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From Cell Biology to Tissue Engineering

Interleukin 6 receptor (IL-6R) was an independent prognostic factor in cervical cancer

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Summary. IL-6 has been found to be associated with poor response to chemoradiotherapy and poor overall prognosis of patients with cervical cancer. However, little is known about the clinicopathological significance of IL-6 receptor (IL-6R) expression in the setting of cervical cancer. To investigate the clinicopathological meaning of IL-6R in cervical cancer, expression of IL-6R was detected using immunohistochemistry in cervical cancer tissue microarray composed of 98 cases of cervical cancer and paired normal controls. As further confirmation of expression trend, western-blotting was conducted in another independent 36 pairs of cervical cancer and matched normal controls. Subsequently, the statistical correlation between IL-6R expression and clinicopathological variables was analyzed, including demographic, TNM stage, clinical grading and overall prognosis. IL-6R expression was shown to be remarkably associated with lymph node metastasis, recurrence and overall prognosis. Moreover, only IL-6R expression was observed to be an independent prognostic factor among these variables that could potentially influence the overall prognosis of patients with cervical cancer. In conclusion, IL-6R was shown to be an independent prognostic factor for patients with cervical cancer.

Keywords: Cervical cancer, Cervical squamous cell carcinoma (CSCC), Cervical adenocarcinoma (CAD), IL-6R, Prognosis

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Introduction

Cervical cancer is the second most common cancer in women (Siegel et al., 2016); with nearly 400 000 new cases of the disease being diagnosed every year, of which approximately half will die (Filippova et al., 2014). When cervical cancer is diagnosed at early or pre-invasive stages, they are often curable with local treatments. However, what is unfortunate is that the large proportion of patients diagnosed with invasive cervical cancer will be subject to chemoresistance and suffer ensuing relapse following initial treatment (Bizzarri et al., 2016). Understanding the underlying reason leading to the chemoresistance in cervical cancer therefore remains a primary priority.

Numerous studies relevant to cancer research have reported that many factors were found to be implicated in the chemoresistance of cancer (Djeu and Wei, 2009; Zhao et al., 2012; Liu et al., 2014). Among which, Interleukin-6 (IL-6) was such a factor (Dijkgraaf et al., 2013; Jinno et al., 2015). IL-6/IL-6 receptor (IL-6R) signaling axis has been found to be heavily involved in the chemoresistance of cancer cells (Jinno et al., 2015). In addition, IL-6/IL-6R has also been found to strongly take part in the promotion of proliferation (Kanazawa et al., 2007), migration (Sun et al., 2014), angiogenesis (Wei et al., 2003), and evasion of tumor immunity (Coward and Kulbe, 2012) other than its engendering of chemoresistance (Bedewy and El-Maghraby, 2014; Jinno et al., 2015; Yadav et al., 2017). In the case of clinicopathological significance of IL-6/IL-6R axis in the setting of cervical cancer, extensive studies focused on IL-6. In contrast, few studies were available regarding IL-6R. Considering this, in our study, we have tried to investigate the clinico-pathological significance

of IL-6R expression in cervical cancer. IL-6R was shown to be pronouncedly up-regulated in cervical cancer tissues in comparison with paired normal control; and IL-6R was also displayed to be an independent prognostic factor for patients with cervical cancer.

Materials and methods

Clinical samples

The present study was approved by the Medical Ethics Committee of QingDao Municipal Hospital (QDMH-2016-010018). Tissue microarray used for immunostaining analysis of IL-6R was from Shanghai Outdo Biotech. Co. Ltd (Catalog Number: HUteA045PG01; OD-CT-RpUtr03-005, Shanghai, China). The tissue microarray consisted of 98 cases of cervical cancer and paired normal controls. Staging and grading were evaluated according to the World Health Organization (WHO) classification and grading system (2015 version). None of the patients received chemoradiotherapy before undergoing hysterectomy. Informed consent was obtained for each participant involved, as claimed by the Shanghai Outdo Biotech company. In addition, 36 cases of fresh cervical cancer tissues and matched normal controls were collected in the department of Gynecology, East Branch of QingDao Municipal Hospital, after obtaining the written informed consent from patients involved.

Immunohistochemistry

Immunostaining was carried out using heat-induced epitope retrieval, an avidin-biotin complex method. The rabbit polyclonal anti- IL-6R antibody (Catalog Number ab85105, Abcam, Cambridge, MA, USA) was diluted 1:150. The negative control when performing immunostaining was set up using non-specific mouse IgG antibody to replace the primary antibody of IL-6R. The sections were evaluated by light microscopic examination, and cellular localization and immunostaining level of IL-6R in each section was assessed by two separate pathologists who were blind to our study throughout. The immunostaining patterns were scored as follows: negative (<15% positive tumor cells), weak (>15% and <30% positive tumor cells), moderate (30-60% positive tumor cells) and strong (>60% positive tumor cells) according to the immunostaining intensity. Both moderate and strong positive expressions were classified into high expression, whereas negative and weak staining were categorized into low expression.

Western-blotting

Tissue proteins were extracted using the RIPA lysis buffer (Bioteke, Beijing, China). The concentration of protein was determined following the accompanying instructions of the Bradford Protein Assay kit (Bio-Rad, Hercules, CA, USA). 50 μg of soluble total protein were subjected to 10% SDS-PAGE separation. After transferring the samples to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore, Boston, MA, USA), 5% skim milk powder was applied for blocking and samples were incubated at 4°C overnight. After washing the membrane with Tris-Buffered saline with Tween 20, the protein samples were incubated with the polyclonal rabbit anti-human IL-6R antibody (dilution at 1:500, Catalog Number ab85105, Abcam, Cambridge, MA, USA) and monoclonal mouse anti-human β-actin antibody (dilution at 1:800, Catalog Number sc-47778; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) at 4°C overnight, followed by horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Catalog Number ZDR-5307; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) at 4°C overnight. Subsequently, the membranes were visualized by chemiluminescence with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, USA), and images were captured with a Bio-Rad camera system (Bio Rad, Hercules, CA, USA). β-actin was used as loading control.

Statistical analysis

Data were expressed as mean±Standard Error of Means (SEM). Mann-Whitney U-tests were used to evaluate differences between unpaired observations. Survival rates were calculated using the Kaplan-Meier method, and comparisons were made using the log rank test. After categorization, each clinical and pathologic variable was entered into the Cox proportional regression analysis to determine which parameter had an independent effect on postoperative survival. The correlations among IL-6R expression and various clinicopathological variables were analyzed by Chi-square test. Statistical analysis was carried out with SPSS statistics 17.0 (SPSS Inc., Chicago, IL) and histogram was plotted using Graphpad Prism software (Graphpad Inc., La Jolla, CA). A two-tailed $P<0.05$ was taken to be statistically significant.

Results

IL-6R was shown to be remarkably up-regulated in cervical cancer relative to paired normal control

To understand the expression level of IL-6R in cervical cancer and paired normal control, Immunohistochemistry (IHC) was used with cervical cancer tissue microarray consisting of 98 cases of cervical cancer tissues and paired normal controls. Of these cervical cancer tissues, totaling 98, 49 cases were CSCC and the remainder was cervical adenocarcinoma (CAD). Before undergoing immunohistochemical analysis of IL-6R expression, all the cases were first subjected to clinical pathological confirmation to make sure all case were eligible and qualified for our needs. Through hematoxylin-eosin (HE) staining, all the cases

enrolled were either CSCC or CAD (data not shown), totally meeting our needs. Based on this, immunohistochemistry of IL-6R was carried out subsequently. In terms of the sublocalization of IL-6R immunostaining, it was displayed to be mainly membranous and to be overwhelmingly up-regulated in cervical cancer tissues relative to paired normal controls (Fig. 2), whether in CSCC (Fig. 1) or in CAD (Fig. 3). The negative control when performing immunohistochemistry was the non-specific mouse IgG without any immunization. The negative control exhibited that the primary antibody to IL-6R was adequately specific in our analysis. To further confirm the trend of IL-6R expression, another independent small cohort consisted of 36 paired cases of fresh cervical cancer tissues consisting of 18 cases of CSCC and 18 cases of CAD and paired normal control tissues were employed to be subjected to western-blotting analysis. It was shown that IL-6R was significantly up-regulated in cervical cancer relative to paired normal control (Fig. 4). These data indicate that IL-6R was pronouncedly up-regulated in cervical cancer compared with paired normal control.

IL-6R was displayed to be significantly associated with lymph node metastasis, recurrence and overall prognosis

Having understood the IL-6R expression at cervical cancer tissue level, subsequently we analyzed the clinicopathological significance of IL-6R expression. Cross-table analysis method was used to analyze the correlation between IL-6R expression versus clinicopathological variables, including demographic, TNM staging, grading, lymph node metastasis, recurrence and overall prognosis. It was shown that there was significant correlation between IL-6R expression versus lymph node metastasis, and recurrence (Table 1). No significant correlation was observed between IL-6R expression and other variables. However, there was a trend towards statistical significance between IL-6R expression and differentiation degree, which may lead to the suggestion that IL-6R expression might be associated with cervical cancer differentiation with a larger sample size. To observe whether there was an association between IL-6R expression and overall prognosis, Kaplan-Meier survival curve was performed. Overall survival analysis showed that there was a statistically significant difference of overall prognosis between patients with high IL-6R expression versus patients with low expression (Fig. 5), suggesting that IL-6R was a prognosis-associated factor in cervical cancer. It should be noted that in our analysis of clinicopathological significance of IL-6R expression, in defining the expression status of IL-6R, we set the cut–off value of intensity between moderate and weak immunostaining. As long as the intensity of immunostaining was scored

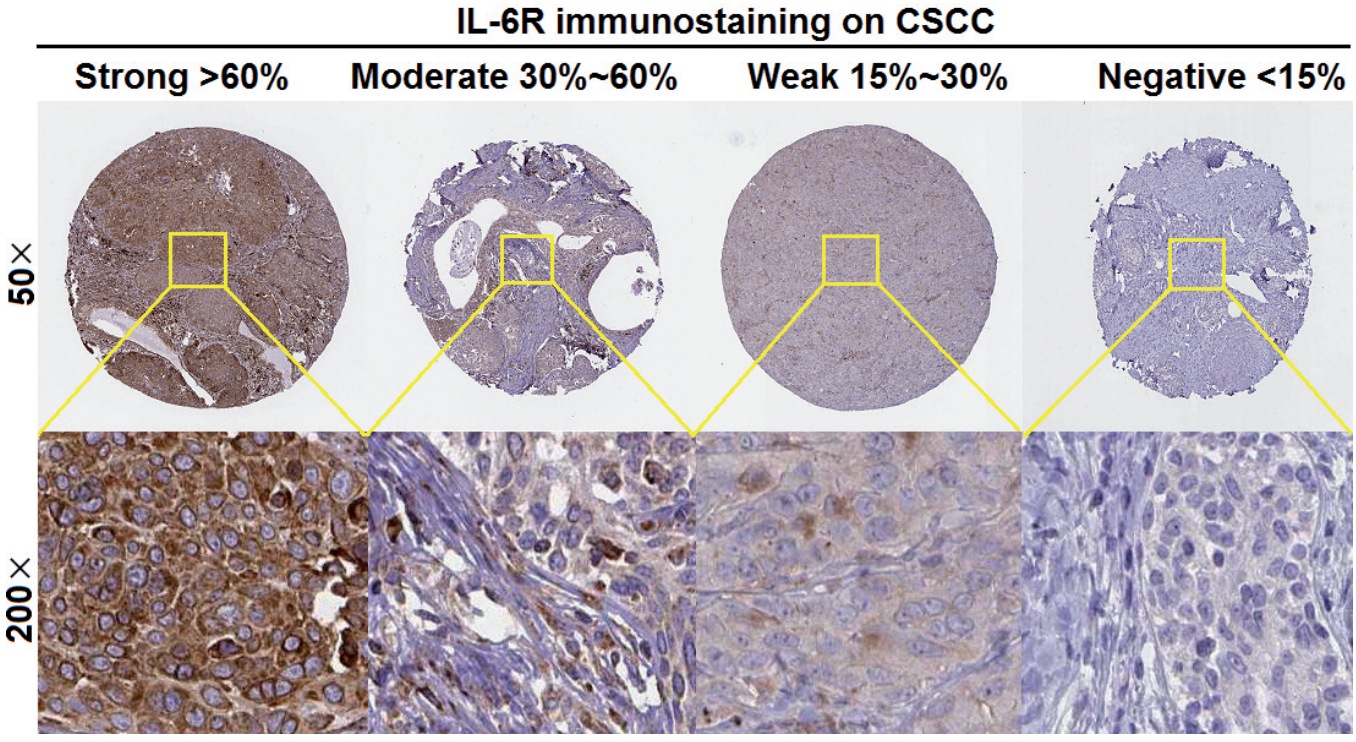

Fig. 1. Expression of IL-6R was detected on cervical squamous cell carcinoma (CSCC). Upper panel, x 50; Lower panel, x 200.

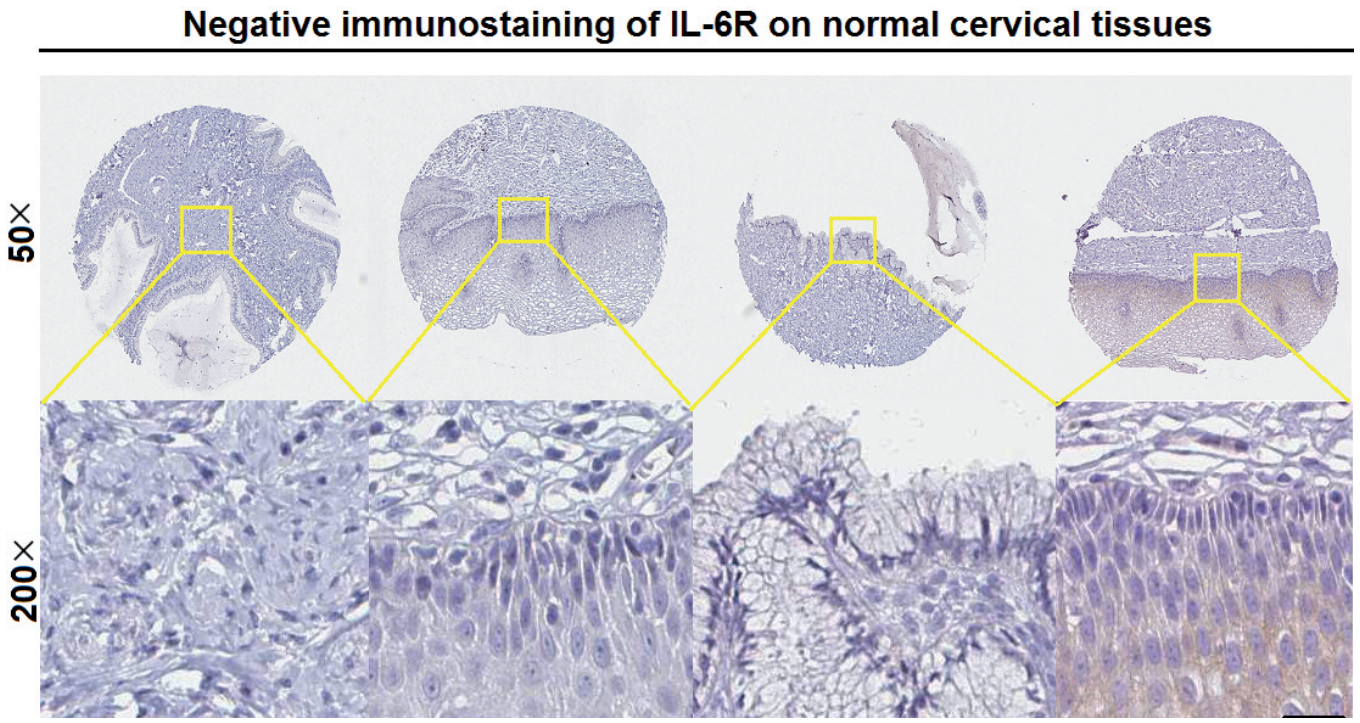

Fig. 2. Expression of IL-6R was detected on normal cervical tissues. Upper panel, x 50, Lower panel, x 200.

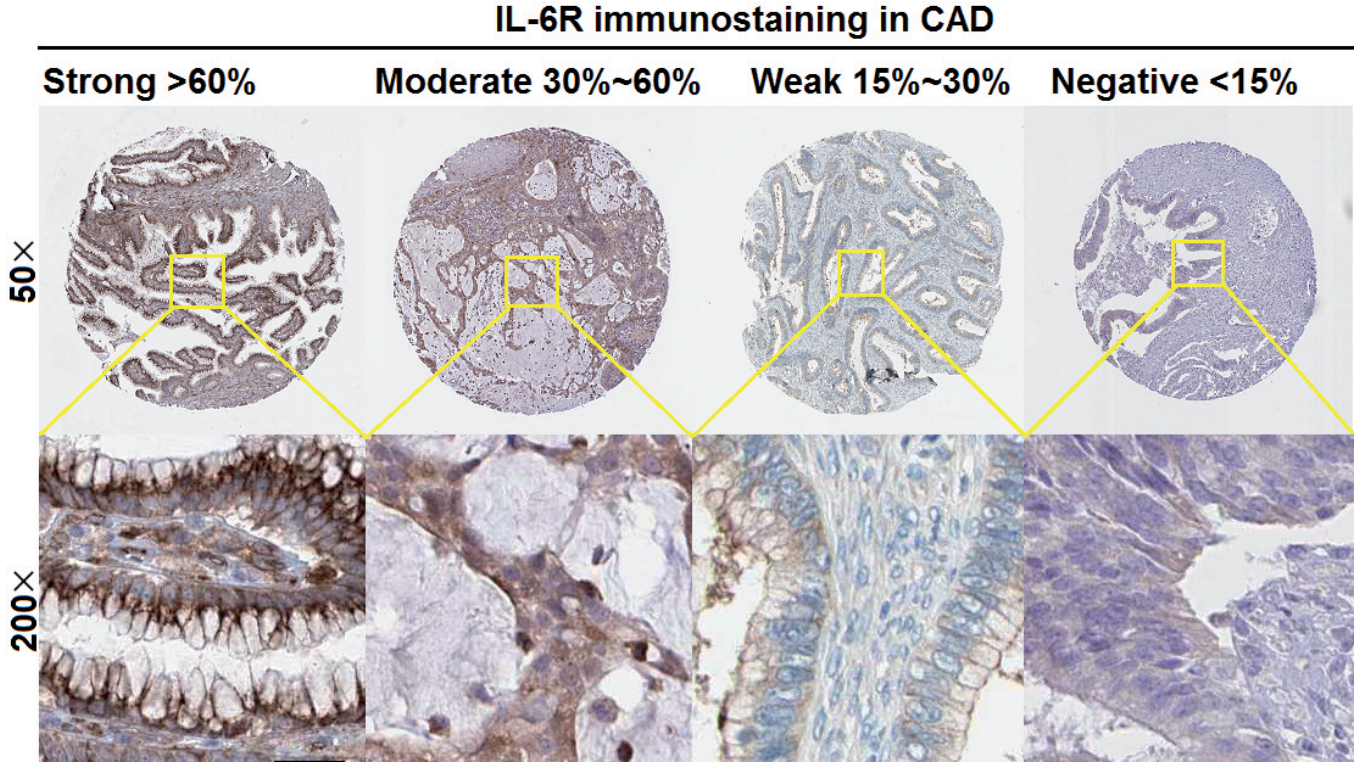

Fig. 3. Expression of IL-6R was detected on cervical adenocarcinoma (CAD). Upper panel, x 50, Lower panel, x 200.

Table 1. Clinicopathological significance of IL-6R expression in cervical cancer tissues.

Clinicopathological variables	Number of cases	IL-6R expression		χ^2	p value
		High	Low		
Cervical cancer	98	55	43	31.701	0.000
Paired normal control	98	17	81		
ECOG					
(0,1)	68	35	33	1.952	0.162
(2,3)	30	20	10		
Age (year)					
50	42	23	19	0.055	0.814
≥50	56	32	24		
T-stage					
I-II	44	26	18	0.286	0.593
III-IV	54	29	25		
Lymph node metastasis					
No	52	20	32	14.032	0.001
Yes	46	35	11		
Differentiation degree					
Weak-Moderate	47	31	16	3.548	0.064
Poor	51	24	27		
Tumor size					
≥6cm	62	42	20	12.477	0.000
6cm	36	13	23		
Treatment				0.345	0.557
RT	42	25	17		
CCRT	56	30	26		
Recurrence					
Yes	33	28	5	16.672	0.000
No	65	27	38		

ECOG is abbreviated from Eastern Cooperative Oncology Group; CCRT represents concurrent chemo radiation therapy; RT stands for radiotherapy.

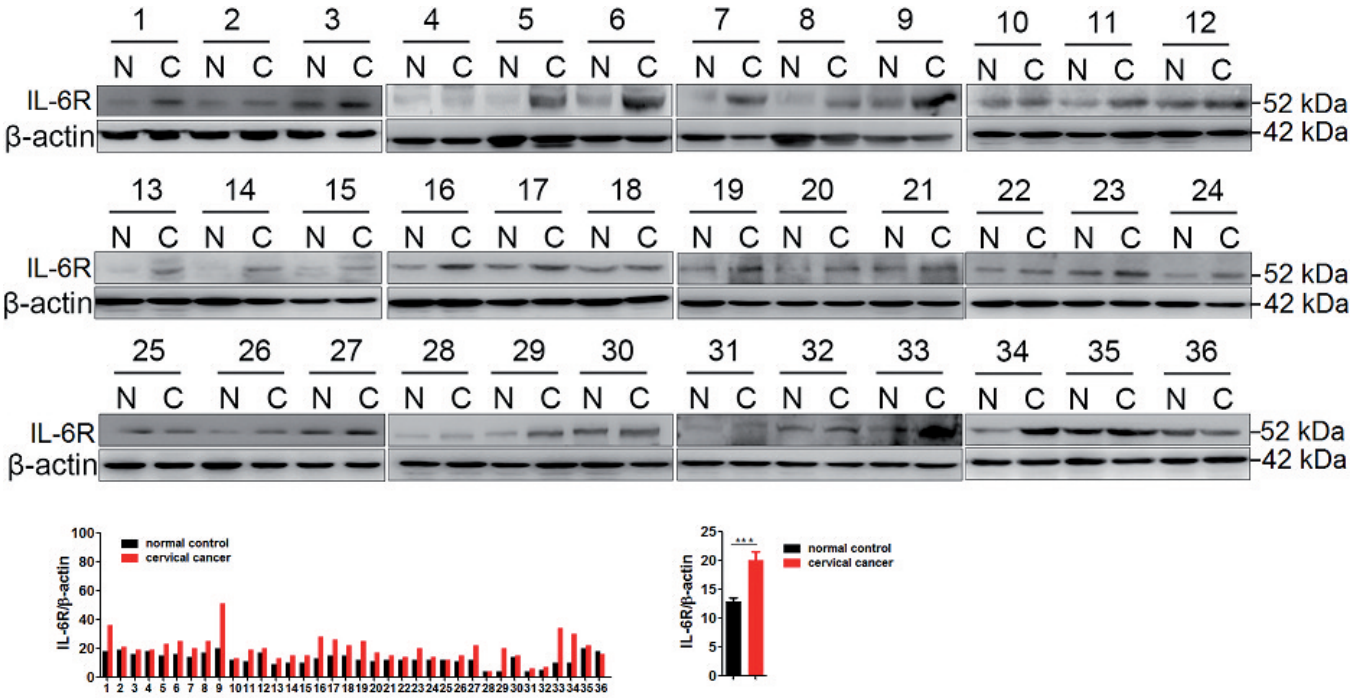

Fig. 4. Expression of IL-6R was detected using western-blotting on another independent cohort consists of 36 paired of fresh cervical cancer tissues and paired normal control. Mann-Whitney U test was used to analyze the statistical difference between the two groups, *** stands for p<0.001.

Table 2. Univariate and multivariate analysis of factors potentially influencing overall prognosis.

Clinicopathological parameters	Univariate analysis		Multivariate analysis	
	RR (95% CI)	p	RR (95% CI)	p
Age (n=98)	1.382 (0.551, 3.468)	0.491		
T-stage (n=98)	3.579 (1.446, 8.860)	0.006	2.169 (0.811, 5.800)	0.123
Lymph-node metastasis	1.934 (0.636, 5.880)	0.245		
Tumor stage	1.211 (0.426, 3.445)	0.72		
IL-6R Immunoscoring	5.089 (1.467, 17.657)	0.01	4.108 (1.135, 14.873)	0.031

RR is abbreviated from relative risk.

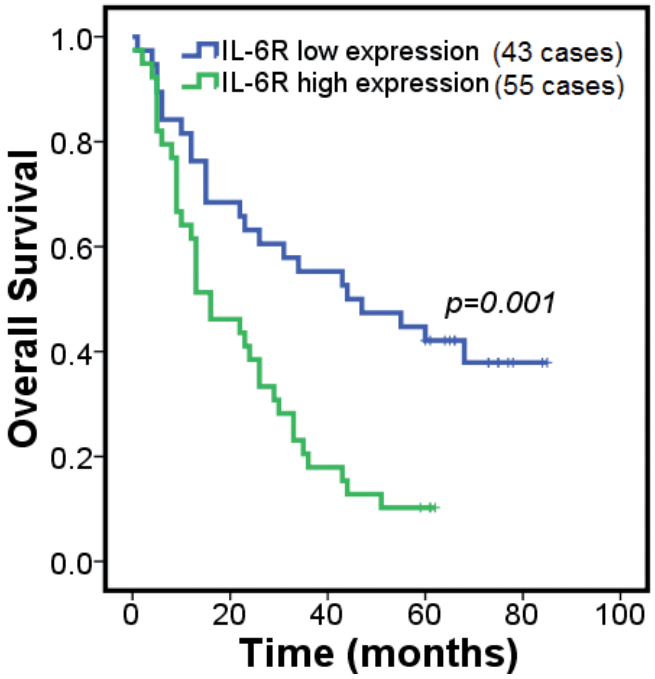

Fig. 5. Overall prognosis of IL-6R expression in patients with cervical cancer was analyzed using Kaplan-Meier survival curve. Log-Rank test was used to analyze the statistical difference between the two groups.

to be moderate or above, it will be defined as high expression of IL-6R; whereas those whose intensity of IL-6R immunnostaining was scored to be weak or negative were defined as low expression. The set of cut-off was also used in the analysis of prognosis.

IL-6R was shown to be an independent prognostic factor for patients with cervical cancer

To further evaluate the effect of IL-6R expression and clinicopathological variables (including demographic, clinical stage, TNM stage and lymph node metastasis) on prognosis, we performed both univariate and multivariate Cox regression analysis. Univariate Cox regression analysis showed IL-6R expression (p=0.01) and tumor stage (p=0.006) were prognostic factors for cervical cancer. By using multivariate analysis, we further examined prognostic parameters of cervical cancer that were shown to be significant in univariate analysis. It can be seen that IL-6R expression (p=0.031) was an independent prognostic factor influencing the 5-year overall survival, indicating that IL-6R expression can be used as an independent prognostic predictor for patients with cervical cancer (Table 2).

Discussion

Although large number of studies have evaluated the biological role and clinical relevance of IL-6, which has been found to be heavily involved in the promotion of metastasis and progression of various cancers, including cervical cancer (Kotowicz et al., 2016; Kyo et al., 2000; Tjiong et al., 1999), breast cancer (Ibrahim et al., 2016), prostate cancer (Wang et al., 2013; Merz et al., 2016), colorectal cancer (Yeh et al., 2010) and some other different types of malignancies. Unfortunately, few studies have focused upon and reported the IL-6 receptor (IL-6R), especially in regard to the clinicopathological significance of IL-6R expression, in cervical cancer. Therefore, the clinical significance of IL-6R has not been fully understood and deserves to be explored. This is the first report, to the best of our knowledge, in which IL-6R was shown to be pronouncedly over-expressed in cervical cancer tissues relative to paired normal control and that elevated IL-6R was observed to be markedly associated with lymph node metastasis, recurrence and overall prognosis. IL-6R was displayed to be an independent prognostic factor after further evaluation using multivariate COX regression analysis.

IL-6R, especially its soluble form, has been reported to be remarkably up-regulated in the serum (Kovacs, 2001; Yeh et al., 2010) or tissue (Okugawa et al., 2010) of patients with cancer, which may lead to the implication that IL-6R may have oncogenic properties in cancer. Nevertheless, there is a lack of direct evidence regarding the clinicopathological significance of IL-6R expression in cancer at clinical tissue level, let alone in cervical cancer with the exception of several mechanistic

studies mainly from cell culture (Moriasi et al., 2012; Ying et al., 2015) and mouse model (Matsumoto et al., 2010), reporting that IL-6R was shown to be heavily implicated in the chemoresistance of cancer. IL-6R has been claimed to be target in the therapy of cancer that was supported by a recent publication concerning clinical trials of patients with epithelial ovarian cancer (Dijkgraaf et al., 2015). In our own setting, IL-6R was displayed to be pronouncedly up-regulated in cervical cancer tissues relative to paired normal control using immunohisto-chemistry method on formalin-fixed paraffin-embedded tissue blocks. As further confirmation, the trend of IL-6R expression was verified using western-blotting in another independent small cohort of fresh clinical tissues consisting of 36 pairs of cervical cancer and matched normal control. The result obtained by western-blotting was totally congruent with what we obtained by immunohistochemistry that IL-6R was shown to be significantly up-regulated in cervical cancer tissues versus normal control. On the grounds of understanding of the expression level of IL-6R in CSCC, we tried to analyze the clinicopathological significance of IL-6R expression. It was found that there was significant correlation between IL-6R expression versus lymph nodes metastasis, recurrence and overall prognosis. The observation we made was partly in line with previously published report by Fu Q and colleagues in which both IL-6 and IL-6R were analyzed to be independent prognostic factors in patients with clear cell renal carcinoma (Fu et al., 2015). However, Okugawa et al. (2010) found using immunohistochemistry in colorectal cancer that expression of soluble IL-6R was significantly inversely correlated with IL-6 expression in the cytoplasm of cancer cells. As for the association between IL-6 and IL-6R in cervical cancer, it remains to be studied. In our setting, we failed to detect the expression level of IL-6 in CSCC. Moreover, the interpretation of our data should be approached with caution in that in our analysis the cut-off of intensity was set between moderate and weak immunostaining. That is, immunostaining intensity above weak was defined as high expression of IL-6R. While those whose intensity was observed to be weak or negative were defined as low expression.

There were several limitations that deserve to be noticed when interpreting our data. First of all, all the conclusions we have drawn were based on the limited sample size, therefore the conclusion may need to be further warranted with larger sample size; secondly, the clinical samples involved were retrospectively archived and retrieved, some important clinical information for instance of chemoresistance from patients involved, could be missing. Consequently, some key statistics cannot be analyzed; thirdly, the specificity of primary antibody to IL-6R we've employed was not subjected to pre-test, which could bias the final conclusion.

In summary, IL-6R was shown for the first time to be an independent prognostic factor for patients with cervical cancer.

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Conflicts of interest. The authors declare that they have no conflict of interest.

Authors' contribution to the study. Shaohong Luan, Data collection; Shaohong Luan, Manuscript writing; Zhijie An and Shuna Bi, Sample collection and data analysis; Long Chen, Reagent preparation; Jun Fan, Responsible for the whole paper.

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