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Genomic features of bladder neuroendocrine carcinoma with composite histology

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Summary. Neuroendocrine carcinoma (NEC) is a rare and aggressive malignancy derived from multiple body parts, with the urogenital organs being the second-largest extrapulmonary sites. The detailed mechanism of bladder NEC pathogenesis remains unknown. We reviewed data from 23 patients diagnosed with NEC from urogenital organs (bladder and prostate) and conducted targeted sequencing of 523 cancer-related genes, focusing on bladder NEC. While 14 cases featured a pure NEC histology, the remaining nine cases included NEC histology mixed with other tumors, such as urothelial carcinoma (UC) or adenocarcinoma. Median overall survival in the entire cohort was 11.1 months, and survival curves were comparable between pure NEC and NEC of mixed appearance. Major mutations detected in the NEC component were in *TP53* (38%), *TERT* promoter (31%), *PIK3CA* (25%), histone-modification genes (19%), and *RB1* (19%). The *BARD1* frameshift variant related to homologous recombination was also detected in one patient. More than half of the patients had a high total mutational burden (TMB; ≥10), including two with a TMB ≥45. Intriguingly, at least one identical gene variant in driver genes was detected between NEC and non-NEC (UC) components in the four bladder specimens analyzed. These results highlight the possibility of shared genetic background between bladder NEC and UC. Additionally, several cases harbored druggable gene alterations as presented by TMB-high. Our presentation of the histopathological and molecular features of NEC may help clarify the underlying mechanisms and contribute to efficient treatment of the disease.

Key words: Urogenital neuroendocrine carcinoma, Bladder, Composite histology, Genome, Total mutational burden

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Introduction

Neuroendocrine carcinoma (NEC) is a rare malignancy that can arise from multiple organs. In principle, treatment strategies for patients with advanced extrapulmonary NEC conform to those for patients with small-cell lung cancer (SCLC), as the two types share histological features. The urinary tract, including the bladder and male genital tract, including the prostate gland, are common extrapulmonary sites secondary to the gastrointestinal tract (Dasari et al., 2018). The percentage of NEC in bladder or prostate malignancies is extremely low (<1% and 0.5-2%, respectively) (Choong et al., 2005; Tan et al., 2014). Morphologically, NEC appears poorly differentiated, and can be classified into small-cell carcinoma (SCC) and large-cell neuroendocrine carcinoma (LCNEC) subtypes. The clinical behavior in metastatic NEC is generally aggressive.

According to analyses using the Surveillance, Epidemiology, and End Results database, the five-year overall survival (OS) rate in localized cases from the urinary or male genital tract excedes 30%, but remains

poor (<5%) for metastatic cases (Dasari et al., 2018). A US multi-center retrospective analysis of urogenital neuroendocrine neoplasms reported a median OS of 3.6 years (95% confidence interval (CI), 2.2-9.2) and one year (95% CI, 0.8-1.3) for localized/regional and metastatic cases, respectively (Le et al., 2023).

The mechanism of NEC onset is presumed to vary among primary organs.Pathogenesis in prostate NEC is unique in that some cases transform from prostate adenocarcinoma during androgen-deprivation therapy in metastatic locations (Conteduca et al., 2019; Beltran and Demichelis, 2021; Rinott Mizrahi., 2022; Shui et al., 2022). Pathologists may encounter NEC coexisting with other histological components. Regarding bladder cancer, mixed histology composed of NEC and non-NEC components accounts for 70% or more of the cases (Abrahams et al., 2005; Wang et al., 2018, 2021; Lim and Sundar., 2019). Neuroendocrine transformation in the bladder typically occurs *de novo* and develops at the primary tumor location (Akbulut and Al-Ahmadie., 2024). However, the pathophysiology underlying bladder NEC has not been fully elucidated.

Next-generation sequencing (NGS) for composite NEC is a useful tool that can offer insights into the pathophysiology of urogenital NEC and can help identify new therapeutic options for patients with advanced disease by investigating druggable gene alterations. In this study, we assessed morphological features and performed targeted sequencing of urogenital NEC specimens, chiefly bladder NEC. Our analysis focused on comparing genomic features between NEC and non-NEC components in composite histology cases.

Materials and methods

Clinical data collection

This study was reviewed and approved by the Institutional Review Board of the Japanese Foundation for Cancer Research (2020-GA-1221) and conducted in accordance with the guidelines established by the Helsinki Declaration. We reviewed the database of the Cancer Institute Hospital of the Japanese Foundation for Cancer Research from 2006 to 2021 and extracted data from patients who developed NEC in urogenital organs. Collectively, patients with NEC derived from the urinary bladder, renal pelvis, or prostate gland were included in this analysis. The following clinical data were collected: sex, age, smoking history, history of any cancer, primary organ, and clinical stage. OS was calculated as the interval from pathological diagnosis to death by any cause or last follow-up. Board-certified pathologists (K.K and K.I) at our institution assessed each resected or biopsied tumor specimen, and only cases with a definitive NEC diagnosis were examined in detail.

Morphological assessment of NEC specimens

Resected or biopsied tumor specimens were formalin-fixed and paraffin-embedded, and paraffin blocks were sectioned at 5-μm thickness for hematoxylin and eosin staining and additional immunohistochemistry. The diagnosis of NEC (SCC or LCNEC) was based on the criteria described in the fifth edition of the World Health Organization Classification of Urinary and Male Tumors (Compérat et al., 2022). Briefly, tumor cells in SCC are small with a high nuclear-to-cytoplasmic ratio and scant cytoplasm, inconspicuous nucleoli, and vesicular chromatin (Akbulut and Al-Ahmedie, 2024). LCNEC has larger polygonal tumor cells with abundant cytoplasm, prominent nucleoli, and high mitoses. A mixture of non-NEC components was evaluated for each sample. For urothelial carcinoma (UC), we evaluated histological variants, divergent histological differentiation, and UC *in-situ* components. Immunostaining for chromogranin A, synaptophysin, CD56, INSM1, and Ki-67 was performed.

Targeted gene sequencing using formalin-fixed and paraffin-embedded specimens

Block sectioning for genomic analysis was conducted at our institution, and subsequent DNA/RNA extraction, library preparation, and targeted sequencing were performed by RIKEN Genesis Co., Ltd. (Tokyo, Japan). Ten slides with 5-μm thick sections of the paraffin-embedded blocks were prepared per case. Phosphate-buffered neutral formalin was used for tissue fixation, and the fixation time was less than 72 hours. DNA and RNA samples were extracted from specimens with tumor cell proportions >20%.

Samples from NEC and other histologies were collected 145 via manual macrodissection. The extracted DNA/RNA was checked, and only qualified samples were included for library preparation. Custom-targeted capture and library preparation were performed using the TruSight Oncology 500 Library Preparation Kit (Illumina, San Diego, CA, USA). The NextSeq System (Illumina) was used for targeted capture sequencing of 523 cancer-related genes (*Supplementary Table 1*). DNA analyses included single nucleotide variants (SNVs), insertions/deletions, copy number variations, total mutational burden (TMB), and microsatellite instability. RNA analyses included gene fusion and splice variants. TMB was calculated by dividing the total number of somatic SNVs and insertions/deletions by the length of the captured region. TMB-high was defined as ≥10 mutations/megabase (mut/Mb).

The pathogenicity of each variant was interpreted by referring to the public databases COSMIC (Catalog of Somatic Mutations in Cancer; https://cancer.sanger.ac.uk/cosmic) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). Non-synonymous variants not listed in these databases were classified based on the predicted

effect on the protein product, where nonsense and frameshift variants were considered as deleterious unless they occurred in the last exon. To predict whether an amino acid substitution affected protein function, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and Mutation Taster (https://www.mutationtaster.org) were used. Based on the above algorithm and a literature review, respective variants were classified as deleterious, benign, or of uncertain significance. The detailed library preparation and procedures related to bioinformatics analysis have been described in our previous publication (Ohmoto et al., 2021). Pathogenicity was assessed onl 168 y for variants with VAF ≥5% based on the TruSight Oncology 500 Library Preparation Kit protocol.

Statistical analysis

Differences in categorical variables between groups were analyzed using the Fisher's exact test. Survival curves were estimated using the Kaplan–Meier method, and P-values were calculated using the log-rank test. Univariate analysis of risk factors for OS was performed using the Cox proportional hazards regression model. Because of the limited number of patients, multivariate analysis was not appropriate in this study. A two-sided $p<0.05$ was considered to indicate statistical significance. All statistical analyses were performed using EZR (v.1.4.1; Saitama Medical Center, Jichi Medical University, Shimotsuke, Japan) (Kanda, 2013).

Results

Study design and patient population

Figure 1 shows the process of clinical data collection and analysis of tumor samples. Thirty-eight patients were pathologically diagnosed with NEC of the urogenital organs. Tumor specimens were unavailable for 15 patients whose transurethral resection of the bladder tumor or biopsy was performed in another hospital; data from the remaining 23 patients were included in subsequent analyses. The clinical characteristics of the 23 patients are summarized in Table 1.

The median patient age was 70 years (range, 54-87 years), and 68% were smokers. Seven patients (30%) had a history of malignancy; one patient was simultaneously diagnosed with renal carcinoma and prostate NEC. The primary organ was the urinary bladder in 12 patients, the prostate gland in 10, and the renal pelvis in one. Ten patients (45%) had metastatic disease at initial diagnosis. As a first-line treatment, 14 patients (61%) received curative treatment comprising surgical resection, or transurethral resection of bladder tumor (TURBT) or prostate (TURP).

Pathological features of NEC specimens

The morphological features of the 23 NEC specimens are presented in Table 1. Tumor specimens were obtained via surgical resection (n=6), TURBT/TURP (n=9), biopsy (n=8), and autopsy (n=1), including cases with both TURBT/TURP and biopsy specimens. Transurethral resection and biopsy samples were available in two cases.

Fourteen samples had pure NEC histology, and nine were composite tumors comprising NEC and other components. The latter included UC (n=5) (Fig. 2), adenocarcinoma (n=2), squamous cell carcinoma (n=1), and sarcoma (n=1). The detailed morphology regarding the NEC component was SCC (n=17), LCNEC (n=2), and composite SCC/LCNEC (n=1). The Ki-67 index in 14 evaluable NEC samples ranged between 40% and 90% (median, 55%). The positivity rate of neuroendocrine markers was 45%, 91%, 82%, and 91% for chromogranin A, synaptophysin, CD56, and INMS1, respectively.

Clinical outcomes

The survival curves of all 23 patients are shown in (Fig. 3A). The median follow-up time for the survivors was 17.5 months. The median OS was 11.1 months, and the 1-year OS rate was 41%. We did not observe any significant difference regarding OS between 10 metastatic and 12 non-metastatic cases (1-year rate: 53% vs. 36%, $p=0.36$) (Fig. 3B); between 13 cases derived from the bladder, including the renal pelvis, and 10 cases from the prostate (one-year rate: 38% vs. 43%, $p=0.27$) (Fig. 3C); or between nine cases with mixed histology and 14 with pure NEC histology (one-year rate: 33% vs. 48%, $p=0.91$) (Fig. 3D).

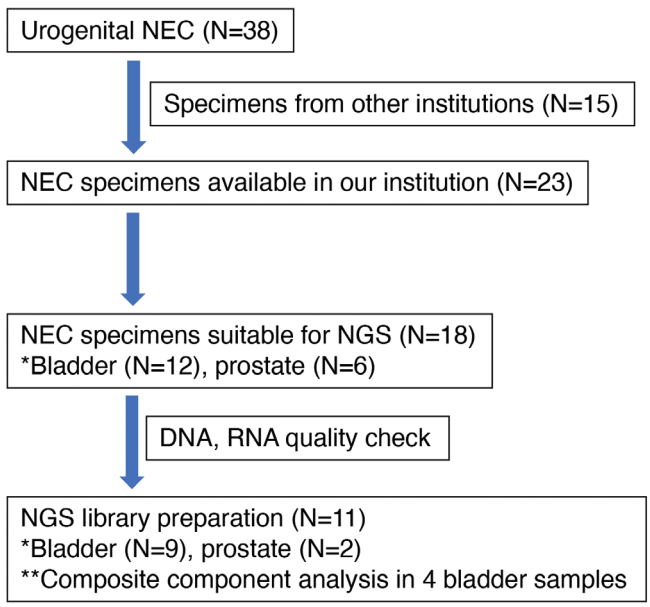

Fig. 1. Flow chart illustrating the process of clinical data collection and analysis of tumor samples. NEC, neuroendocrine carcinoma; NGS, next-generation sequencing.

Gene alteration profiles detected with NGS

The initial quality check of extracted DNA/RNA yielded 14 bladder samples (nine NEC and five non-NEC) from nine patients and two prostate NEC samples from two patients that were used for library preparation. Regarding the bladder specimens with composite histology (patient IDs: 2, 3, 4, and 6), both NEC and non-NEC components were subjected to macro-dissection and submitted as an independent sample.

The average unique coverage depth was 405.9 (range, 128.0-951.9). The gene alterations detected in the 16 samples are presented in (Fig. 4) and (*Supplementary Table 2*). Pathogenic/likely-pathogenic SNVs were detected in 11 (69%) specimens. The major mutated genes in the NEC component were *TP53* (38%), *TERT* promoter (31%), *PIK3CA* (25%), and *RB1* (19%). Mutations in histone-modification genes (*KMT2A* or *NSD1*) were detected in 19% of the cases. The *BARD1* (*BRCA1-associated RING domain 1*) frameshift variant that induces homologous recombination loss was detected in patient ID-9 (Dillon et al., 2022). In terms of gene fusion, known driver fusions were not detected. The median TMB and percent of unstable microsatellite sites in NEC components were 10.2 mut/Mb (range, 2.4-67.8) and 2.6% (range, 1.3-5.2), respectively. Remarkably, more than half (6/11) of the patients had a high TMB (≥10), including two patients with a TMB ≥45. Four of the six patients with a high TMB had a history of smoking, but we did not find any significant correlation between TMB ≥10 and smoking history (p=0.24). In terms of prognosis, OS was similar between the six cases with TMB ≥10 and five with TMB <10 (1-year rate: 33% vs. 27%, p=1.0) (Fig. 3E).

Table 2 presents a comparison of SNVs between NEC and non-NEC components in four specimens (ID-

Table 1. Clinical data in 23 patients with NEC from urogenital organs.

Variables	Number (%)
Age	Median 70 (range, 54-87)
Gender	
Male	18 (78%)
Female	5 (22%)
Smoking	
Yes	13/19 (68%)
Past history of any cancer	7/23 (30%)
Primary organ	
Urinary bladder	13 (57%)
Renal pelvis 1	(4%)
Prostate	9 (39%)
Disease stage at diagnosis	
Metastatic	10/22 (45%)
Initial treatment	
Curative treatment	14 (61%)
Histology	
Pure NEC	14 (61%)
Composite NEC with other histology	9 (39%)
Other histological component (composite NEC)	
Urothelial carcinoma	5
Adenocarcinoma	2
Squamous cell carcinoma	1
Sarcoma	1
NEC morphology	
SCC	17 (85%)
LCNEC	2 (10%)
SCC/LCNEC	1 (5%)
Immunostaining for neuroendocrine markers	
CgA	5 (45%)
SYP	10 (91%)
CD56	9 (82%)
INSM1	10 (91%)

NEC, neuroendocrine carcinoma; SCC, small cell carcinoma; LCNEC, large-cell neuroendocrine carcinoma; CgA, chromogranin A; SYP, synaptophysin; INSM1, insulinoma-associated protein 1.

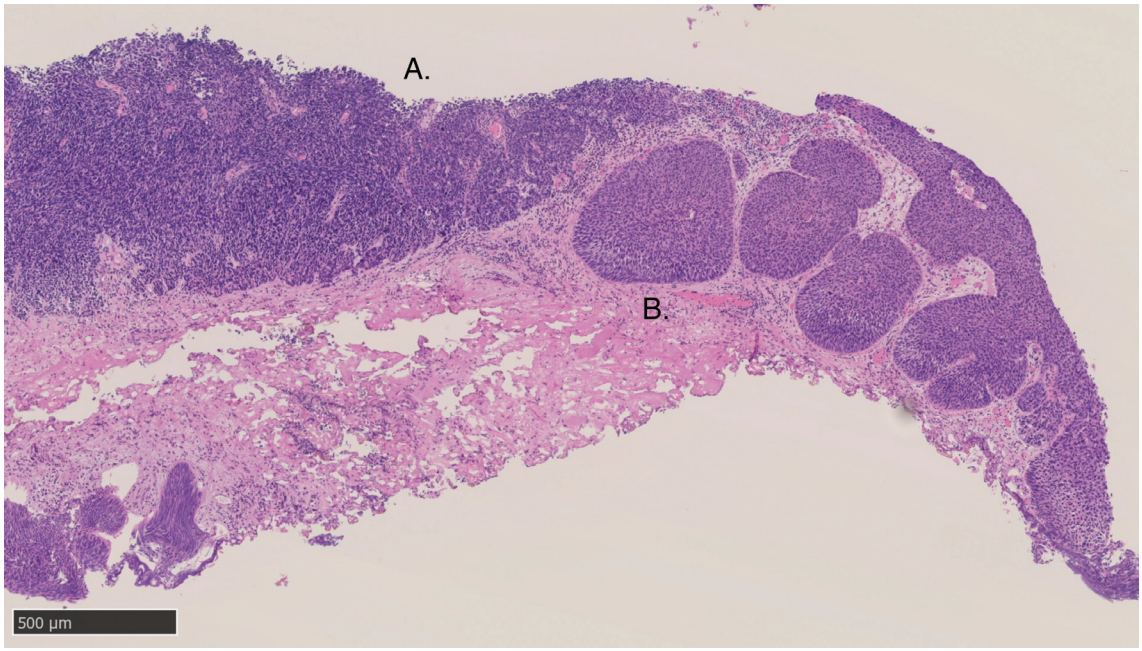

Fig. 2. Composite NEC consisting of SCC **(A)** and UC **(B)** components. SCC consists of diffuse sheets of small malignant cells with inconspicuous nucleoli and scant cytoplasm. NEC, neuroendocrine carcinoma; SCC, small cell carcinoma; UC, urothelial carcinoma.

Table 2. Gene mutation prevalence in NEC and non-NEC components.

Patient ID	1	2.1	2.2	2.3	3.1	3.2	4.1	4.2	5	6.1	6.2	7	8	9	10	11
Primary organ	B	B	B	B	B	B	B	B	B	B	B	B	B	B	P	P
Histology	S/L	S	U	U*	S	U	S	U	S	U*	S	S	L	S	L	S
Gene mutation																
TP53																
TERT																
RB1																
PIK3CA																
KMT2A																
NSD1																
RECQL4																
PTEN																
KMT2D																
BARD1																
TMB	28.89	25.79	2.4	18.06	6.25	7.82	10.15	10.16	67.81	24.99	26.56	7.11	7.82	45.84	2.37	3.91
Median sequence depth	951.9	458.7	128	192.9	636.6	449.4	535.3	562.5	145.6	650.1	576.1	166.5	362.3	153.1	260.3	

Variant type: Missense Nonsense Frameshift Promotor mutation

NEC, neuroendocrine carcinoma; TMB, total mutational burden; B, bladder; P, prostate; S, small-cell carcinoma; L, large-cell neuroendocrine carcinoma; U, urothelial carcinoma. *UC with glandular differentiation

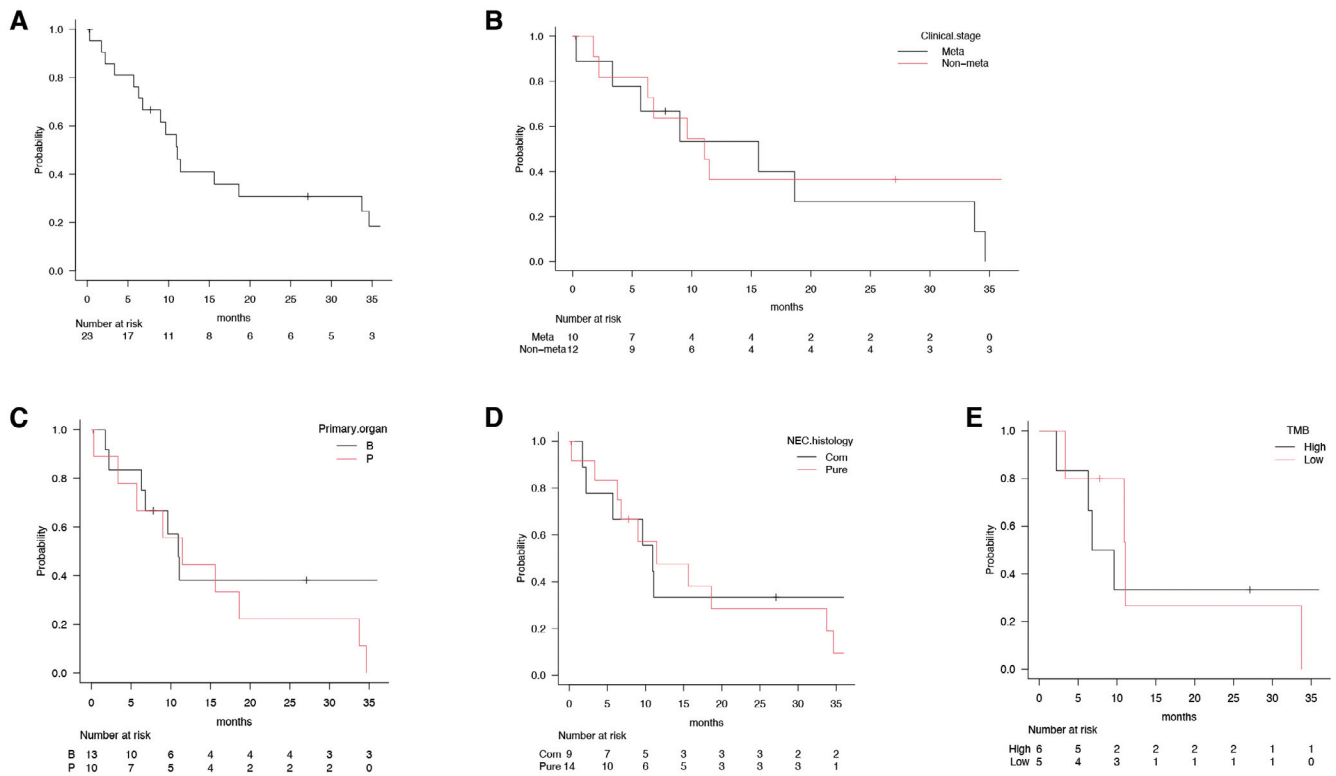

Fig. 3. Survival curves between clinical or histological subgroups. **A.** Entire cohort (n=23). Subgroup analysis in terms of clinical stage **(B)** (metastatic cases vs. non-metastatic cases). **C.** primary organ (urinary bladder NEC vs. prostate NEC). One patient with the renal pelvis phenotype was included in the bladder group. B, bladder; P, prostate. **D.** NEC histology (pure NEC vs. composite NEC). **E.** TMB: high (≥10) vs. low (<10). NEC, neuroendocrine carcinoma; TMB, total mutational burden.

2, 3, 4, and 6). In patient 2, the *PIK3CA* c.3140A>G variant was shared between SCC and UC components. Additionally, the *KMT2A* variant, a chromatin-modifying gene, and *TERT* promoter variant were uniquely detected in the SCC component. In patient 3, the same variant in *NSD1* that was recently recognized as a developmental regulator of the epigenome was shared between SCC and UC components (Choufani et al., 2015). In patient 4, the *TP53* c.853G>A variant was shared between SCC and UC components. In patient 6, two different SNVs in *PIK3CA* (c.1357G>A and 252 c.1633G>A), *PTEN* (c.640C>T and c.860C>G), and *RB1* (c.409G>T and c.751C>T) and a *TERT* promoter variant (c.228 C>T) were shared between SCC and UC components. Overall, the TMB was similar between NEC and non-NEC components, except for one sample with UC histology (ID-2.2).

Discussion

Several studies have compared the clinical behaviors of urogenital pure and mixed NEC. Wang et al. reported worse cancer-specific survival in urinary pure LCNEC tan in mixed LCNEC (median, 3.5 months vs. 40.5 months; p=0.03) (Wang et al., 2021). According to the multi-center analysis by Pasquier et al., pure SCC of the bladder (SCCB) is associated with worse OS than mixed SCCB (2-year OS rate, 45% vs. 22%; p=0.02) (Pasquier et al., 2015). In contrast, Parimi et al. recently demonstrated no significant differences in OS between patients with pure SCCB and those with mixed SCCB (Parimi et al., 2023). In our relatively small-sized cohort, no visible difference was detected in OS between the two histological groups. Thus, any clinical significance remains controversial. It should be noted that our survival analysis may be biased by the small sample size and the low number of surgically resected cases. In our study, the most common histological subtype coexisting with NEC was UC. Similarly, previous studies including mixed NEC showed that conventional UC and UC *in situ* were the major non-NEC components, followed by adenocarcinoma (Wang et al., 2018, 2021). In our study, mixed NEC cases accounted for approximately 40% of all NEC cases. The lower percentage of mixed NECs compared with the literature may be related to the fact that most cases are biopsies (Abrahams et al., 2005; Wang et al., 2018, 2021; Lim and Sundar, 2019).

In our genomic analysis with a focus on bladder NEC, the prevalence of *TP53* and *RB1* mutations was not as extraordinary as that in SCLC harboring these mutations in over 90% of the cases (George et al., 2015). Alternatively, mutations in histone-modification genes and *TERT* promoter, rarely observed in SCLC, were detected in approximately 30% of the cases. A *TERT* promoter mutation is the most common genomic alteration in UC, affecting 60-80% of patients (Necchi et al., 2021). Chang et al. characterized molecular features of SCCB (n=61), with reference to SCLC or UC (Chang et al., 2018). While the prevalence of mutations in *TP53* and *RB1* was high in both SCCB and SCLC, mutations in chromatin-modifying genes or the *TERT* promoter were exclusively observed in SCCB. Mutations in chromatin-modifying genes or the *TERT* promoter were also characteristic of UC. According to a large-scale analysis of 132 SCCB cases based on the Foundation Medicine database, the prevalence of mutations in *TP53*, *RB1*, and *TERT* promoter was 92%, 75%, and 68%, respectively (Hoffman-Censits et al., 2021). Parimi et al. also compared the gene expression profiles and occurrence of mutations between pure and mixed SCCB (Parimi et al., 2023). They showed that the gene expression level of the neuroendocrine signature in mixed SCCB was intermediate between the level in pure SCCB and UC, and that, histologically, UC in mixed SCCB featured a neural signature without neuro-endocrine differentiation.

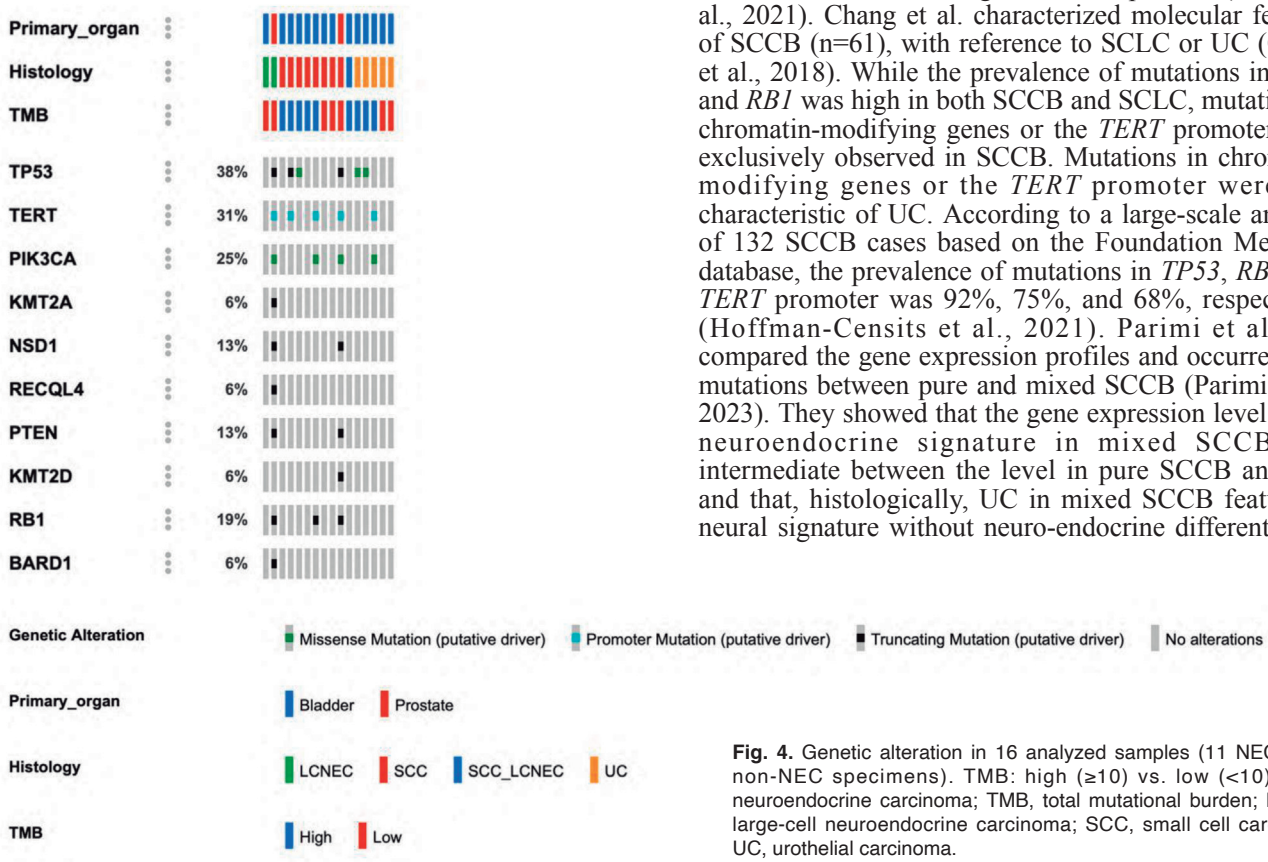

Fig. 4. Genetic alteration in 16 analyzed samples (11 NEC and 5 non-NEC specimens). TMB: high (≥10) vs. low (<10). NEC, neuroendocrine carcinoma; TMB, total mutational burden; LCNEC, large-cell neuroendocrine carcinoma; SCC, small cell carcinoma; UC, urothelial carcinoma.

As for the four specimens in which the SCC and UC components were analyzed separately, the mutation status was concordant in 50% of the cases for *TERT*, 50% for *TP53*, 100% for *ARID1A*, 100% for *RB1*, and 75% for *ATRX*. According to another genomic analysis of 24 SCCB cases, *TERT*, *TP53*, *ARID1A*, and *RB1* mutations were detected in 92%, 75%, 38%, and 38% of the cases, respectively (Feng et al., 2024). The level of neuroendocrine markers in mixed SCCB was intermediate between that of pure SCCB and conventional muscle-invasive bladder cancer. Consistently, our analysis yielded similar mutation profiles between NEC and non-NEC components, despite the different morphologies. This observation suggests that bladder NEC likely has a clonal origin shared with UC or adenocarcinoma. In our analysis, SNVs in *PIK3CA* were also detected in both NEC and UC components. Previous studies have shown that 10-20% of NECs and 20% of UCs harbor these SNVs (Hoffman-Censits et al., 2021; Feng et al., 2024).

In terms of potential druggable targets, the *BARD1* frameshift variant, which encodes a tumor suppressor involved in the DNA damage response, was detected in one patient (ID-9). According to OncoKB™, the first somatic tumor mutation database recognized by the U.S. Food and Drug Administration (Chakravarty et al., 2017), *BARD1* oncogenic mutations in prostate cancer are categorized as Level 1, and administration of olaparib as a PARP inhibitor has been approved by the FDA. In addition, *PIK3CA* p.H1047R/p.E545K are grouped as Level 1 mutations for breast cancer and acute myeloid leukemia, respectively. The evidence for endocrine cancer is not robust, and further investigations are warranted. Remarkably, more than half of our patients with SCCB had a high TMB (≥10 mut/Mb), and are candidates for the immune checkpoint inhibitor pembrolizumab (FDA Label KEYTRUDA®). The study by Hoffman-Censits et al. showed a high TMB (≥10 mut/Mb) in over one-quarter of SCCB samples (Hoffman-Censits et al., 2021).

Some limitations should be acknowledged in this study. First, owing to the rarity of the disease, the number of enrolled patients was small, which complicates in-depth comparisons between molecular subgroups and discussions on prognosis and pathogenesis. Second, the tissue size obtained from prostate NEC was mostly unsuitable for genomic analysis, and only two cases were examined. Third, the sequencing depth was relatively low in several samples associated with minute tissue sizes, and gene alterations with a low variant allele frequency (minor clone) may have escaped detection. Fourth, our genomic analysis focused primarily on SNVs; incorporating copy number variations or splice variants could have increased the percentage of gene alterations. Lastly, further comprehensive studies incorporating epigenetic or transcriptome analyses should be conducted to obtain a fuller understanding of the disease pathophysiology.

In conclusion, based on molecular features, this study suggests mechanisms for the pathogenesis of urogenital NEC. Bladder NEC may originate from clones shared with UC but not from an independent clone. Additionally, some cases harbor druggable gene alterations, including TMB-high, that are applicable to checkpoint inhibitor immunotherapy. These data comprise a useful reference for future clinical developments regarding this rare and intractable malignancy.

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Author Contributions. A.O., K.K., Y.S., K.I., and S.T. conceived and designed the study; A.O. developed the methodology and wrote the paper; K.K., Y.S., N.H., J.Y., K.I, and S.T. reviewed and revised the paper; A.O., K.K., Y.S., N.H. acquired, analyzed, and interpreted the data, and performed statistical analyses. All authors read and approved the final manuscript.

Data Availability. The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

A Conflict of Interest Statement. The authors declare no conflicts of interest.

Ethics Approval and Consent to Participate. This study was approved by the Institutional Review Board of the Japanese Foundation for Cancer Research (2020-GA-1221) and was performed in accordance with the principles of the Declaration of Helsinki.

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