Upregulation of stem cell markers ALDH1A1 and OCT4 as potential biomarkers for the early detection of cervical carcinoma

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Abstract. Previous studies have reported the upregulation of stem cell biomarkers that are associated with tumorigenesis, in particular with cancer infiltration, recurrence and metastasis. Infection by human papilloma virus (HPV) is the main etiopathological factor of cervical carcinogenesis, but the expression of stem cell markers in cervical carcinoma and HPV infection have yet to be investigated so far. A total of 94 cases of fresh cervical tissues, 116 cases of paraffin-embedded cervical specimens and 72 cases of peripheral blood samples were collected from Uighur women who were either diagnosed with cervical squamous cell carcinoma (SCC) or cervical intraepithelial neoplasia (CIN) II-III, or from healthy subjects (negative controls, NC). HPV infection was detected in tissue DNA by polymerase chain reaction (PCR) with a HPV genotyping kit. The mRNA expression levels of aldehyde dehydrogenase 1 family member A1 (ALDH1A1), nanog homeobox (NANOG), POU class 5 homeobox 1 (OCT4), SRY-box 2 (SOX2) and twist family BHLH transcription factor 1 (Twist1) were determined using reverse transcription-quantitative PCR (RT-qPCR). Histological analysis was performed in order to examine the protein expression of ALDH1A1 and OCT4 in paraffin-embedded tissue specimens by immunohistochemical staining and the plasma levels of those two proteins was measured by ELISA. RT-qPCR analysis indicated a significant increase in the mRNA expression of ALDH1A1 and OCT4 in CIN II-III and SCC tissue specimens compared with NC ($P<0.05$). Although the expression levels of NANOG, SOX2 and Twist1 were significantly higher in SCC compared with NC ($P<0.05$), no significant difference was revealed in CIN II-III tissues compared with SCC or NC ($P>0.05$). Subsequent analysis by immunohistochemistry staining confirmed that the upregulation of ALDH1A1 and OCT4 was also significantly increased in SCC and CIN II-III compared with controls at the protein level. Notably, ELISA analysis detected significantly higher levels of ALDH1A1 and OCT4 in the peripheral blood (plasma) of patients with SCC compared with healthy subjects. The upregulation of stem cell markers ALDH1A1 and OCT4 in cervical carcinoma and its precursor lesions, in particular in the peripheral blood, indicates that ALDH1A1 and OCT4 may serve as biomarkers for the early detection of cervical carcinoma or for the monitoring of treatment of patients.

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Introduction

A model that explains the phenotypic and functional heterogeneity of cancer cells within the same tumor is the cancer stem cell (CSC) model. According to the CSC model, a tumor has a hierarchical cellular structure, where a small population of tumorigenic CSCs differentiates into non-stem cancer cells or non-tumorigenic progeny (1). Cancer stem cells are thought to possess stem-like properties of self-renewal, and initiate and drive tumor growth and metastasis, in addition to therapy resistance and recurrence following conventional therapy (2). As genital infection with human papillomaviruses (HPVs), in particular the persistent infection of high-risk species (including HPV16 and 18), promotes disease progression from cervical intraepithelial neoplasia (CIN) to malignancy, the virus may interact with CSCs in the epithelium of the uterine cervix (3,4).

In general, the cervix is composed of hard squamous cells in the ectocervix, soft columnar cells in the endocervix and metaplastic cells in the squamocolumnar junction, also known as the transformation zone (5). Squamous and columnar epithelial cells are regenerated by the active division of stem-like

reserve cells in the transformation zone, where cervical cancer is hypothesized to originate (6,7). It has been suggested that HPV enters the cell by distinct pathways, including clathrin- or caveolar-mediated endocytosis, or alternative routes in which tetraspanin-enriched microdomains are involved (8). The dependence of HPV infection on the activity of cyclin dependent kinases and microtubule reorganization, suggests that the incorporation of the viral genome into the nucleus of reserve cells may require the cell to enter into mitosis (9). HPV may program the cell to undergo symmetric division, by the disturbance of asymmetric division, therefore allowing the cells to proliferate and produce viral particles, but preventing them from differentiation (10,11). Accordingly, reserve cells may be converted to cervical CSCs by the interplay with high risk-HPV viral oncogenes and cellular alterations during cervical carcinogenesis (11). In fact, the presence of CSCs in cervical carcinoma is detectable by using stem cell markers. Early studies suggested that transcription factor tumor protein p63 (p63) and cytokeratin 17 (KRT17) are markers for reserve cells and reserve cell hyperplasia in the epithelium of cervical lesions and are upregulated during HPV infection of epithelial cells (10,12,13). Nanog homeobox (NANOG), POU class 5 homeobox 1 (OCT4) and SRY-box 2 (SOX2) are key regulators of embryonic stem cells and incorporate primarily into the regulatory network responsible for self-renewal and pluripotency (14,15). NANOG is expressed in cervical lesions but may not associate with the prognosis of cervical carcinoma (16). Different isoforms of OCT4 are expressed in cultured cells of cervical carcinoma, with the highest level in HPV-positive cells (17,18). Aldehyde dehydrogenase (ALDH), a stem cell marker for early stem-cell differentiation, is detectable in cervical epithelial lesions and carcinoma in addition to stem-like cells that are isolated from cervical carcinoma cells (19-21). Transcription factor twist family BHLH transcription factor 1 (Twist1) may serve a function in the disruption of E-cadherin-mediated cell-cell adhesion and the induction of epithelial-mesenchymal transition in cervical cancer stem-like cells (20-23). These previous studies suggest that cervical carcinoma or cell lines harbor a small population of CSCs that are characterized by the expression of stem cell markers; however, to date, the expression pattern of stem cell markers in cervical carcinoma and precursor lesions have yet to be well-characterized.

In the present study, the transcription and protein expression of ALDH family member A1 (ALDH1A1), NANOG, OCT4, SOX2 and Twist1 in cervical lesions and blood plasma from patients with squamous cell carcinoma (SCC) and its precursor lesion, CIN, were detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), immunohistochemistry (IHC) and ELISA. The results of the present study may contribute to current knowledge on the association of cervical carcinogenesis with the regulation of stem cell marker expression, and provide potential biomarkers for the early prognosis, diagnosis and monitoring of cancer treatment.

Materials and methods

Tissue specimens. The present study was approved and monitored by the Ethics Committee of Xinjiang Medical University (Xinjiang, China). All procedures performed in the present study were in accordance with the Helsinki Declaration of 1975, as revised in 2000. Written informed consent was obtained from all patients and healthy individuals prior to the study, and all data were analyzed anonymously. Patients diagnosed with cervical SCC, CIN and subjects (negative control, NC) with a normal cervix were enrolled in the present study, according to the diagnostic criteria of the World Health Organization and the Chinese Medical Association as described elsewhere (24). A total of 94 fresh tissue samples were sourced from patients and healthy controls by routine biopsies and surgical operations at the Department of Gynecology at the First Affiliated Hospital and The Third Affiliated Cancer Hospital of Xinjiang Medical University from March 2013 to June 2014. The specimens were pathologically classified, and included 32 cases of cervical SCC, 30 cases of CIN stages II-III and 32 cases of normal controls. CIN II and III are classified as a high-grade squamous intraepithelial lesion, which is considered a significant precancerous lesion, whereas CIN I, as a low-grade squamous intraepithelial lesion, is considered a much more benign lesion since most of these lesions regress; those diagnosed at this stage (CIN I) were excluded from the present study (24). Fresh tissue specimens were frozen quickly in liquid nitrogen following resection and stored at -80˚C for six months or in liquid nitrogen storage for up to two years.

For immunohistochemical (IHC) analysis, 116 cases of paraffin-embedded cervical specimens (3-μm thickness) were obtained from the specimen bank in the Pathology Department of the First Affiliated Hospital of Xinjiang Medical University. These specimens were selected following a case review by two experienced pathologists, and these included 47 cases of SCC, 37 cases of CIN stages II-III and 32 cases of NC.

For ELISA analysis, plasma samples from 85 Uighur women were collected, including 40 cases of SCC, 28 cases of CIN II-III and 17 cases of NC. A total of 3 ml blood samples were obtained from each donor by venipuncture into evacuated blood collection tubes that contained EDTA as an anticoagulant, and the plasma was preserved at -80˚C for further use following centrifugation at 800 x g and 4˚C for 10 min. The median age of patients was 53 years (range 25-65 years), and normal controls were age-matched to patients.

The clinical staging of patients was based on guidelines established by the International Federation of Gynecology and Obstetrics of 1994-1997, as revised in 1999 by Pecorelli *et al* (25). Tumor specimens were collected from patients with cancer who underwent radical surgery for cervical SCC at clinical stages I-IIa. Precancerous lesions were collected from patients with CIN stages II-III as biopsies or from cervical conization. Normal controls were obtained from patients without a history of cervical lesions or any form of cancer who underwent a hysterectomy for nonmalignant reasons during the same time period. Indications for hysterectomy were fibroids, prolapsed uterus or adenomyosis, and primarily a combination of fibroids with prolapse.

DNA extraction and HPV detection. Genomic DNA was extracted from fresh cervical specimens using a QIAamp DNA Mini kit for tissue DNA (cat no. 51306; Qiagen, Inc., Valencia, CA, USA). Concentration and purity of extracted DNA were measured using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Inc., Waltham, MA, USA). HPV

Table I. List of primers used in reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)
ALDH1A1	NM_000689	TTGTCCAGCCCACAGTGTTCTC	TGTCTTTGGTAAACACTCCTGCTGA	168
OCT4	NM_002701.4	GTGCCGTGAAGCTGGAGAA	TGGTCGTTTGGCTGAATACCTT	192
NANOG	NM_024865	CCTGTGATTTGTGGGCCTGA	CTCTGCAGAAGTGGGTTGTTTG	168
SOX2	NM_003106	GTGAGCGCCCTGCAGTACAA	GCGAGTAGGACATGCTGTAGGTG	82
TWIST1	NM_000474	CAGCTACGCCTTCTCGGTCT	CTGTCCATTTTCTCCTTCTCTGG	138
β-actin	NM_001101.3	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA	171

ALDH1A1, aldehyde dehydrogenase 1 family member A1; NANOG, nanog homeobox; OCT4, POU class 5 homeobox 1; SOX2, SRY-box 2; Twist1, twist family BHLH transcription factor 1.

infection of 8 high-risk HPV genotypes, including HPV 16, 18, 45, 31, 33, 52, 58 and 67, was determined by multi-fluorescent PCR assay using the Real Quality RQ-HPV HR kit (cat no. RQ-22-120A; AB Analitica SRL, Padua, Italy) containing specific primers for HPV16, HPV18/45 and HPV31, and general primers for HPV33/45/52/58/67. Following the activation of the polymerase at 95˚C for 1 min, 40 cycles of the denaturation at 95˚C for 15 sec, annealing at 57˚C for 30 sec and elongation at 72˚C for 30 sec, were performed on the ABI-7500 PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The human β-actin gene was used as an internal control for DNA quality.

RNA extraction and RT-qPCR analysis. Fresh frozen tissues (~50 mg) were packed in aluminum foil and pulverized by grinding under liquid nitrogen. Total RNA was isolated from the powder by dissolving with TRIzol® lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) followed by phenol/chloroform extraction and ethanol precipitation. cDNA was synthesized from 1 μg of total mRNA by RT using a RevertAid kit (Fermentas; Thermo Fisher Scientific, Inc.) at 42˚C for 60 min. For RT-qPCR analysis, primer pairs specific to the mRNA sequences of target genes and suitable for use were designed and synthesized by Takara Bio (Takara Biotechnology Co., Ltd., Dalian, China; Table I). Each cDNA sample (20 ng) was analyzed in a 25 μl by RT-qPCR using SYBR Premix Ex Taq™ kit (TIi RNase H Plus; Takara Biotechnology Co., Ltd.) on an iQ5™ PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and synthesis at 60˚C for 30 sec and a final incubation at 4˚C for 10 min. Expression levels of target genes were quantified using the $2^{-\Delta\Delta Cq}$ method (26) using the software iQ5 Standard Edition (Version 1.028; Bio-Rad Laboratories, Inc.), setting β-actin as an internal control. Experiments were repeated in triplicate for each sample.

IHC analysis. The paraffin-embedded tissues were sectioned into 3-μm slices. The IHC staining was performed with the streptavidin peroxidase-conjugated method using primary rabbit polyclonal antibodies recognizing target proteins ALDH1 (cat. no. ab23375; Abcam, Cambridge, UK) and OCT3/4 (cat. no. PA5-27438; Thermo Fisher Scientific Inc.) at 1:200 dilutions, and IHC kits containing biotin-labeled goat anti-rabbit secondary antibody (cat. no. ZB-2010; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.; OriGene Technologies, Beijing, China) at the 1:300 dilution according to titration experiments and the manufacturer's protocol. Briefly, tissue sections were dewaxed in 100% xylene and rehydrated in ethanol at 100, 85 and 75% gradients and each for 5 min at room temperature, and washed with distilled water at room temperature followed by antigen retrieval with heating in the microwave oven for 15 min at 95˚C in EDTA buffer (pH 8.0). Subsequent to cooling and rinsing in distilled water, endogenous peroxidase activity was blocked by incubating sections for 15 min in 3% H_2O_2 at room temperature followed by rinsing in 0.01 M PBS (pH 7.4) for 10 min. Following treatment at room temperature with the protein blocking solution provided by the IHC kits for 10 min, the sections were incubated with the primary antibodies in a humid chamber overnight at 4˚C. Then, the sections were washed with PBS three times and incubated with the secondary antibody for 15 min at room temperature. The staining was visualized with the DAB Kit (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.; OriGene Technologies). Negative controls were treated with PBS instead of primary antibodies. All tissue sections were counterstained using 100% hematoxylin at room temperature for up to 30 sec, in accordance with the preliminary experiments. PBS was used in place of the primary antibody as a negative control. For evaluation, stained tissue sections were scored under a light microscope (magnifications, x20 and x40) by two experienced pathologists. The results were scored on a scale of 0 to 3 by an estimation of the percentage and intensity of positive staining in 5 fields each time using a previously described method for evaluation and interpretation of IHC staining (27). A mean percentage of positive staining was calculated according to the number of positively stained cells in each field and time and scored as 0 for negativity, 1 for 0 to 30% positivity, 2 for >30 to 60% positivity and 3 for >60% positivity. Positive staining intensity was scored as 0 for a negative signal, 1 for weak signal intensity, 2 for moderate signal intensity and 3 for high signal intensity. A consensus number between the two investigators was reached for each tissue slice. An overall score was calculated by the addition of the two scores and the overall scores were classified according

to the following categories: i) 0-2, a loss of expression or weak expression; ii) 3-4, moderate expression and iii) 5-6, strong expression.

ELISA. Plasma levels of ALDH1 and OCT4 were determined in blood samples from patients and controls by 96-well plate sandwich ELISA using commercially available kits for ALDH1 (cat. no. SEE824Hu) and OCT4 (cat. no. SEA424Hu; both Uscn Life Sciences, Inc., Wuhan, China) according to the manufacturer's protocol. The quantification of the relative plasma content was based on the standard curves derived from standard substances (proteins) of ALDH1 and OCT4 supplied with the indicated kits, in which the absolute concentration of each protein was known.

Statistical analysis. Statistical analysis was performed using SPSS (version 17.0; SPSS Inc., Chicago, IL, USA). All P-values were two-sided, and P<0.05 was considered to indicate a statistically significant difference. All data was presented as the mean ± standard deviation. Data derived from the results of RT-qPCR and ELISA tests were compared for statistical differences. Between and within group analysis were carried out using one-way ANOVA followed by Dunnett's post-hoc test. Continuous data derived from IHC scoring was analyzed using Mann-Whitney U test.

Results

Transcription of stem cell markers in cervical carcinoma and its precursor lesions. The present study focused on five stem cell markers, including ALDH1A1, OCT4, NANOG, SOX2 and Twist1, which are considered to be potential biomarkers for cervical carcinoma as previously reported (16-23). The transcription of genes encoding these proteins was analyzed in tissue specimens from patients with SCC, CIN II-III and NC using RT-qPCR using primer pairs specific for mRNA sequences. The data indicated an increase in the transcription of these genes with the progression of cervical cancer (Fig. 1, Table II). Statistical analysis confirmed significant differences in the expression of all five genes in SCC tissues compared with NC tissues (P<0.05). In addition, significant differences were detected in the expression of ALDH1A1, OCT4 and NANOG in CIN II-III tissues compared with NC tissues (P<0.05). There were also significant differences detected in ALDH1A1 and OCT4 expression in SCC tissues compared with CIN II-III tissues (P<0.05). These findings indicate that cervical carcinogenesis may be characterized by the upregulation of stem cell markers, in particular the differential expression of ALDH1A1 and OCT4 in SCC tissues compared with its precursor lesions and normal cervical tissue.

Among the 94 cases of fresh tissue specimens, HPV infection was detected in 64 cases with qPCR using probes for HPV16, 31, 18/45, and 33/52/58/67 (Table III). Of these 64 cases, 32 cases of SCC, 24 cases of CIN II-III and 8 cases of normal controls (NC) were positive for HPV16 infection, including several cases co-infected by HPV16 and other HPV species. Due to high HPV positivity, in particular the predominance of HPV16 infection in SCC (32/32) and CIN II-III (24/30) and the HPV negativity of NC (24/32), the data of

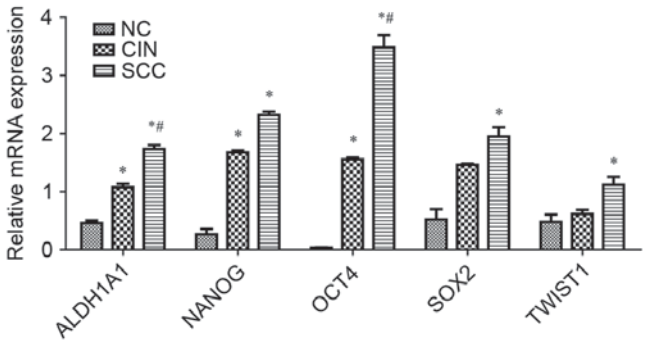

Figure 1. mRNA expression patterns of five genes, including ALDH1A1, NANOG, OCT4, SOX2 and Twist1 in SCC, CIN II-III and NC tissues analyzed by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. NC; #P<0.05 vs. CIN. ALDH1A1, aldehyde dehydrogenase 1 family member A1; NANOG, nanog homeobox; OCT4, POU class 5 homeobox 1; SOX2, SRY-box 2; Twist1, twist family BHLH transcription factor 1; SCC, cervical squamous cell carcinoma; CIN, cervical intraepithelial neoplasia; NC, negative controls.

HPV detection was not suitable for analyses for the association of gene expression with HPV16 infection. Nevertheless, due to the contrast between HPV16 positive cervical lesions and HPV-negative normal controls, it is hypothesized that a regulation of stem cell markers independent of HPV16 infection was integrated into the result of RT-qPCR (Table II).

Verification of ALDH1A1 and OCT4 expression by immunohistochemistry. To validate the results of the RT-qPCR analysis, the protein expression of ALDH1A1 and OCT4 in 116 cases of formalin-fixed and paraffin-embedded tissue specimens of SCC, CIN II-III and NC were analyzed by immunohistochemical analysis. Positive staining for ALDH1A1 and OCT4 was localized in the cytoplasm of cervical epithelial or carcinoma cells (Fig. 2). Statistical analysis revealed that the strong and moderate positive staining of ALDH1A1 was significantly increased in CIN II-III and SCC compared with NC and from CIN II-III to SCC (P<0.05; Table IV). Although a weak staining of OCT4 was observed in a number of the specimens from SCC, CIN II-III and NC, moderate staining of OCT4 was significantly increased in SCC compared with CIN II-III and NC (P<0.05). These results indicated that the transcription and protein expression of ALDH1A1 and OCT4 is increased during the development of cervical carcinoma and its precursor lesions compared with normal tissues.

Upregulation of ALDH1A1 and OCT4 in circulating blood plasma with the development of cervical carcinoma. To evaluate the potential of ALDH1A1 and OCT4 as biomarkers for non-invasive diagnosis, the levels of these proteins in 85 cases of plasma samples from patients with SCC and CIN and healthy subjects were detected (Table V). The ELISA analysis indicated significantly higher levels of ALDH1A1 and OCT4 in the plasma of patients with SCC compared with patients with CIN II-III and healthy controls (P<0.05), but there was no significant difference between patients with CIN II-III and healthy controls (P>0.05; Fig. 3). Therefore, the quantitative increase of these proteins in plasma may serve as biomarkers for cervical carcinoma.

Table II. mRNA expression levels of five stem cell markers in cervical lesions as determined by reverse transcription-quantitative polymerase chain reaction.

	Relative mRNA expression level (mean ± standard deviation)			One-way analysis of variance (P-value)				
Markers	NC (n=32)	CIN II-III (n=30)	SCC (n=32)	F-value	P-value	SCC vs. NC	CIN vs. NC	SCC vs. CIN
ALDH1A1	0.458±0.047	1.076±0.055	1.733±0.068	10.943	<0.0001	<0.0001	0.031	0.022
NANOG	0.267±0.090	1.676±0.036	2.318±0.058	6.825	0.003	0.001	0.019	0.272
OCT4	0.034±0.006	1.555±0.031	3.482±0.216	19.723	<0.0001	<0.0001	0.009	0.001
SOX2	0.521±0.176	1.461±0.022	1.949±0.156	4.281	0.020	0.006	0.069	0.338
TWIST1	0.476±0.130	0.615±0.072	1.114±0.142	3.384	0.043	0.017	0.613	0.061

ALDH1A1, aldehyde dehydrogenase 1 family member A1; NANOG, nanog homeobox; OCT4, POU class 5 homeobox 1; SOX2, SRY-box 2; Twist1, twist family BHLH transcription factor 1; SCC, cervical squamous cell carcinoma; CIN, cervical intraepithelial neoplasia; NC, negative controls.

Table III. HPV genotyping of cervical specimens.

Samples	HPV16	HPV16/ 18/45	HPV 16/31	HPV16/33/ 52/58/67
NC (n=32)	6	2	n.d.	n.d.
CIN II-III (n=30)	20	4	n.d.	n.d.
SCC (n=32)	20	3	3	6

SCC, cervical squamous cell carcinoma; CIN, cervical intraepithelial neoplasia; NC, negative controls; n.d., not detected; HPV, human papilloma virus.

Table IV. Immunohistochemical staining analyses of ALDH1A1 and OCT4 expression in cervical lesions.

A, ALDH1A1

Samples	-	+	++	+++	P-value	
NC (n=33)	8	18	7	0	<0.0001[a]	
CIN II-III (n=37)	11	4	19	3	0.028[b]	
SCC (n=47)	5	0	25	17	<0.0001[b]	<0.0001[c]

B, OCT4

Samples	-	+	++	+++	P-value	
NC (n=33)	20	12	1	0	0.001[a]	
CIN II-III (n=37)	19	13	5	0	0.138[b]	
SCC (n=47)	16	14	17	0	<0.0001[b]	0.029[c]

-, negative expression; +, weak positive expression; ++, moderate positive expression; +++, strong positive expression; [a]Comparison between all three groups; [b]Comparison with NC; [c]Comparison with CIN II-III. ALDH1A1, aldehyde dehydrogenase 1 family member A1; OCT4, POU class 5 homeobox 1; SCC, cervical squamous cell carcinoma; CIN, cervical intraepithelial neoplasia; NC, negative controls.

Discussion

The concept of cancer stem cells in human tumors opens up novel research directions on how cancer is initiated and how cancer cells are capable of switching from dormancy to malignancy, and challenges previous understanding of tumor recurrence, angiogenesis, metastasis and drug resistance (28-30). Accordingly, the studies on stem cell markers have notably shifted from focusing on the function of the markers in embryonic development to their involvement in tumorigenesis (31-33). In previous studies, cervical CSCs were most frequently identified and characterized in cell lines of cervical carcinoma by detection of stem cell markers, including p63, KRT17, ALDH1, OCT4, NANOG, SOX2 and Twist1 (12,16,17,21,22). However, the expression profile of these stem cell markers in cervical lesions has yet to be intensively studied (5). Furthermore, the application of stem cell markers for clinical diagnosis is limited due to the lack of sufficient validation studies. In the present study, it was revealed that the transcription and protein expression of ALDH1A1 and OCT4 were significantly upregulated in cervical lesions from patients with CIN II-III and SCC compared with normal controls, and also significantly higher in the plasma of patients with SCC compared with healthy controls. In the case of NANOG, SOX2 and Twist1, the transcription of these genes in SCC tissues was significantly higher compared with NC, but no significant difference was identified in CIN II-III compared with SCC or NC.

As ALDH1 and OCT 4 are considered as biomarkers for embryonic and/or cancer stem cells (14,20), the results of the present study indicate that the deregulation of these two proteins in cervical lesions reflects the function of CSCs or cancer cells with stem-like properties during cervical carcinogenesis (14,21). However, the outcome of the association analysis between the gene expression and HPV infection may be limited due to the high HPV16 positivity of SCC and CIN specimens in comparison with normal controls negative for HPV infection. Nevertheless, as the expression levels of stem cell markers were analyzed in SCC and CIN compared with

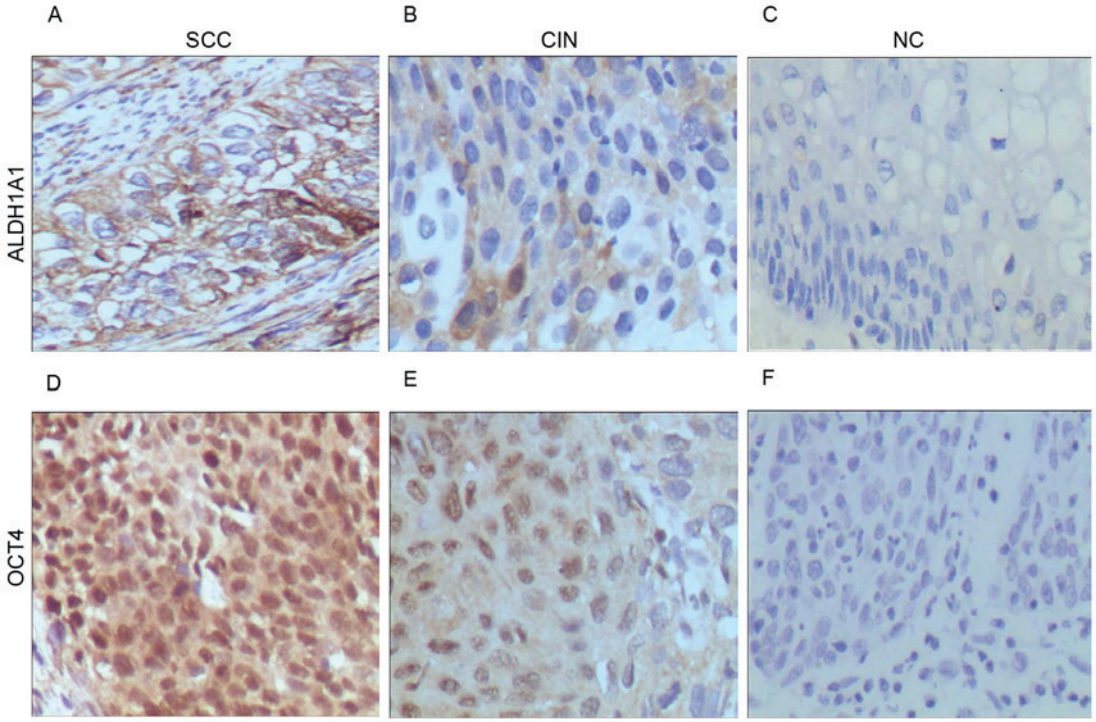

Figure 2. Expression pattern of ALDH1 and OCT4 proteins in cervical lesions as detected by immunohistochemical staining. Expression of ALDH1A1 in (A) SCC, (B) CIN and (C) NC tissues. Expression of OCT4 in (D) SCC, (E) CIN and (F) NC tissues. Magnification, x400. ALDH1A1, aldehyde dehydrogenase 1 family member A1; OCT4, POU class 5 homeobox 1; SCC, cervical squamous cell carcinoma; CIN, cervical intraepithelial neoplasia; NC, negative controls.

the normal controls in the present study, it was suggested that the expression of stem cell markers potentially regulated by HPV16 infection may be reflected in the RT-qPCR results, and presumably in IHC analyses of tissue specimens that were not tested for HPV infection. Therefore, independent analyses, in particularly *in vitro* studies, associated with HPV16 infection are required to validate the outcome of the present study.

The ALDH family has 19 different isoforms that are localized in the cytoplasm, mitochondria or nucleus, and serve crucial functions in cellular protection by the oxidation of intracellular aldehydes, which induces resistance to a number of alkylation agents used in cancer therapy (34). Among them, ALDH1A1, ALDH1A2, ALDH1A3 and ALDH8A1 participate in the oxidation of retinol to retinoic acid in the cytoplasm, which in turn translocate into the nucleus and initiates the transcription of genes involved in early stem-cell differentiation (35,36). In addition, ALDH1A1 may contribute to the majority of ALDH activity in CSCs (37). The predominant expression of ALDH1A1 has been associated with ALDH activity in prostate and thyroid cancer (38,39). In the case of 6 ALDH1 isoenzymes, ALDH1A1 may be a major contributor of ALDH1 activity and a biomarker for predicting the poor survival of patients with breast cancer (40). High levels of ALDH1A1 and ALDH1A3 expression are associated with malignant transformation to lung adenocarcinoma (41). Little is known about the regulation of ALDH1A1 expression in cervical lesions, but the function of ALDH in cervical CSCs or cervical carcinoma has been described in a number of studies: CSCs that are isolated and enriched from cervical carcinoma cell lines with high ALDH activity or ALDH1 positivity exhibit enhanced stem-like properties of self-renewal, high tumorigenicity and resistance to cisplatin treatment compared with those with low ALDH activity or negative for ALDH1 expression (20,21). ALDH-positivity and expression levels are increased in tumor specimens of the uterine cervix, and therefore ALDH may serve as a predictive marker for poor prognosis, poor clinical outcome and resistance to chemotherapy in patients with cervical carcinoma (42-44). Corresponding with these results, the results of the present study also revealed that the upregulation of ALDH1A1 in the progression of precancerous lesions (CIN II-III) to cervical carcinoma. This indicates that ALDH1A1 may be a potential biomarker for the early diagnosis of cervical carcinoma.

OCT4 is a well-established stem cell factor that cooperates with SOX2 in maintaining the self-renewal and pluripotency of human and mouse embryonic stem cells (45,46). The loss of OCT4 expression or downregulation is associated with stem cell differentiation (47). OCT4 is detected in tumor-initiating cells or stem-like cancer cells enriched from tumor tissues or cell lines, indicating its function in tumorigenesis, metastasis and resistance to anticancer therapies (48). The co-expression of OCT4 with SOX2 has been detected in CIN but not in SCC, and the elevated expression of OCT4 in the absence of SOX2 is associated with the poor prognosis of patients with cervical carcinoma (49). However, the upregulation of both OCT4 and SOX2 in cervical carcinoma and its precursor lesions was detected in the present study by RT-qPCR. Consistent with the findings in the present study, a number of previous studies suggest that SOX2 functions cooperatively with other dosage-sensitive transcription factors, including OCT4 and NANOG, in order to maintain the regulatory networks responsible for self-renewal and to repress differentiation programs in embryonic stem cells (50,51). These studies indicate that the expression profile of OCT4 and SOX2 and

Table V. Plasma levels of ALDH1A1 and OCT4 in healthy controls and patients with cervical lesions as analyzed using ELISA.

	Plasma protein level (ng/ml)			One-way analysis of variance (P-value)				
Markers	NC (n=17)	CIN II-III (n=28)	SCC (n=40)	F-value	P-value	SCC vs. NC	CIN vs. NC	SCC vs. CIN II-III
ALDH1A1	9.923±0.585	11.432±0.818	14.287±0.069	3.449	0.034	0.034	0.759	0.034
OCT4	3.170±0.808	3.885±0.033	5.143±0.914	5.946	0.004	0.032	0.553	0.002

ALDH1A1, aldehyde dehydrogenase 1 family member A1; OCT4, POU class 5 homeobox 1; SCC, cervical squamous cell carcinoma; CIN, cervical intraepithelial neoplasia; NC, negative controls.

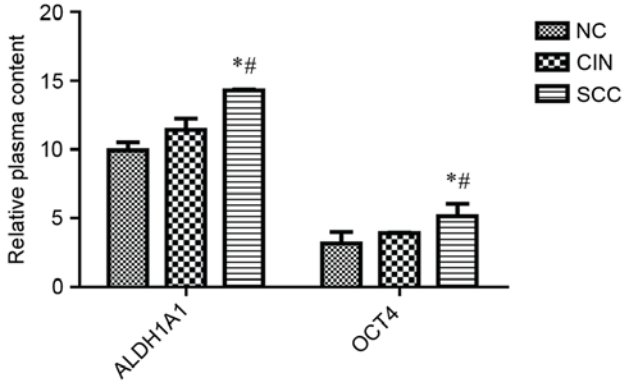

Figure 3. Plasma levels of ALDH1 and OCT4 in controls and patients with cervical lesions as determined by ELISA. *P<0.05 vs. NC; #P<0.05 vs. CIN. ALDH1A1, aldehyde dehydrogenase 1 family member A1; OCT4, POU class 5 homeobox 1; SCC, cervical squamous cell carcinoma; CIN, cervical intraepithelial neoplasia; NC, negative controls.

NANOG may serve as biomarkers for the prognosis of cervical carcinoma.

Genital infection by HPVs, in particular by oncogenic high-risk HPV types (including HPV 16 and 18), is generally known to be the primary etiological factor in cervical carcinogenesis (52). However, the effects of HPV detection in cervical specimens are constantly debated in studies on cervical carcinoma, as the majority of HPV infections are transient and harmless or eliminated by the host immunity (4). Even persistent infections have a relatively long latent period prior to the induction of cancer, indicating that HPV testing alone is not sufficient in the prognosis of cervical carcinoma (3). Therefore, HPV-based cancer prognoses may require the use of auxiliary diagnostic biomarkers that are present during HPV-induced carcinogenesis (53). However, based on the results of HPV genotyping performed in the present study, it was difficult to define the association between the expression of target genes and HPV infection as the majority of cases of SCC and CIN II-III were positive for HPV and most of the normal controls were HPV-negative. Nevertheless, the potential effects of HPV infection on the expression of stem cell markers have been described in previous studies. In cervical cancer cells, HPV16 may activate OCT4 expression, which in turn induces the expression of miR-125b that targets BCL2 antagonist/killer 1 and consequently leads to the suppression of apoptosis (54,55). Stem-like cancer cells isolated from primary cervical tumors or cell lines express OCT4 together with NANOG and SOX2 and have sphere-forming and self-renewal abilities, which are abolished by the suppression of HPV-coding E6 protein (56). This suggests that independent analyses are required in order to reveal the potential mechanism of the regulation of stem cell markers by HPV infection.

In biomarker discovery, the sensitivity and specificity as well as the convenience of diagnosis for physicians should be taken into account. Due to its invasive and metastatic potential, CSCs may be able to spread from primary tumors and survive in the circulating blood by evading immune surveillance, and subsequently form metastases (57). Accordingly, the determination of a stem cell marker that is released from circulating CSCs may become a fast and direct approach to plasma-based diagnosis of cancer.

A previous study reported that ALDH1 is elevated in the blood of patients with non-small-cell lung cancer prior to surgery but is not detectable in post-operative samples (58). The detection of serum ALDH1A1 has been suggested to have a predictive value in monitoring the chemotherapy of patients with primary and metastatic breast cancer (59). Additionally, high levels of serum OCT4 and NANOG are detectable in patients with hepatocellular carcinoma and hepatitis B virus infection (60).

It is also important for the detection of stem cell markers with a tumor origin to take into account the complexity of stem cells, multipotent stem-like cells or progenitor cells in the blood circulation. Notably, the blood contains highly heterogenic populations of vascular-resident stem or progenitor cells with proliferative capacity and clonogenicity, including mesenchymal stem cells, pericytes, endothelial progenitor cells and smooth muscle progenitor cells, which serve important functions in vascular homeostasis (61). These cells are either derived from the bone marrow and other sources or frequently released from the vascular endothelium to the blood by hemodynamic forces, including fluid shear stress and cyclic strain or pathologic processes during heart and vascular diseases (62,63). In addition, mesenchymal stem cells are multipotent and are able to differentiate into vascular smooth muscle cells and endothelial cells (64). High ALDH activity may be detected in human bone marrow-derived mesenchymal stromal cells with multipotent stromal and pro-vascular regenerative functions (65). Consistent with these findings, the results of the present study indicate that the increased levels of ALDH1A1 in the blood may, to a high extent, contribute to cervical carcinogenesis, and therefore ALDH1A1 may be used as a marker to distinguish patients with cervical carcinoma from normal controls.

To the best of our knowledge, the number of previous studies on the detection of OCT4 in the blood of patients with cancer is limited. In the present study, there were no significant differences between the plasma levels of ALDH1 and OCT4 in patients with CIN II-III and normal controls. However, the plasma levels of ALDH1 and OCT4 significantly increased in patients with cervical carcinoma compared with CIN II-III and controls. These results were consistent with the findings from IHC, which revealed that in specimens from normal subjects, the expression of ALDH1 and OCT4 proteins was negative, whereas the expression of these proteins was moderate or strongly positive in cervical carcinoma. Additionally, the differences in expression of ALDH1 and OCT4 proteins between specimens from normal subjects and patients with cervical carcinoma were statistically significant. Therefore, OCT4 may additionally contribute to the diagnosis and prognosis of cervical carcinoma, when its detection is combined with the detection of ALDH1 and other biomarkers.

NANOG expression is detectable in embryonic stem cells, embryonic limb bud cells and embryonal carcinoma cells (66,67). The expression of NANOG is elevated at advanced clinical stages of breast cancer, glioma and bladder cancer, which may be associated with poor prognosis (68). The results of the present study revealed that NANOG transcription was significantly increased in tissues from patients with SCC or CIN compared with controls, but the expression was not significantly different between specimens from patients with SCC and CIN. In addition, a previous study also indicated that the high expression of NANOG in cervical lesions may not be associated with the prognosis of cervical carcinoma (16). Accordingly, NANOG expression was not additionally analyzed by IHC and plasma-based ELISA experiments. However, the combined detection of ALDH, OCT4 and NANOG markers may improve the accuracy and specificity of the biomarker profile for diagnosis of cervical carcinoma.

In conclusion, it was demonstrated that ALDH1A1 and OCT4 were upregulated in cervical carcinoma tissues and its precursor lesions at the level of mRNA and protein, as well as in blood plasma, which represents the protein levels in the whole body. Notably, the samples subjected to analysis by RT-qPCR, IHC and ELISA were obtained from three independent populations of patients and controls, which may increase the feasibility and accuracy of quantitative verification. Despite the limited prognostic potential of ALDH1A1 and OCT4 in the blood-based detection, it may be suggested that the combined detection of these proteins at the level of transcription, protein expression and in the blood circulation may provide an auxiliary profile for the prognosis, diagnosis and monitoring of the treatment of cervical carcinoma.

Cervical cancer is a challenge for the Uighur female population in Xinjiang, China, where there is a high incidence rate (490-560/100,000) accompanied with the prevalence of HPV infection (69,70). In accordance with preliminary data, the case detection rate of cervical carcinoma accounts for ~20% of all patients diagnosed with cancer in Xinjiang (71). Therefore, further validation of this profiling, and further in-depth studies on the association of the altered expression of stem cell markers with HPV infection by other approaches, will contribute to establishing an auxiliary diagnostic profile for HPV-based cancer prognosis and greatly benefit the women in areas with a high prevalence, including Uighur women in China.

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Availability of data and materials

The datasets generated and/or analysed during this study are available from the corresponding author on reasonable request.

Authors' contributions

AA designed the research. WT, RY and LS performed the research. MR and RY were responsible for clinical diagnosis and sample collection. DL contributed to data collection and statistical analysis. AA and WT wrote the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

Te present study was approved and monitored by the Ethics Committee of Xinjiang Medical University (Urumqi, China). All procedures performed in this study were followed in accordance with the Helsinki Declaration of 1975, as revised in 2000 (5). Written informed consent was obtained from all patients and healthy individuals prior to the study, and all data were analyzed anonymously.

Patient consent for publication

Written informed consent was obtained from all individual participants included in the present study.

Competing interests

All authors declare that they have no competing interest.

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