Developing a Nomogram for Preoperative Prediction of Cervical Cancer Lymph Node Metastasis by Multiplex Immunofluorescence

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Abstract

Objective: We aimed to explore the potential mechanism of pelvic lymph node (pLN) metastasis of cervical cancer (CC) by multiplex immunofluorescence (mIF) and construct a nomogram for preoperative prediction of pLN metastasis in patients with CC. Methods: A series of 90 patients with CC (2009 FIGO, IB1-IIA2) was retrospectively assayed with metastatic pLN or not. Tissue microarray (TMA) were prepared and tumor-infiltrating immune markers were assessed by mIF. Pearson correlation coefficients (R), linear regression and spatial proximity analysis were applied to study the potential mechanism of these markers. Multivariable logistic regression analysis and nomogram were used to develop the predicting model. Results: We concluded that T cells may interact with NK cells and macrophages through PD-1 and PD-L1 to promote pLN metastasis of CC. Multivariable logistic regression analysis and nomogram construct a predictive model and area under curve (AUC) can reach 0.843. By internally validation with the remaining 40 percent of cases, a new ROC curve was emerged and the AUC reaching 0.888. Conclusions: This study presents an immune nomogram, which can be conveniently used to facilitate the preoperative individualized prediction of LN metastasis in patients with CC. Keywords: cervical cancer, multiplex immunofluorescence, nomogram, internally validation

Introduction

Cervical cancer (CC) is one of the leading malignant tumors in United States [1,2]. Lymph node (LN) metastasis have been considered as a vital factor in the development of CC [3-6]. Patients with this stage IB1-IIA2 CC (2009 International Federation of Gynecology and Obstetrics (FIGO) stage) will undergo pelvic LN (pLN) resection [7] and cause a variety of complications, including bleeding, nerve injury, lower pelvic lymphocele and extremity lymphedema [8,9] [10,11]. Thus, it will be particularly important to further study the mechanism of CC pLN metastasis. Furthermore, preoperative assessment of pLN status in CC patients is therefore critical for clinical decision making.

Increasing evidence has demonstrated that pLN metastasis is a complex process involving tumor immune milieu [12-15]. However, molecular mechanisms behind metastasis processes remain obscure. Traditional procedures have been used to elucidate the mechanism by regularly exploring molecular pathways [14,16,17]. However, due to the tissue-destructive nature of most of these methods, the spatial distribution and temporal distribution of immune milieu in situ will not be preserved [18]. Although morphological examination including conventional immunohistochemistry (IHC) or immunofluorescence (IF) can reveal some information, its effect is extremity limited because of high inter-observer variability and the capacity to label only one marker per tissue section [19].

Multiplex immunohistochemistry / immunofluorescence (m-IHC/IF) has emerged and provides high-throughput multiplex staining and further standardized quantitative analysis for highly efficient, reproducible and cost-effective tissue studies [19-21]. It can show up to seven targets simultaneously on a single

slide. Afterwards, HALO (Indica Labs, Albuquerque, USA), an image analysis system not only can be used for quantitative tissue analysis, but also can reveal the spatial location of each target [22-26].

In the current study, we sought to comprehensively compare the quantification of immune markers and their spatial orientation interrelation between CC in situ tissue with positive pLN and negative pLN. Based on differential expression of immune-related indicators, a clinical prediction model was constructed for individual preoperative prediction of pLN metastasis and an internal validation was assessed. In the future, we can predict pLN metastasis by cervical biopsy, thus avoiding its dissection and improving patients' quality of life.

Methods

Microarray Dataset Collection and Data Process

To systematically analyze the differential immune makeup between cervical cancer patients with positive LN and negative LN, the cancer genome atlas (TCGA), a comprehensively database for inferring the relative abundance of diverse cell infiltrates was conducted.

Patient Cohort

All procedures were ethically approved by the institutional Ethics Review Committee of Fudan University Shanghai Cancer center (FDCC). Appropriate written informed consent was obtained from all patients prior to sample collection.

A retrospective cohort study was conducted in the Department of Gynecology Oncology, FDCC, which included 180 patients with the 2009 FIGO stage IB1-IIA2 who underwent radical abdominal hysterectomy with or without bilateral salpingooophorectomy and pelvic ± para-aortic lymphadenectomy from 2009 to 2012. All the enrolled patients had undergone standard pelvic lymphadenectomy by experienced gynecological oncologist. All the microscopic slides were reviewed by the same professional gynecologic pathologist and were reconfirmed by another experienced gynecologic pathologist. A total of patients (90 with positive pLN and 90 with negative pLN) were assayed and their tissue specimens and clinical records were retrospectively studied.

Making Tissue Microarray (TMA)

The 180 CC tissues (in situ) with pLN positive metastasis or negative metastasis were prepared into TMA as previously described [27,28].

M-IF Staining Protocol

Opal 7-colour kit (NEL811001KT, PerkinElmer) was used for mIF. TMAs were dewaxed and rehydrated. In the first step, antigen was retrieved at 125 for 3 min and then cooled to room temperature (RT). Washed with TBST three times for 5min, incubated in $H_2O_2$for 10 min. Repeated washed and blocked with blocking buffer. Primary antibody, PDL-1 (ab237726, abcam, 1:500, dye 480) was incubated at RT for 30min. Slides were washed and an HRP-conjugated secondary antibody was incubated at RT for 10min. TSA dye (1:100) was applied for 10min after washes. The procedures were repeated six times using the following antibodies, CD3 (ab16669, abcam, 1:200, dye 690; used as T lymphocyte cell marker [29]), CD8 (ab93278, abcam, 1:100, dye 570; used as cytotoxic T cell marker [30]), CD56 (ab75813, abcam, 1:500, dye 620; used as NK cell marker [31]), CD68 (ab213363, 1:1000, abcam, dye 780; used as pan-macrophage marker [32]), programmed death-1 (PD-1) (ab237728, abcam, 1:300, dye 520), programmed death ligand-1 (PD-L1) (ab237726, 1:500, dye 480) [33]. Secondary antibodies anti-mouse (NEF822001EA, PerkinElmer) or anti-rabbit (NEF812001EA, PekinElmer) were used at a 1:1000 dilution.

Constructing a DiagnosticPrediction Model

By comparing the difference of quantification and spatial distribution of immune markers, we can construct a diagnostic prediction model by randomly selected sixty percent of the cases [34,35].

Validation of the Prediction Nomogram

Internal validation was performed using the rest 40 percentage data set.

Statistical Analysis

Statistical analysis was performed with Graphpad Prism 8 and R language. Unsupervised hierarchical clustering was conducted to define the immune subtypes by different markers. Person correlation analysis was used to test the associations between different markers. Comparisons between two conditions were based on two-sided Student's test. P value of ¡0.05 were judged to be statistically significant.

Results

TCGA of Cervical Squamous Cell Carcinoma (CESC)

Our TCGA data have systematically analyzed the differential gene makeup in the two groups. The expression profiles of fragments per kilobase of transcript per million fragments mapped (FPKM) based on TCGA Cervical Squamous Cell Carcinoma (CESC) was used to analyze the expression differences of non-metastasis and metastasis in CC patients. The differential screening criteria is set at $|\log2(FC)|>1$ and $p<0.05$, forty-two genes upregulated and sixty-seven genes downregulated (Figure 1A and Figure 1B) (Table S1). Further data mining revealed that LN metastasis of CESC may be related to immune infiltration (Figure 1C) (Table S2).

Patient Characteristics

The study cohort contained 180 cases of CC with high quality TMA. The median age of the patients was 46 years (range, 23-71 years). The histological diagnosis was all squamous cell carcinoma and based on the FIGO 2009 guidelines, nineteen (21.1%) subjects were stage IB1, twenty (22.2%) subjects were stage IB2, twenty-nine (32.3%) subjects were stage IIA1, and twenty-two (24.4%) subjects were stage IIA2. The median follow-up time was 61.05 months (range, 7.93-78.50 months) while thirteen (14.4%) subjects relapsed and seven (7.8%) subjects died.

Immune Infiltrates in CC

By mIF and HALO system was further applied to scan the relative ratio of each target on each tissue on the TMA [36,37]. The results can be graphically presented in heatmaps and its color can be digitized (Figure 2).

In order to better understand the complex immune characteristics in CC, we quantified the immune stains by HALO system. These six immunologic markers revealed distinct positive staining ratios in 180 samples. We observed CD8 marker (19.87% +- 3.47% vs 29.65% +- 3.31%;P ¡0.05), CD68+ macrophages (1.23% +- 0.38% vs 0.35% +- 0.94%;P ¡0.05), PD-1 (39.23% +- 3.37% vs 19.06% +- 2.43%;P ¡0.0001), PD-L1 (51.41% +- 3.47% vs 11.62% +- 1.67%;P ¡0.0001) in CC with positive pLN and negative pLN respectively (Figure 3).

In a word, our results demonstrated that the positive proportion of CD68, PD-1 and PD-L1 immune cell infiltration were significantly up-regulated with pLN metastasis, while CD8 was significantly decreased, CD3 and CD56 did not changed.

Correlation of Immune Infiltrates in CC

We further assessed the interrelationships of immune stains by analyzing the correlation of pairwise markers. By unsupervised hierarchical clustering, Pearson correlation coefficients (R) was showed in the two groups heatmaps which respectively revealed a dominant array of co-regulated markers, including CD3, CD8, CD56, CD68, PD1 and PD-L1 (Figure 4AC).

With pLN metastasis, the correlation were enhanced between CD3 and CD8 (R =0.51, P ¡0.0001), CD3 and CD68 (R =0.38,P ¡0.001), CD8 and CD56 (R =0.92, P ¡0.001), CD8 and CD68 (R =0.47, P ¡0.001), CD8 and PD-1 (R =0.42,P ¡0.001), CD68 and PD-1 (R =0.35, P ¡0.001) (Figure 4BD).

Distinct Spatial Distribution of Immune Infiltration

The HALO can not only quantify the number of immune cells on a panel, but also locate their position, measure their spatial distance [20,38,39]. By this way, we can further find the relative number and location of immune cells in situ tissue of CC after pLN metastasis more visually and thereby draw the relationship of immune cells which would promote LN metastasis.

The average distance (um) of CD8 to CD56 was 8.33 +- 1.26% in the pLN-negative group, 3.86 +- 1.08% in the pLN-positive group (pLN-negative versus pLN-positive, $P < 0.05$) (Figure 5A). The average distance (um) of CD8 to CD68 was 75.08 +- 21.91% in the pLN-negative group, 5.56 +- 1.62% in the pLN-positive group (pLN-negative versus pLN-positive, $P < 0.01$) (Figure 5B). The average distance (um) of CD8 to PD-1 was 4.12 +- 0.76% in the pLN-negative group, 1.30 +- 0.08% in the pLN-positive group (pLN-negative versus pLN-positive, $P < 0.001$) (Figure 5C). The average distance (um) of CD8 to PD-L1 was 5.58 +- 0.81% in the pLN-negative group, 1.50 +- 0.16% in the pLN-positive group (pLN-negative versus pLN-positive, $P< 0.0001$) (Figure 5D). In these data, CD8+T cells were significantly close to NK cells, macrophages and tumor cells with pLN metastasis.

The average distance (um) of CD56 to PD-1 was 2.11 +- 0.24% in the pLN-negative group, 0.91 +- 0.09% in the pLN-positive group (pLN-negative versus pLN-positive, $P< 0.0001$) (Figure 5E). The average distance (um) of CD56 to PD-L1 was 2.57 +- 0.32% in the pLN-negative group, 1.26 +- 0.18% in the pLN-positive group (pLN-negative versus pLN-positive, $P< 0.0001$) (Figure 5F). Thus, NK cells were significantly close to tumor cells with pLN metastasis.

The average distance (um) of PD-1 to PD-L1 was 2.09 +- 0.10% in the pLN-negative group, 1.74 +- 0.09% in the pLN-positive group (pLN-negative versus pLN-positive, $P< 0.05$) (Figure 5G).

Development of an Individualized Prediction Model

Multivariable logistic regression analysis began with the following immune variables: CD3, CD8, CD56, CD68, PD-1, PD-L1, the distance between CD8 and CD56, CD8 and CD68, CD8 and PD-1, CD8 and PD-L1, CD56 and PD-1, CD56 and PD-L1, PD-1 and PDL-1. Immune signature was applied to develop a diagnostic model for pLN metastasis by using the two groups. An ROC curve is made for each meaningful marker and every cutoff value was emerged. Thereafter, these cutoff values are divided into two groups. At this point, we can make a diagnostic ROC curve and the AUC reach 0.843 (Figure 6A). The model that incorporated these above independent predictors was developed and presented as the nomogram (Figure 6B).

Internally Validation of the Prediction Model

With the nomogram, we then tested the remaining 40 percent of cases, and obtained a ROC curve with an AUC reaching 0.888. (Figure 6C).

Discussion

Main findings and Interpretation

Patients with the 2009 FIGO stage of IB1-IIA2 CC will undergo pLN resection and impair the patient's quality of life [8,40].Begin with this study, our TCGA data originated from bioinformatics analysis demonstrated that LN metastasis of CESC may be related to immune infiltration. Results stressed that the major immune infiltration cell types were natural killer (NK) cells, macrophages and T cells.

In addition, traditional procedures have been used to elucidate the mechanism by regularly exploring such molecular pathways which would destroy the spatial structure[41,42]. By mIF and HALO system, their spatial orientation interrelation of immune cells and immune markers would be preserved [19,43]. Our results further revealed that immune infiltration, including CD68, PD-1 and PD-L1 were significantly up-regulated and CD8 was significantly down-regulated with pLN metastasis of CC. This can be explained as macrophages and the immune molecule PD-1, PD-L1 may promote pLN metastasis of CC.

Thirdly, by Pearson correlation coefficients (R) and linear regression, we further found a significant positive correlation between CD3 and CD8, CD3 and CD56, CD3 and CD68, CD8 and CD56, CD8 and PD-1, CD8 and PD-L1, CD56 and CD68, CD56 and PD-1, cd56 and PD-L1, PD-1 and PD-L1 in CC without pLN metastasis. With pLN metastasis, these correlations were enhanced between CD3 and CD8, CD8 and CD56, CD8 and CD68. While these correlations became weaken between CD3 and CD68, CD8 and PD-1, CD8 and PD-L1, CD56 and PD-1, CD56 and PD-L1, PD-1 and PD-L1. These results indicated that a large number of T cells are activated, and enhanced communication with macrophages and NK cells may be involved in pLN metastasis of CC.

Fourthly, by spatial proximity analysis, the average distance (um) between CD8 and CD56, CD8 and CD68, CD8 and PD-1, CD8 and PD-L1, CD56 and PD-1, CD56 and PD-L1 were significantly closer with pLN metastasis. These data have demonstrated that CD8+ T cells, NK cells and macrophage may be involved in pLN metastasis of CC. To sum up to above two points, these data further proved that the interaction between T cells and NK cells, as well as T cells and macrophages may promote the metastasis of pLN metastasis in CC.

Lastly, based on randomly selected sixty percent patients, a diagnostic prediction model was established and the AUC can reach 0.843. The nomogram incorporates five items of PD-1, PD-L1, the average distance of CD56 to PD-1, the average distance of CD56 to PD-L1, and the average distance of PD-1 to PD-L1. By internally validation with the remaining 40 percent of cases, a new ROC curve was emerged and the AUC reaching 0.888.

Summary with this information, T cells interact with NK cells and macrophages through PD-1 and PD-L1 to promote pLN metastasis of CC.

Our nomogram can serve as an effective preoperative predictive tool to assess pLN status in CC patients.

Cervical biopsy is an essential step before the diagnosis of CC. In the process, we can take part of cancer tissue through cervical biopsy for mIF detection. HALO system is further applied in quantitative and spatial analysis. By the diagnostic prediction model, we can predict pLN metastasis preoperatively, thus avoiding unnecessary routine pLN dissection. In this way, we can avoid additional surgical trauma and possible complications for patients.

Strengths and limitations

The limitations of our study include external validation for the model. External validation is needed to acquire high-level evidence for clinical application.

Conclusion

This study presents that T cells may interact with NK cells and macrophages through PD-1 and PD-L1 to promote pLN metastasis of CC. In addition, we construct an immune nomogram that incorporates five items of PD-1, PD-L1, the average distance of CD56 to PD-1, the average distance of CD56 to PD-L1, and the average distance of PD-1 to PD-L1, which can be conveniently used to facilitate the preoperative individualized prediction of pLN metastasis in patients with CC.

Author Contributions

JC W, QH G, and J Z: Conceptualization, Data curation, Investigation, Writing - original draft. Y W: Conceptualization, Data curation, Investigation, Writing - original draft, Formal analysis, Methodology, Resources, Software, Validation, Visualization. SY L: Conceptualization, Project administration, Supervision, Writing - original draft. SY C, SM W: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing. XZ J, XH W: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Approval and Consent to Participate

All study surgical procedures and experiment protocols have been approved by the Ethics Committee of Experimental Research, Shanghai Medical College, Fudan University. All participants have been informed the potential risks and benefits and each patient has signed the informed consent form.

Data Availability Statement

The data that support the results of this study are available from the corresponding author upon reasonable request.

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