Whole-exome sequencing in cervical adenocarcinoma in mainland Chinese patients

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\textbf{Background:} Cervical cancer is the most common gynecological malignancy worldwide. Adenocarcinoma is an important pathological type of cervical cancer. In recent years, the incidence of adenocarcinoma is rising in some countries and the prognosis of it remains poor. A precise description of the mutational landscape in cervical adenocarcinoma may provide insights into a better selection of treatments and improve prognosis.

\textbf{Methods:} In this study, we conducted whole-exome sequencing (WES) for cervical adenocarcinomas and matched blood samples from a cohort of 24 mainland Chinese patients. Additionally, the Human-Papilloma virus (HPV) infection statuses of these tumor samples were detected, and the genes that were enriched in both HPV positive and negative samples were also analyzed.

\textbf{Results:} The results of WES revealed the gene expression profile of cervical adenocarcinoma of women in mainland China and identified multiple genes/pathways, which are frequently mutated in these tumors, including the PI3K-AKT (\textit{KRAS}, \textit{PIK3CA} and \textit{PTEN}), estrogen signaling (\textit{KRAS}, \textit{PIK3CA} and \textit{GNAS}) and NK cell-mediated antibody-dependent cellular cytotoxicity pathways. Besides, seven patients had HPV infection, and the mutated genes in HPV-positive tumor tissues were relatively consistent, while the mutation profiles of HPV-negative tumor tissues were relatively scattered.

\textbf{Conclusions:} Taken together, these findings provide novel insights into the pathogenesis of cervical adenocarcinomas. They suggest the potential for individualized treatment of cervical adenocarcinoma according to genomic information.

\textbf{Keywords:} Cervical adenocarcinoma; whole-exome sequencing (WES); somatic mutations

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\textbf{Introduction}

With the promotion and popularization of cervical cancer screening, the global incidence of cervical cancer has increased in recent years (1,2). Cervical cancer markedly contributes to the cancer burden in China, with 110,000 new cases and 34,000 related deaths in 2018 (3). Among the different pathological types of cervical cancer, adenocarcinoma is second only to squamous cell carcinoma, accounting for 10–25% of cervical cancer cases. Furthermore, adenocarcinoma of the cervix is an
aggressive tumor and can progress to lymph node or distant metastases at an early stage. With poor sensitivity to the radiation therapy, cervical adenocarcinoma is considered by most researchers to have a poor prognosis. Even though the pathogenesis of cervical cancer has been extensively studied, the exact mechanisms of carcinogenesis remain unclear (4). It is widely believed that persistent infection with oncogenic types of human papillomavirus (HPV), especially HPV18, represents the most important risk factor for the development of cervical adenocarcinoma (5,6). Nevertheless, HPV infection alone is not sufficient for neoplastic transformation, and other genetic events, independent or in combination with HPV infection, are required (7). Therefore, a deeper understanding of the molecular basis of cervical adenocarcinoma, and the development of new and more effective therapy, remain unmet medical needs.

In the current study, we sequenced the exomes of a large cohort of fresh-frozen cervical adenocarcinoma tissue, and determined the gene expression profile of cervical adenocarcinoma of women in mainland China. The results identified alterations in multiple genes of the PIK3CA/AKT/mTOR, estrogen signaling, and NK (natural killer) cell–mediated antibody-dependent cellular cytotoxicity pathways.

We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/tcr-19-2930).

Methods

Clinical material

Pretreatment tumor tissue and matched blood samples from 24 mainland Chinese patients with cervical adenocarcinoma were collected from the Department of Gynecologic Oncology, Shandong Cancer Hospital, from January 2018 to January 2019. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee of Shandong Cancer Hospital (No.: SDSZLYY20190315), and individual consent for this retrospective analysis was waived. All hematoxylin-eosin-stained sections of tumor tissues were reviewed by a pathologist to confirm the histological type. Genomic DNA isolated from peripheral blood was obtained from each patient as a corresponding control.

Whole exome-sequencing (WES)

DNA extraction and quality control

Genomic DNA was extracted from tumor samples and matched blood cells using the DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Careful quality control steps were applied to ensure that high-quality DNA samples were obtained. These steps included agarose gel electrophoresis tests to confirm that there was no obvious DNA degradation or RNA contamination, testing of DNA fragment sizes (Agilent Technologies, Santa Clara, CA, USA), and testing of OD values using a NanoDrop (Thermo Scientific, MA, USA).

Library construction, target capturing and sequencing

Matched tumor and blood cell samples from 24 cervical cancer patients were subjected to exome sequencing using the Illumina platform. Exome capture and paired-end sequencing of DNA was performed using the Paired-End Sample Preparation Kit (Illumina, San Diego, CA, USA) followed by in-solution capture of genomic DNA using the SureSelect Human All Exon V6 Kit (Agilent Technologies, Santa Clara CA, USA), targeting 37 Mb of exonic sequence. For each sample, captured DNA was sequenced by multiplexed paired-end sequencing using 2 pools of samples on the Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA), which yielded 10 Gb of reads with a read length of 150 bp.

Identification of somatic mutation sites and downstream analysis

Raw reads were filtered using fastp (v0.19), and the resulting clean reads were mapped to the human reference genome version 38 (hg38) using bwa (v0.7). Then, GATK (v4.1) was used to call somatic variants for each sample pair. Somatic mutations were filtered by criterion of frequency <0.01 in 1,000 Genomes, and nonsynonymous mutations in coding regions. These high-quality somatic mutation sites were annotated using ANNOVAR (v201804) with the refGene, 1,000 Genomes, dbSNP and COSMIC databases. Sites matching relevant records in COSMIC were used for further analysis. The flowchart of result analysis is indicated in Figure S1. The PATHVIEW platform was used to visualize tumor pathways related to the corresponding genes.
**HPV infection status and integrating locus**

HPV genotypes for each sample were determined by polymerase chain reaction (PCR) and reverse dot hybridization. Fifteen high-risk HPV types \([16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82] \), three likely high-risk HPV types \([26, 53, 66] \), and ten low-risk HPV types \([6, 11, 40, 42, 43, 44, 54, 61, 81, 83] \) were examined. In the PCR step, 20 μL of extracted genomic DNA was centrifuged at 6,000 rpm for 5 s and then amplified with the following thermal profile: 3 min of preparation at 50 °C; 15 min of activation at 95 °C; 40 cycles of 40 s of denaturation at 94 °C, 40 s of annealing at 55 °C, and 40 s of extension at 72 °C, and a 7-min final extension at 72 °C. After removal from the thermal cycler, samples were stored at 4 °C for more than 2 min. In the hybridization step, the corresponding HPV nylon membrane strip, 4 mL of hybrid solution I and 40 μL of the PCR products were mixed in a well and reacted in a shaking (100 rpm) water bath at 42 °C for 1 h. Following hybridization, the strips were briefly rinsed and mixed with 3 mL of prewarmed (42 °C) solution II in another well, and the trays were incubated in a shaking water bath at 42 °C for 10 min. After the stringent wash, the buffer was removed, 6 mL of prewarmed (42 °C) solution II was added to each well, and the tray was placed in a shaking water bath at 42 °C for 10 min. After the final wash, the buffer was removed, and the strips were rinsed and photographed within 2 h of color development in a sealed plastic bag in the dark. HPV genotyping results were obtained by scanning the photographed strips.

To detect the genomic integrating locus for each HPV-positive sample, WES read pairs with one segment mapped and the mate unmapped were filtered using Samtools. The unmapped mate sequences were used as BLAST queries to search for homologous fragments in 180 HPV genomes obtained from the GenBank database. Hits with E-values lower than 10-10 and query coverage more than 80% were regarded as positive hits, and the positions of their mate reads were regarded as putative HPV integrating loci.

**Statistical analysis**

R 3.6.0 statistical software was used for data analysis. Statistical analysis was performed with Wilcoxon Rank Sum Tests and Fisher’s Exact Tests. P<0.05 was considered statistically significant.

**Results**

**Clinical material**

Tumor tissue and matched blood samples from 24 mainland Chinese women with cervical adenocarcinoma [9 stage I, 6 stage II, 8 stage III, and 1 stage IV, according to the International Federation of Gynecology and Obstetrics (FIGO) staging system] aged between 25 and 71 years at the time of diagnosis were included in the present study. Of the 24 patients, 7 had HPV infection, and among these patients, 3 were positive for HPV16, 3 were positive for HPV18, and 1 was positive for HPV16 and HPV18. In 3 of the patients, the HPV was integrated into the genome. The demographic details of these patients are summarized in Table 1.

**Quality control and comparison results**

According to the description in the Materials and Methods, the quality control results of the 24 cervical adenocarcinomas and matched blood cell samples met the requirements. Similarly, the alignment of the above specimens also met the requirements, indicating that these samples were not contaminated (Figure S2).

**Exome sequencing and somatic variations**

The peripheral blood control of each patient was used to filter out the germline variations, and further quality filtering led to the detection of 23,139 somatic variations falling within coding regions, of which 19,954 (86.2%) were nonsynonymous single-nucleotide variants (SNVs), 1,742 (7.5%) were stop-gain mutations, and the remaining variations were mainly frameshift mutations and non-frameshift variations in the 24 tumors (Figure 1A). Further analysis found that the mutation count of HPV-positive patients was far lower than that of the HPV-negative cases in stage I; however, there was only a slight difference in mutation count between HPV-positive and HPV-negative patients in stage II and III patients (Figure 1B,C,D). Venn diagram was used to compare the mutated genes among these patients in each stage (Figure 2).

**Gene mutation frequency in patients**

Figure 3A indicates that 70 genes were found to be mutated
Table 1 Demographic details of the 24 patients included in this study

| NO  | Histology       | FIGO stage | Age (years) | HPV type       | Integration locus |
|-----|-----------------|------------|-------------|----------------|------------------|
| T1  | Adenocarcinoma  | IIIB       | 62          | HPV18          | Unknown          |
| T2  | Adenocarcinoma  | IB1        | 50          | Not detected   | –                |
| T3  | Adenocarcinoma  | IIIB       | 41          | Not detected   | –                |
| T4  | Adenocarcinoma  | IB2        | 56          | Not detected   | –                |
| T6  | Adenocarcinoma  | IIIB       | 62          | HPV18          | 16q21            |
| T8  | Adenocarcinoma  | IB         | 38          | Not detected   | –                |
| T9  | Adenocarcinoma  | IIA        | 31          | Not detected   | –                |
| T12 | Adenocarcinoma  | IIIA       | 66          | Not detected   | –                |
| T13 | Adenocarcinoma  | IIIB       | 58          | Not detected   | –                |
| T14 | Adenocarcinoma  | IB         | 43          | Not detected   | –                |
| T16 | Adenocarcinoma  | IB         | 41          | Not detected   | –                |
| T18 | Adenocarcinoma  | IIIB       | 71          | Not detected   | –                |
| T23 | Adenocarcinoma  | IB         | 34          | HPV16, HPV18   | 1p36.12, 1p32.3, 10q22.2, 12p13.31, 17p12, 17q12, 17q22, 3p21.1, 6p25.2, 9q21.12, 9q22.33, 9q34.13, |
| T26 | Adenocarcinoma  | IV         | 66          | Not detected   | –                |
| T27 | Adenocarcinoma  | IIIB       | 62          | HPV16          | Unknown          |
| T28 | Adenocarcinoma  | IB         | 25          | Not detected   | –                |
| T30 | Adenocarcinoma  | IIIB       | 61          | Not detected   | –                |
| T32 | Adenocarcinoma  | IIIB       | 47          | Not detected   | –                |
| T33 | Adenocarcinoma  | IIIB       | 66          | HPV16          | Unknown          |
| T34 | Adenocarcinoma  | IB1        | 43          | HPV18          | Unknown          |
| T36 | Adenocarcinoma  | IIIB       | 71          | Not detected   | –                |
| T37 | Adenocarcinoma  | IIIB       | 50          | HPV16          | 12q22, 18q21.33  |
| T39 | Adenosquamous   | IB1        | 49          | Not detected   | –                |
| T41 | Adenocarcinoma  | IIIB       | 30          | Not detected   | –                |

FIGO, International Federation of Gynecology and Obstetrics; HPV, human papillomavirus.

in at least 3 tumors. Figure 3B shows that the mutated genes in HPV-positive tumor tissues were relatively consistent, with 17 genes enriched in these samples (Table 2), while the mutation profiles of HPV-negative tumor tissues were relatively scattered, with only 1 gene, HUWE1, enriched in HPV-negative tumor tissue (Table 3). The top 10 most frequently mutated genes among these tumor sample are summarized in Table 4.

**Somatic mutations associated with cervical cancer**

The Catalogue of Somatic Mutations in Cancer (COSMIC) database was used to annotate the variation sites of each sample, and the variation of cervical cancer was screened by linking each mutation site. Screening results showed that 8 sites in 6 genes (KRAS, NRAS, PIK3CA, PTEN, GNAS, HLA-A) were associated with cervical adenocarcinoma.
Figure 1 Somatic mutations in cervical adenocarcinoma. (A) Somatic mutation types in tumors of 24 cervical adenocarcinoma patients. (B-D) Somatic mutation counts of HPV-negative and HPV16-positive patients with stage I, II, and III cervical adenocarcinoma. FIGO, International Federation of Gynecology and Obstetrics; HPV, human papillomavirus; SNV, single-nucleotide variants.

Figure 2 Venn diagram of somatic mutated genes between HPV negative and HPV positive patients with stage I, II and III cervical adenocarcinoma. FIGO, International Federation of Gynecology and Obstetrics; HPV, human papillomavirus.
Figure 3 Gene mutation frequency in patients. (A) Heat map of frequently mutated genes. In all, 70 genes were found to be mutated in at least 3 tumors. (B) The most mutated genes in all the samples (orange), genes with high mutation rates in HPV-positive tissues (green), and genes with high mutation rates in HPV negative tissues (blue).
Table 2 Genes enriched in HPV-positive samples

| Gene  | HPV positive Samples with mutations | HPV positive Samples without mutations | HPV negative Samples with mutations | HPV negative Samples without mutations | P value |
|-------|------------------------------------|---------------------------------------|------------------------------------|---------------------------------------|---------|
| DPY19L3 | 4 | 3 | 2 | 15 | 0.038 |
| FAM110A | 3 | 4 | 0 | 17 | 0.017 |
| ITGB3 | 3 | 4 | 0 | 17 | 0.017 |
| KDM4B | 3 | 4 | 0 | 17 | 0.017 |
| MCF2L2 | 3 | 4 | 0 | 17 | 0.017 |
| MYOM1 | 4 | 3 | 0 | 17 | 0.003 |
| NUMA1 | 5 | 2 | 2 | 15 | 0.009 |
| PVR | 3 | 4 | 0 | 17 | 0.017 |
| RSBN1 | 3 | 4 | 0 | 17 | 0.017 |
| TEFM | 3 | 4 | 0 | 17 | 0.017 |
| TRIP11 | 3 | 4 | 0 | 17 | 0.017 |
| TWSG1 | 3 | 4 | 0 | 17 | 0.017 |
| TYW1B | 3 | 4 | 0 | 17 | 0.017 |
| USP19 | 3 | 4 | 0 | 17 | 0.017 |
| UTP20 | 4 | 3 | 2 | 15 | 0.038 |
| VWA8 | 3 | 4 | 0 | 17 | 0.017 |
| ZFYVE26 | 4 | 3 | 2 | 15 | 0.038 |

HPV, human papillomavirus; DPY19L3, dpy-19 like C-mannosyltransferase 3; FAM110A, family with sequence similarity 110 member A; ITGB3, integrin subunit beta 3; KDM4B, lysine demethylase 4B; MCF2L2, MCF2 cell line derived transforming sequence-like 2; MYOM1, myomesin 1; NUMA1, nuclear mitotic apparatus protein 1; PVR, PVR cell adhesion molecule; RSBN1, round spermatid basic protein 1; TEFM, transcription elongation factor, mitochondrial; TRIP11, thyroid hormone receptor interactor 11; TWSG1, twisted gastrulation BMP signaling modulator 1; TYW1B, tRNA-γW synthesizing protein 1 homolog B; USP19, ubiquitin specific peptidase 19; UTP20, UTP20 small subunit processome component; VWA8, von Willebrand factor A domain containing 8; ZFYVE26, zinc finger FYVE-type containing 26.

Table 3 Gene enriched in HPV-negative samples

| Gene  | HPV positive Samples with mutations | HPV positive Samples without mutations | HPV negative Samples with mutations | HPV negative Samples without mutations | P value |
|-------|------------------------------------|---------------------------------------|------------------------------------|---------------------------------------|---------|
| HUWE1 | 1 | 6 | 12 | 5 | 0.018 |

HUWE1, HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1. HPV, human papillomavirus.

(Table 5). Further analysis showed that these six were mainly involved in the PIK3CA/AKT/mTOR pathway, estrogen signaling, and natural killer (NK) cell–mediated antibody-dependent cellular cytotoxicity (Figure 4). Therefore, these signaling pathways are the major alterations in cervical adenocarcinoma.

Discussion

This study conducted whole-exome sequencing (WES) of fresh-frozen tumors from a cohort of mainland Chinese patients with cervical adenocarcinoma. The results revealed a heterogeneous mutation spectrum, and identified some frequently altered genes, including KRAS/NRAS, PIK3CA/
Table 4: The top 10 most mutated genes in all samples

| Gene   | Samples with mutations | Proportion  |
|--------|------------------------|-------------|
| TTN    | 22                     | 91.67%      |
| MUC16  | 14                     | 58.33%      |
| OBSCN  | 14                     | 58.33%      |
| SYNE1  | 14                     | 58.33%      |
| HUWE1  | 13                     | 54.17%      |
| PLEC   | 13                     | 54.17%      |
| FRAS1  | 12                     | 50.00%      |
| MUC3A  | 12                     | 50.00%      |
| SYNE2  | 12                     | 50.00%      |
| VPS13D | 12                     | 50.00%      |

TTN, titin; MUC16, mucin 16, cell surface associated; OBSCN, obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF; SYNE1, spectrin repeat containing nuclear envelope protein 1; HUWE1, HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1; PLEC, plectin; FRAS1, Fraser extracellular matrix complex subunit 1; MUC3A, mucin 3A, cell surface associated; SYNE2, spectrin repeat containing nuclear envelope protein 2; VPS13D, vacuolar protein sorting 13 homolog D.

Table 5: Variations data related to the cervical adenocarcinoma detect by whole exome sequencing

| Chromosome | Location | Gene   | Variation type | Variation | Rs No.       | Sample mutation abundance | COSMIC ID         | FATHMM evaluation |
|------------|----------|--------|----------------|-----------|--------------|---------------------------|-------------------|-------------------|
| chr20      | 58909365 | GNAS   | Nonsynonymous SNV | c.2530C>T p.R844C | rs11554273 | T33:16.6%; T37:29.6% | COSM123397; COSM27887 | Pathopoiesis      |
| chr6       | 29942967 | HLA-A  | Stop gain       | c.284C>G p.S95  | rs121913529 | T8:10.6%               | COSM4847247; COSM4847248 | Neutrality         |
| chr12      | 25245350 | KRAS   | Nonsynonymous SNV | c.35G>A p.G12D | rs112445441 | T36:25.8%; T4:27.2% | COSM1135366; COSM532 | Pathopoiesis      |
| chr12      | 25245347 | KRAS   | Nonsynonymous SNV | c.38G>A p.G13D | rs11554290 | T14:21.9%               | COSM584           | Pathopoiesis      |
| chr1       | 114713908| NRAS   | Nonsynonymous SNV | c.182A>G p.Q61R | rs121913279 | T33:10.1%               | COSM94986; COSM775 | Pathopoiesis      |
| chr3       | 179234297| PIK3CA | Nonsynonymous SNV | c.3140A>G p.H1047R | rs121909224 | T14:24.3%               | COSM5154         | Pathopoiesis      |
| chr10      | 87933147 | PTEN   | Stop gain       | c.388C>T p.R130 | rs121909219 | T4:49.6%               | COSM5154         | Pathopoiesis      |
| chr10      | 87957915 | PTEN   | Stop gain       | c.697C>T p.R233 |             |                           |                   |                   |

COSMIC, Catalogue of Somatic Mutations In Cancer; SNV, single-nucleotide variants; FATHMM, Functional Analysis Through Hidden Markov Models; GNAS, GNAS complex locus; HLA-A, major histocompatibility complex, class I, A; KRAS, KRAS proto-oncogene, GTPase; NRAS, NRAS proto-oncogene, GTPase; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PTEN, phosphatase and tensin homolog.
Figure 4 Mutant genes related to cervical adenocarcinoma detected by whole-exome sequencing and the associated signaling pathways, including the PI3K-AKT (KRAS, PIK3CA and PTEN), estrogen signaling (KRAS, PIK3CA and GNAS), and NK cell-mediated antibody-dependent cellular cytotoxicity pathways.

...and metabolism (17). PIK3CA and PTEN have been established as the main genes involved in alterations in this signaling cascade (18). Our results indicated that 4.5% of tumor samples harbored nonsynonymous SNVs of PIK3CA, and 9.1% harbored stop-gain mutants of the PTEN gene. The recently reported mutations in ERBB2 were not detected in this study, possibly because of the low sample size. These results suggest the involvement of this signaling cascade in cervical adenocarcinoma. Moreover, preclinical data showed that patients with PIK3CA mutations exhibited a high response rate to PI3K/AKT/mTOR pathway inhibitors (19). Similarly, loss of enzyme activity induced by somatic missense mutation of PTEN could be predictive of the efficacy of the aforementioned therapy. These findings indicate that a subgroup of cervical adenocarcinomas might greatly benefit from PI3K/AKT/mTOR axis inhibitors.

Two tumors harbored nonsynonymous SNVs in GNAS. Previous studies have shown that GNAS mutations are detected in intraductal mucinous carcinoma of the pancreas and in mucinous endocervical adenocarcinoma. Furthermore, activation of GNAS via mutation has been found to induce high adenyl cyclase activity and improve the level of adenosine 3',5'-monophosphate (cAMP) (20-22). The GPCR pathway is a known primary target for pharmaceutical research, and a medicine targeting GPCRs has been indicated to inhibit the malignant phenotypes of various human tumor cells. The results of this study provide potential treatment options for patients with cervical adenocarcinoma.

To our knowledge, this is the first study in which WES...
has been applied to define the mutational landscape of cervical adenocarcinoma in mainland Chinese patients, and the results identified multiple genes/pathways that are frequently mutated in these tumors. These findings will help guide further study and targeted therapies against this cancer worldwide.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee of Shandong Cancer Hospital (No.: SDSZLYY20190315), and individual consent for this retrospective analysis was waived.

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