

RESEARCH ARTICLE

Elevated Expression of Ox2R in Cervical Cancers and Placentas of Uyghur Women in Xinjiang, China

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Abstract

Objective: Cervical cancer is one of the major causes of mortality of Uyghur women in Xinjiang, China. Although increased expression of orexin receptor (OxR), known to be strongly expressed in human placenta, has a proven relation to some cancers, there have been few studies of cervical cancer. Thus, we explored this question by evaluating the expression of orexin receptor as a biomarker for screening early stage of cervical cancer in Uyghur women with highest occurrence rate of cervical cancer in China. **Study Design:** We used polymerase chain reaction (PCR) and immunohistochemical staining to determine the expression of both Ox1R and Ox2R in cervical cancer and cervicitis biopsies collected from Uyghur women infected with human papilloma virus (HPV)16. The expression rate was compared between cervical cancers of low, intermediate and high differentiation and cervicitis. **Results:** Although there was no significant difference in the expression rate of Ox1R between groups, Ox2R was significantly overexpressed in cervical cancer patients when compared to the cervicitis group. Ox1R was negative in normal human placenta while Ox2R was positive. **Conclusions:** While expression of Ox1R had no correlation with invasion or metastatic potential, Ox2R demonstrated elevation in cervical cancer with heterogeneity in groups with different metastatic potential, in the human placenta as well, implying that it might serve as an indicator of invasive capacity along with other indices.

Keywords: Ox1R- Ox2R- cervical cancer- HPV16- human placenta

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Introduction

Cervical cancer is the second most common malignant tumor in women worldwide, with 500 thousand new cases and 275 thousand deaths occurring each year (Parkin et al., 2005). Uyghur women lived in Xinjiang (China) are one of the highest risk groups of cervical cancer, whose morbidity and mortality are both higher than that of other ethnic groups in the same region and the mortality is the highest (Jiang et al., 2006). And the high HPV infection rate (459 per 100,000) was believed to be a vital reason, of which the high-risk genotype HPV 16 was the most dominant type (Jiang et al., 2006). Furthermore, cervical cancer tends to develop in younger ages among Uyghur women in recent years (Abudulimu et al., 2014). Most Uyghur women with cervical cancer were diagnosed at late stage when they came to the hospital, therefore develop a simple and convenient way to make early diagnosis is urgent.

The regulatory peptides orexin A and B, acting via G protein-coupled receptors orexin-1 receptor (Ox1R) and orexin-2 receptor (Ox2R), control sleep-wakefulness, feeding, and a variety of neuroendocrine functions (Cataldi et al., 2012). Although orexins are primarily expressed in the lateral hypothalamus, the distribution of orexins has

been also showed outside the central nervous system such as adrenals, gastrointestinal tract, or endocrine pancreas (Voisin et al., 2003). Recently, Başar (2013) found that OxRs' expression was widespread in prostate cancer and in patients with a higher Gleason score. They concluded that OxR might be considered as a marker for poor prognosis in prostate cancer (PCa) and was associated with poorly differentiated malignancy. From their research we assumed that there might also be some link between OxRs and cervical cancer.

Aggressive behavior of cells occurs both in tumor metastasis and mammalian embryo implantation (Fang et al., 2010). Nowadays, scientists found these two processes are analogous in many aspects by researching the interaction of physiological and pathological processes (Chen et al., 2011). We believed that if OxRs expressed in both cervical cancer and human placenta, it might reflect that they show cell's invasion ability in some degree.

The objective of the present study was to examine the association between the OxRs' expression in cervical cancer and the degrees of differentiation as well as the invasiveness of cervical cancer, and to examine whether these receptors can serve as a biomarker for screening early stage of cervical cancer along with other indexes.

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Materials and Methods

Patients and Tissue Samples

To examine changes of the expression of Ox1R and Ox2R, a total number of 62 patients (average age: 47 years, range: 23-80 years) were enrolled in this study, of whom 47 were with cervical cancer (11 was low differentiation, 27 was intermediate differentiation and 9 was high differentiation) and 15 were with cervicitis which was set as the age-matched control group. This study was approved by the Institutional Review Board of Xinjiang Medical University and the First Peoples' Hospital of Kashgar, and the tissues were collected upon obtaining signed consent forms. All patients involved in this study were residents of the Xinjiang region and had not received any form of preoperative radiotherapy or chemotherapy prior to the time of sample collection. All samples were collected during clinical observations of these patients between 2002 and 2007 in the First Affiliated Hospital of Xinjiang Medical University (Urumqi, Xinjiang, China) and the First People's Hospital of Kashgar District (Kashgar, Xinjiang, China). All the tissues were detected positive for HPV16, which was the most common type of HPV to infect Uyghur women, by Polymerase Chain Reaction (PCR) as showed in Figure1.

To study the relationship between OxRs' expression and human placenta, we collected placenta tissues from a woman who gave birth in the First Affiliated Hospital of Xinjiang Medical University.

The use of clinical materials for this study was approved by the Medical Ethics Committee of Xinjiang Medical University (20160828-01). All the tissues were formalin-fixed and paraffin-embedded, and the diagnosis was histologically confirmed by experienced pathologists from the Xinjiang Medical University.

Reagents

The rabbit polyclonal antibodies used in this study were Anti-Orexin Receptor 1 antibody (ab68718, abcam, Shanghai, China) and Anti-Orexin Receptor 2 antibody (ab104701, abcam, Shanghai, China). The other major chemicals and kits were: QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), TaKaRa Ex Taq Polymerase (DRR001A, TaKaRa Biotechnology (Dalian) Co. Ltd, Dalian, China), dNTP mix (Life Technologies, Beijing, PR China), agarose gel (A600014-0050, Sangon Biotech, Shanghai, China), Permout Mounting Medium (Thermo Fisher Scientific, Waltham, MA, U.S.A.), citrate-buffered solution (MVS-0066, MAIXIN Bio, Fuzhou, China), phosphatebuffered saline (PBS-0060, MAIXIN Bio, Fuzhou, China), the endogenous peroxidases (SPKIT-A2, MAIXIN Bio, Fuzhou, China), DAB (Diaminobenzidine) Horseradish Peroxidase Color Development Kit (DAB-0031, MAIXIN Bio, Fuzhou, China), biotinylated goat anti-rabbit antibody (KIT-5004, MAIXIN Bio, Fuzhou, China), etc.

Detection of HPV Infection

Small pieces of tissue samples were cut from the paraffin-embedded sections. Then the DNA was isolated from the cervical tissue samples using the QIAamp DNA

Mini Kit following the instructions provided by the manufacturer. The quantities of the DNA samples were determined by the optical density at 260 nm and the quality of DNA samples was evaluated by amplification of a 110-bp fragment of the hemoglobin beta-chain(HBB) gene (Malczewska-Lenczowska et al., 2014). A Gp consensus primers which was specific for high-risk HPV16 were used to examine the samples isolated from the paraffin-embedded cervical tissues through PCR. The sequences of primers were:

- Forward 5'-GGTCGGTGGACCGGTCGATG-3'
- Reverse 5'-GCAATGTAGGTGTATCTCCA-3'

The main steps of PCR were as follow: the PCR reaction system's whole volume was 50 μ L, which contained DNA (500 ng/ml, 5 μ L), TaKaRa Ex Taq Polymerase (5 U/ μ L, 3 μ L), Mg²⁺ (5 μ L), dNTP Mixture (2.5 mmol/ml, 4 μ L), as well as 2 μ L of each primer (20pmol/ml), and then added DEPC to 50 μ L. The cycling conditions were denaturation at 95°C lasted for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds. Then primer was annealed at 60°C for 20 seconds, and extension at 72°C for 25 seconds, followed by a final extension at 72°C for 7 minutes (Biedermann et al., 2004). The cycling protocol for the Gp consensus primers (Gp5 and Gp6) was 40 cycles, which included denaturation at 94°C for 30 seconds, primer annealing at 45°C for 30 seconds, and extension at 72°C for 30 seconds (Husnjak et al., 2000). To analyze the PCR results, 5 μ L of each PCR reaction product was resolved by electrophoresis in 1.5% agarose gel, and the images were recorded through Gel Doc 2000 system (BioRad, Hercules, CA, U.S.A.). Each PCR experiment was replicated seven times.

Immunohistochemical Staining

Briefly, 4- μ m sections were dewaxed in xylol and alcohol. Antigenic epitopes were unmasked by microwave heating at 98.5°C for 15 minutes in 0.01 M citrate-buffered solution (pH 6.0), and cooled to room temperature in the same solution for 30 minutes. The sections were then rinsed in wash buffer (PBS with 0.05% polysorbate 20 surfactant) for 20 minutes. After that, the endogenous peroxidases were blocked by keeping the slides for 15 minutes in methanol with 3% H₂O₂. The sections then rinsed in PBS for 20 minutes again. For immunohistochemical detection of proteins, the sections were pre-incubated with 10% normal goat serum for 10 minutes and then incubated with the respective antibodies diluted in PBS with 1% goat serum to an optimal concentration based on pilot experiments. After an overnight incubation at 4°C, the sections were washed with wash buffer and then incubated with biotinylated goat anti-rabbit antibody for 15 minutes. After washing with wash buffer, the slides were incubated with DAB (Diaminobenzidine) Horseradish Peroxidase Color Development Kit for 5 minutes at room temperature. The sections were then counterstained with hematoxylin and mounted in Permout Mounting Medium.

Each experiment was replicated three times. The sections with known positive tissue sections were used as positive controls, and the sections stained with isotype-matched immunoglobulin molecules as well as cervicitis tissues disposed by this method with an excess of the

respective antigen were used as negative controls.

Evaluation of Immunohistochemistry

Ox1R and Ox2R are expressed in cervical cells' cytoplasm, and brown immunohistochemical staining in the cytoplasm under the microscope was defined as positive for the target protein. The images of all the stained tissues sections were taken using Nikon microscope (Nikon Corporation, Tokyo, Japan), and analysed by Image Pro Plus software Version 6.0, which is capable of taking information obtained by photograph or scanned documents and collecting intensity data from the area of interest. This method can offer good results with less operator dependency and systematically increasing efficiency, therefore has been used in many studies (Aleksandra et al., 2016).

Statistical Analysis

The statistical analysis was implemented using the SPSS software version 17.0 (Chicago, IL, U.S.A.). The data were first evaluated using the Homogeneity of variance test. The significant standard was set at $p > 0.05$. And then comparisons between groups were carried out with two-independent-samples test and Kruskal-Wallis test. The significant standard was set at $p < 0.05$.

Results

We used PCR to detect the HPV Infection of cervical tissues, all samples used in this study were proved to be infected with HPV16 as was showed in Figure A.

Figure B shows typical presentations of orexin1 and orexin2 antigens. The target proteins in the tissue sections of both cervical cancer and cervicitis are successfully visualized through the immunohistochemical staining method. The positive cells in the cervicitis and cervical cancer tissues were stained brown in cytoplasm, and the stained cells were either gathered at the epithelium, glands and surrounding areas or diffusely distributed. Deserve to be mentioned, compared to the epithelium and glands' surface, the basilar part was stained more strongly in both cervical cancer and cervicitis. Most cells were stained in the cytoplasm, but a few cells were also stained in the cell nuclei as well as intercellular space. In general, the cancer tissues were stained much stronger than the cervicitis.

To compare further in detail, the positive samples were graded into five levels based on the positive areas occupied by the target antigens at 400× magnification: less

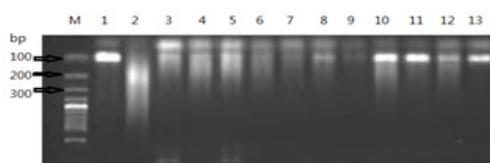


Figure A. PCR Amplification of HPV16 from the DNA Samples Extracted from the Cervical Cancer Lesions. The lanes from left to right: M: molecular marker, 1: positive control sample (96bp), 2: negative control sample (96bp), 3-13: examined tissue samples (96bp), and of them 8,10-13 were positive for HPV 16.

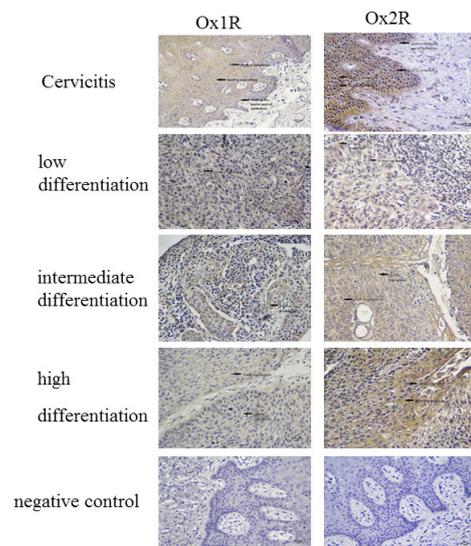


Figure B. Expression of Ox1R and Ox2R in the Cervical Tissues from Patients with Cervical Cancers of Different Differentiation Grades and Cervicitis (400× Magnification).

than 5% was regarded as negative(-); 5%–25%, as weak positive (+); 26%–50%, as positive (++); 51%–75%, as strong positive (+++); and more than 75%, as extreme strong positive (++++).

For Ox1R, because of sample loses during the staining process, results were obtained from 32 cervical cancer tissues and 15 cervicitis tissues. Our results demonstrated that, the positive expressions of Ox1R were increased in cervical cancer lesions than in cervicitis, but the differences were not statistically significant ($z = -0.36$, $p > 0.05$) (Table A).

For Ox2R, the results were obtained from 47 cervical cancer tissues and 14 cervicitis tissues (Table B). Regardless of differentiated degrees, positive expressions of Ox2R were apparently increased in cervical cancer sections (95.7%) compared with cervicitis sections (50.0%) as a whole ($z = -14.01$, $p < 0.05$) (Table A). The homogeneity of variance test showed $p < 0.05$, so we used the two-independent-samples test and Kruskal-Wallis test. The Kruskal-Wallis test showed $p < 0.01$, which meant there were meaningful distinctions among the different differentiation groups and the cervicitis group. As could

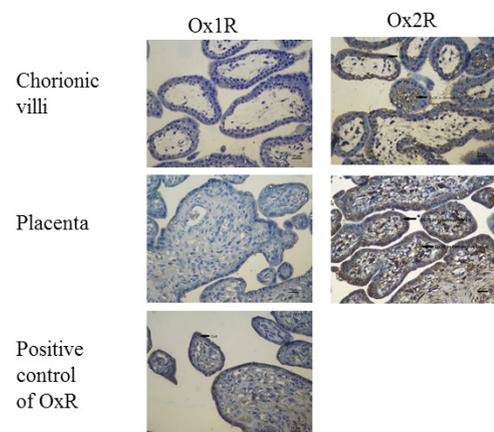


Figure C. Expression of Ox1R and Ox2R in Chorionic Villi and Placenta (400× Magnification)

Table A. The Comparison of Ox1R and Ox2R's Expression between Cervicitis and Cervical Cancer Sections (N, $\bar{x}\pm s$)

	Comparison	N	IOD($\bar{x}\pm s$)	z	P
Ox1R	Cervical cancer	32	26,871.2±48578.0	-0.36	0.72
	Cervicitis	15	19,947.3±19036.4		
Ox2R	Cervical cancer	47	5,437.2±4919.9	-14.01	<0.01
	Cervicitis	14	7,876.6±23978.1		

Table B. Expression of Ox2R in Cervicitis and Cervical Cancer Sections from Uighur Women

	Low differentiation	Intermediate differentiation	High differentiation	Cervicitis
-	1 (9.1%)	0	1 (11.1%)	7 (50.0%)
+	2 (18.2%)	4 (14.8%)	1 (11.1%)	1 (7.1%)
++	0	2 (7.4%)	2 (22.2%)	3 (21.4%)
+++	0	2 (7.4%)	2 (22.2%)	2 (14.4%)
++++	8 (72.7%)	19 (70.4%)	3 (33.4%)	1 (7.1%)
Total	11	27	9	14

see from Table B, most samples of the cervicitis group were negative (50.0%) while cervical cancer groups ranged from (+) to (++++): of all 47 samples, 7 showed (+) (14.9%), 4 showed (++) (8.5%), 4 showed (+++) (8.5%), 30 showed (++++) (63.8%). The most obvious differences were in the extreme strong positive (++++) group as could see from Table B: the low differentiation showed 72.7% for positive, the intermediate differentiation showed 70.4%, the high differentiation showed 33.4%, while cervicitis was 7.1%, which meant positive rate got higher as the differentiated degree was less.

As for the placenta tissue samples, the expressions of Ox1R and Ox2R were summarised in Figure C. After analyzing, we found that the expression of Ox1R in chorionic villi and placenta tissues were negative. While Ox2R was obviously positive in Chorionic villi and placenta tissues, especially strong in the cytoplasm of cytotrophoblast and chorion frondosum in placenta, as well as in the connective tissues in both tissues. (Due to it was difficult to get human placenta in China by law, our samples were all collected from one woman. So in this study, we just provided immunohistochemical stained pictures, more information should be taken from larger scale studies.)

Discussion

Although had been discovered for years, studies of orexins and orexin receptors were mainly focused on the central nervous system of the brain. Recently it has been shown that the orexins have a broader role in peripheral organs and in cancer (Dehan et al., 2013).

In this study, we used immunohistochemical method to figure out whether there was some specific relations between the expression of orexin receptors and the differentiated degrees of cervical cancer. Our results described here provided the first evidence that the expression rates of orexin receptors were associated with the differentiation grades of the cervical cancer.

In our study, we found that although Ox1R and Ox2R belong to the class I subfamily within the superfamily of GPCRs with seven transmembrane domains and

share 64% aminoacid identity in humans (Voisin et al., 2003), the two showed difference in their expression associated with cervical cancer. The positive expressions of Ox1R were increased in cervical cancer sections than in cervicitis, but the differences had no significance ($p>0.05$). In contrast, Ox2R was stained positively mostly in the epithelium and glands in both cervical cancer and cervicitis, and assembled in the basilar part where cell division was most active. Moreover, from the statistic analysis, we found that the Ox2R was significantly increased in its expression in cervical cancers compared to that in cervicitis. Half of the cervicitis samples (50%) were showed negative expression of Ox2R while cervical cancers were mostly positive. The major differences were in the extreme strong positive (++++) samples, which uncovered the trend that the positive expression rates were raised correspond with the stronger of cervical cancers' potential invasive capacities. Meanwhile, we also took carcinoma in situ in consideration, but its capability of invasiveness had not been completely showed yet for it hadn't destroyed the basement membrane, and the sample size was relatively small, so it was hard to find it in this stage, so we didn't include those in this study.

Invasion process occurs both in mammalian embryo implantation during development and malignant cancer cell metastasis (Cataldi et al., 2012). The invasion of endometrium by trophoblast cells is similar to the invasion and metastasis of malignant tumor, and uncontrolled invasion of trophoblast cells may caused the aggressive tumor, chorioepithelioma (Voisin et al., 2003). Previous studies showed that Orexins caused a caspase-dependent cell death by apoptosis and a drastic cell growth inhibition in Chinese hamster ovary cells transfected with Ox2R cDNA (Voisin et al., 2003). So we may regard Ox2R as a marker or indicator of cell's invasive capacity, since the lower cancer's degree of differentiation, the stronger Ox2R's inhibiting effect (KISHIDA et al., 2012). And Ox2R was considered to be a potential target for immunotoxin or antibody-drug conjugate (ADC) cancer therapy on Ox2R-positive cancer cells such as human colon cancer cells (KISHIDA et al., 2012; Laburthe et al., 2010), which showed it may play an important role

in cancer detect and treat in the future. To establish this, further studies with a large number of samples and more detailed approaches are needed in the future.

For screening of cervical cancer, the current method is Pap smear test (Mo et al., 2015). Because of the lack of professionals and relatively limited inspection technique, the results of this test sometimes were not so reliable, if we set Ox2R as a complementary examination test, we can not only raise the veracity of the result but also preliminarily judge the grades of the malignancy of cervical cancer although cervical biopsy remains the gold standard technique for diagnosis of cervical cancer.

Our study demonstrated that increased expression of Ox2R in cervical cancer lesions. The expression rate of Ox2R was related with cervical cancer's differentiated degrees and it may do some help as a new biomarker for detecting cervical cancer and its differentiation in some degree along with other indexes. The mechanisms underlying the increased expression of Ox2R and the association with cervical cancer need to be addressed in future studies.

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Conflict of interest disclosures

The authors declare that they have no financial conflicts of interest.

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