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Original Article

Genetic analysis of cervical cancer with lymph node metastasis

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ABSTRACT

Objective: To find out the differences in gene characteristics between cervical cancer patients with and without lymph node metastasis, and to provide reference for therapy.
Methods: From January 2018 to June 2022, recurrent cervical cancer patients 39 cases with lymph node metastasis and 73 cases without lymph node metastasis underwent testing of 1,021 cancer-related genes by next-generation sequencing. Maftools software was used to analyze somatic single nucleotide/insertion-deletion variation mutation, co-occurring mutation, cosmic mutation characteristics, oncogenic signaling pathways.
Results: EP300 and FBXW7 were significantly enriched in lymph node-positive patients. Lymph node-positive patients with EP300 or FBXW7 mutations had lower overall survival (OS) after recurrence. Both lymph node-positive and -negative patients had plenty of co-occurring mutations but few mutually exclusive mutations. Lymph node-positive co-occurring mutation number ≥6 had lower OS, while lymph node-negative co-occurring mutation number ≥3 had lower OS after recurrence. The etiology of SBS3 was defects in DNA double strand break repair by homologous recombination, which exclusively exist in lymph node-positive patients. There was no difference in median tumor mutation burden (TMB) between positive and negative lymph nodes, but TMB was significantly associated with PIK3CA mutation.
Conclusion: The somatic SNV/Indels of EP300 and FBXW7, SBS3 homologous recombination-mediated DNA repair defect were enriched in lymph node-positive patients. For lymph node-positive patients, EP300 or FBXW7 mutations predicted poor prognosis. No matter lymph node-positive or negative, more co-occurring mutation number predicted poor prognosis. PIK3CA mutation may account for the higher TMB and help identify patients who benefit from immunotherapy.

Keywords: Genetic Profile; Cervical Cancer; Lymph Node Metastasis

Synopsis

Cervical cancer patients underwent genetic testing of 1,021 cancer-related genes by next-generation sequencing. The gene mutation characteristics were different between lymph node-positive and -negative patients. These different features might be potential molecular markers to predict lymph node metastasis and prognosis in cervical cancer.

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Author Contributions

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INTRODUCTION

Cervical cancer is the fourth most common cancer in women. There are 106,000 new cases of cervical cancer in China every year, and 48,000 deaths [1]. The 80% of new cases are locally advanced cervical cancer [1]. Lymph node metastasis is commonly observed in locally advanced cervical cancer, which is significantly correlated with prognosis. Apparently, lymph node metastasis indicates a worse survival prognosis in cervical cancer, with a 3-year survival rate of only 64% compared with 94% of those with negative metastasis [2]. The patients with lymph nodes metastasis have a poorer prognosis.

At present, the sensitivity and specificity of imaging examination for detecting cervical cancer lymph node metastasis cannot meet clinical needs, and the biopsy through surgical resection of lymph nodes is traumatic and cannot be widely applied [3]. More methods for detecting or predicting lymph node metastasis in cervical cancer are needed.

The accuracy of lymph node metastasis assessment and the potential risk of lymph node metastasis can be improved by analyzing the differences in clinical characteristics, tumor markers, high-risk human papillomavirus (HPV) types, immunohistochemical results and genetic profiles between patients with lymph node-positive and -negative disease during initial treatment [4,5]. Then the comprehensive treatment of cervical cancer can be guided.

In this study, on account of the different prognosis of lymph node-positive and -negative cervical cancer, we characterized the genomic features by performing 1,021-gene next-generation sequencing (NGS) in patients respectively. Our results might provide reference for clinical practice and insights that will ultimately improve the prediction of lymph node metastasis and optimize personalized cancer therapy.

MATERIALS AND METHODS

1. Study design and population

This was a retrospective study. Patients with recurrent cervical cancer treated in the Gynecological Oncology Center of the Cancer Hospital Affiliated to Chongqing University from January 2018 to June 2022 were analyzed, and tissue samples were collected for gene detection. Patients were grouped according to the presence or absence of lymph node metastasis at the time of recurrence, no matter the condition of initial treatment. Lymph node metastasis includes pelvic lymph nodes, para-aortic lymph nodes, mediastinal lymph nodes, supraclavicular lymph nodes, cervical lymph nodes, and inguinal lymph nodes, etc. This study was approved by the ethics committee of Cancer Hospital Affiliated to Chongqing University (ethic No.: CZLS2023018-A). Informed consent were obtained from the patients.

2. Data collection

The clinical characteristics were collected, including age, pathological type, high-risk HPV state, stage at initial treatment, recurrence site, specimen source, initial treatment, and treatment received after recurrence. The International Federation of Gynecology and Obstetrics 2018 cervical cancer staging criteria were adopted for the staging at the time of initial treatment, and the stage was not changed after recurrence. The tissue samples of the lymph node-positive group were from the lymph nodes at the time of recurrence. The tissue samples of the lymph node-negative group were from cervix, vagina, bladder, lung, and bone at the time of recurrence.

Data of patients with cervical cancer from The Cancer Genome Atlas (TCGA) database were collected to validate our results.

3. NGS procedures

Tumor tissues were collected at surgery and made into formalin-fixed, paraffin-embedded (FFPE) specimens. Five milliliters of peripheral blood samples from each patient were collected by using a Streck Vacutainer tube (Streck Company, La Vista, NE, USA) as control. All samples were sent to Geneplus (Beijing, China) for testing of 1,021 tumor-related genes. The **Fig. S1** list these 1,021 sequenced genes.

Briefly, genomic DNA was extracted from peripheral blood using the CWE9600 blood DNA kit (China Kangwei Century Company, Beijing, China). Genomic DNA was extracted from FFPE samples using Maxwell 16 FFPE Plus LEV DNA Purification Kit (Promega, Madison, WI, USA). Genomic DNA was fragmented into 200–300-bp fragments with a Covaris S2 ultrasonic instrument (Covaris, Woburn, MA, USA). NEBNext Ultra™ II DNA Library Construction Kit (NEB, Cold Spring Harbor, NY, USA) was used for library construction. The library DNA was hybridized with custom biotinylated oligonucleotide probes (Integrated DNA Technologies, Coralville, IA, USA) covering 1,021 genes. The targeted capture library was sequenced using the Geneplus-2000 sequencing platform (Suzhou Geneplus Medical Engineering Co., Ltd., Suzhou, China), with a sequencing depth of 150× for peripheral blood and 500× for tumor tissues.

Data generated by NGS were firstly filtered out of low-quality reads and sequencing adapters using fastp software (version number 0.19.5). Then the BWA software (version 0.7.12-r1039) was used to compare the filtered data to the human reference genome (hg19). The somatic single nucleotide variants (SNVs) were obtained by MuTect software (version 1.1.4) and NChot software (version 0.1.0). Small insertion and deletion mutations (Indels) were obtained using GATK software (version 3.4–46-gbc02625). Somatic copy number variants (CNVs) were obtained using CONTRA software (version 2.0.8).

4. Statistical analysis

Data analysis was performed using the R 4.1.2 software. The R package maftools (version 3.16; R Foundation for Statistical Computing, Vienna, Austria) was used to analyze the frequency of somatic SNV/Indels mutations, difference in mutation frequency, mutually exclusive or co-occurring mutations, and mutational signatures. Somatic CNV differences were analyzed using the Fisher's exact test. Kaplan-Meier curve was used for survival analysis. The p-value less than 0.05 was considered statistically significant.

RESULTS

1. Clinical characteristics

A total of 112 patients were enrolled, including 39 patients with lymph node-positive cervical cancer and 73 with lymph node-negative cervical cancer. For 112 patients, the median age was 52 years (range, 28–75). There were 81 (72.3%) patients with cervical squamous cell carcinoma, 16 (14.3%) with adenocarcinoma, 5 (4.5%) with small cell carcinoma, 5 (4.5%) with mucinous adenocarcinoma, 3 (2.7%) with adenosquamous cell carcinoma, and 2 (1.8%) with clear cell carcinoma. The case of high-risk HPV infection was 92 (82.1%). There were 26 (23.1%) patients with tumor size ≤2 cm, 51 (45.4%) patients with tumor size <2 cm and ≤4 cm, 35 (31.5%) patients with tumor size >4 cm. There were 17 (15.2%) patients with stage

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I disease, 15 (13.4%) with stage II disease, 57 (50.9%) with stage III disease, and 23 (20.5%) with stage IV disease at the initial treatment. The initial treatment included radical surgery, staging surgery, radiotherapy and chemotherapy according to National Comprehensive Cancer Network guidelines. The treatment received after recurrence included radiotherapy, chemotherapy, and immunotherapy. There was no difference in clinical characteristics baseline levels between patients with positive and negative lymph nodes (p>0.05) (**Table 1**).

Table 1. Clinical characteristics

Clinical characteristics	Total	Positive lymph node	Negative lymph node	p-value
No. of patients	112	39	73	
Age (yr)				0.926
Median (range)	52 (28–75)	54 (28–73)	51 (26–75)	
Pathological type				0.395
Squamous cell carcinoma	81 (72.3)	24 (61.5)	57 (78.1)	
Adenocarcinoma	16 (14.3)	7 (17.9)	9 (12.3)	
Adenosquamous carcinoma	3 (2.7)	2 (5.1)	1 (1.4)	
Clear cell carcinoma	2 (1.8)	1 (2.6)	1 (1.4)	
Small cell carcinoma	5 (4.5)	2 (5.1)	3 (4.1)	
Mucinous adenocarcinoma	5 (4.5)	3 (7.7)	2 (2.7)	
High-risk HPV type				0.309
Positive	92 (82.1)	34 (87.2)	58 (79.5)	
Negative	20 (17.9)	5 (12.8)	15 (20.5)	
Tumor size				0.902
≤2 cm	26 (23.2)	10 (25.6)	16 (21.9)	
<2 cm and ≤4 cm	51 (45.5)	17 (43.6)	34 (46.6)	
>4 cm	35 (31.3)	12 (30.8)	23 (31.5)	
Stage at initial treatment				0.603
I	17 (15.2)	5 (12.8)	12 (16.4)	
II	15 (13.4)	6 (15.4)	9 (12.3)	
III	57 (50.9)	17 (43.6)	40 (54.8)	
IV	23 (20.5)	11 (28.2)	12 (16.4)	
Recurrence site*				
Lymph nodes	39	39	0	
Cervix	41	13	28	
Vagina	31	7	24	
Pelvic and abdominal implants	9	3	6	
Bladder	11	3	8	
Intestine	4	1	3	
Lung	31	11	20	
Liver	9	3	6	
Bone	16	5	11	
Specimen collection source*				
Lymph nodes	39	39	0	
Cervix	28	0	28	
Vagina	24	0	24	
Pelvic and abdominal implants	0	0	0	
Bladder	4	0	4	
Intestine	0	0	0	
Lung	11	0	11	
Liver	0	0	0	
Bone	6	0	6	
The initial treatment*				0.999
Radical surgery	24	8	16	
Staging surgery	55	18	37	
Radiotherapy	94	30	64	
Chemotherapy	83	27	56	
The treatment received after recurrence*				0.261
Radiotherapy	23	13	10	
Chemotherapy	65	24	41	
Immunotherapy	37	16	21	

Data shown are number (%) not otherwise specified.
*Possible to multiple selection.

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2. Spectrum analysis of single-site mutations

Of 112 patients, 87 had mutations, and the mutation rate was 77.7%. The 5 genes with the highest frequency of somatic SNV/Indels were PIK3CA (39%), MLL3 (26%), MLL2 (21%), EP300 (15%), and FBXW7 (13%) (**Fig. 1A**). The mutation rate was 87.2% in the lymph node-positive group and 76.7% in the lymph node-negative group. In patients with positive lymph nodes, the 5 genes with the highest frequency of somatic SNV/Indels were PIK3CA (46%), MLL3 (33%), EP300 (26%), FBXW7 (23%), and MLL2 (21%) (**Fig. 1B**). In patients with negative lymph nodes, the 5 genes with the highest frequency of somatic SNV/Indels were PIK3CA (36%), MLL3 (22%), MLL2 (21%), TP53 (14%), and ARID1A (10%) (**Fig. 1C**). Further differential analysis found that the mutation frequencies of EP300 and FBXW7 were significantly different between lymph node-positive and -negative patients (p=0.030), and the mutation rates were higher in lymph node-positive patients (**Fig. S2**).

3. Difference in gene alterations

In patients with positive lymph nodes, the number of gene amplifications was 27, and the number of deletions was 2. There were 45 gene amplifications and 8 deletions in lymph node-negative patients. Somatic CNV frequency did not differ between lymph node-positive and -negative patients (**Table S1**).

4. Co-occurrence and mutual exclusivity analysis feature

Co-occurrence and mutual exclusivity analysis found a large number of co-occurring mutations in both lymph node-positive and -negative patients, while mutually exclusive mutations were rare (**Fig. 2**). There were extremely significant pairs (p<0.001) of co-occurring mutations in the lymph node-positive group: PIK3CA-LRP1B, CASP8-LRP1B, MLL3-BCOR, APC-BRCA1, MLL3-MLL2, and ARID1A-BCOR. The most significant pairs of co-occurring mutations in the lymph node-negative group were: PIK3CA-TERT, PIK3CA-EP300, PIK3CA-MLL2, PIK3CA-MLL3, MLL3-EP300, MLL2-EP300, NOTCH-MLL, ARID1A-NSD1, and APC-FBXW7. Gene co-occurrence and mutual exclusivity exclusion patterns differed between the 2 groups.

5. Mutation signatures analysis

Mutational signatures are reflections of the mutational processes that have been active throughout someone's life [6]. The mutational signature analysis was performed on all cases using the non-negative matrix factorization method implemented in MutationalPatterns R package [7] to infer the underlying mutational processes. Comparing the mutational patterns between patients with positive and negative lymph nodes, the cosine similarity was only 0.459 which suggested there was potential difference between 2 groups (**Fig. 3**). This signatures was further compared with the previously defined COSMIC signatures (https://

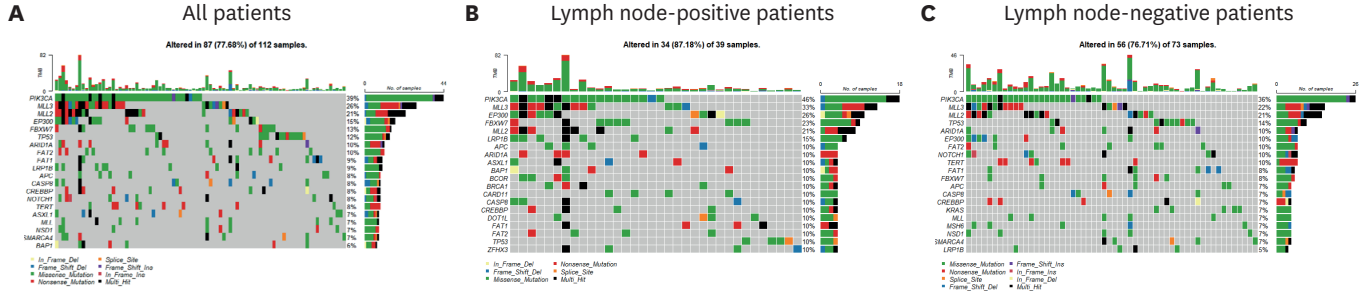

Fig. 1. Waterfall diagram of gene SNV/Indels mutations. (A) All patients, (B) lymph node-positive patients, and (C) lymph node-negative patients.
Indels, insertion and deletion mutations; SNV, single nucleotide variant.

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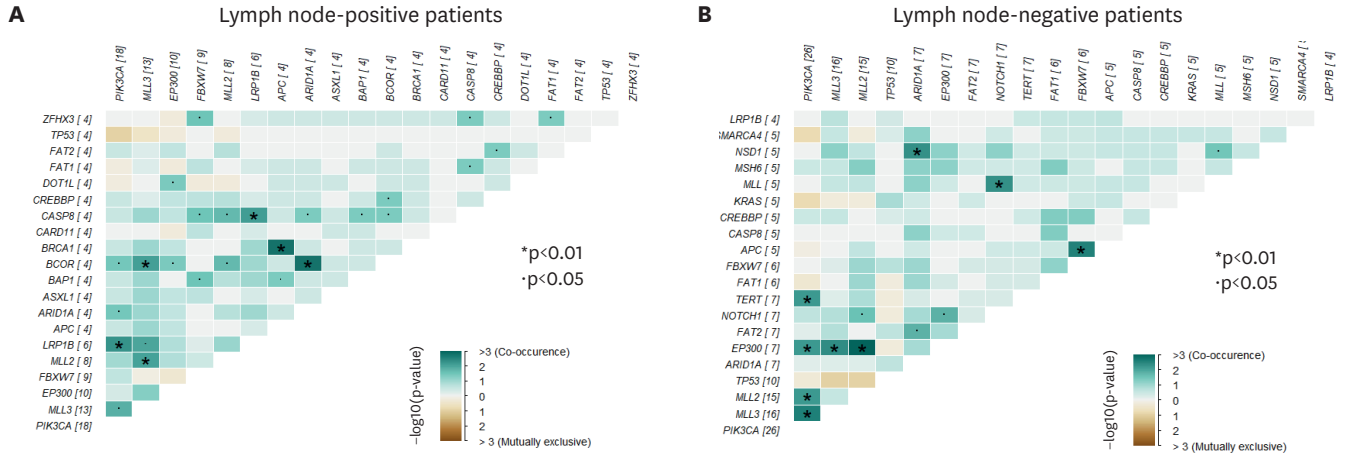

Fig. 2. Coexistence-mutual exclusion features of gene SNV/Indels mutations in lymph node-positive and -negative patients. (A) Lymph node-positive patients and (B) lymph node-negative patients.
Indels, insertion and deletion mutations; SNV, single nucleotide variant.

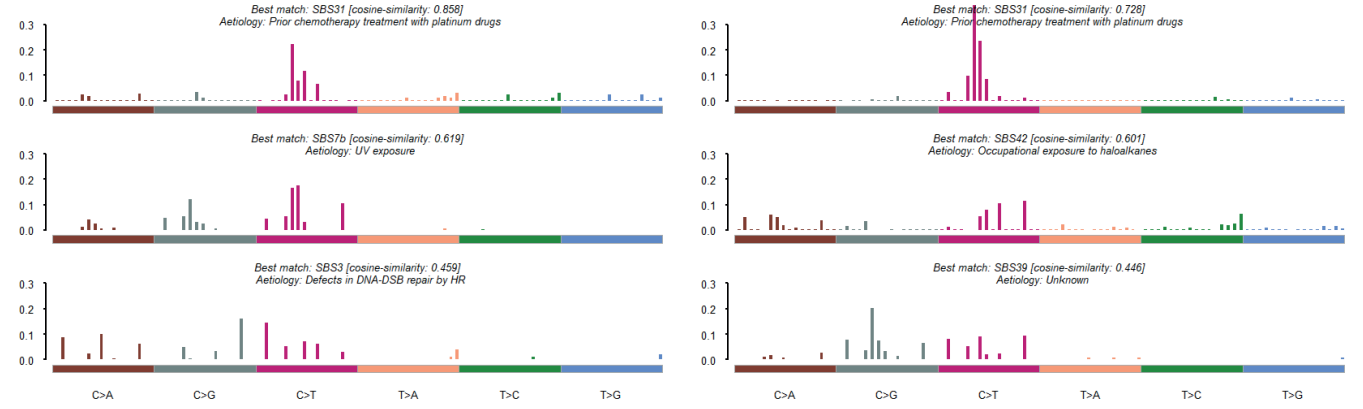

Fig. 3. Gene SNV/Indels cosmic mutation characteristics in lymph node-positive and -negative patients.
Indels, insertion and deletion mutations; SNV, single nucleotide variant.

cancer.sanger.ac.uk/cosmic/signatures_v2) in order to determine the clinical relevance. The etiology of SBS3 was defects in DNA-double strand break repair by homologous recombination, which exclusively exist in lymph node-positive patients. The significantly reduced capacity of DNA double-strand break-repair may indispensably participate in lymph node metastasis.

6. Enrichment of somatic mutations by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

To further determine whether these genomic alteration landscapes could lead to difference in cancer-related signaling pathways and biological processes, we performed pathway-level analysis using the KEGG and GO database. For both groups, the genes affected by somatic mutations and/or CNVs were concatenated and tested the potential enrichment against each KEGG pathway. Based on gene count and p-value, The figures showed the top 15 pathways in each group enriched by KEGG (**Fig. S3A and B**) and GO (**Fig. S3C and D**). Altered signaling pathways included central carbon metabolism in cancer, PI3K-Akt signaling, FoxO signaling pathway, Rap1 signaling pathway, Ras signaling pathway, p53 signaling, ErbB signaling, VEGF signaling, and other well-known pathways. There was no difference in KEGG and GO pathway between patients with positive and negative lymph nodes.

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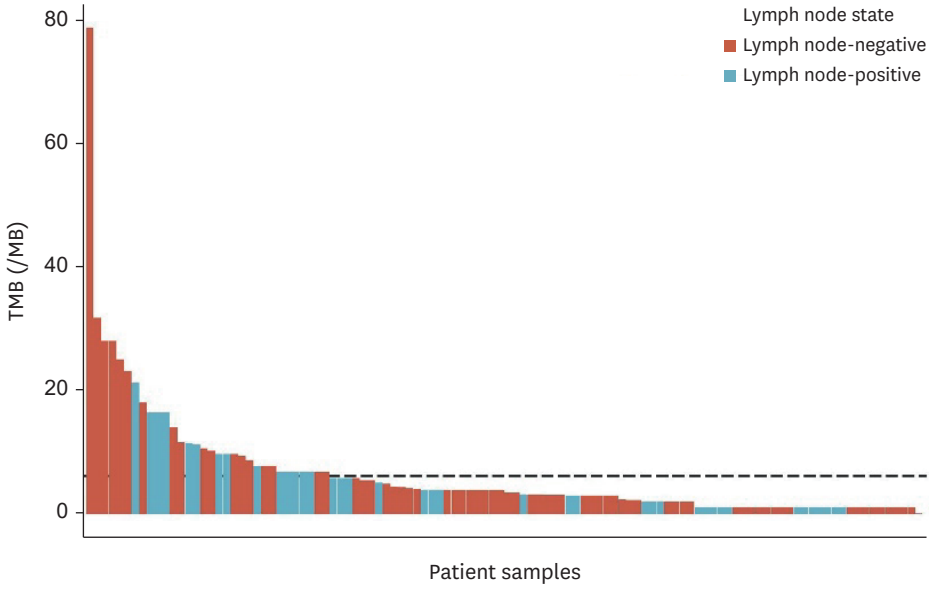

Fig. 4. Distribution of TMB in lymph node-positive and -negative patients.
TMB, tumor mutation burden.

7. Tumor mutation burden (TMB) and its clinical correlation

TMB is defined as the number of somatic mutations and indels per megabyte of coding regions. A TMB score of ≥10 mutations/Mb has been proposed as a threshold with a high likelihood of neoantigen formation [8], which was based on the recent Food and Drug Administration approval for immunotherapy in solid tumors [9]. We divided TMB values into 2 categories: TMB-H (defined as ≥10 mutations/Mb) and TMB-L (defined as <10 mutations/Mb). The median TMB was 9.3 mutations/Mb (range, 0.9–78.7) in the lymph node-positive group and 6.2 mutations/Mb (range, 0.9–43.2) in the lymph node-negative group, without statistical difference (p=0.100) (**Fig. 4**). We investigated the association of high-frequency mutated genes with TMB, including PIK3CA, MLL3, EP300, FBXW7, and MLL2. Patients were divided into wild-type and mutant-type subgroups according to the mutation status for specific genes. Particularly, the most common mutation in lymph node-positive patients is PIK3CA, which may be a useful biomarker for predicting the response to immunotherapy in different cancer types [10]. The median TMB for PIK3CA-mutant tumors was 5.76 mutations/Mb, which was significantly higher than that for PIK3CA-wild-type tumors (4.00 mutations/Mb, p=0.033). There were no TMB differences in the other 4 genes between mutant-type and wild-type.

8. Overall survival (OS) after recurrence

There was no difference in OS between patients with positive and negative lymph nodes. Interestingly, lymph node-positive patients with EP300 mutations had lower OS than EP300 mutations-free after recurrence(p<0.001) (**Fig. 5A**), while there were no difference in OS between lymph node-negative patients with EP300 mutations and EP300 mutations-free after recurrence(p=0.66) (**Fig. 5B**). lymph node-positive patients with FBXW7 mutations had lower OS than FBXW7 mutations-free after recurrence (p=0.001) (**Fig. 5C**), while there were no difference in OS between lymph node-negative patients with FBXW7 mutations and FBXW7 mutations-free after recurrence (p=0.44) (**Fig. 5D**). Both lymph node-positive and -negative patients had a large number of co-occurring mutations but few mutually exclusive mutations. Lymph node-positive co-occurring mutation number range in 0–7 (median=3),

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lymph node-negative co-occurring mutation number range in 0–6 (median=2). Lymph node-positive patients with co-occurring mutation number ≥6 had lower OS after recurrence (p=0.024) (**Fig. 5E**), while lymph node-negative patients with co-occurring mutation number ≥3 had lower OS after recurrence (p=0.045) (**Fig. 5F**).

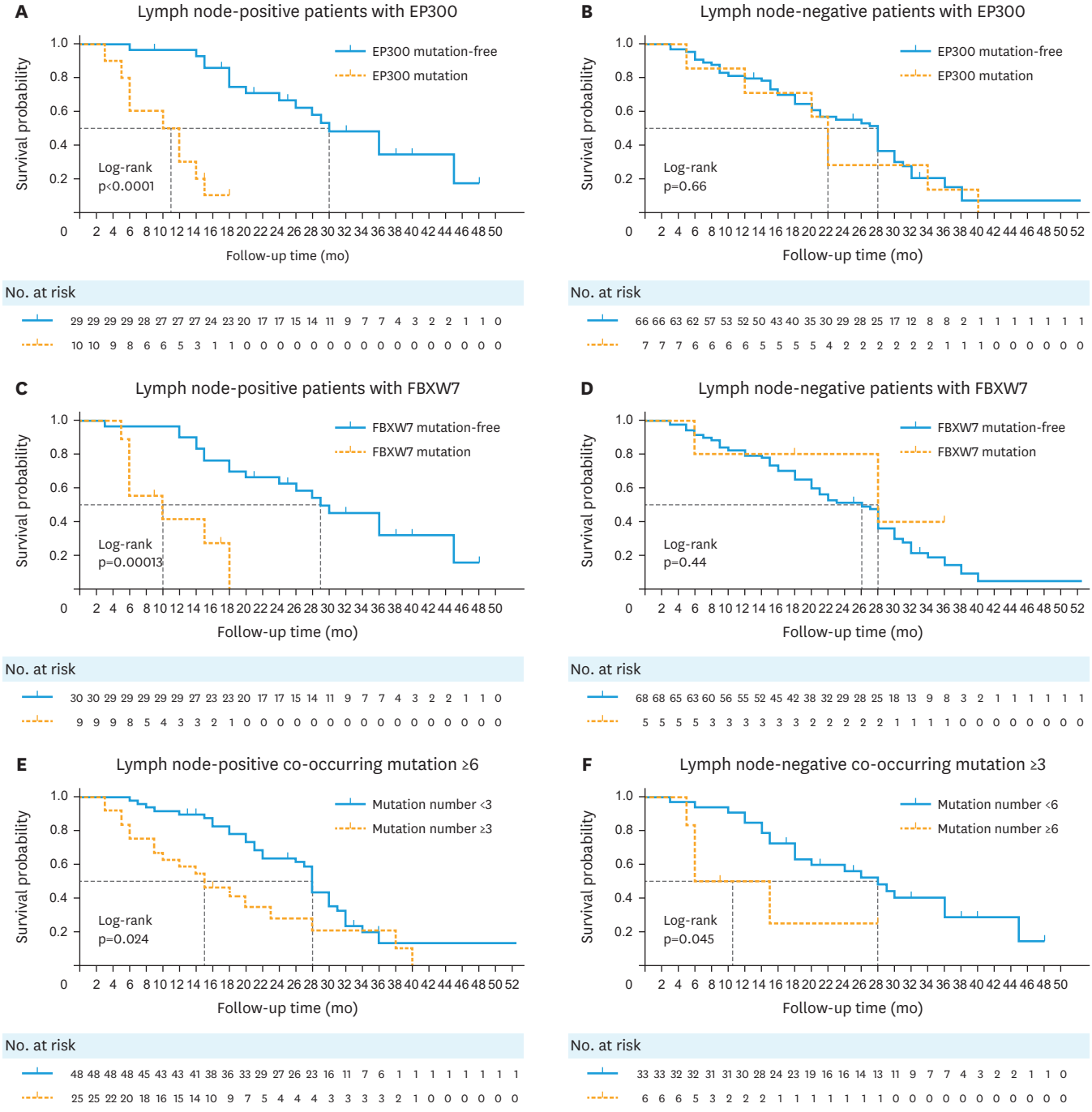

Fig. 5. Overall survival between patients with positive and negative lymph nodes. (A) Lymph node-positive patients with EP300, (B) lymph node-negative patients with EP300, (C) lymph node-positive patients with FBXW7, (D) lymph node-negative patients with FBXW7, (E) lymph node-positive co-occurring mutation ≥6, and (F) lymph node-negative co-occurring mutation ≥3.

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9. Analysis of cervical cancer whole exome sequencing data in TCGA database

Data of 180 patients with cervical cancer were collected from the TCGA database, including 57 patients with positive lymph nodes and 123 patients with negative lymph nodes. A total of 153 patients had mutations, with a mutation rate of 85.0% (**Fig. S4A**). The mutation rates of lymph node-positive and -negative patients were 91.2% and 87.8%, respectively (**Fig. S4B and C**). EP300 and FBXW7 had high mutation frequencies in lymph node-positive patients, but there was no significant difference in mutation frequencies between lymph node-positive and -negative patients (**Fig. S4D**). Mutation coexistence-mutual exclusion analysis found a large number of co-occurring mutations in both lymph node-positive and -negative patients, while mutually exclusive mutations were rare.

DISCUSSION

In this study, a total of 112 patients with cervical cancer were analyzed by 1,021-gene NGS in our center, including 39 patients with positive lymph nodes and 73 with negative lymph nodes. The differences in the gene characteristics of the 2 groups could be used to further study the mechanism of cervical cancer lymph node metastasis at the genetic level. Therefore, we investigated genomic profiles of positive lymph nodes and explored its differences from negative lymph nodes in somatic mutations, pathway-level analysis, mutational signature, therapeutic targets, and TMB by NGS to facilitate precise diagnosis and therapy.

Single-site mutation spectrum analysis found EP300 and FBXW7 gene mutations in cervical cancer, and mutation frequency difference analysis found that there were differences between patients with positive and negative lymph nodes. As a histone acetyltransferase, EP300 regulates transcription through chromatin remodeling, and all four core histones in nucleosomes are acetylated, providing epigenetic marks for transcriptional activation. EP300 mediates cAMP gene regulation by specifically binding to phosphorylated CREB protein [11], and mediates acetylation of histone H3 at Lys-122, a modification localized to the surface of histone octamers that may stimulate transcription by promoting nucleosome destabilization. Lys-27, which mediates histone H3 acetylation, also acts as an acetyltransferase on non-histone targets such as ALX1, HDAC1, PRMT1, and SIRT2. Lys-27 acetylates Lys-131 of ALX1 and acts as its coactivator. Acetylation of SIRT2 indirectly increases p53/TP53 transcriptional activity through acetylation and subsequent attenuation of SIRT2 sirtuin function. After DNA damage, a stress-responsive p53/TP53 coactivation complex with JMY is formed, mediating p53/TP53 acetylation, and increasing p53/TP53-dependent transcription and apoptosis. In other literature reports, EP300 is also involved in the invasion and metastasis of oral squamous cell carcinoma, breast cancer, pancreatic cancer and other tumors. EP300 plays a central role in the regulation of cell proliferation, which has prompted the development of specific inhibitors of EP300's enzymatic activity and protein-protein interaction [12], but the current research is relatively scattered and most of them are preclinical researches. In our OS analysis, lymph node-positive patients with EP300 mutations had lower OS than EP300 mutations-free after recurrence ($p<0.05$),while there were no difference in OS between lymph node-negative patients with EP300 mutations and EP300 mutations-free after recurrence($p>0.05$).EP300 mutations predicted poor prognosis in lymph node-positive patients. Regulating the activity of EP300 may be potential avenues for the treatment of lymph node metastasis in cervical cancer.

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FBXW7 is a substrate recognition component of the SCF (SKP1-CUL1-F-box protein) E3 ubiquitin-protein ligase complex, mediating ubiquitination and subsequent proteasomal degradation of target proteins. FBXW7 recognizes and binds phosphorylation sites/phosphorylons in the target protein, and then brings it to the SCF complex for ubiquitination. FBXW7 acts as a negative regulator of JNK signaling by binding to phosphorylated JUN, promoting its ubiquitination and subsequent degradation. FBXW7 involves in negative regulation of bone homeostasis and osteoclast differentiation. Through the ubiquitination and proteasomal degradation of its transcriptional repressor NR1D1, FBXW7 regulates the circular expression amplitude of liver core clock genes and lipid and glucose metabolism-related genes. As an important tumor suppressor gene, FBXW7 participates in the regulation of multiple biological processes in cells, and its mutation or deletion can promote the occurrence and development of tumors [13]. In other literature reports, FBXW7 mutation is also involved in the invasion and metastasis of endometrial cancer, ovarian cancer, oral squamous cell carcinoma, esophageal cancer and other tumors. FBXW7 is lowly expressed in cervical cancer tissues, but highly expressed in normal cervical tissues, and the low expression of FBXW7 is related to vascular tumor thrombus, tumor size, lymphatic metastasis, histological grade and squamous cell carcinoma antigen. Kitade et al. [14] found only one patient with single FBXW7 mutation among 57 patients with ovarian cancer, indicating that the mutation of FBXW7 in ovarian cancer is rare. The expression of FBXW7 in ovarian borderline tissues was significantly higher than that in ovarian cancer patients, indicating that FBXW7 negatively correlates with tumor malignancy [14]. In our OS analysis, lymph node-positive patients with FBXW7 mutations had lower OS than FBXW7 mutations-free after recurrence ($p<0.05$), while there were no difference in OS between lymph node-negative patients with FBXW7 mutations and FBXW7 mutations-free after recurrence($p>0.05$). FBXW7 mutations predicted poor prognosis in lymph node-positive patients.

Co-occurrence and mutual exclusivity analysis found a large number of co-occurring mutations in both lymph node-positive and negative patients, while mutually exclusive mutations were rare. Co-occurrence reflect the interaction mechanism between gene mutations. There may be a functional synergistic mechanism between the co-occurring mutant genes, and there may be a mutual antagonism mechanism between the mutually exclusive mutant genes [15]. A large number of co-occurring genes was associated with a poor prognosis, while a large number of mutually exclusive mutations was associated with good prognosis. This phenomenon was common in cancers such as lung cancer and leukemia [16], which may have important significance for predicting the prognosis of cervical cancer. In our OS analysis, co-occurring mutation predicted poor prognosis in both lymph node-positive and negative patients, the co-occurring mutation number cut-off value were 6 and 3, which could predict prognosis.

Mutational signatures analysis found that SBS3 was detected in lymph node-positive patients but not in lymph node-negative patients, which indicated defects in DNA-double strand break repair by homologous recombination. The significantly reduced capacity of DNA double-strand break-repair was also a genetic signature of lymph node metastasis in cervical cancer. Future studies linking this signature of unknown origin with cancer risk factors may provide insights into mechanisms and offer avenues for further research into lymph node metastasis.

Analysis of oncogenic signaling pathways found that RTK-RAS, PI3K, and NOTCH were the most enriched pathways, but there was no difference in oncogenic signaling pathways between patients with positive and negative lymph nodes. KEGG pathway, GO enrichment

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analysis, and CNVs showed no differences between lymph node-positive and -negative patients. In other studies, many signal pathways related to lymph node metastasis of cervical cancer have been reported, such as fatty acid-binding protein 5, fatty acid synthase, receptor for activated C kinase 1, ligand 1, C kinase 1, hematological and neurological expressed 1 gene, etc. The pathway of lymph node metastasis in cervical cancer was identified and promoted lymph node metastasis, but these studies did not control lymph node-negative patients [17-24].

Aside from targeted therapy, immunotherapy has also expanded the therapeutic regimens for cervical cancer. Previous studies have identified TMB as a promising biomarker for immunotherapy response [25]. In our study, PIK3CA mutation was significantly positively correlated with TMB, which might help identify specific patients who benefit from immunotherapy. More clinical studies are needed to confirm this relationship.

In our study, we also searched the relevant data of cervical cancer lymph node metastasis from the TCGA database. The mutation frequencies of EP300 and FBXW7 in lymph node-positive patients were high, but no significant differences were observed between lymph-node positive and -negative patients. This discrepancy between the TCGA data and our results might be due to the difference in detection method or patient's baseline characteristics. In addition, mutation coexistence was also found in the TCGA database. A large number of co-occurring mutations was found in both node-positive and -negative patients, while mutual exclusion mutations were rare, consistent with our findings.

Our study has several limitations. This was a single-center retrospective study and the sample size was small. Thus, the potential bias might exist. Despite the results were validated using the data from the TCGA database, the sample size from the TCGA database was still small. A large-scale study is still needed for further investigation. The imbalance of sample size between lymph node-positive and negative group may result in statistical bias. The molecular mechanism related to the occurrence and development of lymph node metastasis found in this study was still lacking in further exploration and verification. The sequencing tissue samples were from lymph node or other recurrence lesion rather than primary cervical tumor. As a result that this study focus on lymph node metastasis of cervical cancer, at the point of recurrence, lymph node sample was accessed directly for lymph node metastasis case, the other metastasis lesion was accessed directly for lymph node negative case, which could make sure fresh tumor tissue and pertinence for lymph node. If we selected initial primary cervical tissue after recurrence, the long preserve interval sample could influence the gene test result. There were still 52 cervical or vagina tissue samples for negative lymph node group, which was the most sample resource. We also made peripheral blood samples as control, and germ line mutation frequency was extremely low (3.6%).

To our knowledge, this study comprehensively demonstrated the different gene characteristics of cervical cancer patients with lymph node metastasis. We deeply analyzed the differences in somatic mutations and gene expression patterns between lymph node-positive or negative patients, as well as biological pathways that may play an important role in progress of lymph node metastasis. Our finding of survival analysis may provide new insights into personalized treatment for lymph node metastasis patients. The somatic SNV/Indels of EP300 and FBXW7, SBS3 homologous recombination-mediated DNA repair defect signature were enriched in lymph node-positive patients. For lymph node-positive patients, EP300 or FBXW7 mutation predicted poor prognosis. No matter lymph node-positive or

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negative, more co-occurring mutation number predicted poor prognosis. PIK3CA mutation may account for the higher TMB and help identify patients who benefit from immunotherapy. These different features might be potential molecular markers to predict lymph node metastasis and prognosis in cervical cancer.

SUPPLEMENTARY MATERIALS

Table S1

Difference in gene alterations between lymph node-positive and -negative patients

Fig. S1

Gene list of the 1,021-gene panel.

Fig. S2

Differences in gene SNV/Indels mutation frequency between lymph node-positive and -negative patients.

Fig. S3

Analysis of KEGG pathway and GO enrichment in lymph node-positive and -negative patients. (A) KEGG in lymph node-positive patients, (B) KEGG in lymph node-negative patients, (C) GO enrichment in lymph node-positive patients, and (D) GO enrichment in lymph node-negative patients.

Fig. S4

Cervical cancer whole exome sequencing data of 180 patients with cervical cancer from TCGA database. (A) All patients, (B) lymph node-positive patients, (C) lymph node-negative patients, and (D) differences in gene mutations.

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