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Research Article

Prognostic Impact of Coexpressed CXCL12 and CD44v5 on Human Cervical Cancer After Radiotherapy: Stem-Like Hallmark and Radioresistance

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Abstract

Objectives: The aim of this study is to investigate the prognostic impact of CXCL12 and CD44v5 on patients with advanced cervical cancer.

Methods: Paraffin specimens from 130 advanced cervix cancer before radiotherapy were examined using immunohistochemistry to test the expressions of CXCL12 and CD44v5. The correlations between the expressions of CXCL12 and CD44v5 and the five-year survival rate were analyzed. The expression changes of CXCL12 and CD44v5 in residual cancer tissues after a total radiotherapy dose of 50 Gy were tested by Real-Time PCR.

Results: In the 130 patients, a significant correlation was found between CXCL12 and CD44v5 (P=0.028). The coexpression occurred in 34 patients with lower five-year survival rate of 22.9%.There was no correlation between the expression of CXCL12 and CD44v5 and age, tumor stage, size, pelvic lymph node involvement and therapeutic schedule. Log-rank and multi-factor survivals analysis showed that tumor stage, lymph node involvement, CXCL12 expression, CD44v5 expression, CXCL12 and CD44v5 co-expression were independent prognostic factors. The expressions of CXCL12 and CD44 were significant elevated in residual tumor tissues, when compared to pre-radiotherapy, ($p<0.05$).

Conclusion: A significant positive correlation occurred between the expression of CXCL12 and CD44v5. The coexpression might be informative regarding poor prognosis in patients with radical radiotherapy.

Keywords: Cervical cancer, coexpression, CXCL12, CD44v5, prognosis, radiotherapy

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Cervical cancer, the second most common cancer in women, is a significant cause of death in China. As reports, the number of cervical cancer is estimated to have increased by 14% from 2000 to 2005 in our country.[1] As lack of earlier screening, the incidence of advanced cervical cancer in some rural area is higher than that in the urban area as a result of lacking earlier screening.[2] The standard therapy of advanced cervical cancer (stage IIb-IV) is a combination of external pelvic radiation and brachytherapy. But the traditional treatment could not always satisfactorily improve the prognosis with a poor 5-year survival rate.[3] In recent years, there are about 35% women diagnosed as recurrent cervical cancer. 90% of them are found within 3 years after the initial treatment.[4] Prior reports had indicated that therapeutic failure might be associated with radioresistance and radiation-induced metastasis.[5] Thus

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the improved radiosensitization strategies are necessary to reduce the mortality.

Recently, a pool of self-renewing malignant cells known as cancer stem-like cells was reported. These cells expressed specific stem markers and participated in numerous cellular functions, including carcinogenesis and proliferation, resulting in an enhanced risk of relapse and metastasis.[6] Various surface stem markers have been used to identify cancer stem-like cells. CD44v5, an isoform of CD44, has been regarded as a marker to screen cervical cancer stem cell.[7]

Chemokine superfamily, a vertebrate-specific group of small molecules, plays a key role in cancer. CXC chemokine ligand 12 and its receptor (CXCR4) regulate multiple tumorigenesis including breast, Pancreas, prostate.[8-10] The fundamental role of CXCL12–CXCR4 interactions in the metastatic cascade in breast cancer had led to an intensive research of vast malignant diseases.

In this study, using biopsy specimens of cervical squamous cell carcinoma treated with/without radiotherapy, we evaluated the predictive and prognostic values of CXCL12 and CD44v5. The possible association of the stem-like property and radioresistance is also investigated.

Methods

Patients and Treatment

From January 2005 to October 2007, 135 women with cervical squamous cell carcinoma were treated with radiotherapy at the Department of Radiotherapy, The 900th Hospital of the Joint Logistics Team. Patients without integrated follow-up were excluded. A total of 130 patients were included in this study. Approval by the Institutional Review Board of Fu Zhou General Hospital was obtained in advance, and the informed consent requirement was waived because the current study was performed by retrospective review. But the informed consent requirements of the other 5 patients with staged III in 2012 were obtained because the data of these patients were analyzed prospectively. None of the enrolled had underlying disease that would influence survival.

RT protocols were as follows: For patients with stage IIB, IIIA, IIIB, or IVA, whole pelvic radiotherapy delivering 30 Gy was followed by subsequent 20 Gy with a central shield. Intracavity irradiation delivered 30-36 Gy to Point A in 5-6 sessions. Concurrent chemoradiation was conducted by 2 cycles of platinum-based chemotherapy in all patients.

Tumor samples, obtained by punch biopsy for histologic diagnosis prior to RT, were fixed in 10% formalin, embedded in paraffin, and processed for routine diagnosis. Residual tumor tissues of 5 patients undergoing a total radiotherapy dose of 50 Gy were also obtained by punch biopsy. One part of these tumor tissues were used for RNA detection and the other part were processed as those pre-radiotherapy. Routinely processed formalin-fixed, paraffin embedded tissue blocks from all patients' specimens were cut into 5-m sections on lysine-coated glass slides (Muto Pure Chemicals, Tokyo, Japan). One section was stained by routine hematoxylin & eosin (HE) and duplicate serial sections were used for histochemical and immunohistochemical studies.

The follow-up interval is 3 months in the first and second year and 6 months in the third year and one year thereafter. Patients were checked for local recurrences at the outpatient clinic. Chest X-ray and abdominal ultrasonography were performed every 3 or 6 months for examination of lung and liver metastasis, respectively. Local recurrence was confirmed histologically and distant metastases were diagnosed either radiologically, histologically, or both.

Clinical, pathologic, and survival data were obtained from hospital records. Clinical staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO) staging system.

RNA Preparation and RT-PCR

Total RNA was extracted using RNeasy (QIAGEN, Venlo, Netherlands) according to the manufacturer's protocol. The sequences of the forward and reverse primers are as follows: CXCL12: (F)5'-GAAGTGGAGCCATAGTAATGCC -3'; (R)5'-TCCAAGTGGAAAAATACACCG - 3' (2)CD44: (F) 5'-TTGTGGCATTTATTCATCAG-3'; (R) 5'-AGAGGGTAGACAGGGAGG-3'. β-actin: (F)5'- CTG TAT GCC TCT GGT CGT AC-3',(R):5'- TGA TGT CAC GCA CGA TTT CC- 3' PCR was performed using TAKARA Ex Taq (TaKaRa) according to the manufacturer's protocol. PCR was performed as follows: 95 ˚C for 5 min; 40 cycles of 95 ˚C for 30 s, annealing temperature 56-58 ˚C for 90 s, and 72 ˚C for 60 s. The PCR productswere separated on a 2 % agarose gel, visualized with ethidium bromide staining, and photographed with FAS-III Series (NIPPON Genetics Co., Ltd., Tokyo, Japan).

Quantitative Real-Time RT-PCR

We used the MiniOpticon Real-Time PCR Detection System (Bio-Rad, Hercules, CA) for real-time PCR using iQTM SYBR Green Supermix (Bio-Rad). Relative quantification of PCR products was calculated after normalization to b-actin.

Histochemical and Immunohistochemical Analyses

The sections from tissue blocks were deparaffined in xylene, and rehydrated in a descending ethanol series, ending in water. They were then incubated for 30 min in 0.3% hydrogen peroxide in methanol. The serial sections were incubated with primary antibodies (CXCL12 and CD44v5) in a humid chamber at 4°C overnight. They were then rinsed in PBS, and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody.

Immunohistologic expression was assessed by two expert pathologists independently without knowledge of clinical outcome. The positive cell degree was expressed using a scale from 0-4: (-) represents 0%; (+) represents 1%-25%; (++) represents 26%-50%; (+++) represents 51%-75% and (++++) represents 76%-100%.

Statistical Analysis

SPSS 18.0 for windows were performed. Survival curves for patient groups were drawn using the Kaplan-Meier method. The difference between the survival curves was examined by means of the log-rank test. Logistic regression analysis was used to identify predictors of clinical radioresistance. The prognostic significance of individual parameters was calculated by Cox's regression analysis. Differences in proportions were evaluated by χ^2 test and Fisher's exact test. A value of $p<0.05$ was considered to indicate statistical significance.

Results

Patients' Characteristics

The characteristics of 130 patients were listed in Table 1. Patients' ages ranged from 35 to 78 with a mean of 53.7 years. The median follow-up time in surviving patients was 68 months.

Table 1. Patients' characteristics

Characteristics	n (%)
Age (year)	
<50	53 (40.8)
>50	77 (59.2)
Stage (FIGO)	
IIb	33 (25.4)
III	58 (44.6)
Iva	39 (30.0)
Tumor size	
<4cm	53 (40.8)
>4cm	77 (59.2)
lymphatic metastasis	
Yes	82 (63.1)
No	48 (36.9)
Tumor classification	
Exogenous	35 (26.9)
Endogenous	30 (23.1)
Cervical canal	30 (23.1)
Ulcerative	35 (26.9)
Adjuvant therapy	
None	59 (45.4)
Concurrent chemoradiation	71 (54.6)

The Expression of CXCL12 and CD44v5 and Its Association with Clinicopathologic Factors in 130 Patients Before Radiotherapy

Immunolocalization with anti-CXCL12 antibody largely showed positive staining in the cell membrane and cytoplasm of cancer cells, while CD44v5 protein mainly in cell membrane (Fig. 1). The CXCL12 and CD44v5 positive cell radio was 61.5% and 42.3%, respectively.

The correlation analysis showed that no association occurred between the single expression or coexpression of CXCL12 and CD44v5 in cervical cancer tissues of 130 patients and several clinicopathological factors including age, FIGO stage, tumor size, tumor type, lymph node metastasis and treatment program (Table 2).

The Correlation Between the of Expression CXCL12 and the Expression of CD44v5 (Table 3)

The Spearson correlation test showed that the positive correlation occurred between the expression of CXCL12 and the expression of CD44v5 in primary cervical tumor tissues ($r=0.038$, $p=0.028$).

Univariate and Multivariate Survival Analyses of the Patients

During the follow-up period, 62 patients died. The 5-year survival rate was 62/127 (52.3%). The univariate analysis revealed that age ($p=0.131$) and tumor size ($p=0.511$) were not related to survival in this cohort. A multivariate analysis, including the prognostic factors determined by the univariate analysis to have statistical significance, revealed a positive finding for FIGO stage ($p=0.017$), lymph node metastasis ($p=0.013$), treatment program ($p=0.014$), CXCL12 expression ($p=0.038$), CD44v5 expression ($p=0.039$), CXCL12 and CD44v5 coexpression ($p=0.000$) to be independent significant prognostic factors (Table 4). The mobility risk of patients underwent radiotherapy is 2.06 times that of patients underwent concurrent radiochemotherapy ($p=0.020$). The death risk radio of patients with positive CXCL12 expression to negative expression is 3.07. There was a significant difference between the groups ($p=0.035$,

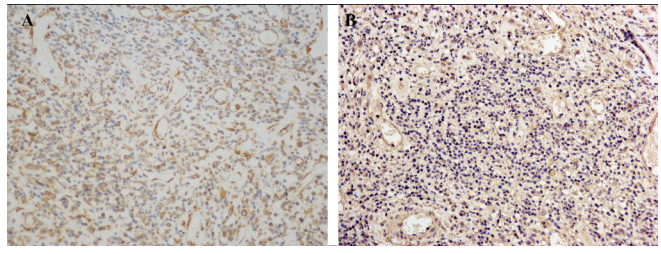

Figure 1. Immunohistochemical results of CXCL12 and CD44v5 expression.

Table 2. Correlation between CXCL12 and CD44v5 expression and clinicopathological factors in cervical cancer of 127 patients

	CXCL12				CD44v5				CXCL12 and CD44v5			
	Positive	Negative	χ^2	P	Positive	Negative	χ^2	P	Positive	Others	χ^2	P
Age (years)												
<50	27	21			29	19			15	33		
>50	53	29	0.899	0.343	36	46	3.303	0.069	18	54	3.442	0.064
FIGO stage												
II	22	14			15	21			8	28		
III	31	24			29	26			12	43		
IV	27	12	1.600	0.449	21	18	1.394	0.498	13	26	0.610	0.737
Lymph node												
Positive	30	18			30	18			16	32		
Negative	49	33	0.096	0.757	36	46	3.091	0.081	18	64	0.073	0.787
Tumor size												
<4 cm	34	19			23	30			12	41		
>4 cm	45	32	0.429	0.512	43	34	1.946	0.163	22	55	0.006	0.938
Treatment												
Radiotherapy	34	25			30	29			14	45		
CCRT	45	26	0.447	0.504	35	36	0.031	0.860	19	52	0.375	0.540

CCRT: Concurrent chemoradiation.

Table 3. Correlation between CXCL12 and CD44v5 expression in primary cervical cancer

	CD44v5			
	Positive	Negative	χ^2	p
CXCL12				
Positive	34	45		
Negative	32	19	4.816	0.028

Fig 2a). The estimated 5-year survival rate was 43.2% for patients with negative CD44v5 expression, 35.5% for those with positive CD44v5 expression. There was a significant difference between the groups (p=0.039, Fig. 2b). Among the 130 patients, there were 34 women with positive CXCL12 and CD44v5 coexpression. The 5-year survival rate was only 22.9%, the other was 45.5% (p=0.005, Fig. 2c). The death risk radio of patients with positive CXCL12 and CD44v5 coexpression to negative expression is 6.639. Kaplan-Meier survival curve showed that CXCL12 and CD44v5 coexpression revealed significantly poor prognosis.

Expression of CXCL12 and CD44v5 in Tumor Tissues Before Radiotherapy and Residual Tumor Tissues After a Radiotherapy Dose of 50 Gy

RNA was extracted from residual tumor tissues of 5 patients with stage IIIB cervical cancer before radiotherapy and after a radiotherapy dose of 50 Gy. The expression of CXCL12 and CD44v5 was detected. As shown in Figure 3, the increasing mRNA expression of CXCL12 and CD44v5 occurred in residual tissues, with the radio of 35.3 and 3.53, respectively.

Discussion

In the world, there is about half a million new cases of cervical carcinoma every year. The incidence in resource-poor countries is higher than that in developed countries. Radiotherapy or concurrent chemoradiotheray is a valid management for patients with advanced stage cervical cancer.[11] But there was still 50–70 % recurrence rate for patients with locally advanced stage.[12] Until now, the mechanisms of radioresistance are still unclear and remain challenges for the further improvement of oncologic outcomes.

In recent years, there are some reports showing the occurrence of stem-like cells in cancers. These cells are described as cancer stem cells with the capability of self-renewing and differentiation. This hypothesis provides the explanation for the origins of tumor heterogeneity. Since the discovery of human hematopoietic cancer stem cell, some solid tumor putative stem cells had been identified.[13-19] Until now, only few studies had focus on cervical cancer stem cells that have the specific stemness markers including p63, CK17, Nanog, Musashi-1, Nucleostemin, ALDH1, CD44, CD49f, Oct-4, and CD133. Similar to other cancer stem cells, cervical cancer stem cells play an important role

Table 4. Univariate and multivariate Cox regression analysis of prognostic factors

Clinicopathological characteristics	n (n=130)	5-year survival rate	Kaplan-Meier analysis		Cox regression model analysis	
			χ^2	p	χ^2	p
Age(years)						
<40	48	31.6				
≥40	82	44.0	2.284	0.131	0.147	0.702
FIGO stage						
II b	36	53.5				
III	55	40.6				
IV a	39	25.2	8.108	0.017	6.272	0.012
Lymph node						
Positive	48	26.5				
Negative	82	46.5	6.118	0.013	4.392	0.025
Tumor size						
<4cm	53	41.2				
≥4cm	77	39.3	0.432	0.511	0.228	0.633
Treatment						
Radiotherapy	59	28.4				
CCRT	71	48.1	5.983	0.014	5.423	0.020
CXCL12 expression						
Positive	79	30.0				
Negative	51	52.7	4.305	0.038	4.451	0.035
CD44v5						
Positive	66	35.5				
Negative	64	43.2	4.251	0.039	4.259	0.039
CXCL12 and CD44v5						
Positive	34	22.9				
Negative	96	45.5	16.291	0.000	8.048	0.005

CCRT: Concurrent chemoradiation.

in cancer treatment for the resistance of conventional chemotherapy, radiotherapy or radiochemotherapy. Kumazawa et al.[20] isolated cancer stem-like cells from Hela cells and found that these cells were associated with resistance to radiation-induced apoptosis. The stronger resistance to radiotherapy is relevant to better initiation of DNA damage response. Ghanbarnasab et al.[21] showed that cells with radioresistance were in the G0 and G1 cell cycle phases, while cells with radiosensitivity were in G2 and M phases. Unlike differentiated tumor cells, most cancer stem cells are in the

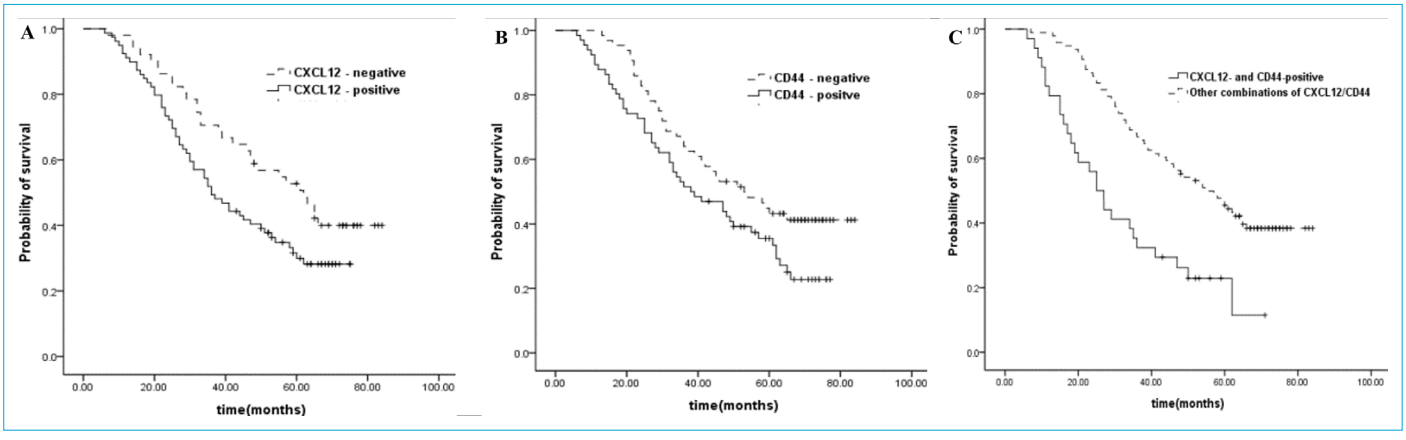

Figure 2. Kaplan-Meier survival analysis. CXCL12 and CD44v5 coexpression revealed significantly poor prognosis.

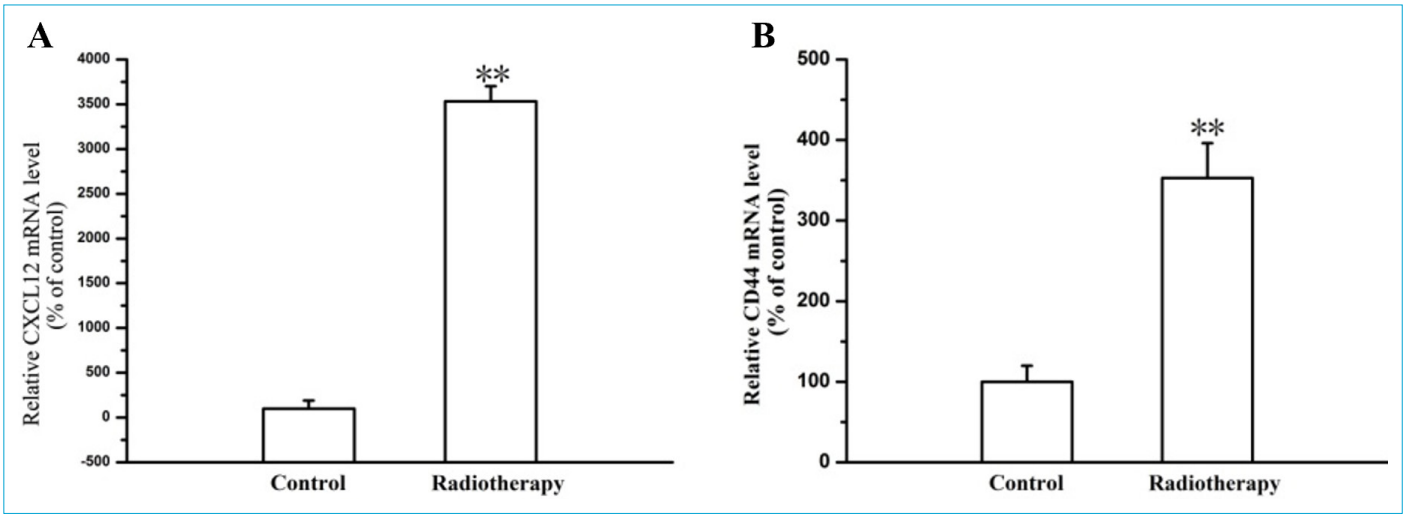

Figure 3. Expression of CXCL12 and CD44v5 in Tumor Tissues of the control group and the radiotherapy group.

G0/G1 phases. On the other hand, epithelial-mesenchymal transition (EMT) is a process observed during embryonic development. In the period, cells lose epithelial characteristics including the downregulation of adhesion molecules to increase the ability of motility and invasion. Radiotherapy could also elevate the expression of transforming growth factor-β(TGF-β), an inducer of EMT, with the result of development of treatment resistance. Epithelial cancer cells would gain stemness, motility, invasiveness and metastatic ability when they underwent EMT.

CD44, first described by Gallatin et al.,[22] played important role in the progression of many tumor types. There are two types of CD44: CD44s and CD44v. CD44s is a standard isoform, while CD44v is a variant isoform. Until now, the specific functions of CD44v remain unclear. The malignant cells often express unique patterns of CD44 isoforms. CD44v5 was used to screen the cervical cancer stem cells as a stem cell marker in 2009 by Feng et al. In this study, we found the rate of CD44v5-positive cells increased significantly in residual tumor tissues after a radiation dose of 50 Gy. The possible reason might be that most of the differentiated tumor cells radiosensitive to X-ray died while cancer stem cells radioresistant to X-ray survived. Another reason might be the radiation promoted the acquisition of CD44+ stem-cell phenotype and induced the cancer cell radioresistance.

Solid tumors are regarded as "organs" comprised of cancer cells and tumor microenvironment including the extracellular matrix (ECM), mesenchymal stem cells (MSCs), cytokines and growth factors. Similar to stem cells, cancer stem cells also require a special microenvironment, namely "CSC niche", to regulate their stemness and proliferation and save CSCs from deletion. Hypoxia is an essential feature of the tumor microenvironment in solid tumors and can enhance the phenotypes of CSCs.[23] Most evidences propose that CSCs exist in hypoxic microenvironment. There are reports that hypoxia could provide a niche for quiescent, drug resistant cells, which might be identical to CSCs. In our study, the increasing expression of CD44+ stem-cell in residual tissues might be the results of hypoxia-induced radioprotection.

CXCL12, a member of superfamily of small pro-inflammatory chemoattractant cytokines, was first cloned from a bone marrow-derived stromal cell line.[24] CXCL12 plays an important role in the homing of hematopoietic stem cells to the bone marrow, and also could mediate the survival and proliferation of human progenitor cells. The secretion of CXCL12 within or around injured tissues is an important event that may create a microenvironment facilitating the homing of circulating endothelial tissue-committed stem cells. Interestingly, DNA-damaging agents such as irradiation or chemotherapeutics could increase CXCL12 expression. The CXCL12 promoter contains two HIF-1α binding sites, thus the increasing expression of HIF-1α expression in either hypoxic or damaged tissues results in the elevation of CXCL12 levels.[25] We found the expression of CXCL12 increased significantly in residual tumor tissues after an irradiation dose of 50 Gy.

According to the above results, we thought that the elevated expression of CXCL12 and the survival of CD44v5-positive stem cells might be the poor prognosis factor for cervical cancer. Thus we test the association of the 5-survival rate and the expression of CXCL12 and CD44v5 in 130 patients with locally advanced cervical cancer before radiotherapy. The results showed that the coexpression of CXCL12 and CD44v5 occurred in 34 patients with a lower 5-survival rate of 22.9%. While the CXCL12 or CD44v5 alone expression was also associated with the lower survival rate.

Similar to other reports, we also found that there was no

correlation between the age, tumor size, tumor classification and the overall survival rate in patients with locally advanced cervical squamous carcinoma. But therapeutic results of concurrent chemoradiotherapy were significantly more effective than those of radiotherapy alone. We also found the correlation between the expression of CXCL12 and CD44v5. We thought that CXCL12 might be a protective factor for CD44v5-positive cancer stem cells and play an important role in radioresistance.

Conclusion

As we know, this might be the first analysis of CXCL12 and CD44v5-positive in prognosis of advanced cervical cancer by studying the clinical characteristics. In this study, we found that the expression of CXCL12 and CD44v5 were the poor prognosis factor for cervical cancer. But there are some limits in our study: what is the receptor of CXCL12? What is the mechanism? What is the regulation mechanism of CXCL12 and CD44v5? How the research results used for medical treatment? All of these questions are our work in next.

Disclosures

Ethics Committee Approval: First: Hospital: Fu Zhou General Hospital ; Date:2012/12/06, Number: 2012126. As the Hospital name were changed on request, all evidence of documentations were changed accordingly. Hospital Name: The 900th Hospital of the Joint Logistics Team. Date: 2020/03/18. Number: 202318.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – F.W., Z.F.; Design – F.W., Z.F.; Supervision – F.W., Z.F.; Materials – L.G.; Data collection &/or processing – N.Z.; Analysis and/or interpretation – N.Z.; Literature search – L.G.; Writing – F.W.; Critical review – F.W., Z.F.

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