proteins, termed CD44 variant isoforms. These proteins have been implicated in several biological processes, including cell adhesion, cell to cell interactions for example in lymphocyte homing hemopoiesis, cell migration and metastasis. These abilities are important in chronic inflammation and in cancer. In cancer, deregulation of the adhesion mechanisms increases the ability of tumor cells to metastasize. CD44 may function in certain cells through interactions with type I receptor tyrosine kinases, including...
erbB2 (2.7-8). Published data have demonstrated that CD44 mediates constitutive type I receptor signaling in cervical carcinoma cells (2). The assessment of CD44 isoform expression may be of clinical value in deciding upon adjuvant therapy, resulting in a more individualized management of therapy (9). In oral tongue squamous cell carcinoma, reduced expression of CD44 may be an indicator of high tumor invasiveness by increasing cervical lymph node metastasis (10). One common confounder for the analysis of clinical tumor specimens is the cellular heterogeneity of CD44+/CD44- cells. Flow cytometry sorting technology is able to overcome this problem and obtain pure CD44+ or CD44- cells for mechanistic study (11-14). Furthermore, FOS-like antigen 1 (Fra-1) is a proto-oncogene, located on chromosome 11q13, encoding a 1.7 kb mature mRNA. It is a negative inhibitor of activator protein-1 activity and has transforming activity.

The present study used flow cytometric analysis to examine the expression levels of ATP-binding cassette sub-family G member 2 (ABCG2), CD24, CD44, CD133, cytokeratin (CK) 14 and CK19 between cervical cancer tissues and corresponding normal non-tumor tissue. In addition, the CD44+ or CD44- cells from cervical cancer tissues were sorted.

Materials and methods

Tumor samples. In total, 12 participants were recruited at The Third Xiangya Hospital, Central South University (Changsha, China). Consent forms were obtained from individual patients and experimental protocols were approved by the Institutional Review Board of The Third Xiangya Hospital. The 12 participants were females with histologically-confirmed cervical cancer (Table I). All subjects enrolled in the present study were Chinese. Cervical cancer tissues and corresponding non-tumor normal tissues were collected, and each biopsy sample was divided into two sections, one was submitted for routine histological diagnosis, and the remaining section was subjected to flow cytometric analysis and cell sorting.

Flow cytometric analysis of ABCG2, CD24, CD44, CD133, CK14 and CK19. Single-cell suspensions of cervical cancer tissue or corresponding normal non-tumor tissue were prepared as follows: The cancer tissue or non-tumor normal tissue was sliced into 1-mm3 sections and digested in serum-free Dulbecco's modified Eagle's medium containing 1 mg/ml collagenase IV (InVitrogen Life Technologies, Carlsbad, CA, USA), 1% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) and 0.25% DNase I (Merck & Co., White House Station, NJ, USA). Enzymatic digestion was incubated at 37°C until fully with oscillation every 10-15 min prior to the substrate being passed through a 70-µm cell strainer. The resulting cell suspension was centrifuged at 500 x g for 10 min and resuspended in saline.

Single-cell suspensions were stained and incubated at 4°C for 30 min with the following antibodies, respectively: Fluorescein isothiocyanate (FITC)-conjugated CD44, phycoerythrin-conjugated ABCG2 and FITC-conjugated Ck14. Isotype controls were performed with FITC-conjugated rabbit anti-human IgG (negative control) All antibodies were purchased from Beckman Coulter (Miami, FL, USA) and used according to the manufacturer's instructions. The cells were washed twice and examined by fluorescence-activated cell sorting (FACS) using a MoFlo<sup>™</sup> XDP High-Performance Cell sorter (Beckman Coulter). Data were acquired and analyzed using Summit v5.2 software (Beckman Coulter, Inc., Fullerton, CA, USA).

CD44+ cell sorting by FACS. The expression levels of ABCG2, CD24, CD44, CD133, CK14 and CK19 were examined as described above. At the same time, the CD44+/CD44- cells were sorted for all cervical cancer samples. The parameters of sorting were conducted according to the manufacturer's instructions. CD44+/CD44- cells were pooled from every four samples. The sorted cells were immediately stored in TRIzol reagents or liquid nitrogen.

RNA extraction and quantitative PCR (qPCR). Total RNA was extracted from the sorted cells using an RNasy<sup>®</sup> kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions. The total RNA samples (1 µg) were used to generate cDNA. Reverse transcription was performed as previously described (12). Following the RT reaction, the PCR reaction was preceded by 94°C for 5 min, then 30 cycles for lactotransferrin (LTF) of 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min followed by 72°C for 7 min. All reverse transcription-PCR reactions were repeated at least three times at different numbers of the extension cycle to avoid false results of the PCR. GAPDH was used as an endogenous control for normalization. The sequences of the primers used for reverse transcription-PCR were synthesized (Table II). The expression of mRNA was assessed by evaluated threshold cycle (CT) values. The CT values were normalized with the expression levels of GAPDH and the relative quantity of mRNA specific to each of the target genes was calculated using the 2<sup>-ΔΔCT</sup> method (15,16).

Western blot analysis. The proteins of the sorted cells were prepared using lysis buffer. The protein concentrations were determined using the bicinchoninic acid (Pierce Chemical, Rockford, IL, USA) protein assay method. Extracts containing 50 µg of proteins were separated in 10% SDS-PAGE gels and electroblotted onto nitrocellulose membranes (HyClone Laboratories, Logan, UT, USA). The membranes were incubated using Tris-buffered saline/Tween-20 (25 mM Tris-HCl, 150 mM NaCl, pH 7.5 and 0.05% Tween-20) containing 5% non-fat milk followed by overnight incubation at 4°C with primary antibodies (Rabbit anti-Fra-1 antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:500). Following three washes, the membranes were incubated with horse-radish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) and the specific signals were visualized using an ECL detection system. Anti-β-actin antibody (Santa Cruz Biotechnology, Inc; 1:3,000) was used as a loading control.

Intracellular protein level detection by FACS. Following washing in Dulbecco's phosphate-buffered saline (D-PBS), the sorted cells were permeabilized with detergents (Triton X-100). The cells were washed twice with D-PBS and then the single-cell suspensions were stained and incubated at 4°C for 30 min with FITC conjugated Fra-1 (Biorbyt, Cambridge, UK), respectively.
Isotype controls were performed with FITC-conjugated rabbit anti-human IgG (negative control; Biorbyt). All the antibodies were used according to the manufacturer's instructions. The cells were washed twice and examined by FACS using a MoFlo™ XDP High-Performance Cell sorter (Beckman Coulter). Data were acquired and analyzed using summit v5.2 software.
Expression analysis of miR-19a and miR-19b in cervical cancer. As described above, total RNA was extracted from the sorted cells using an RNeasy® kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 2 mg of total RNA with M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) in a 25 ml volume containing 2 mg total RNA, 400 mM reverse transcription primer [oligo(dT)]18 for random primers for U6 RNA and miR-19a and miR-19b specific primers; Bulge-Loop™ miRNA qPCR primers purchased from Guangzhou RiboBio, Co., Ltd. (Guangzhou, China); for miRNA 4 U/ml M-MLV, 1 U/ml inhibitor and 0.4 mM dNTP mix. qPCR was performed using the reagents of SYBR-green I mix (Takara, Dalian, China) in a 20 ml reaction volume (10 ml SYBR-green I mix, 200 mM forward and reverse primer, and 2 ml cDNA template) on an MJ Opticon Monitor chromo4 instrument (Bio-Rad, Hercules, CA, USA) using the following protocol: 95°C for 20 sec, 40 cycles of 95°C for 10 sec, 60°C for 20 sec, 70°C for 1 sec. Data analysis was performed using the 2^(-ΔΔCT) method (15-16).

Statistical analysis. Differences of nonparametric variables were analyzed by the Fisher's exact test using the EPI software (EPI Info, version 3.2.2; www.CDC.gov/epiinfo/). Differences of the quantitative variables between groups were analyzed by Student's t-test using SPSS 11.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of ABCG2, CD24, CD44, CD133, CK14 and CK19 in cervical cancer and corresponding non-tumor normal tissues. In the present study, all 12 cervical cancer tissue samples were diagnosed as squamous cell cancer. There was a 75% (9/12) infection rate of HPV 16 or 18. Other HPV types included HPV 33, 35, 52 and 58. In addition, there were 50% peasants (subsistence farmers and the farm labourers; 6/12; Table I).

In total, 12 pairs of cervical cancer and corresponding non-tumor normal tissues were analyzed by FACS. The positive rate (%) of the marker CD44 in cervical cancer tissues and corresponding non-tumor normal tissues was 6.74 and 1.29%, respectively. The expression level of CD44 in cervical cancer tissues was higher than in the corresponding non-tumor normal tissues (t=3.12; P=0.0102). The positive rate (%) of the marker CD44 in cervical cancer tissues and corresponding non-tumor normal tissues was 2.82 and 1.08%, respectively. No significant differences between these genes and CD44, CD44+ and CD44- cells were sorted using a MoFlo™ XDP High-Performance Cell sorter (Beckman Coulter). Then, RNA extraction and qPCR were performed with the sorted CD44+/CD44- cells. Compared with the CD44+ cells, nestin, NR4A2 and OCT4 genes were highly expressed in CD44+ cells. The fold changes were 3.55, 2.46 and 2.87, respectively (P<0.05). However, Bmi1 and ck5 genes had low expression levels in CD44+ cells. The fold changes were 0.39 and 0.23, respectively (P<0.05). No significant differences between other genes, including SOX2, CD133 and Nanog, were identified in CD44+ and CD44- cells (Fig. 2).

Figure 1. Expression of ABCG2, CD44 and CK14 in cervical cancer and corresponding non-tumor normal tissues by flow cytometry. Flow cytometric description of ABCG2, CD44 and CK14 in cervical cancer and corresponding non-tumor normal tissues. Single-parameter histograms show the expression of markers ABCG2, CD44 and CK14. (A, C and E) Corresponding normal non-tumor tissues and (B, D and F) cervical cancer tissues. ABCG2, ATP-binding cassette sub-family G member 2; CK, cytokeratin.

mRNA expression levels of other tumor-associated genes in CD44+ and CD44- cells sorted from cervical cancer tissues. Fra-1, tumor protein p53 (TP53), Raf kinase inhibitor protein (RKIP), LTF, ubiquitin associated protein 1, (UBAP1), protein phosphatase 1, regulatory subunit 13 like (lasp), metaladherin (MTDH), metastasis associated 1 family, member 2 (m2a2), short palate, lung, and nasal epithelial clone-1 (Splencl), bromodomain containing 7 (BRD7) and p63 are all genes 5 (ck5), Nanog, nestin, END_1, nuclear receptor subfamily 4, group A, member 2 (NR4A2), OCT4 and ABCG2 genes are associated with cancer stem cells. These genes are important in various types of tumor. To investigate the expression levels of these genes in CD44+ cervical cancer cells and the effects between these genes and CD44, CD44+ and CD44- cells, (Fig. 2).

mRNA expression levels of genes associated with cancer stem cells in CD44+ and CD44- cells sorted from cervical cancer tissues. The results demonstrated that SOX2, Bmi1 polycomb ring finger oncogene (Bmi1), CD133, cytokeratin
that are associated with several types of tumor. To investigate these genes in CD44+ cervical cancer cells and the effect between these genes and CD44, CD44+ and CD44- cells were sorted using a MoFlo™ XDP High-Performance Cell sorter (Beckman Coulter). Then, RNA extraction and qPCR were conducted with the CD44+/CD44- sorted cells.

The results demonstrated that the TP53 and LTF genes exhibited a low expression in CD44+ cells compared with CD44- cells. The fold changes were 0.36 and 0.42 to TP53 and LTF genes, respectively (P<0.05). However, Fra-1 and p63 genes were highly expressed in CD44+ cells. There was 3.55 fold to Fra-1 gene and 2.56 fold to P63 gene fold in CD44+ cells compared with CD44- cells, respectively (P<0.05). No significant differences were identified in RKIP, UBAP1, Iaspp, MTDH, mta2, Splunc1 and BRD7 genes between CD44+ and CD44- cells (Fig. 3).

Analysis of protein expression levels of Fra-1 between CD44+ and CD44- cells sorted from cervical cancer tissues using western blot analysis. To verify whether the Fra-1 gene had a higher expression level of CD44+ cells than the CD44- sorted cells from cervical cancer tissues, its protein expression levels were further examined (Fig. 4A). In comparison to the CD44- cells, the expression level was high in the CD44+ cells. This corresponded with the results of qPCR and confirmed that Fra-1 was highly expressed in CD44+ cells sorted from cervical cancer tissues.

Analysis of protein expression levels of Fra-1 in CD44+ and CD44- cells sorted from cervical cancer tissues by FACS. To confirm that Fra-1 was expressed highly in CD44+ cells sorted from cervical cancer tissues, the protein expression levels of Fra-1 were further examined by FACS (Fig. 4B). In comparison to the CD44- cells, the expression level was high in the CD44+ cells. This corresponded with the results of qPCR and confirmed that Fra-1 was expressed highly in CD44+ cells.

Expression of miR-19a and miR-19b are downregulated in CD44+ cells in cervical cancer tissues. The open access program TargetScan (http://www.targetscan.org/) was used to predict the targets of miR-19a and miR-19b. Fra-1 was found to be a potential target of miR-19a and miR-19b. The endogenous expression of miR-19a and miR-19b was compared in CD44+/CD44- cells sorted from cervical cancer tissues by qPCR. As
Table VI. Identification of the expression of miR-19a and miR-19b in CD44+ CD44- cells sorted from cervical cancer tissues.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Sample</th>
<th>U6 CT (mean ± SD)</th>
<th>miRNA CT (mean ± SD)</th>
<th>ΔCT (mean ± SD)</th>
<th>ΔΔCT (mean ± SD)</th>
<th>Folda</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-19a</td>
<td>CD44+</td>
<td>19.84±0.83</td>
<td>29.77±1.05</td>
<td>9.93±0.99</td>
<td>0.88</td>
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<tr>
<td></td>
<td>CD44-</td>
<td>19.97±0.92</td>
<td>29.02±1.11</td>
<td>9.05±1.04</td>
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<tr>
<td>miR-19b</td>
<td>CD44+</td>
<td>19.91±0.89</td>
<td>31.45±1.06</td>
<td>11.54±1.01</td>
<td>0.93</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>CD44-</td>
<td>20.03±0.96</td>
<td>30.64±1.15</td>
<td>10.61±1.07</td>
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</table>

*aCompared with CD44- cells. The mean fold change in expression of the target gene was calculated using the following formula: \( \Delta \Delta CT = (C_{T,\text{Target}} - C_{T,GAPDH})_{\text{CD44}+} - (C_{T,\text{Target}} - C_{T,GAPDH})_{\text{CD44}-} \). At least three replicates of each reaction were performed. Sample spreadsheet of data analysis using the 2^\(-\Delta\Delta CT\) method. CT, threshold cycle; SD, standard deviation.

Figure 3. Expression levels of other tumor-associated genes between CD44+ and CD44- cells. The mRNA expression levels were analyzed by qPCR for Fra-1, TP53, RKIP, LTF, UBAP1, Iaspp, MTDH, mta2, Splancl, BRD7 and P63 genes. CD44+ and CD44- cells were sorted from cervical cancer tissues. The mean fold change in expression of the target gene was calculated using the following formula: \( \Delta \Delta CT = (C_{T,\text{Target}} - C_{T,GAPDH})_{\text{CD44}+} - (C_{T,\text{Target}} - C_{T,GAPDH})_{\text{CD44}-} \). At least three replicates of each reaction were performed. Sample spreadsheet of data analysis using the 2^\(-\Delta\Delta CT\) method. CT, threshold cycle; SD, standard deviation.

Figure 4. Expression levels of the Fra-1 protein in CD44+ and CD44- cells sorted from cervical cancer tissues. (A) Western blot analysis. (B) Fluorescence-activated cell sorting. Purple indicates the result of samples dyed with FITC-conjugated Fra-1 antibody; green indicates the result of samples dyed with FITC-conjugated rabbit anti-human IgG (negative control); red indicates the result of samples dyed without FITC-conjugated Fra-1 antibody. Data are one representative of three independent experiments. Fra-1, FOS-like antigen 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FITC, fluorescein isothiocyanate.
shown in Table IV, the expression of miR-19a and miR-19b was downregulated by ~50% in CD44+ cells compared with CD44− cells (Table IV). These results confirmed that Fra-1 was highly expressed in CD44+ cells.

Discussion

Cervical cancer that has been verified to be associated with the human papillomavirus (HPV), is the second most common type of cancer in females worldwide and is a leading cause of cancer-related mortality in females in developing countries (17,18). The most common high-risk HPV types in cervical cancer are HPV 16 and 18, and the most common low-risk types causing genital warts are HPV 6 and HPV 11 (19-22). In the present study, there was a 75% (9/12) infection rate of HPV 16 or 18. Other HPV types included HPV 33, 35, 52 and 58. This corresponded with previous studies (17,19).

CD44 has been implicated in numerous biological processes, including cell adhesion and cell to cell interactions for example lymphocyte homing hemopoiesis, cell migration and metastasis. Published data have demonstrated that CD44 mediates constitutive type I receptor signaling in cervical carcinoma cells (2). The assessment of CD44 isoform expression may be of clinical value in deciding upon adjuvant therapy, resulting in a more individualized management of therapy. CD44 may function in certain cells through interactions with type I receptor tyrosine kinases, including erbB2 (2.7-8). However, one common confounder for the analysis of clinical tumor specimens is the cellular heterogeneity. Sorted pure CD44+/CD44− cells were used to reveal the mechanisms of cervical cancer. To the best of our knowledge, there are no studies in which the cell sorting of CD44+ technology was used to study the mechanisms of cervical cancer.

In the present study, the CD44+ or CD44− cells of cervical cancer tissues were sorted by flow cytometry. Then, the mRNA expression levels of 21 genes were analyzed and the expression levels of miR-19a and miR-19b were detected by qPCR. The results demonstrated that the expression level of CD44 in cervical cancer tissues was higher than in the corresponding non-tumor normal tissues. Compared with the CD44+ cells, the Fra-1, nestin, NR4A2, OCT4 and P63 genes were highly expressed in CD44+ cells. The fold changes were 3.55, 3.55, 2.46, 2.87 and 2.56, respectively (P<0.05). Bmi1, ck5, TP53 and LTF genes were expressed at low levels in CD44+ cells. Bourguignon et al (23) confirmed that hyaluronan-CD44v3 interaction with Oct4-Sox2-Nanog promotes miR-302 expression leading to self-renewal, clonal formation and cisplatin resistance in cancer stem cells from head and neck squamous cell carcinoma. A study by Dhingra et al (24) demonstrated that nestin and CD44 are significantly expressed in a subset of gastric adenocarcinoma, particularly co-expression of nestin and CD44, and are highly expressed in Lauren intestinal histological subtypes. Overexpression of chromatin assembly factor-1 p60, poly (ADP-ribose) polymerase 1 and nestin predicts metastasizing behavior of oral cancer (25). CD44 is a key tumor-promoting agent in transformed tumor cells lacking p53. It was also suggested that the derepression of CD44 resulting from inactivation of p53 may potentially aid the survival of immortalized, premalignant cells (26). Immunostaining of p53 family members may aid the diagnosis and monitoring of high-risk pre-malignant lesions of the oral epithelium. The combination of staining patterns of p63, p73α and CD44v6 enabled us to isolate phenotypic undifferentiated or transient amplifying areas, reflecting the immaturity of the tumour cell lineage (27). The results from the present study and other studies suggested that CD44 affected the expression of important genes, including TP53 and nestin.

Fra-1 was highly expressed in CD44+ cells. Fra-1 was a potential target of miR-19a and miR-19b. The expression of miR-19a and miR-19b was downregulated by ~50% in CD44+ cells compared with CD44− cells. The results suggested that CD44 dysregulated the activation of numerous genes with important functions. Ramos-Nino et al revealed that Fra-1 is associated with cell migration in human malignant mesothelioma (MMs) and that Fra-1 modulation of CD44 may govern the migration of selected MMs (28). The results from the study by Kajanne et al demonstrated that Fra-1 was an important molecule in prostate cancer (29). Thus, interaction of Fra-1 and CD44 may be important in cervical carcinoma.

The findings of the present study suggested that CD44 dysregulated the activation of the Fra-1 gene and may exhibit an important role.

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References


