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The next generation sequencing of cancer-related genes in small cell neuroendocrine carcinoma of the cervix

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HIGHLIGHTS

- Mutated genes in SCNEC were clustering in RTK/RAS (43%), PI3K-AKT (39%), p53 pathway (23%) and MYC family (20%).
- Nearly 14% of SCNEC patients harboring at least one mutation in homogenous recombination repair genes.
- Several novel targetable mutated genes, including *IRS2* (15%) and *SOX2* (6%) were identified with SCNEC patients.
- Two out of 51 SCNCE patients were presented with MSI-H/dMMR.

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ABSTRACT

Objective. Small cell neuroendocrine carcinoma of the cervix (SCNEC) is a lethal malignancy and little treatment progress has been made for decades. We sought to map its genetic profiles, and identify whether SCNEC harbor mutations and potential targets for therapeutic interventions.

Methods. Primary tumor tissue and blood samples were obtained from 51 patients with SCNEC. The next-generation sequencing was carried out to detect mutations of 520 cancer-related genes, including the entire exon regions of 312 genes and the hotspot mutation regions of 208 genes. Quantitative multiplex PCR was performed for the detection of seven high-risk HPV types.

Results. Of the 51 detected patients, 92.16% were positive for HPV 18. Ninety-eight percent of cases harbored genetic alterations. Two cases were observed with hypermutated phenotype and determined as MSI-H/dMMR. Genetic mutations were clustering in RTK/RAS(42.86%), PI3K-AKT(38.78%), p53 pathway(22.45%) and MYC family(20.41%). Mutations in genes involved in the p53 pathway indicate a poorer prognosis (3-year OS, 33.5% vs 59.9%, $p = 0.031$). A total of seven patients harboring mutations in homogeneous recombination repair (HRR) genes were reported. In addition, *IRS2* and *SOX2* were amplified in 14.9% and 6.12% of SCNEC patients, respectively.

Conclusions. SCNEC is specifically associated with HPV 18 infection. Its genetic alterations are characterized by a combined feature of high-risk HPV driven events and mutations observed in common neuroendocrine carcinoma. We identified several targetable mutated genes, including *KRAS, PIK3CA, IRS2, SOX2,* and HRR genes, indicating the potential efficacy of target therapies in these patients. MSI-H/dMMR individuals may benefit from checkpoint blockade therapies.

1. Introduction

Neuroendocrine neoplasias (NENs) are aggressive malignancies derived from neuroendocrine cells. The term neuroendocrine refers to the fact that the tumor cells originate from the embryonic neuroectoderm and display an immunohistochemical profile consistent with endocrine

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glandular cells [1]. Neuroendocrine of the cervix is a highly lethal gynecologic malignancy accounting for 1–1.5% of all cervical cancers [2]. Among all histologic subtypes of cervical neuroendocrine cancer, including small cell, large cell, low-and high-grade carcinoid, small cell neuroendocrine of the cervix (SCNEC) is the most common one. Unlike its squamous cell carcinoma (ACC) and adenocarcinoma (AC) counterparts, SCNEC shows much more aggressive behavior for its wide involvement of lymph node (41.6–67%) and metastatic propensity at an early stage. As a consequence, the prognosis of SCNEC is far worse than common cervical cancer, and the 5-year overall survival is poorer with nearly 30% compared to more than 65% for ACC and AC [3,4].

Due to the rarity of this neoplasm, there has been no one prospective clinical study concerning the standard treatment algorithm of SCNEC. Given the similar histologic signatures and aggressive nature of different originating neuroendocrine carcinomas, the treatment strategies for SCNEC derived from the therapy of small cell lung cancer, the most common NENs, as well as cervical cancer in general. Both documents published by the Society of Gynecologic Oncology (SGO) in 2011 and the Gynecologic Cancer InterGroup (GCIG) in 2014 proposed a multimodality therapeutic strategy for SCNEC including surgery, chemotherapy, and radiotherapy. However, little treatment progress has been made for decades. The capacity of targeted therapies in this malignancy and the underlying molecular features remain investigated.

Distinct from NENs originating from lung or other rare sites, SCENC is highly associated HPV infection, providing a rationale to specifically study the molecular characteristics of SCNEC. A recent systematic literature review showed the mutations most identified in SCNEC were in the *TP53* (22/86; 26%), *KRAS* (7/60; 12%), *PIK3CA* (8/44; 18%), and *MYC* (8/15; 53%) genes, respectively. Loss of heterozygosity (LOH) was found to be present in 16/53 (30%) cases [2]. To date, genetic studies of SCNEC have mainly covered a narrow gene panel or assessed a small number of samples, which provided limited insight into biological signatures of this disease. Furthermore, the existent researches lacked the comprehensive evaluation of HPV infection status and clinical association [5–8]. To provide a broader molecular profile of SCNEC, we carried out 520-gene based next-generation sequencing (NGS), containing tumor mutation burden (TMB) and microsatellite instability (MSI) analyses, in a large Chinese SCNEC cohort to advance the knowledge of this malignant entity.

2. Methods

2.1. Patients collection and follow-up

Primary tumor tissue and blood samples were obtained from 55 patients with small cell neuroendocrine carcinoma treated at Fudan University Shanghai Cancer Center (FUSCC). These patients underwent radical surgery between 2007 and 2018. Four patients failed for quality control were excluded and a final cohort of 51 patients was available for analysis. Clinicopathological characteristics were recorded, including age, FIGO stage, tumor size, histological type, and lymph node status.

The end of the follow-up period within this cohort was August 30, 2020. The median follow-up period was 59 months. Overall survival (OS) was defined as the period from the completion of surgery to the date of death of any causes. Patients without events were censored from the time point of the last follow-up. The study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (NO.050432–4-1212B) and was implemented according to the approved guidelines. Written informed consent was obtained from all patients.

2.2. Pathological assessment

Pathological slides were independently reviewed by two pathologists specialized in gynecological oncology. Positive staining for synaptophysin (DAK-SYNAP, 1:100; DAKO), chromogranin (LK2H10 + PHES, 1:100, Maixin Biotech), cytokeratin (AE1/AE3, dilution 1:50; DAKO), and P16 (E6H4, Predilute, Roche) was in the diagnosis but was not a requirement. Patients with pure carcinoid or large cell neuroendocrine carcinoma were ruled out from this study. Samples involving mixed components with small cell carcinoma were included and termed “Mixed”.

2.3. DNA extraction

DNA was extracted using a QIAamp DNA FFPE tissue kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA concentration was measured using Qubit dsDNA assay (Thermo Fisher Scientific, Waltham, MA, USA). DNA fragmentation was performed using a Covaris M220 Focused-ultrasonicator (Woburn, MA, USA), followed by end repair, phosphorylation, and adaptor ligation. Fragments of 200–400 bp were selected using AMPure beads (Agencourt AMPure XP Kit, Beckman Coulter, CA, USA), followed by hybridization with capture probe baits, hybrid selection with magnetic beads, and PCR amplification. Subsequently, a high-sensitivity DNA assay was performed to assess the quality and size of all fragments.

2.4. Quantitative multiplex PCR assay

Quantitative multiplex PCR for human papillomaviruses(*HPV*)detection was performed as previously described with minor modifications [5]. In brief, PCR primers and corresponding TaqMan probes were synthesized for HPV 16, 18, 31, 45, 52, and 58. Primers and probes are listed in Supplementary Table S1. Two PCR reactions were performed: one to detect HPV 16, 18, 31, and 45; another to detect HPV 32, 52, 58, and GAPDH , using ABI Q7 (Applied Biosystems). The PCR reaction comprises template DNA(up to 50 ng), 12.5 μl Premix Ex Taq™ (Probe qPCR)(Takara), 10 pmol of each primer, probe mix-1(HPV16 5 pmol, HPV18 15 pmol, HPV31 7.5 pmol, HPV45 2 pmol) or probe mix-2 (HPV33 5 pmol, HPV52 1.5 pmol, HPV58 5 pmol, GAPDH 5 pmol) and ddH_2O to a final volume of 25 μl. Positive result is defined as ΔCT (CT_{hpv}-CT_{GAPDH}) <20.

2.5. The next-generation sequencing assay

The NGS genetic testing panel OncoScreen PlusTM (Burning Rock Dx Ltd., Guangzhou, China) selects 520 genes closely related to cancer mechanisms and targeted therapies, using probe hybridization and high-throughput sequencing to detect the entire exon regions of 312 genes, and the hotspot mutation regions of 208 genes (exon, intron and promoter regions) [6]. It detects variations including point mutations, amplifications and rearrangements of genes that are clinically relevant to cancer.

The sequencing data in the FASTQ format were mapped to the human genome (hg19) using Burrows–Wheeler Aligner 0.7.10. Local alignment optimization, variant calling, and annotation were performed using GATK 3.2, MuTect, and VarScan, respectively. DNA translocation analysis was performed using both Tophat2 and Factera 1.4.3. Gene-level copy number variation (CNV) was assessed using a statistic after normalizing read depth at each region by total read number and region size and correcting GC-bias using a LOESS algorithm. The tumor mutational burden (TMB) was defined as the number of somatic, coding, base substation, and indels per megabase of genome examined. Fusions, CNVs, and noncoding mutations were not counted. Synonymous mutations were counted to reduce sampling noise. White blood cells were used to filter germline mutations. Working flow and case selection are shown in Supplementary Fig. S1.

2.6. Statistical analysis

The relevance between gene mutations and HPV infection was analyzed by Pearson's chi-square test or Fisher's exact test. Survival curves

were constructed using the Kaplan–Meier method and compared with the log-rank test. All tests were two-sided, and a p value <0.05 was considered statistically significant. All analyses were performed with SPSS 23.0 (SPSS Inc.) software.

3. Results

3.1. Clinicopathologic characteristics and prognosis

Of the 51 patients, the median age of diagnosis was 40 years old (range, 23–68). Patients with histologically pure small cell carcinoma were accounted for 70.6%. Staging was in accordance with FIGO staging system (2009). Two patients were present with FIGO IV. Other clinicopathologic characteristics were shown in Table 1.

High-risk HPV infections were detected in all cases (Supplementary Fig. S2). HPV 18 was the most common type and was detected in 47 cases (92.16%). HPV 16 was positive in 22 cases (43.14%). HPV 16 and HPV 18 infections co-occur in 18 patients (35.29%). Interestingly, one case was triple positive for HPV 16, HPV18, and HPV52.

After a median follow-up period of 59 months, twenty-seven patients(52.9%) died. The median OS was 45 months. The 3-year OS and 5-year OS were 59.6% and 43.4%, respectively.

3.2. Genomic findings

Ninety-eight percent (50/51) of patients were positive for somatic genomic alterations. Collectively, the samples contained 267 somatic mutations, including 38 missenses, 17 stop gained or loss, 10 spice sites, 7 protein altering insertions and/or deletions(indel), 32 frameshifts, 41 synonymous, and 22 intron variants. Two patients with extremely high somatic mutation burdens were excluded from overall analyses and were stated elsewhere. As displayed in Fig. 1, the most frequently mutated genes were *TP53* (12.24%), followed by *KRAS* (10.20%), *PIK3CA* (10.20%), *KTM2D* (8.16%), *PTEN* (6.12%), *ATM* (6.12%), *ATRX* (6.12%), *PRKDC* (6.12%). Five out of six patients with *TP53* mutation died within 4 years. Patients with *PIK3CA* mutation were more likely infected with HPV16 compared with *PIK3CA* wild type(66.7% vs 39.5%, $p = 0.414$) though statistically insignificant due to small samples.

Chromosomal copy-number analysis identified recurrent copy gains and losses in 61.22% of patients (30/49) (Fig. 2). We focused on copy number gains in reported oncogenes and losses in tumor suppressor genes. *LATS1* is the only tumor suppressor gene with copy number loss and *LATS1* deletion is detected in one patient. The most commonly amplified genes were *MYC* (14.29%) and *IRS2* (14.29%), followed by *TERT* (12.24%). The *IL17R*, *RICTOR* genes located on chromosome 5p13 were frequently co-amplified with an incidence of 10.20%. *CDK8* is also amplified in 10.20% of patients, along with several genes adjacently located on chromosome 13q12 (*FLT3, FLT1, BRCA2*). In addition, we identified *SOX2* amplification in 6.12% of cases. Structural arrangements were identified in three patients with EPHA3-DTNA fusion, SLX4-TP53 fusion, and NOTCH3-BRD4 fusion, respectively. Of note, patients with *MYC* amplification is significantly younger than those without ($p = 0.04$) (Supplementary Fig. S3).

Table 1
Clinicopathological features of SCNEC patients.

Characteristics	Cases	%
Age		
≤40	28	54.9
>40	23	45.1
FIGO stage		
IB1	14	27.5
IB2	6	11.8
IIA1	12	23.5
IIA2	17	33.3
IV	2	3.9
Histological homology		
Pure	36	70.6
Mixed	15	29.4
Tumor size		
≤4 cm	32	62.7
>4 cm	19	37.3
LN metastasis		
No	25	49.0
Yes	26	51.0

Abbreviations: LN: lymph node;

3.3. Mutations were clustering in RTK/RAS, PI3K-AKT, MYC, and TP53 pathway

Notably, genetic alterations were clustering in particular gene families and pathways (Fig. 3), including RTK/RAS pathway (*KRAS, ERBB2, FLT3, ROS1*, etc), as well as PI3K-AKT-mTOR (PAM) pathway (*PIK3CA, PTEN, AKT1, AKT2, RICTOR*, etc). Interestingly, 42.86% ($n = 21$) of patients had some mutations among recurrent RTK/RAS pathway and 38.78% ($n = 19$) of patients had at least one alteration among recurrent PAM pathway. Other recurrent mutations include those in the p53 pathway (*TP53, ATM, MDM4*) and MYC family (*MYC, MYCN, MYCL*). Patients harboring mutants in p53 pathway genes showed a worse prognosis compared with those with wild-type p53 pathway genes (3-year OS, 33.5% vs 59.9%, $p = 0.031$) (Supplementary Fig. S4). While no significant difference in survival rates was found between patients with mutational and wild-type genes involved in RTK/RAS, PI3K-AKT, MYC pathway (Supplementary Table S2).

3.4. Identification of mutants of BRCA2 and HRR genes

BRCA2 somatic mutations were observed in two patients (p.S36C, p.P2827fs). No *BRCA1* genomic alterations were found. Besides, genetic alterations were also observed in other homogeneous recombination repair (HRR) genes, including *ATM*, *PALB2*, *FANCA*, *FANCL*, *FANCF*. In total, we identified nine HRR gene mutations in seven patients. Fig. 4 shows the detailed mutation spectrum of these HRR genes. These Patients were more likely infected with HPV16 (71.4% vs 38.2%, $p = 0.216$). The mutational status of these HRR genes failed to show significant correlations with prognosis (Supplementary Table S2).

3.5. Identification of patients with MSI-H/dMMR

In this cohort, two tumor samples were determined as microsatellite instability high (MSI-H). They exhibited extremely high somatic mutation burdens of 50 mutations/Mb and 26.19 mutations/Mb, respectively. One was found to have two pathogenic somatic mutations in the DNA mismatch repair gene *MSH2* (p.G162R, p.A256V), whereas another harbored *PMS2* missense mutation (p.F105V). Defects in *MSH2* and *PMS2* serve as the mechanism underlying the observed hypermutated phenotype and MSI-H. Moreover, HPV18 was positive in both cases, and one of them was with HPV16/HPV18 dual infection. Both patients were alive at the end of the follow-up point (censored at 25, 55 months, respectively).

4. Discussion

SCNEC is among the most lethal gynecological malignancies but little treatment progress has been made for decades. Its histologic counterparts, small cell lung cancer, are studied in depth because of its relatively high incidence. Currently, several targeted therapies and checkpoint blockade immunotherapies have been exploited in SCLC [7] Identification of genetic alterations of SCNEC provides opportunities for the application of the targeted therapies and immunotherapies.

Distinct from SCLC and NENs originating from other sites, SCNEC is characterized by the ubiquitous existence of HPV when diagnosed. A systemic review and meta-analysis reported that the prevalence of HPV in SCNEC is nearly 85% [8] The predominant subtypes are HPV18 whereas

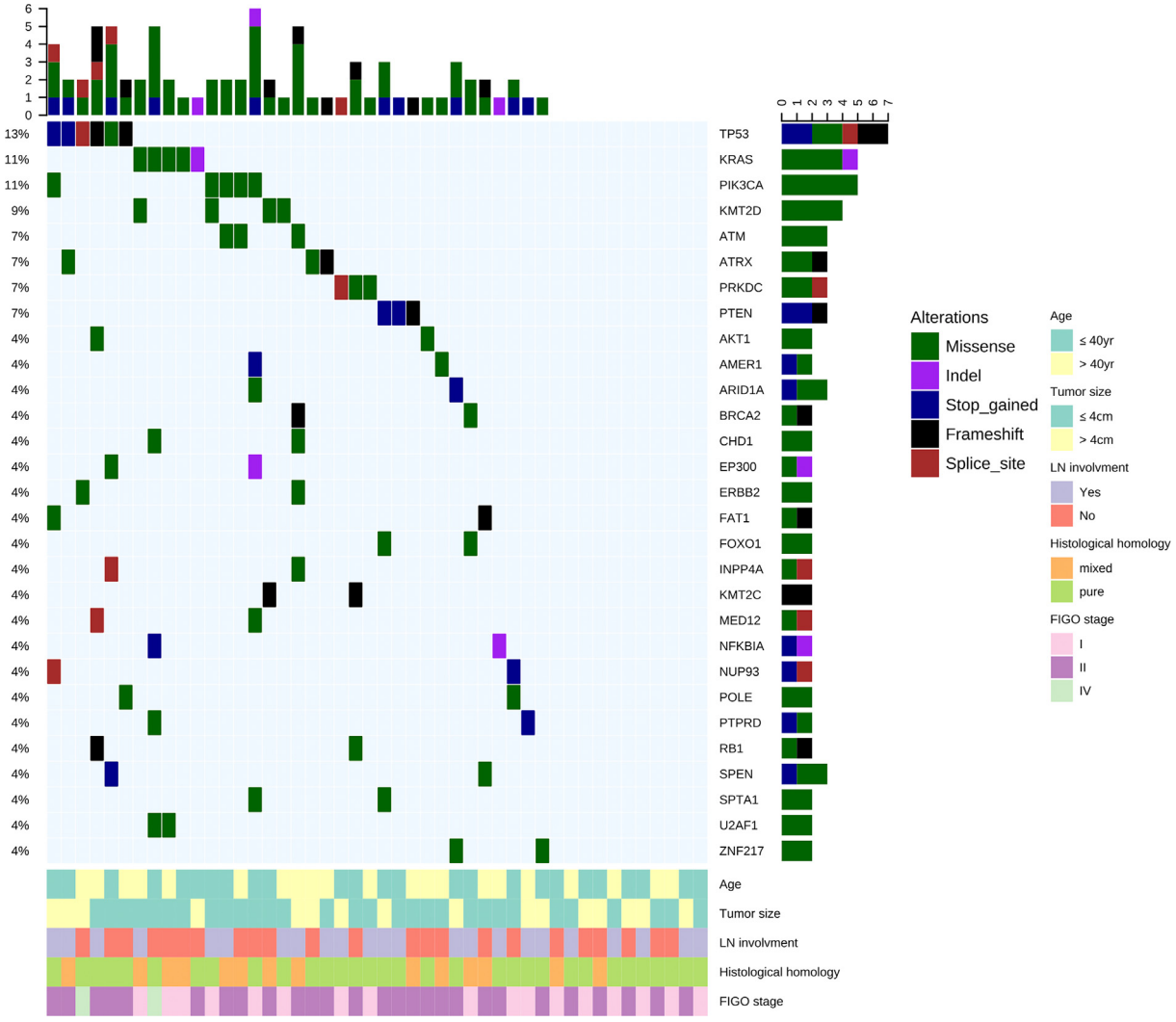

Fig. 1. Mutation spectrum in SCNEC tumors. Forty-eight SCNEC samples with mutational data are displayed with clinical features annotated below(two samples with extremely high somatic mutation burden were excluded). The somatic mutation frequencies for each gene are listed on the left panel. Mutation types are displayed on the right panel. The results are shown only for the gene which was mutated in $n \geq 2$ patients.

HPV16 is more commonly seen in SCC and AC, suggesting that HPV 18 is a viral type specifically associated with SCNEC. This is consistent with the observation in our study. Hence, HPV infection should be taken into consideration when discussing the distinctive genetic features of SCNEC.

Interestingly, none of genes detected here appeared to recurrently alter in more than 15% of SCNEC patients, implying no one particular driver gene was recognized in the carcinogenesis of SCNEC. Mutated genes tend to cluster in several signaling pathways. The receptor tyrosine kinase (RTK)-RAS pathway was the most commonly involved one, nearly half of the cases had at least one mutation in the genes of this pathway. *KRAS* was the most frequently mutated gene among them. Three *KRAS* p.G12D mutants, one p.G12A mutant, one p.G12_G13insAG inframe insertion, and one *KRAS* amplification was identified in 6 patients (12.24%) in this study. Consistently, Frumovitz M et al. reported *KRAS* mutations(G12D, G12V) in 14% of patients in their study [9]. Xing D et al. found one patient (1/10) with a *KRAS* mutation c.35G > T (G12V) based on the NGS within a 637-gene panel. This patient was alive for >10 years [10]. *KRAS* p.G12A has not been found previously in SCENC but it is a common *KRAS* pathological mutation in other tumors [11]. However, mutations in *KRAS* is scarcely observed in SCLC based on several comprehensive genomic analysis [12,13], as well as other NENs [14]. Lyons et al. used the mitogen-activated protein kinase 1 (MEK)-inhibitor trametinib in a woman with recurrent SCENC who harbored *KRAS* mutation (c.35G > A, p.G12D). She had a complete radiologic response after 3 cycles of treatment [15]. Targeted therapy directly targets *KRAS* is difficult. MEK inhibitor is an example of targeting downstream KRAS-RAF-MEK-ERK signaling pathway. Other strategies include targeting proteins that promote KRAS binding to the plasma membrane, components that support KRAS-dependent metabolic processes, and synthetic lethal interactors important for mutant KRAS but not wild-type KRAS [16]. Recently, covalent inhibitors targeting the cysteine residue of KRAS (G12C) mutant such as AMG510 shows great efficacy in preclinical and clinical settings [17].

In this study, *PIK3CA* mutations were detected in 6 patients (12.24%). Three *PIK3CA* mutants p.E542K, P.E545K, p.K111E were detected in 5 patients (11%) and *PIK3CA* amplifications were found in 2 patients. Among them, two harbored p.E542K, two had p.E545K, one had p.K111E, respectively. p.E542K, p.E545K are the most common mutations of *PIK3CA* in cervical cancer. While p.K111E is less common but has been reported previously [18]. The patients with *PIK3CA* p.K111E also had amplified *PIK3CA*. Similar to our results, Frumovitz M et al. found that *PIK3CA* mutated in 18% (8/44) of tested SCNEC samples in their hotspot-sequencing based study [9]. Another NGS sequencing within a 637-gene-panel based study in 10 SCENE patients identified *PIK3CA* mutations in 3 tumors [10]. *PIK3CA* is one of the most frequently mutated genes in SCC and AC. In the previous study, we found 13.6% of surgically

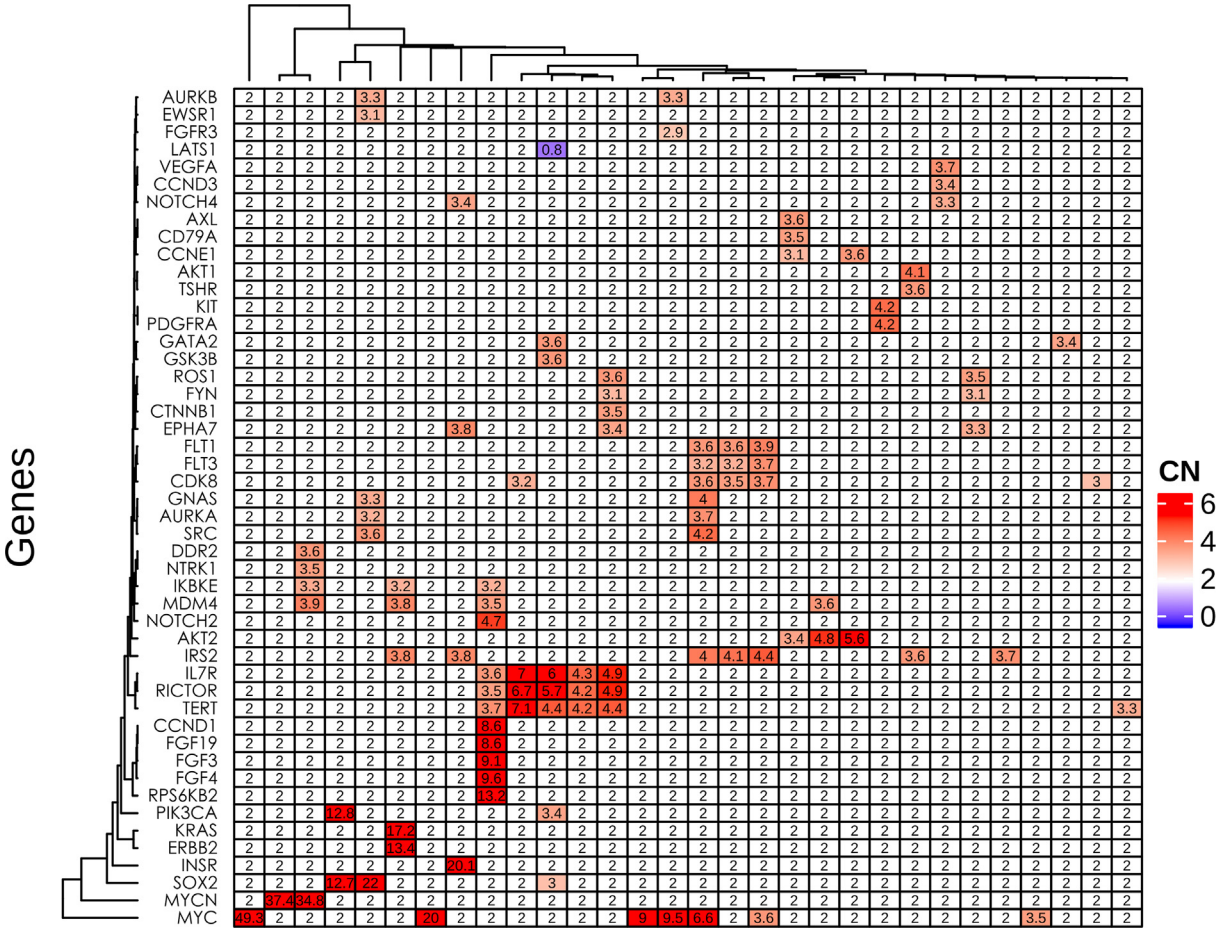

Fig. 2. Distribution of recurrent copy number variation in SCNEC tumors. Only amplifications in known oncogenes and deletion in tumor suppressor genes are shown.

resected cervical cancer samples had *PIK3CA* mutations [19]. Other HPV-related carcinomas, such as HPV positive neck and head carcinomas and anal cancer, also have a high prevalence of mutants in *PIK3CA* [20]. In contrast, rare mutations (<5%) in *PIK3CA* were identified in SCLC and other NENs [12]. *PIK3CA* encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K). The activation of PI3K results in PIP3 production and further activation of downstream targets such as AKT, which could phosphorylate multiple substrates including mTOR [21]. The tumor suppressor gene *PTEN* dephosphorylates proteins in PI3K pathway and negatively regulates it. A total of 19 SCNEC patients (38.78%) in this study have at least one mutation in PI3K-AKT pathway related genes, implying a paramount role of this pathway in the development of SCNEC. Similarly, a whole exome sequencing (WES) based study in 5 paired tumor-normal SCNEC samples did not detect any *PIK3CA* mutation but genes in the AKT-mTOR pathway were the most recurrently mutated [22]. Activation of this pathway by HPV E6, E7, and E5 is critical for viral infection and survival [23].

Insulin-like growth factor-1 receptor (IGF-1R) and insulin receptor (IR) pathway could also activate PI3K-AKT signaling. *IRS2* is located on 13q.34 and encodes for insulin receptor substrate2 (IRS2), a key mediator of both IGF-1R and IR signaling. We identified *IRS2* amplification in 14.29% of SCNEC samples, which has not been reported elsewhere. Besides, mutations in *PIK3CA*, *PTEN*, and *IRS2* were mutually exclusive, implying various mutated genes contribute to the abnormal activation of PI3K-AKT pathway in SCNEC. Several strategies targeting the PI3K-AKT pathway has been proposed, including mTOR, mTORC1/2, AKT, PI3K inhibitors [24]. Taken together, PI3K-ATK signaling related genes are frequently mutated in SCENC, and patients bearing these mutations may benefit from small molecules targeting this pathway.

Moreover, *IRS2* is thought to be a candidate driver oncogene on 13q34 in colorectal cancer. Ampification of *IRS2* was observed in 2–5% of SCLC patients [13,25], and ceritinib, a dual inhibitor of ALK/IGF-1R, could efficiently inhibit the growth of SCLC with *IRS2* amplification in vitro and in vivo. Moreover, We found *KARS* mutation and *IRS2* amplification co-occurrence in 3 patients. It has been reported that the combination of IGF-1R and MEK inhibitors appears to be a promising novel strategy against *KRAS*-mutant lung cancer [26]. Conditional ablation of *irs1/irs2* in murine lung with Kras activation and p53 loss strongly suppresses lung tumorigenesis, providing evidence that IRS1/2 is required for KRAS mutant lung cancer initiation, and targeting of the IGF-1R signaling pathway may represent an effective way in treating KRAS mutant non-SCLC (NSCLC) [27]. Therefore we postulate it a rational and promising treatment strategy to inhibit IGF-1R/IR in SCNEC patients with *IRS2* amplification. Moreover, in patients harboring mutated *KRAS* and amplified *IRS2*, the efficacy of combined KRAS targeted therapy with IGF-1R/IR inhibitors is worth considering.

Poly-(ADP)-ribose polymerase enzymes (PARP) function to repair DNA single-strand breaks (SSB). Mutations in breast cancer 1 (*BRCA1*) and *BRCA2* lead to deficiencies in homologous recombination (HRD), leaving the mutational cancers highly dependent on PARP-mediated repair and sensitive to PARP inhibitors [28]. PARP inhibitors have been successfully used for the treatment of germline *BRCA1/2* mutant ovarian and breast cancer patients in the first place. Notably, patients with a somatic *BRCA* mutant and mutants in other HRR genes could also benefit from PARP inhibitors [29]. *BRCA2* somatic mutants were observed in two patients in this study. *BRCA2* p.S36C has not been reported before or in the cosmic database and is with uncertain significance. *BRCA2* p.P2827fs leads to frameshift variant and is likely pathogenic. Totally,

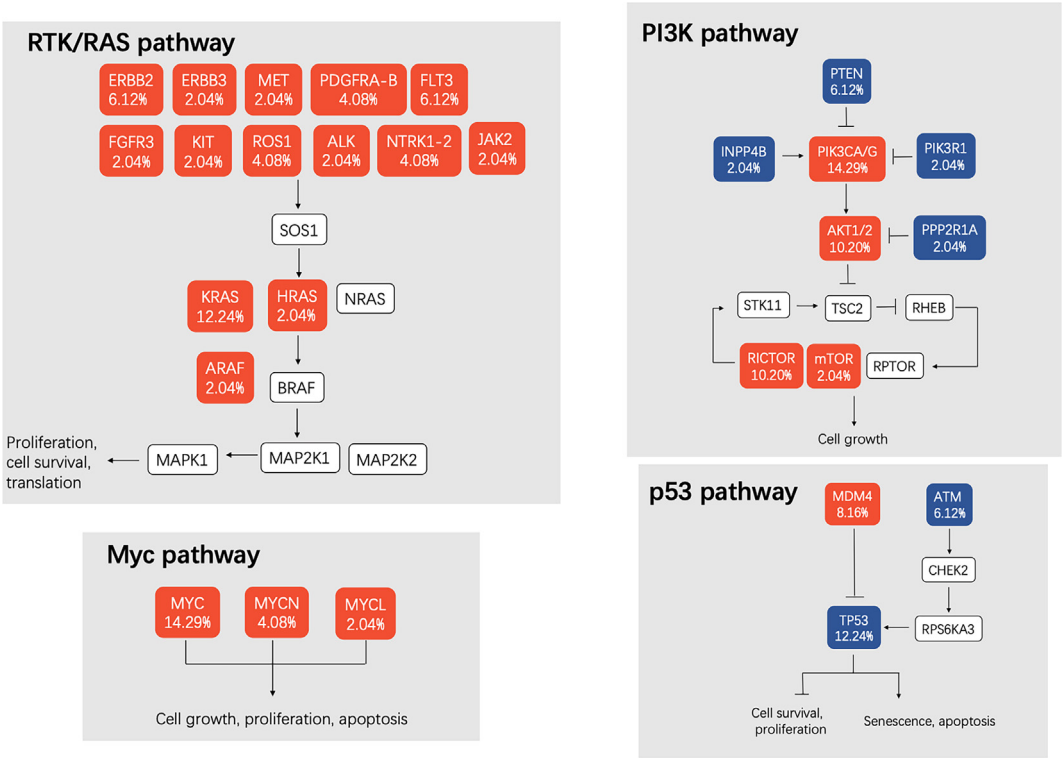

Fig. 3. Signaling pathways recurrently affected in SCNEC. Red and blue boxes represent genes with activating and inactivating alterations, respectively. Genes shown in white boxes are not included in this study or without any mutations.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

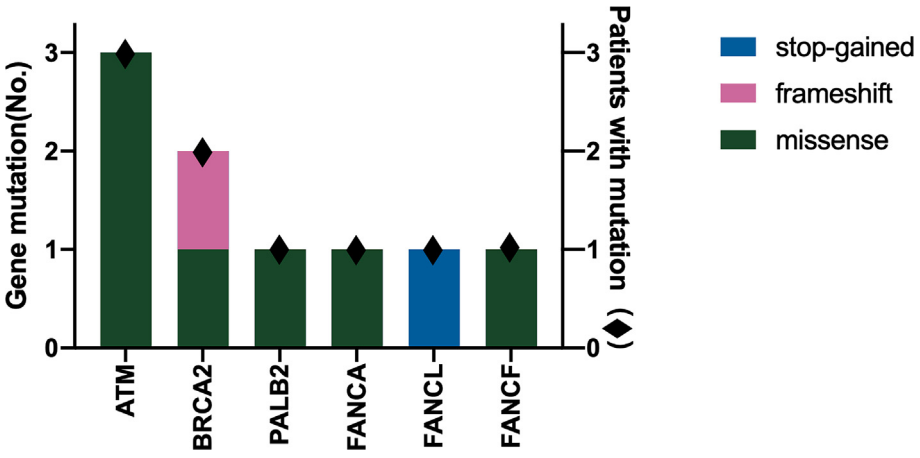

Fig. 4. The number and type of somatic mutation(s) in individual HRR gene.The total number of mutation(s) detected for a given gene is shown on the left axis. The number of patients with any type of mutation in a given gene is shown on the right axis and labeled as a diamond above the histogram.

genetic alterations in HRR genes including *BRCA2, ATM*, *PALB2*, *FANCA*, *FANCL*, and *FANCF* occurred in a considerable proportion (7/49) of SCNEC patients in this study. Xing D et al. reported that *BRCA1* and *BRCA2* somatic mutations were individually detected in two (2/10) SCNEC patients [10]. Rose et al. reported a metastatic SNCEC patient with *BRCA2* loss exons 17–27. This patient was treated with a PARP inhibitor rucaparib following carboplatin and etoposide chemotherapy. She had stable disease and was progression-free for long intervals (15 months) [30]. Although homologous recombination deficiency(HRD) or mutants in *BRCA1/2* are rare in SCLC(≤3%) based on comprehensive genomic analyses. PAPR inhibitors have shown promising efficacy in SCLC preclinical models and early-phase trials. Recently a phase I/II trials indicated substantial clinical activity of combination olaparib, a PARP inhibitor, with temozolomide in patients with previously treated SCLC [31]. Temozolomide is an alkylating agent that could increase SSBs and is used for second-line therapy in SCLC. The overall response rate was 41.7%. PARP trapping may be the underlying mechanism for synergy between PARP inhibitors and DNA damaging drugs which could increase the incidence of single-strand breaks. Taken together, identification of somatic mutations in *BRCA2* as well as other HRR genes in patients with SCNEC endows rationale for designing clinical trials of PARP inhibitors in selected patients. Furthermore, the combined use of PARP inhibitors with DNA damaging agents such as temozolomide in SCNEC patients even without HRD is worth evaluating.

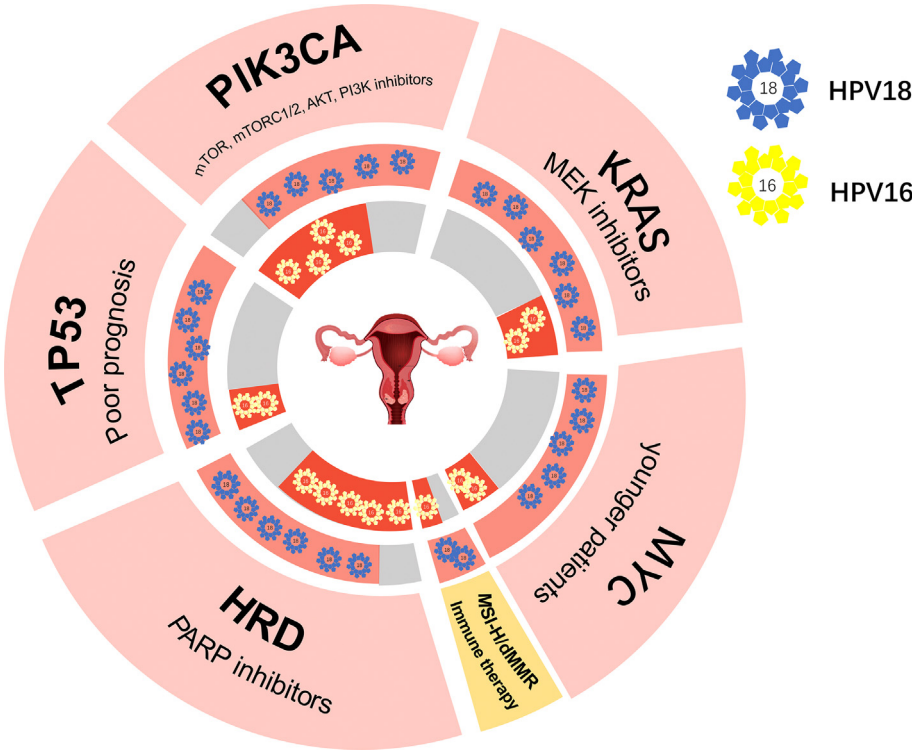

Fig. 5. The schematic figure of the genetic/infectious characteristic and potential targets in SCNEC.

In contrast to a high incidence of *TP53* (75–90%) and *RB1* (60–90%) inactivation in SCLC, mutations in *TP53*(13%) and *RB1*(4%) are less common in SCNEC indicated by our results, implying distinct molecular profiles of NENs arising from lung and cervix. *TP53* mutations have been observed in 11%–62.5% of SCNEC in three studies [9,10,32]. In contrast, several studies didn't recognize TP53 mutations in SCNEC [22,33,34].

We found *SOX2* amplification in 6.12% (3/49) of the samples. *SOX2* is dispensable in stabilizing embryonic stem cells in a pluripotent and self-renewing state. Overexpression of *SOX2* has been demonstrated to promote tumorigenesis in several tumors including lung and cervical cancer [35]. Recently, a genomic analysis in small-cell lung cancer identified *SOX2* as a frequently amplified gene with an amplification rate of ~27%. Compared with adjacent normal tissues, the majority of the SCLC samples had a higher *SOX2* mRNA expression level. The functional experiments further supports the idea of SOX2 as a putative lineage-survival oncogene in SCLC [12]. Strategies targeting *SOX2* are promising in cancer treatment. The use of miRNA 145 delivery in glioblastoma multiforme showed the ability to inhibit tumorigenesis and enhance sensitivity to radiotherapy and temozolomide in vitro and in vivo by downregulating SOX2 and Oct4 [36]. Thus, the oncogenic role of *SOX2* in SCNEC is worth further elucidation. *SOX2* silencing might be a novel therapeutic approach for those harboring *SOX2* amplification.

ATRX is a gene involved in the gene regulation at interphase and chromosomal segregation in mitosis. Mutations in *ATRX*, previously identified in 30.23% small intestine neuroendocrine tumors [37] and 17.65% well-differentiated pancreatic neuroendocrine tumors [38], were found in three SCNEC samples in our study. Surprisingly, the WGS based sequencing of SCNEC study identified 4 out of 5 patients had *ATRX* mutations [22]. These findings indicate *ATRX* mutations are recurrent molecular events in NENs including SCNEC. But the biological role of *ATRX* in NENs is poorly elucidated and understanding of this gene may advance the knowledge of the common pathogenesis of NENs.

Unexpectedly, two SCNEC patients with MSI-H and hypermutated phenotypes were present in this study. MSI is a major predictive biomarker for the efficacy of immune checkpoint inhibitors. Pembrolizumab, a PD-1 inhibitor, has been approved by the FDA for the treatment of metastatic solid tumors with MSI-H/dMMR, including colorectal cancer and SCLC. Recently one large-scale phase II studies: KEYNOTE-158 valued the efficacy of Pembrolizumab in patients with 27 non-colorectal MSI-H/dMMR cancers including cervical cancer, further demonstrating Pembrolizumab is effective in patients with MSI-H/dMMR cancers [39]. In our study, the MSI-H samples with *PMS2* mutation simultaneously harbored three *PRKDC* missense variants. *PRDKC* has been reported as a new candidate for checkpoint blockade immunotherapy. *PRKDC* mutation was found in ~60% of lung and melanoma cancer patients who responded to immunotherapy. Besides, *PRDKC* mutation is significantly correlated with high mutation load in several solid cancers [40]. However, in the current study, we failed to show a relationship between high mutation burden with *PRKDC* mutation ($p = 0.28$). Taken together, a subset of small cell cervix tumors exhibiting MSI-H/dMMR phenotype are potentially sensitive to immune checkpoint blockades. The predictive role of *PRKDC* mutation in SCNEC immunotherapy needs to be further investigated.

This study, to our knowledge, comprised one of the largest cohort patients and the next generation sequencing was performed in SCNEC patients within a large panel of 520 cancer-related genes , whereas some of genes analyses were based on hotspot sequencing only. We plan to further expand our knowledge in molecular signatures of SCNEC through RNA, whole-exome sequencing and whole-genome sequencing.

To sum up, the genetic profile of SCNEC provided here has shown a higher prevalence of activating mutations in *KRAS* and *PIK3CA,* as well as a lower incidence of inactivating mutations in *TP53* and *RB1* compared with those of SCLC. Alterations were also found in *SOX2* amplification and *ATRX* which characterize the genetic features of NENs deriving from other sites (lung, pancreas). The similar pathological background of all NENs and the specific HPV-related microenvironment of cervix may account for the combined molecular signature. We identified a proportion of SCNEC patients harboring actionable mutants in

KRAS, PIK3CA and genes of corresponding pathways as reported in other studies. We proposed a number of low-frequency, novel targetable mutations or predictive biomarkers, including *IRS2*, *SOX2* amplification and mutations in HRR genes. Notably, individuals with MSI-H/dMMR may benefit from immune checkpoint blockades(Fig. 5). This dataset proposed several candidate targets and provided unrecognized therapeutic possibilities. Moreover, the comprehensive genetic profiles in this study could serve as an important reference for further exploration of the biology of SCNEC.

Author contributions

HJY and LBX conceived the project and modified the manuscript. XP conducted the expreiments, anaylized data and drafted the mamuscipt. WC and LNY comlepleted patients' follow-up. LBX, XXS and WJ collected clinical samples. XYZ modified the manutscript.

Declaration of Competing Interest

The authors report no conflict of interest.

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Appendix: Supplementary Data

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