Transcriptome analysis indentifies KRT23 as a immunotherapeutic target in cervical cancer

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Research Article

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Abstract

Background: Cervical cancer (CC) is one of the most common malignancies in females worldwide. Traditional treatments have been used widely, but the prognosis remains poor. Therefore, new strategies are needed to improve outcomes. Immunotherapy has been used to treat various types of solid tumors. Subtypes of the tumor microenvironment (TME) are associated with the response to immunotherapy, so understanding complexity of TME is pivotal for immunotherapy.

Methods: In this study, we used two methods, “ssGSEA” and “xCell”, to estimate the immune profile in CC. R packages “Corrplot” was used to analysis the correlation of immune cells. “ConsensusClusterPlus” was used to cluster CC based on infiltration of immune cells. “Limma” was used to identify differentially expressed genes (DEGs), and “clusterprofile” was used to perform enrichment analysis. “survival” and “glmnet” was used to construct prediction model. RT-PCR was used to detect Keratin, type I cytoskeletal 23 (KRT23) expression. Cytometric bead arrays and ELISA were used to detect CCL5 expression. Transwell assay was used to detect migration of CD8+ T cells.

Results: We subdivided CC into “hot” and “cold” tumors, in which hot tumors had infiltration of more immune cells and longer survival. Enrichment analyses of DEGs revealed that the number of activated immune signaling pathways was higher in hot tumors. KRT23 showed high expression in cold tumors and its expression was negatively correlated with infiltration of immune cells. In vitro experiments, knockdown of KRT23 expression promoted secretion of CCL5, and promoted recruitment of CD8+ T cells. We also constructed a model based on DEGs that had high efficacy for predicting the survival of CC and patients receiving immunotherapy.

Conclusion: Our study provides deep insights in infiltration of immune cells into CC. KRT23 may act as an immunotherapeutic target. Our model can predict the prognosis of CC patients and may guide immunotherapy.

Background

Cancer has the highest clinical, social, and economic burdens in terms of cause-specific disability-adjusted life years (1). Cervical cancer (CC) is ranked fourth for incidence and mortality in women worldwide (2). Invasion and metastasis by CC cells are the most prevalent causes of cancer-associated deaths among patients with CC, and are accompanied with a poor prognosis (3, 4).

Luckily, excellent primary and secondary prevention strategies are available for CC, such as human papilloma virus (HPV) vaccination and annual cytology smears. Almost all CC patients suffer from a persistent infection of high-risk types of the HPV (5–7). Surgery, chemotherapy, or radiotherapy show satisfactory efficacy for early-stage and low-risk CC (8–10). However, 5-year survival for metastatic cervical cancer (mCC) is 16.5% (11). In addition, side effects caused by chemotherapy and radiotherapy limit the efficiency. Therefore, exploration of the biological mechanisms and development of new therapeutic targets and strategies for CC to improve the survival of patients are needed.
In recent years, increasing numbers of studies have focused on the crucial role of immunotherapy in CC. Considering expression of programmed cell death-1 and programmed cell death ligand-1 (PD-1/PD-L1) in advanced CC, pembrolizumab (a humanized monoclonal anti-PD1 antibody) has demonstrated durable antitumor activity and manageable safety in clinical trials for treatment of recurrent CC or mCC (12–14). Notably, several studies have reported that cisplatin-based chemotherapy can increase PD-L1 expression in CC (15–17). Combinations of immune-checkpoint inhibitors with chemotherapy, radiotherapy, or other novel approaches may improve the results of CC treatment. Even though immunotherapy has achieved remarkable efficacy, accumulated data in recent years have demonstrated that many patients experience minimal or no clinical benefit if provided with identical treatment. This phenomenon could be attributed to the complexity and uniqueness of the tumor microenvironment (TME).

The TME is a complex, plastic, and dynamic system “sculpted” by tumor cells and surrounding cells (18, 19). Cells from the innate immune system and adaptive immune system are important components of tumor stroma, can be reprogrammed according to the TME, and may be involved (positively or negatively) in the survival and progression of tumor cells (20, 21). For example, tumor-associated macrophages (TAMs) are usually the largest population of myeloid cells infiltrating in most solid tumors (22). TAMs display a high degree of functional plasticity if exposed to various microenvironmental conditions, and can be classified as “M1-like” (pro-inflammatory and usually anti-tumor) or “M2-like” (anti-inflammatory and pro-tumor) (23, 24). Accumulating evidence suggests the critical roles of the TME on promoting tumor progression. However, how the TME affects the efficacy of immunotherapy is not known. Immunotherapy harnesses or restores the immune system to kill tumor cells, but this requires the infiltration of immune cells to the tumor site. Studies have demonstrated that the type of the TME is associated with the clinical efficacy of immunotherapy. A “hot” tumor that has sufficient tumor-infiltrating lymphocytes and antigen-presenting cells can respond robustly to immunotherapy. A “cold” tumor lacking immune cells, in general, cannot elicit an effective response to immunotherapy (25). Therefore, understanding and distinguishing the unique classes of the TME is useful for predicting and guiding immunotherapy.

We undertook a comprehensive analysis to explore the infiltration of immune cells in CC using two methods and constructed a prediction model. We observed that CC patients with infiltration of a higher number of immune cells survived longer. To uncover the underlying mechanisms of infiltration of immune cells, we subdivided tumors into hot and cold types and ascertained the differentially expressed genes (DEGs) between them. In addition, our model performed well in predicting the overall survival (OS) of patients with CC and the OS of patients receiving immunotherapy.

Materials And Methods

Ethics statement

CC specimens were obtained after surgical treatment in the First Affiliated Hospital of Zhengzhou University. Specimens were frozen in the biobank of Zhengzhou University. All participants provided
written informed consent for their specimens to be used in this study. The study protocol was approved by the ethics committee of First Affiliated Hospital of Zhengzhou University.

**Cell culture**

A human cervical cell line (HeLa) was purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were sustained in RPMI1640 medium with 5% fetal bovine serum and an atmosphere of 5% CO\textsubscript{2} in a humidified incubator at 37°C.

**Acquisition and normalization of data**

Level-2 mRNA-sequencing data (fragment per kilobase of transcript per million mapped reads) of CC were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) and transformed to transcripts per million for further analyses. The clinical data of CC were downloaded from Xena within University of California Santa Cruz (http://xena.ucsc.edu/). The GSE78220 dataset was downloaded from the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/). A dataset of patients with metastatic urothelial cancer treated with anti-PDL1 agents was downloaded on online website supplied in the article(http://research-pub.gene.com/IMvigor210CoreBiologies/)

**Estimation of the immune profile**

The immune profile (i.e., the number and type of immune cells) was estimated by the packages “ssGSEA” and “xCell” within R (R Institute for Statistical Computing, Vienna, Austria). For xCell, we selected samples with p <0.05 and we included only immune cells for further analyses. The Immune Score, Stromal Score and tumor purity were calculated by the R package “ESTIMATE”.

**Identification and functional annotation of DEGs**

Tumor samples were divided into two subtypes: “cold” and “hot”. DEGs were calculated by the R package “Limma” and visualized by volcano plots constructed by the R package “ggplot2”. DEGs with log fold change>1 and p<0.05 were selected for annotation using the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/) and Gene Ontology (GO; http://geneontology.org/) databases by the R package “clusterprofiler”. A protein–protein interaction (PPI) network was constructed using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; www.string-db.org/) and visualized by Cytoscape3.6.1 (https://cytoscape.org/).

**Correlation and survival analyses**

The R package “corrplot” was used to analyze the correlation of immune cells. The correlation of keratin, type I cytoskeletal 23 (KRT-23) and C-X-C motif chemokine ligand 9 (CXCL9), and CXCL10 and C-C motif chemokine ligand 5 (CCL5), in TCGA dataset was analyzed through cbioportal(www.cbioportal.org/). Correlation in tumor tissues from patients was conducted by Prism7
(GraphPad, San Diego, CA, USA). For survival analyses, samples were divided into four clusters of hot and cold tumors. The R package “survival” was used to assess the survival difference using the logrank test.

**Construction and validation of prediction model**

The DEGs between hot and cold tumor were utilized to perform unicox analysis using R package “survival” to select survival-related DEGs with p<0.05 and followed with LASSO regression to optimize the gene sets using R package “glmnet”. The risk formula was calculated by multicox in R package “survival”. The R package “survivalROC” was used to analyze the sensitivity and specificity of the model.

**Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was extracted by TRIzol® Reagent according to manufacturer (TaKaRa Biotechnology, Shiga, Japan) instructions and the concentration was measured using a spectrophotometer (NanoDrop™ 2000; Thermo Fisher, Waltham, MA, USA). RNA (1 µg) was used to reverse DNA using the PrimeScript™ RT Reagent kit (TaKaRa Biotechnology). The primers for KRT23 were constructed by PrimerBank (https://pga.mgh.harvard.edu/primerbank/index.html/) and synthesized by Sangon Biotech (Shanghai, China) (Table 1). Glyceraldehyde-3-phosphate dehydrogenase was used for data normalization.

**Small interfering RNA (siRNA) transfection**

Knockdown of KRT23 expression was achieved using the jetPRIME® Transfection Reagent kit (Polyplus-transfection, Illkirch-Graffenstaden, France). HeLa cells (1×10⁵) were seeded in six-well plates with RPMI1640 medium. Before transfection, siRNA of KRT23 was diluted to 20 µM according to manufacturer instructions. Then, 200 µL of transfection buffer and 4 µL of jetPRIME reagents were mixed and incubated for 10 sat room temperature. After that, 50nM of siRNA was added and incubation allowed for 15 min at room temperature. siRNA efficacy was analyzed by RT-qPCR after 48 h. The sequence of siRNA synthesized by Gene Pharma (Shanghai, China) is listed in Table 2.

**Transwell™ assay**

Migration of CD8⁺ T cells was analyzed through the Transwell assay. CD8⁺ T cells (2×10⁴) isolated by microbeads from healthy donors were activated with CD3/CD28 beads and seeded in the upper chamber of the Transwell apparatus with serum-free medium (Millipore, Billerica, MA, USA). HeLa cells (2×10⁴) were seeded in the lower chamber with RPMI1640 medium. The number of CD8⁺ T cells was calculated using flow cytometry.

**Enzyme-linked immunosorbent assay (ELISA)**

Tumor cells were transfected with siRNA for 48 h. Then, supernatants were collected and centrifugation (1500rpm, 5 minute) to remove debris. The CCL5 concentration was measured by the LEGEND MAX™ Human CCL5 (RANTES) ELISA kit according to manufacturer (Biolegend, San Diego, CA, USA) instructions. Briefly, standard dilutions and samples were prepared, followed by addition of 50 µL of
Assay Buffer B to each well. Then, 50 μL of the standard or sample was added to the appropriate well. This action was followed by incubation of the plate at room temperature for 2 h with agitation at 200 rpm. Then, 100 μL of Human CCL5 Detection Antibody solution was added, followed by 100 μL of Avidin-HRP A solution, to each well. Results were read at an optical density of 450 nm.

Detection of multiple chemokines

We used the LEGENDplex™ kit (Biolegend) to detect the chemokines secreted by tumor cells. This kit can detect 13 types of chemokines. First, 25 μL of assay buffer was added to the standard or sample in each tube. Second, we added 25 μL of mix beads (A and B) and permitted incubation at room temperature for 2 h with agitation at 500 rpm. Third, we added 25 μL of antibodies to each tube and allowed incubation at room temperature for 1 h with agitation at 500 rpm. Next, we added 25 μL of SA-PE to each tube and washed with washing buffer. The fluorescence intensity was detected by a flow cytometer and analyzed by LEGENDplex v8.0.

Statistical analyses

Statistical analyses were undertaken using Prism 7 (GraphPad) and R 3.6.3. Two-tailed unpaired t-tests and the Wilcoxon test were used to compare the difference in data between two groups. Spearman’s rank correlation coefficient was used to evaluate correlation. p < 0.05 was considered significant.

Results

Infiltration pattern of immune cells in adjacent tissue and tumor tissue

We carried out a multistep analysis to explore the infiltration of immune cells into CC (Fig. 1). First, we estimated the number of immune cells in each sample by ssGSEA and xCell, which have various algorithms and focus on different immune cells between adjacent tissue and tumor tissue. The number of each cell type infiltrated into the TME was different, which revealed the complexity of the TME. ssGSEA and xCell showed consistent results. In general, the number of the most adoptive immune cells in tumor tissue was higher than that in adjacent tissue, such as activated CD4+ T cells, effector memory CD4+ T cells, type-17 T-helper (Th17) cells, and Th2 cells, which indicated an activated immune response in tumor tissue. The number of CD8+ T cells tended to be higher in tumor tissues, but not significantly. Cells from the innate immune system showed high infiltration in normal tissues (Fig. 2A, B). Tumor tissues had a lower Immune Score, but there was no significant difference in the Stromal Score (Fig. 2C, D). We also compared the difference in immune cells in patients who received radiotherapy. Pro-B cells and Th1 cells tended to accumulate in tumor tissue after radiotherapy (sFig 1A, B). These results revealed distinct patterns of infiltration between adoptive immune cells and innate immune cells.

Characterization of immune clusters in CC tissue
Activation of an efficacious anti-tumor immune response requires the synergistic action of multiple cells. To explore the relationships between different cell types, we undertook a correlation analysis of infiltrating cells in tumor tissues. Most infiltrating cells showed a high correlation with each other, especially activated CD8⁺, CD4⁺ T, dendritic, and B cells. We observed a higher correlation of immunosuppressive cells and immune cells, such as regulatory T cells, myeloid-derived suppressor cells, and M2 macrophages, which suggested immune suppression induced by tumor cells after activation of the immune system. Cells of the innate immune system, such as monocytes, neutrophils, and natural killer cells, showed a weak association with other cells, which demonstrated a unique anti-tumor immune process (Fig.3A,B).

Next, we performed consensus clustering of all samples based on the proportions of immune cells to identify the subtypes of infiltrating immune cells. The consensus matrix heatmap showed four clearly identified groups estimated by two methods, respectively (Fig.3C,D). We observed a gradual increase in infiltration of immune cells in tumor tissue from groups 1 to 4, in which group 1 and group 2 lacked infiltration of immune-related cells, group 3 had modest infiltration, and group 4 showed abundant infiltration of immune cells (sFig.2A,B). In accordance with these results, group 4 had the highest Immune Score (sFig.2C,D). To further characterize the clusters of CC cells, we took the intersection of each group of the two methods, denoted as clusters 1 to 4 (sFig.3A). In accordance with the results stated above, cluster 4 had a high Immune Score (Fig.4A). Next, we analyzed expression of genes involved in the immune response, immune tolerance, and antigen presentation in the four clusters. Expression of immune checkpoint-related genes (CD276, CD274, CD40, CTLA4, HAVCR2, LAG3, PDCD1), antigen presentation-related genes (B2M, HLA-B, HAL-C, HLA-DQA1, TAP1, TAP2, HLA-DQA2), cytokine-related genes (GZMB, GZMH, IFNG, PRF1, TNF) and chemokine-related genes (CCL5, CXCL10, CXCL13, CXCL9) increased gradually from cluster 1 to cluster 4 (Fig.4B, sFig.3C, D). Survival analyses revealed that cluster 4 had the longest survival relative to that in clusters 1, 2, and 3 in terms of OS and progression-free interval (Fig.4C,D).

Survival status and signaling alterations between hot tumors and cold tumors

To further explore the mechanisms of immune-cell infiltration, we redefined cluster 1, cluster 2, and cluster 3 as cold tumors and cluster 4 as hot tumors based on immune cells and survival status. Hot tumors had longer OS and progression-free interval compared with those for cold tumors (Fig. 5A,B). Next, we analyzed the difference between the two groups at the transcriptional level. Hot tumors and cold tumors showed different transcription patterns according to volcano plots (Fig. 5C). Finally, 657 messenger (m)RNAs with upregulated expression in hot tumors and 55 mRNAs with upregulated expression in cold tumors were identified. To further explore DEGs function, enrichment analyses using GO and KEGG databases were done. The GO database revealed that DEGs in hot tumors were enriched primarily in “T cell activation”, “regulation of lymphocyte activation”, “leukocyte cell–cell adhesion”, “regulation of T cell activation” and “leukocyte proliferation”; none of these GO terms were enriched in cold tumors (Fig 5D). Analyses of enrichment of DEGs using the KEGG database revealed that DEGs in cold tumors were significantly enriched in “apical part of cell”, “actin-based cell projection” and “apical
plasma membrane" (Fig. 5E). In hot tumors, DEGs were enriched mainly in "cytokine–cytokine receptor interaction", "chemokine signaling pathway" and "cell adhesion molecules", which indicated an activated immune response in hot tumors (Fig. 5F). These results suggested that immunity was activated comprehensively in hot tumors, particularly the T cell-mediated immune response. PPI networks also revealed that the "hub genes" in DEGs of hot tumors were mainly immune-related chemokines and cytokines (sFig. 4A,B).

**Inhibition of KRT23 expression promotes infiltration of CD8⁺ T cells**

The results stated above revealed a correlation between infiltration of many immune cells and longer survival. Therefore, promoting infiltration of immune cells in cold tumors may enhance antitumor immunity and prolong survival. KRT23 showed the highest expression in cold tumors as compared with hot tumors. Enrichment analyses using the KEGG and GO databases revealed that KRT23-related genes were negatively correlated with the immune response (Fig. 6A,B). Knockdown of KRT23 expression in HeLa cells inhibited cell proliferation (Fig. 6C, D), which suggested an important role of KRT23. To explore how KRT23 affected infiltration of immune cells, we measured expression of KRT23 and CD8⁺ T cell-related chemokines in tumor samples (CCL5, CXCL9, CXCL10). KRT23 expression was negatively correlated with these chemokines, and this result was confirmed using TCGA database (Fig. 6E, sFig. 5). Furthermore, knockdown of KRT23 expression promoted CCL5 expression at the mRNA level. Cytometric bead arrays and ELISA confirmed that inhibition of KRT23 expression increased CCL5 secretion (Fig. 6G, H) and recruitment of CD8⁺ T cells (Fig. 6I).

**Construction and validation of a prediction model based on DEGs**

Next, we used DEGs to construct a prediction model. We undertook a univariate Cox analysis followed by a lasso regression analysis (sFig. 6A). To optimize the model, we carried out a multivariate Cox analysis and, finally, identified 11 genes to construct our model (sFig. 6B). Heatmaps revealed expression of these genes in high and low risk groups in a training cohort and testing cohort. Survival analyses showed that patients with a high risk of CC had shorter survival in the training cohort and testing cohort (Fig. 7A, B). To explore the accuracy of our model, we analyzed receiver operating characteristic (ROC) curves in the training cohort and testing cohort at 1, 3, and 5 years. Our model had a higher area under the ROC curve (AUC) in the training cohort and testing cohort (Fig. 7C, D). Our model was constructed by DEGs in hot tumors and cold tumors, so we hypothesized that this model may predict the response to immunotherapy. Hence, we used two external cohorts of patient sreceiving immunotherapy. Patients with a higher risk of CC had shorter survival in the two cohorts (Fig. 7E, F). These results suggested that our model could not only predict the survival of patients with CC, it could also be used to predict the response to immunotherapy.

**Discussion**
Most cases of CC are caused by HPV infection (26, 27). Chemotherapy for CC is limited. The optimal regimen against recurrent CC or mCC is a combination of cisplatin, paclitaxel, and bevacizumab. This regimen is associated with an overall response rate of 48% and median survival of 17 months (28). The side-effects caused by radiotherapy restrict its clinical application in CC (29). Therefore, new and efficacious strategies for CC are needed urgently.

Immunotherapy has shown sustainable clinical response and is first-line treatment for various types of tumors (30). “Cancer immunotherapy” is a general term describing harnessing of the immune system of a patient to elicit antitumor effects (31). Antibodies against PD-1 and PD-L1 are used commonly for cancer immunotherapy. They work by releasing the “inhibitory brakes” of T cells, resulting in robust activation of the antitumor immune response (32).

The major risk factor for CC is HPV infection (33). High-risk HPV types, such as 16 and 18, are more likely to persist and integrate into the host genome to enable excess expression of the oncoproteins E6 and E7. These oncoproteins interfere with the immune response (34). Therefore, therapies targeting the HPV have been attempted, but the effects have been suboptimal (12). However, the retained viral antigens in CC make immunotherapy an attractive option because they could be recognized as foreign. Indeed, several clinical trials have used antibodies against PD-1 or PD-L1 (31, 35). Efficacious immunotherapy is reliant on the infiltration of lymphocytes and antigen-presenting cells. In general, the TME can be divided into two broad categories: “T cell-inflamed” and “non-T cell-inflamed” (36). Several methods have been used to estimate the immune profile in the TME: ssGSEA, CIBERSORT, TIMER, MCP-counter, and xCell (37-41). ssGSEA and MCP-Counter use specific cell-maker genes and score the immune profile through expression of these genes. CIBERSORT focuses on the ratios of each cell type using Nu-support vector regression. xCell is an integration of these methods and expands the cells that can be evaluated to 64 types. In order to more accurately reflect the level of immune cells in the TME of CC, we used two methods to score the immune cells. Results from the comparison between tumor and adjacent tissues as well as correlation analysis of estimated immune cells showed constant findings of the two methods, suggesting that these two methods can be used to estimate immune levels. Based on the consensus clustering, the CC can be divided into 4 clusters, and clusters with higher immune infiltration had a better survival. In line with previous studies, Wang J et.al, also reported that CD4+ T cells are independent prognostic factor of CC estimated by CIBERSORT (42). Meanwhile, infiltration of immune cells also correlated with the response of chemotherapy (43). We further re-divided the 4 clusters into 2 subtypes and donated as “hot” and “cold” tumor based on the immune levels, which “hot” tumor is T cell-inflamed and “cold” tumor is Non-T cell-inflamed. Pathway enrichment analysis confirmed that hot tumor is a state of active immune response.

Cold tumors are characterized by infiltration of few immune cells. They are the most challenging to eradicate and are invariably associated with a poor prognosis (44). Several strategies have been used to covert cold tumors to hot tumors: radiotherapy, chemotherapy, targeted therapy, and adoptive-cell therapy (45–49). We compared the difference between hot tumors and cold tumors, and identified KRT23 as most upregulated gene in cold tumors. Keratin is the main component of epithelial cells, and malignant tumor cells originate from these epithelial cells. KRT23 is a newly identified gene in the KRT
Studies have reported that KRT23 overexpression promotes the migration of ovarian cancer cells via epithelial–mesenchymal transition. KRT23 promotes proliferation of colorectal tumor cells through increasing expression of telomerase reverse transcriptase. Although the oncogenic role of KRT23 has been explored, how KRT23 affects the immune response is not known. We observed that KRT23 expression was negatively correlated with the immune response. Knockdown of KRT23 expression in tumor cells results in increased secretion of CCL5 (a potent chemokine that recruits CD8+ T cells) and inhibits the proliferation of tumor cells. Our results indicate that inhibition of KRT23 expression not only inhibits tumor growth but also enhances the anti-tumor response. Hence, a potential combination strategy of targeting KRT23 and anti-PD1 and PD-L1 could be a rational approach against CC.

The large difference in survival between hot tumors and cold tumors prompted us to construct a prediction model based on the DEGs of the two types of CC. This model performed well in the training cohort, test cohort, and an external-validation cohort. Hence, our model was reliable and could be used to guide clinical treatment. Several studies have tested a prediction model for CC. Mei and colleagues estimated the immune profile through ssGSEA and identified four immune-related prognostic gene signatures. Ding et al. screened survival-related immune genes and constructed a prediction model containing 13 genes. The results from those studies further support our study findings. Of note, our model can predict the survival of patients receiving immunotherapy for CC.

Conclusion

In summary, we undertook a comprehensive analysis of the infiltration of immune cells in CC. We identified hot tumors and cold tumors of CC, and observed the former to have a favorable outcome. We demonstrated KRT23 to be a negative regulator of the immune response, and that knockdown of KRT23 expression promoted CCL5 secretion. In addition, we constructed a prediction model based on DEGs between two types of CC. This model performed well in predicting the survival of CC patients receiving immunotherapy. We provided new insights into the infiltration of immune cells into CC and highlighted KRT23 as a potential target to enhance immunotherapy against CC.

Abbreviations

CC: cervical cancer; TME: tumor microenvironment; DEGs: differentially expressed genes; KRT23: type I cytoskeletal 23; KEGG: the Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; PPI: protein–protein interaction; PD-1/PD-L1: programmed cell death-1/ligand-1.

Declarations

Acknowledgements

Not applicable

Authors’ contribution
Xia Li designed and performed the experiments, wrote the manuscript, analyzed the data. Yanmei Cheng prepared figures 1-6 and Yanyan Jia performed figures 1-6. Huirong Shi supported, designed the study, analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

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**Data availability statement**

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The data that support the findings of this study are openly available on the online website UCSCXena (https://xenabrowser.net/). GSE77280 are available from on the GEO database (https://www.ncbi.nlm.nih.gov/geo/). and patients with metastatic urothelial cancer treated with anti-PDL1 agents were downloaded as online website supplied in the article (http://research-pub.gene.com/IMvigor210CoreBiologies/)

**Ethics approval and consent to participate**

All participants provided written informed consent for their specimens to be used in this study. The study protocol was approved by the ethics committee of First Affiliated Hospital of Zhengzhou University.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests

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Tables

Table1: Primers used in this study

| Gene  | forward          | reverse                        |
|-------|------------------|--------------------------------|
| KRT23 | TACTAGGCGGAAATGGAAGG | TCTTACCATCCACTATCTGCTCC       |
| CXCL9 | CCAGTAGTGAGAAAGGTCGC | AGGGCTTGGGGCAAAATTGTT     |
| CXCL10| GTGGCATTCAAGGAAGTACCTC | TGATGGCTTTCGATTCTGGATT  |
| CCL5  | CCAGCAGTCGTCTTTGTCAC | CTCTGGGTTGGCACAACACTT     |

Table2: the sequence of KRT23 siRNA
SiRNA-KRT23

|   |              |              |
|---|--------------|--------------|
| Si-1 | AUGAGAAUGAGCUCUUUCCUC | GGAAAGAGCUCAUUCUCAUGA |
| Si-2 | GGAGGAUAUGAGACAAGAAUA | UUCUUGUCUCAUAUCCUCCAG |

**Figures**

**Figure 1**

*Multiple step analysis of this study*

**Figure 2**

*Infiltration pattern of immune cells in adjacent tissue and tumor tissue.* (A-B) Level of immune cells in normal and tumor tissues estimated by ssGSEA and xCell. (C-D) Immune and Stromal score in normal and tumor tissues estimated by ESTIMATE.

**Figure 3**

*Correlations of immune cells.* (A-B) Correlation of immune cells in tumor tissues estimated by ssGSEA and xCell. (C-D) Heatmap showing the consensus clustering of CC based on levels of immune cells estimated by ssGSEA and xCell.

**Figure 4**

*Characterization of immune clusters of CC.* (A) Expression of immune score, stromal score and tumor purity in four subtypes. (B) Expression of cytotoxicity-related cytokines in four subtypes. (C-D) Kaplan and Meier curve showing the OS and PFI of 4 clusters.

**Figure 5**

*Survival and transcriptome characteristics of cold and hot tumor.* (A-B) Kaplan and Meier curve showing the OS and PFI of cold and hot tumor. (C) Volcano plot showing the difference of gene expression in cold
and hot tumor. (D) KEGG enrichment analysis in cold tumor. (E) GO enrichment analysis in hot tumor. (F) KEGG enrichment analysis in hot tumor.

**Figure 6**

**KRT23 promotes CD8⁺ T cells recruitment through secretion of CCL5.** (A-B) GO and KEGG analysis of KRT23 related genes. (C) qPCR analysis showing the knockdown efficacy of KRT23. (D) Proliferating rate of tumor cells with knockdown of KRT23. (E) Correlation of KRT23 and CCL5, CXCL9 and CXCL10 in tumor tissues of CC. (F) qPCR analysis showing CCL5, CXCL9 and CXCL10 expression of tumor cells with knockdown of KRT23. (G) Heatmap showing the concentration of cytokine and chemokine secreted by tumor cells with knockdown of KRT23. (H) ELLISA analysis showing the CCL5 secretion by tumor cells with knockdown of KRT23. (I) Number of CD8⁺ T cells recruited by conditioned-medium derived from tumor cells with knockdown of KRT23.

**Figure 7**

**Construction and validation of predicting model.** (A-B) Kaplan and Meier curve showing the OS in high and low risk group in tanning and testing cohort. (C-D) ROC curve analysis showing the AUC of predicting model in tanning and testing cohort. (E-F) Validation of predicting model using dataset of patients with metastatic melanoma and urothelial cancer receiving immunotherapy treatment.

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