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Original contribution

Targeted next-generation sequencing of well-differentiated rectal, gastric, and appendiceal neuroendocrine tumors to identify potential targets☆,☆☆

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Summary Rectal neuroendocrine tumors (NETs) are the most common gastrointestinal (GI) NETs with an uncertain malignant potential despite their small size. There are limited data about driver mutations in rectal NETs, which may explain the tumors' unexpected behavior or common histologic morphology with other GI-NETs. Here, we investigated the clinically and pathologically relevant mutations of rectal and nonrectal NETs and compared the frequency and clinical significance of detected mutations between them. We sequenced 84 primary GI-NETs (69 rectal, 7 gastric, 5 appendiceal, and 3 sigmoid colon NETs) and 3 metastatic GI-NETs using targeted next-generation sequencing. Twenty-one rectal NETs (30.4%) showed at least 1 mutation in 24 cancer-related genes; the most common

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mutations were *TP53* (10.1%) and *FBXW7* (7.2%), of which 73% were pathogenic/likely pathogenic mutations. *TP53* (p.R337C and p.R213*), *PTEN* (p.W111*, p.Q214*), *CDKN2A* (p.W110*), *FBXW7* (p.R465H), and *AKT1* (p.R23Q) were repetitive mutations found exclusively in rectal NETs, whereas *SMAD4* (p.R361C) and *STK11* (p.D176N) were repetitive mutations found only in gastric NETs. *PTEN* (p.G129K), *EGFR* (p.E709K), and *KIT* (p.V555I) were shared mutations between rectal and appendiceal NETs, whereas *SMAD4* (p.R361C), *ALK* (p.G1202R), *VHL* (p.Q132*), and *IDH1* (p.R132H) were concurrently detected between rectal and gastric NETs. GI-NETs with higher histologic grades, lymphovascular invasion, or recurrence tended to have higher numbers of mutation variants than other tumors; however, there was no significant difference. In conclusion, rectal NETs commonly carried pathogenic/likely pathogenic mutations. Because most mutations were identified in nonhotspot positions, next-generation sequencing is useful in identifying potential drug targets in rectal NETs.

1. Introduction

Neuroendocrine tumors (NETs) are uncommon, heterogeneous groups of neoplasms with a malignant potential, most of which develop in the gastrointestinal (GI) tract [1,2]. The incidence of GI-NETs depends on the primary tumor site (the foregut, midgut, and hindgut), with the highest frequencies observed in the rectum [1,2]. These neoplasms display a large spectrum of biologic behavior with survival ranging from 6 months to >20 years [1,2]. A wide range of somatic genetic alterations have also been described for various NETs, mainly in the pancreas. Except for pancreatic NETs, there have been few studies on molecular profiling of GI-NETs.

Rectal NETs are the most common GI-NETs, with the majority having a small size (<10 mm) [3-5] and with a 5-year overall survival of 88% [6,7]. However, rectal NETs rarely behave in an expected manner. This uncertain malignant potential of rectal NETs, despite their small size and confinement to the mucosa and submucosa, is of clinical concern especially with regard to treatment [8]. Lymph node metastasis occurs in 3% of tumors of <10 mm in size [9]. The Food and Drug Administration recently approved a few targeted agents for pancreatic NETs, including sunitinib, a multi–tyrosine kinase inhibitor, and everolimus, an inhibitor of the PI3K/AKT/mTOR signaling pathway [8]. However, there are limited data about the incidence of driver mutations in rectal NETs, which may explain the tumors' unexpected behavior or common histologic morphology with other GI-NETs. Thus, we conducted this retrospective study to determine the frequency of clinically and pathologically relevant mutations in rectal and other GI-NETs to identify potential targets for treatment. Additionally, this is the first report stratifying the clinical significance of detected mutational profiling in rectal NETs using the ClinVar database [10], a publicly available archive for interpretations of the clinical significance of variants for reported conditions, which may provide practical information about therapeutic assessment in GI-NETs.

2. Materials and methods

2.1. Patients and tumor samples

An electronic search in the database of the pathology department of Hallym University Sacred Heart Hospital revealed 141 consecutive patients with primary GI-NETs who underwent endoscopic or surgical resection in the hospital between 2005 and 2015. We excluded 57 patients whose formalin-fixed, paraffin-embedded (FFPE) tissue blocks contained too few tumor cells for the assay or who had not been treated with chemotherapy or targeted drug therapy at the time of tumor excision. Finally, 84 patients with adequate FFPE tumor blocks for molecular analysis and with complete clinical and follow-up data were enrolled in this study; 60 of these patients were included in previous studies [8,11]. All patients provided informed consent before endoscopic or surgical resection. This study was approved by the institutional review board of Hallym University Sacred Heart Hospital (2017-I020).

Clinicopathological data, including age, sex, tumor location, lymph node or distant metastasis, radiological data, treatment details, tumor recurrence, and survival were recorded. The first follow-up for patients was performed using colonoscopy and abdominal computed tomography 6 months after the endoscopic or surgical resection. Thereafter, endoscopy and abdominal computed tomography were performed yearly. *Follow-up duration* was defined as the interval between endoscopic or surgical resection day and last outpatient visit day. Patients were followed up in the outpatient department every 6 months for the first 2 years after surgery and then annually. The last follow-up was in September 2017.

2.2. Histologic evaluation

All hematoxylin and eosin–stained slides were reviewed by a GI pathologist (M. J. K.) to confirm the diagnosis and evaluate histopathological characteristics, including tumor size, mitotic count, tumor grade, resection margins, depth of invasion, lymphatic invasion, venous invasion, perineural invasion, and

resection margin status. Immunohistochemical (Ki-67 and D2-40) and histochemical Elastica van Gieson (EVG) stainings and their analyses had been performed for proliferative index and lymphatic or vascular invasion in the previous studies [8,11], of which established findings were used in this study. Mitotic rates on hematoxylin and eosin stain were determined in 50 high-power fields (HPFs), and the mean mitotic count was calculated as the number of mitoses per 10 HPFs [11]. Ki-67 labeling index was assessed using a GenASIs capture and analysis system (Applied Spectral Imaging, Carlsbad, CA), as previously described [11]. Briefly, the highest labeled region at low magnification was captured at ×200 magnification, and Ki-67 labeling index was automatically calculated. Either higher mitotic count or Ki-67 labeling index was determined for tumor grading. Therefore, tumors were classified into grade 1 (mitotic count of <2 per 10 HPFs and/or <3% Ki-67), grade 2 (mitotic count of 2-20 per 10 HPFs and/or 3%-20% Ki-67), and grade 3 (mitotic count of >20 per 10 HPFs and/or >20% Ki-67) according to the 2016 World Health Organization (WHO) classification and the North American Neuroendocrine Tumor Society guidelines [12]. Resection margin status was classified according to the extension of tumor cells into the resection margin: complete resection (R0), in which the lateral and vertical resection margins were free of tumor, and microscopic incomplete resection (R1), in which the tumor extended into the lateral or vertical resection margin.

2.3. DNA extraction

A tissue microtome (Leica Biosystems, Wetzlar, Germany) was used to obtain two 10-μm sections on the glass slides from each FFPE tissue block. Tumor areas on the glass slides were manually macrodissected from unstained tissue sections to enrich for a tumor cell population of >50%. DNA was extracted and purified using the Ion AmpliSeq Direct FFPE DNA Kit (Thermo Fisher Scientific, Waltham, MA, USA) and QIAamp DSP DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), respectively. The yield of purified genomic DNA was estimated using the Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) before library preparation for sequencing.

2.4. Library preparation and sequencing

The next-generation sequencing (NGS) platform used in this study was the Ion Personal Genome Machine Sequencer (Thermo Fisher Scientific). Library preparation for each sample was performed using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific) and Ion AmpliSeq Cancer HotSpot Panel v2 (Thermo Fisher Scientific) according to the manufacturer's instructions. This targeted cancer panel sequences hotspot mutations in 207 amplicons covering >20 000 bases of 50 oncogenes and tumor suppressor genes with known cancer associations, including *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAS*, *GNAQ*, *HNF1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR/VEGFR2*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, and *VHL*. Approximately 10 ng of genomic DNA from each sample was used to prepare barcoded libraries using the IonXpress Barcode Adapters (Thermo Fisher Scientific). Libraries were combined to a final concentration of 100 pmol/L using the Ion Library Universal Quantification Kit (Thermo Fisher Scientific), and emulsion polymerase chain reaction was performed using the Ion Torrent OneTouch 2 System. Each pool was loaded onto an Ion 318v2 Chip (Thermo Fisher Scientific) for single-end sequence analysis with the Ion Personal Genome Machine Sequencer using 500 flows (125 cycles) for 200-base-read sequencing.

2.5. Data analysis

Raw sequence reads were mapped against human reference genome hg19 and cleaned up before variant calling. The Ion Torrent platform-specific pipeline software was used throughout the variant calling process. Annotation was performed using Variant Effect Predictor [13]. To compare with cancer genome studies and check hotspot mutations, we downloaded all cancer genome studies from cBioPortal [14] using the CGDS-R package. The number of variants in each spot from the cancer genome studies was manually annotated to our results.

To identify confident putative somatic variants, annotated raw variants were filtered according to the following criteria: (1) nonsynonymous single-nucleotide variant (SNV) or short insertion or deletion in coding regions; (2) coverage ≥50× and variant allele frequency (VAF) ≥5%, or coverage ≥1000× and VAF ≥3%; (3) minor allele frequency <1% in the gnomAD [15] and 1000 Genomes Project [16]; and (4) annotated as "Pathogenic," "Likely pathogenic," or "Drug response" in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/; accessed October 2018) [10] or reported at least once in any of the cancer genome projects archived in cBioPortal. The resulting list of variants was manually reviewed and visually confirmed using the Integrated Genomics Viewer (http://www.broadinstitute.org/igv/). R and ProteinPainter were used in the visualization.

Of the final gene list, 14 genes with hotspot mutations underwent gene set enrichment analysis (GSEA) [17] using Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb/annotate.jsp), and we selected 3 gene databases for analysis: KEGG, REACTOME, and BIOCARTA gene sets. A result was considered significant if the cutoff of false discovery rate was ≤0.01 and the P was < .05.

3. Results

3.1. Patient characteristics

A total of 84 Korean patients (53 men and 31 women) with a median age of 48 years (range, 10-73 years) were included

in this study (Table 1). According to the WHO classification, 59 patients had grade 1 NETs (51 in rectum and 8 in nonrectal areas), 24 had grade 2 NETs (18 in rectum and 6 in nonrectal areas), and 1 patient had a grade 3 NET in the stomach. The most frequent site for GI-NETs was the rectum (n = 69, 82.1%) followed by the stomach (n = 7, 8.3%), appendix (n = 5, 6.0%), and colon (n = 3, 3.6%).

Table 1 Demographic and clinical features of patients with gastrointestinal neuroendocrine tumors

Characteristic	N = 84 (%)
Sex	
Male	53 (63.1)
Female	31 (36.9)
Age (y), median	48 (range, 10-73)
<60	69 (82.1)
≥60	15 (17.9)
Procedure	
Surgical resection	12
Endoscopic resection	72
Site of tumor origin	
Rectum	69
Stomach	7
Appendix	5
Sigmoid colon	3
Tumor size, mean ± SD (cm)	0.77 ± 0.50 (range, 0.2-3.5)
≤1 cm	77 (91.7)
>1 cm	7 (8.3)
Tumor depth	
Mucosa/submucosa	80
Muscle layer	1
Adipose tissue	3
Resection margin status	
R0	72 (85.7)
R1	12 (14.3)
Follow-up	
Recurrence	10 (11.9)
Died	5 (5.9)
Grade	
G1	59 (70.2)
G2	24 (28.6)
G3	1 (1.2)
Mitosis/10 HPF	
<2	62 (73.8)
2-20	21 (25.0)
>20	1 (1.2)
Ki-67 LI (%)	
<3	78 (92.9)
3-20	5 (5.9)
>20	1 (1.2)
Vascular invasion	
Positive	21 (25.0)
Negative	63 (75.0)
Lymphatic invasion	
Positive	17 (20.2)
Negative	67 (79.8)
Perineural invasion	
Positive	5 (5.9)
Negative	79 (94.1)

Abbreviation: LI, labeling index.

3.2. Genetic alterations in GI-NETs

We sequenced 84 primary GI-NETs (69 rectal, 7 gastric, 5 appendiceal, and 3 sigmoid colon NETs) and 3 metastatic GI-NETs. There was an average of 405 114 reads (range, 128 509-711 217) per case. An average of 96.4% of reads (range, 81.9%-99.6%) per case was mapped to the intended targeted regions of the human genome. All regions had an average coverage of 1770× (range, 817-3281×) (Supplementary Table 1).

After multiple filtering steps described in the sections of materials and methods, 114 variants of 30 genes (94 missense, 16 nonsense, and 4 splice-site SNVs) were finally sorted in 27 (32.1%) GI-NETs. Three cases of sigmoid colon NETs were all filtered out in the final list of variants. Seven genes, namely, *TP53*, *PTEN*, *SMAD4*, *EGFR*, *ATM*, *CDKN2A*, and *KIT*, were commonly mutated among rectal, gastric, and appendiceal NETs. Among them, the most frequently mutated gene was *TP53* (11/84, 13.1%) followed by *PTEN* (6/84, 7.1%) and *SMAD4*, *EGFR*, and *CDKN2A* (5/84, 6.0%). Genes with variants found in more than 1 sample are depicted in Fig. 1.

3.3. Genetic alterations in rectal NETs

Sixty-six different variants in the 24 genes were identified in rectal NETs. Twenty-one rectal cases (30.4%) showed more than 1 mutation in the 24 cancer-related genes (*TP53* [7/69, 10.1%], *FBXW7* [5/69, 7.2%], *PTEN* [4/69, 5.8%], *CDKN2A* [4/69, 5.8%], *EGFR* [3/69, 4.3%], *ATM* [3/69, 4.3%], *SMARCB1* [3/69, 4.3%], *KIT* [2/69, 2.9%], *IDH1* [2/69, 2.9%], *KRAS* [2/69, 2.9%], *ALK* [2/69, 2.9%], *VHL* [2/69, 2.9%], *AKT1* [2/69, 2.9%], *PIK3CA* [2/69, 2.9%], *RET* [2/69, 2.9%], *SMAD4* [1/69, 1.4%], *STK11* [1/69, 1.4%], *FLT3* [1/69, 1.4%], *BRAF* [1/69, 1.4%], *CTNNB1* [1/69, 1.4%], *ERBB2* [1/69, 1.4%], *EZH2* [1/69, 1.4%], *HNF1A* [1/69, 1.4%], and *SMO* [1/69, 1.4%]). Among these 24 genes, repetitive mutations in both codons and peptides in rectal NETs were identified in *TP53* (p.R337C and p.R213*), *PTEN* (p.W111* and p.Q214*), *CDKN2A* (p.W110*), *FBXW7* (p.R465H), *KIT* (p.V555I), *IDH1* (p.R132H), and *AKT1* (p.R23Q). The representative diagrams of repetitive mutations, together with histology, between rectal NETs and gastric or appendiceal NETs were summarized in Fig. 2. In the histologic findings, P7 and P12 rectal cases sharing *TP53* (R337C) and P38 and P40 rectal cases carrying *TP53* (R213*) showed mucosal invasion and infiltrative border. P68 and P69 rectal cases sharing *PTEN* (W111*) showed no invasion into mucosa and well-defined border. The cases harboring *KRAS* or *BRAF* mutations were not accompanied with any adenomatous epithelium overlying the NETs.

As for clinical significance in ClinVar, 48 (72.7%) of the 66 different variants identified in rectal NETs carried a pathogenic/likely pathogenic assertion of clinical significance

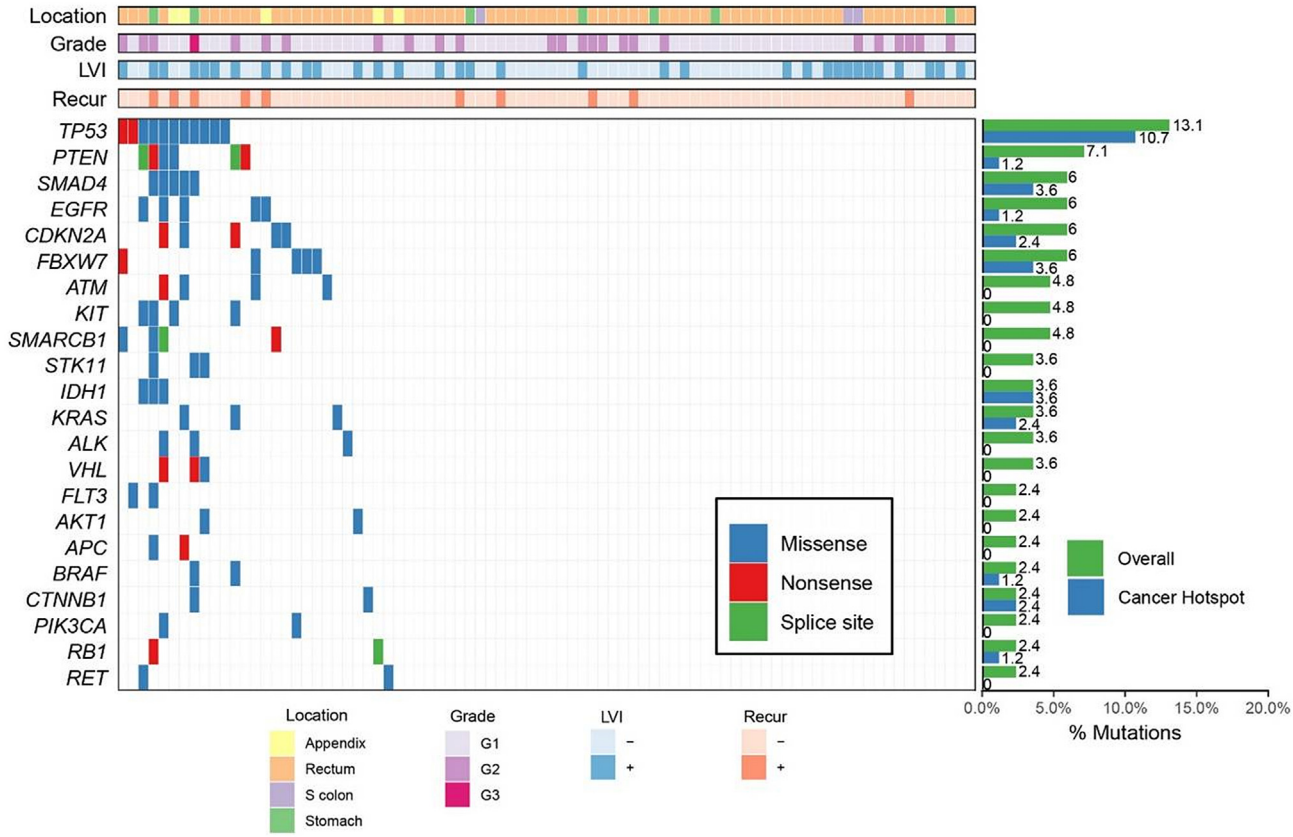

Fig. 1 Schematic overview of overall mutational profile of 84 primary GI-NETs, followed by targeted NGS and analysis. Each column represents a case. The top 4 panels show the location of tumors, WHO grade, LVI, and presence of recurrence. The bottom panel shows the distribution of mutations. The 3 mutation types are distinguished by different colors. The right panel represents the overall frequency and the cancer hotspot mutation frequency (≥20 cases in previous cancer genome studies archived in cBioPortal) in the 84 cases. Abbreviations: LVI, lymphovascular invasion; Recur, recurrence.

in ClinVar, whereas 3 variants (4.5%) (*TP53* [pR337H and E180K] and *STK11* [P179L]) had conflicting interpretation of pathogenicity or were of uncertain significance. *EGFR* (p.E709K and p.T751I) showed drug response in ClinVar. Although listed on ClinVar, 16 variants (24.2%) had no assertion of clinical significance provided (Table 2). In the above-mentioned 7 repetitive mutations, *TP53* (p.R337C and p.R213*), *PTEN* (p.W111* and p.Q214*), and *IDH1* (p.R132H) were pathogenic, whereas *CDKN2A* (p.W110*) and *FBXW7* (p.R465H) were likely pathogenic. *KIT* (p.V555I) and *AKT1* (p.R23Q) had no assertion of clinical significance provided in ClinVar.

3.4. Genetic alterations in gastric and appendiceal NETs

In gastric NETs, 2 cases (2/7, 28.6%) showed more than 1 mutation in 15 cancer-related genes (*TP53*, *SMAD4*, *STK11*, *IDH1*, *PTEN*, *KIT*, *SMARCB1*, *ALK*, *VHL*, *APC*, *BRAF*, *CTNNB1*, *RB1*, *FLT3*, and *GNAS*), where 21 different variants were identified. Among these 15 genes, repetitive mutations in both codons and peptides in gastric NETs were identified in *SMAD4* (p.R361C) and *STK11* (p.D176N). Meanwhile, 6 genes (*EGFR*, *ATM*, *CDKN2A*, *FBXW7*, *KRAS*, and *PIK3CA*) were not detected in gastric NETs. As for clinical significance in ClinVar, 19 (90.5%) of the 21 different variants identified in gastric NETs carried a pathogenic/likely pathogenic assertion of clinical significance (Table 3).

In appendiceal NETs, 4 cases (4/5, 80.0%) showed more than 1 mutation in 13 cancer-related genes (*TP53*, *PTEN*, *SMAD4*, *EGFR*, *ATM*, *CDKN2A*, *KIT*, *KRAS*, *APC*, *RB1*, *ERBB4*, *HRAS*, and *MET*), where 17 different variants were identified. No repetitive mutations in both codons and peptides were identified in appendiceal NETs. Regarding clinical significance in ClinVar, 11 (64.7%) of the 17 different variants were pathogenic/likely pathogenic (Table 4).

3.5. Comparisons between rectal and nonrectal NETs

The mean numbers of mutations were 3.4 for rectal NETs and 7.2 for nonrectal NETs (4.8 for appendiceal NETs and 12 for gastric NETs). Variants of *FBXW7*, *PIK3CA*, *AKT1*, and *RET* were detected only in rectal NETs.

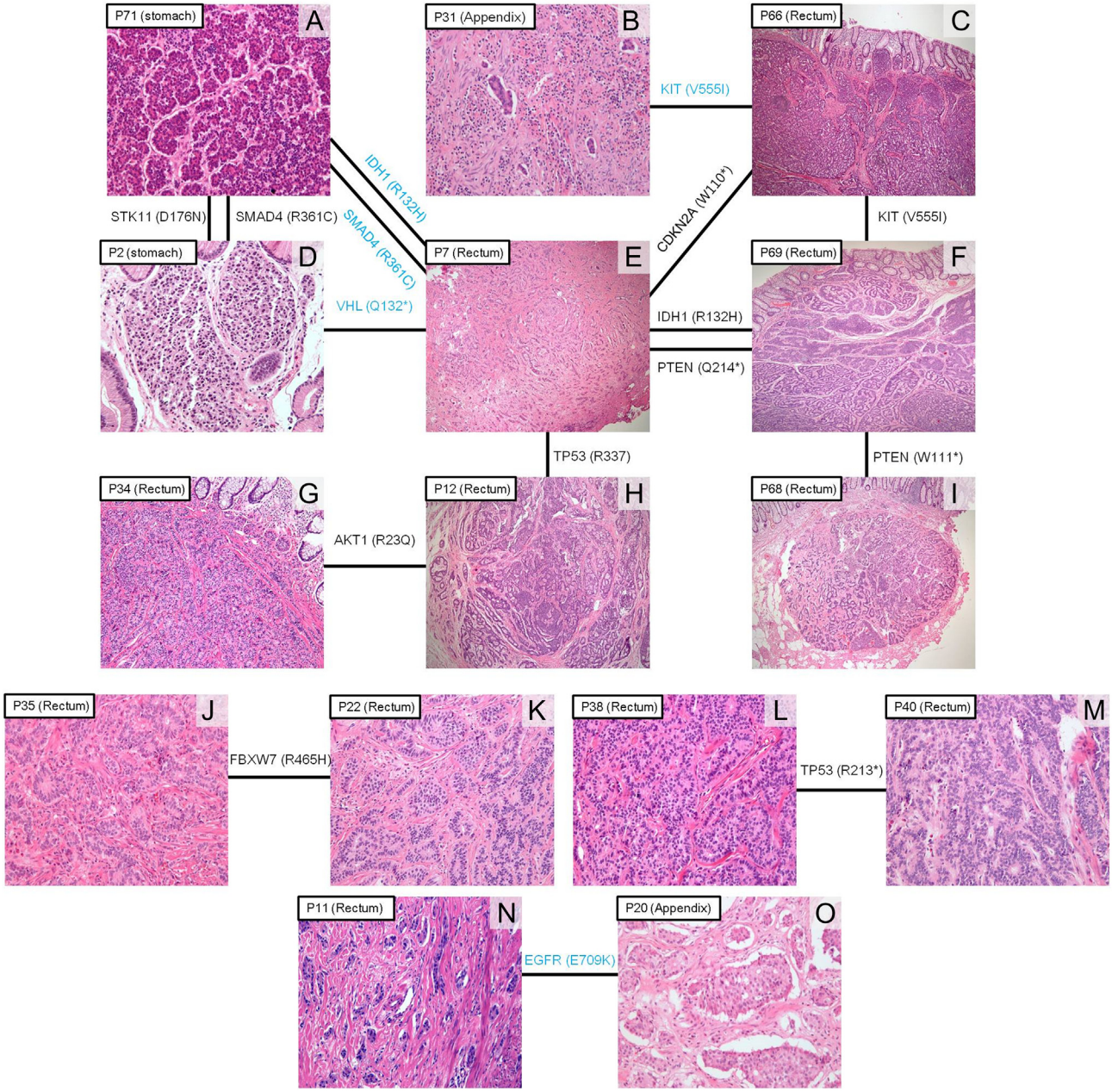

Fig. 2 Schematic overview of histologic findings of repetitive mutations in both codons and peptides between rectal NETs and gastric or appendiceal NETs. (A, B, D, J-N: original magnification, ×100; C, E-I: ×40; O: ×200).

The number of patients having *TP53* mutations was higher for rectal NETs than for nonrectal NETs (7 for rectal, 2 for gastric, and 2 for appendiceal NETs). However, the proportion was lower in rectal NETs than in nonrectal NETs (10.1% for rectal, 28.6% for gastric, and 40.0% for appendiceal NETs).

Although mutually shared mutations among gastric, appendiceal, and rectal tumors were not identified, 3 mutations were shared between rectal and appendiceal NETs and 4 mutations between rectal and gastric NETs. *PTEN* (p.G129R), *EGFR* (p.E709K), and *KIT* (p.V555I) were concurrently found between rectal and appendiceal NETs. On the other hand, *SMAD4* (p.R361C), *ALK* (p.G1202R), *VHL* (p.Q132*), and *IDH1* (p.R132H) were shared between rectal and gastric NETs.

3.6. Associations of number of genomic variants with poor clinicopathological factors

The number of variants tended to increase with increasing histologic grade. The mean numbers of variants were 3.5, 5.3, and 10 for grades 1, 2, and 3, respectively (P = .380).

Table 2 Clinical significance of rectal NET-associated variants in ClinVar

	Case n (Variant n)	Clinical significance			
		Pathogenic	Likely pathogenic	VUS	Not provided
TP53	7 (11)	5 (7) R337C (x2) [a], R213* (x2) [a], R337H, H179Y, V173M	2 (2) R280K, R267W	2 (2) R202C, E180K	0
PTEN	4 (10)	4 (7) G129R, W111* (x2) [a], Q214* (x2) [a], D24N, Splice	3 (3) Splice, C124Y, G165E	0	0
SMAD4	1 (4)	1 (3) R361C, G386D, R445*	0	0	1 (1) G510R
EGFR	3 (6)	3 (3) E709K (DR), G719S, T751I (DR)	1 (1) T790M	0	2 (2) D587N, G724S
ATM	3 (3)	1 (1) R3008C	1 (1) E848K	0	1 (1) W3055*
CDKN2A	4 (4)	1 (1) M53I	2 (2) W110* (x2)	0	1 (1) A68T
FBXW7	5 (5)	0	3 (3) R465H (x2), R505C	0	2 (2) R441Q, R278*
KIT	2 (2)	0	0	0	2 (2) V555I (x2) [a]
STK11	1 (1)	0	0	1 (1) P179L	0
IDH1	2 (2)	2 (2) R132H (x2) [a]	0	0	0
KRAS	2 (3)	2 (2) P34L, G13D	0	0	1 (1) K147T
SMARCB1	3 (3)	2 (2) R40*, R377H	0	0	1 (1) R374W
ALK	2 (2)	0	1 (1) G1202R	0	1 (1) G1201R
FLT3	1 (1)	0	0	0	1 (1) V592I
VHL	2 (2)	2 (2) Q132*, R167W	0	0	0
AKT1	2 (2)	0	0	0	2 (2) R23Q (x2) [a]
BRAF	1 (1)	1 (1) V600M	0	0	0
CTNNB1	1 (1)	0	1 (1) G34R	0	0
PIK3CA	2 (2)	0	1 (1) V344M	0	1 (1) D1045N
RET	2 (2)	1 (1) R897Q	0	0	1 (1) S891L
ERBB2	1 (1)	1 (1) V842I	0	0	0
EZH2	1 (1)	1 (1) V621M	0	0	0
HNF1A	1 (1)	1 (1) T260M	0	0	0
SMO	1 (1)	0	0	0	1 (1) V321M

Abbreviations: VUS, variant of uncertain significance; DR, drug response.
[a] (x2) indicates the 2 numbers of same variants.

The mean number of variants was higher in samples with lymphovascular invasion than in those without lymphovascular invasion (5.1 versus 3.4; $P = .404$) and higher in those with recurrence than in those without recurrence (7 versus 3.6; $P = .174$). However, these findings were not statistically significantly different.

3.7. Genomic alterations in metastatic NETs

We also sequenced 3 metastatic NETs to lymph nodes; 2 of them were matched with primary rectal NETs and 1 with gastric NET. Most of the variants in metastatic NETs showed VAFs <5% (44/47, 94%), whereas 3 variants, namely, *KDR* (p.W206*), *APC* (p.A1582P), and *ATM* (p.2869D), had VAFs >5%. These 3 variants had unknown clinical significance in the ClinVar annotation and were all filtered out in the final list of variants. As a result, no significant genetic alterations in 50 cancer-related genes were found in all 3 metastatic NETs.

3.8. Comparisons with previous cancer genome studies

We further compared the mutational variants detected in our study with "hotspot positions" frequently reported in previous cancer studies (positions shared in ≥20 samples in cancer genome studies in cBioPortal). We recognized that 45 variants (39.5%, 45/114) occurred in the hotspot positions of mutation sequences; 91% (10/11) of *TP53*, 60% of *SMAD4* (3/5) and *FBXW7* (3/5), and all *IDH1* (4/4) detected in our study were identified in the hotspot positions of those mutation sequences. All hotspot *SMAD4* SNVs occurred in the MH2 domain, which encodes p.P356L, p.R361C, and p.R445* (Fig. 3A). Most of the *FBXW7* variants were identified in the WD40 repeat domain, including hotspot SNVs encoding p.R465H and p.R505C (Fig. 3B). All *IDH1* SNVs occurred at hotspots (p.R132H, p.R132C) in the IDH domain (Fig. 3C). Compared with findings of previous cancer genome studies, all hotspot variants of *SMAD4* were reported predominantly in studies of the GI tract; however, variants of *FBXW7* and

Table 3 Clinical significance of 7 gastric NET-associated variants in ClinVar

	Case n (Variant n)	Clinical significance			
		Pathogenic	Likely pathogenic	VUS	Not provided
TP53	2 (5)	2 (2) E286K, L194F	2 (2) E285K, R283H	1 (1) R267Q	0
PTEN	1 (1)	1 (1) Q17*	0	0	0
SMAD4	2 (3)	2 (2) R361C (x2)[a]	1 (1) G508D	0	0
KIT	1 (1)	0	1 (1) D52N	0	0
STK11	2 (2)	0	2 (2) D176N (x2)[a]	0	0
IDH1	1 (2)	1 (2) R132H, R132C	0	0	0
SMARCB1	1 (1)	1 (1) R374Q	0	0	0
ALK	1 (1)	0	1 (1) G1202R	0	0
FLT3	1 (1)	1 (1) D835N	0	0	0
VHL	1 (1)	1 (1) Q312*	0	0	0
APC	1 (1)	1 (1) G1120E	0	0	0
BRAF	1 (1)	0	0	0	1 (1) S467L
CTNNB1	1 (1)	1 (1) G34E	0	0	0
RB1	1 (1)	1 (1) R579*	0	0	0
GNAS	1 (1)	1 (1) R201C	0	0	0

[a] (x2) indicates the two numbers of same variants.

IDH1 had similar reported incidences in GI tract and non-GI tract NETs or a higher incidence in non-GI tract NETs.

3.9. Identification of gene signatures using gene set enrichment analysis

We performed GSEA with 14 genes (*TP53*, *PTEN*, *SMAD4*, *EGFR*, *CDKN2A*, *FBXW7*, *IDH1*, *KRAS*, *BRAF*, *CTNNB1*, *RB1*, *ERBB2*, *GNAS*, and *HRAS*) with hot spot mutations. The top 50 enriched gene sets were listed in Supplementary Table 2. Several types of cancer gene sets including colorectal or pancreatic cancers were significantly enriched. And the genes in cell cycle, adhesion, ERBB, ERK/MAPK, and PI3K signaling pathways were mostly associated with the 14 genes detected in GI-NETs.

4. Discussion

Our study showed that mutations in gene coding sequences were a relatively common event in GI-NETs. Overall, 27 (32.1%) GI-NETs including rectal (30.4%, 21/69), gastric (28.6%, 2/7), and appendiceal (80%, 4/5) cases had at least 1 mutation in at least 30 cancer-related genes. Seven genes, namely, *TP53*, *PTEN*, *SMAD4*, *EGFR*, *ATM*, *CDKN2A*, and *KIT*, were commonly mutated among rectal, gastric, and appendiceal NETs. These genes were linked to the cancer gene sets of colorectal or pancreatic cancers, cell cycle, adhesion, ERBB signaling, ERK/MAPK signaling, and PI3K signaling pathways. Approximately 73% of 66 different variants identified in rectal NETs were pathogenic/likely pathogenic mutations, suggesting that about three-fourths of rectal

Table 4 Clinical significance of 5 appendiceal NET-associated variants in ClinVar

	Case n (Variant n)	Clinical significance			
		Pathogenic	Likely pathogenic	VUS	Not provided
TP53	2 (3)	0	2 (2) A161T, R110H	1 (1) V197 M	0
PTEN	1 (1)	1 (1) G129R	0	0	0
SMAD4	2 (2)	0	0	1 (1) R283H	1 (1) R283H
EGFR	2 (2)	1 (1) E709K (DR)	1 (1) R108K	0	0
ATM	1 (1)	0	1 (1) D2708N	0	0
CDKN2A	1 (1)	0	0	0	1 (1) D74N
KIT	1 (1)	0	0	0	1 (1) V555I
KRAS	1 (1)	1 (1) A146T	0	0	0
APC	1 (1)	1 (1) Q1096*	0	0	0
RB1	1 (1)	1 (1) Splice	0	0	0
ERBB4	1 (1)	0	0	0	1 (1) R306H
HRAS	1 (1)	1 (1) G12S	0	0	0
MET	1 (1)	0	1 (1) T1010I	0	0

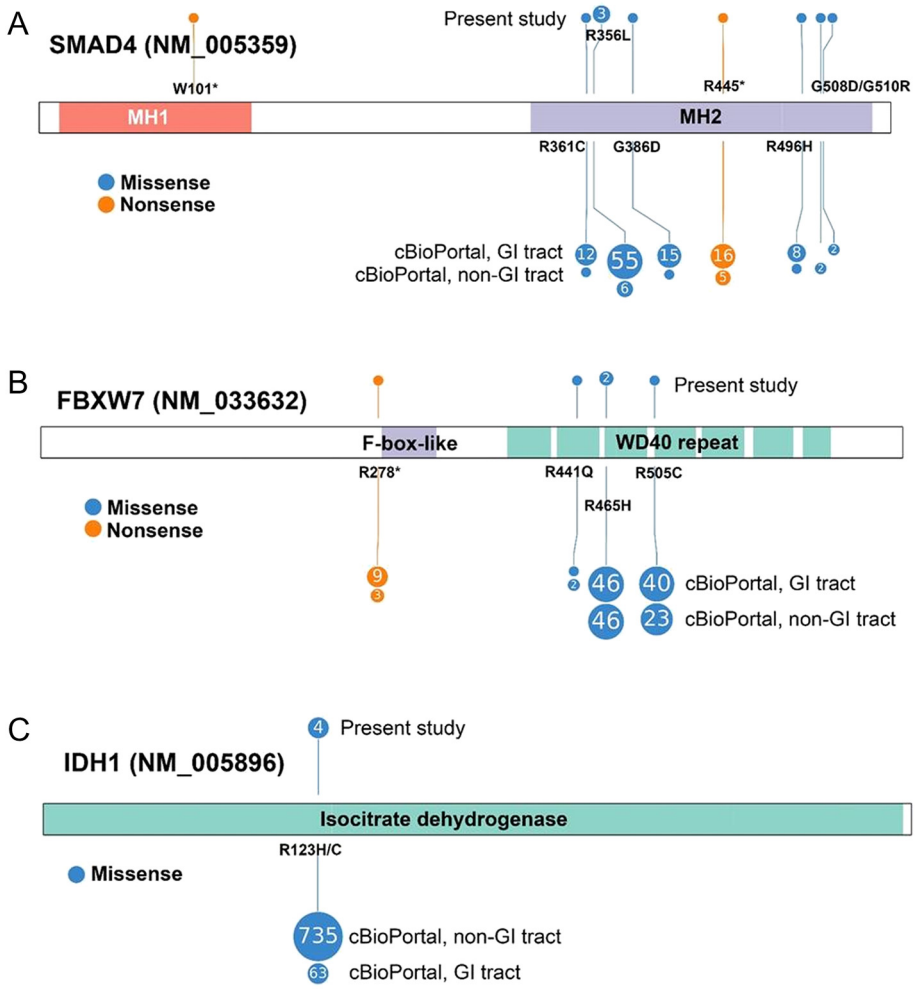

Fig. 3 Locations of *SMAD4* (A), *FBXW7* (B), and *IDH1* (C) mutations. Numbers in circles represent cases with corresponding mutation. Blank circles represent presence of a single mutation case. Upper portions show cases in the present study, and lower portions show cases in previous cancer genome studies archived in cBioPortal.

NET patients may benefit from a drug targeting those related pathways.

The molecular profiles of sporadic rectal NETs and their clinical or pathological relevance are poorly understood. There are only few prior studies of molecular changes in rectal NETs, where almost all rectal cases were a few series [18-22]. The most commonly mutated gene in rectal NETs (10.1%) is *TP53*, for which mutations were also highly identified in gastric and appendiceal NETs in our study. *TP53* mutation has been reported in gastric, small intestine, or pancreatic NETs in the GI tract and even in pancreatic and bronchopulmonary NET cell lines [18,23-26]. In an in vitro study, cancer cells with mutated *TP53* showed accelerated tumor growth associated with increased VEGF expression and neovascularization [25]. Because NETs are highly vascular tumors [27], a clinical trial demonstrated that 3 gastric or pancreatic patients with *TP53* mutations showed a durable response to an antiangiogenesis inhibitor such as pazopanib, suggesting that the presence of *TP53* mutation in GI-NETs may be treated using an antiangiogenesis inhibitor [18,27].

In our study, *FBXW7* mutation was the second most common mutation (7.2%) in rectal NETs and was detected exclusively in rectal NETs. Moreover, *FBXW7* (p.R465H) had a likely pathogenic clinical significance and was a repetitive mutation in rectal NETs. *FBXW7* mutation has been rarely reported in grade 1 small intestine NETs (1.9%) [28]. Thus, an *FBXW7* mutation may be involved in the tumorigenesis of GI-NETs. *FBXW7* is a tumor suppressor gene on human chromosome 4q, and its missense mutations in hotspot positions of the WD40 domain cause a selective loss of function [29]. However, currently, there are no available therapeutic options targeting *FBXW7* in the treatment of NETs.

As mentioned above, 5 repetitive mutations, namely, *TP53* (p.R337C and p.R213*), *PTEN* (p.W111* and p.Q214*), *CDKN2A* (p.W110*), *FBXW7* (p.R465H), and *AKT1* (p.R23Q), were found exclusively in rectal NETs, whereas 2 pathogenic/likely pathogenic mutations, namely, *SMAD4* (p.R361C) and *STK11* (p.D176N), were repetitive mutations found only in gastric NETs. These repetitive mutations may be driver mutations contributing to the pathogenesis

of rectal NETs and gastric NETs, respectively. Indeed, these mutations were all pathogenic/likely pathogenic except for *AKT1* (p.R23Q), which was not provided in the ClinVar database. Meanwhile, we could not find any repetitive mutation detected only in appendiceal NETs. Because of limited data regarding rectal and gastric NETs, those variants are rarely reported in NETs in the COSMIC database. Only *SMAD4* (p.R361C) was reported in appendiceal goblet-cell carcinoid in the COSMIC database [30].

Most GI-NETs show a common histologic feature of well-differentiated tumors arranged into trabeculae, acini, or solid nests, showing cytologically bland uniform cells with indistinct nucleoli, exhibiting a slow growth [12]. Because the stomach, appendix, and rectum represent the foregut, midgut, and hindgut, respectively [3], the commonly shared genetic mutations among gastric, appendiceal, and rectal NETs may be the candidates for driver mutations leading to the unique histology of GI-NETs regardless of the primary tumor origins. Although mutually shared mutations across the 3 locations (gastric, appendiceal, and rectal) were not identified, 3 mutations were commonly shared between rectal and appendiceal NETs and 4 mutations between rectal and gastric NETs. *PTEN* (p.G129K), *EGFR* (p.E709K), and *KIT* (p.V555I) were concurrently found between rectal and appendiceal NETs. *PTEN* (p.G129R) and *EGFR* (p.E709K) were reported as pathogenic mutations, whereas *KIT* (p.V555I) was not provided in ClinVar. On the other hand, *SMAD4* (p.R361C), *ALK* (p.G1202R), *VHL* (p.Q132*), and *IDH1* (p.R132H) were all pathogenic/likely pathogenic mutations, commonly shared between rectal and gastric NETs. As for clinicopathological correlations for specific mutations, we could not find any association between a specific mutation and histologic grade, lymphovascular invasion, or recurrence. However, GI-NETs with higher histologic grades tended to have higher numbers of mutation variants than those with lower histologic grades. Additionally, GI-NETs with lymphovascular invasion or recurrence showed a tendency toward higher numbers of mutation variants than those without lymphovascular invasion or recurrence. As a result, although rectal and appendiceal NETs had low numbers of mutation variants, in contrast to gastric NETs, the individual mutations and the repetitive mutations were widely distributed across almost all chromosomes in these GI-NETs.

Earlier studies have reported no mutations in *BRAF*, *KRAS*, *NRAS*, or *PIK3CA* in rectal NETs using targeted pyrosequencing or direct sequencing targeting *KRAS* (codon 12, 13, 61, or 146), *BRAF* (codon 600), and *PIK3CA* (exon 9 or 20) [21,22], concluding that neither the RAS/RAF/MAPK pathway nor the PI3K/AKT pathway did not play a pathologic role in rectal NETs [21]. However, we found alterations in *KRAS* (2.9%), *BRAF* (1.4%), and *PIK3CA* (2.9%) genes, indicating that the RAS/RAF/MAPK pathway may be a potential therapeutic target. Moreover, *EGFR* (p.E709K and p.T751I) carrying clinically relevant drug-responsive mutations was identified in rectal and appendiceal NETs. *EGFR* mutation has rarely been reported in NETs or neuroendocrine carcinoma [18,31], which makes the efficacy of tyrosine kinase inhibitors uncertain for these conditions [31]. We also noted the presence of *PIK3CA*, *AKT1*, and *PTEN* mutations in the PI3K/AKT/mTOR signaling pathway in rectal NET patients (n = 7, 10.1%), suggesting that targeted therapy might be directed at this particular signal transduction pathway for a subset of rectal NETs. This discrepancy in results might be because the majority of *KRAS*, *BRAF*, and *PIK3CA* mutations we detected in this study were not identified in the hotspots of those respective genes. In contrast, only *TP53*, *SMAD4*, *FBXW7*, and *IDH1* were frequently mutated in the cancer-related hotspot positions in GI-NETs; otherwise, most of the genes were usually altered in nonhotspot positions. However, the NGS analysis in our study successfully allowed analyses for the very low incidence of mutational sites, implicating that this high-throughput genomic test is useful in identifying drug targets in GI-NETs and that the NET-specific NGS panel can also be useful in assessing a wide spectrum of genetic alterations in NETs. In this context, the NGS test may be more cost-effective in the screening of molecular-targeted genes in GI-NETs.

Interestingly, *IDH1* (p.R132H) was detected in rectal and gastric NETs. Although mutations in *IDH1* R132 are common (50%-94%) in grade 2 and 3 gliomas and secondary glioblastomas and rarely reported in acute myeloid leukemia (8%), acute lymphoid leukemia (2%), prostate cancer (3%), and colorectal cancer (9%) [32,33], the *IDH1* mutations in NETs are poorly described. A single isolated case has been reported showing an association between NETs and gliomas [34]. NETs have gene expression profiles surprisingly similar to glial brain tumors (oligodendroglioma and high-grade astrocytoma) [35]. These molecular similarities and presumed common tumoral origin of both NETs and gliomas have raised the possibility of a unique molecular abnormality driving the development of the 2 tumors [35], where *IDH1* mutation may suggest a potential relationship between these 2 rare tumors. In our results from GSEA, the 14 genes frequently found in GI-NETs were associated with the gene signatures of gliomas, confirming the similar gene signatures. We also noted that 2 variants (p.R132H and p.R132C) of *IDH1* mutations were concurrently detected within 1 gastric NET. Although the small number of cases precludes definitive assessment, this finding possibly suggests that NETs may be molecularly heterogenous within the same tumor mass.

One limitation of this study is that we performed a limited cancer-related gene panel that lacked information on copy number alterations. The small number of high-grade rectal, gastric, or appendiceal NETs and the limited information on their variable clinical manifestation are also limitations. Lack of small intestinal NETs in our study cohort may be additional limitation for analysis of midgut NETs. Furthermore, we did not find clinically relevant mutations in either the GI-NETs or metastatic cases, which may decrease the prognostic relevance of the genetic mutations we detected. Nevertheless, we demonstrated high incidences of pathogenic/likely pathogenic mutations in rectal NETs. *TP53*, *PTEN*, *SMAD4*, *EGFR*, *ATM*, *CDKN2A*, and *KIT* were commonly involved

across the rectal, gastric, and appendiceal NETs. Moreover, we identified repetitive mutations of *TP53* (p.R337C and p.R213*), *PTEN* (p.W111* and p.Q214*), *CDKN2A* (p.W110*), *FBXW7* (p.R465H), and *AKT1* (p.R23Q) only in rectal NETs and repetitive mutations of *SMAD4* (p.R361C) and *STK11* (p.D176N) only in gastric NETs. As all genetic mutations except *TP53*, *SMAD4*, *FBXW7*, and *IDH1* were not identified in the hotspots of their respective genes, the production for sequencing primer and its interpretation warrant caution, and a comprehensive NGS panel can be effective in identifying clinical drug targets in rectal NETs.

Author contributions

Mi Jung Kwon and Ha Young Park designed the study, interpreted the data, wrote the manuscript, and drafted of the manuscript; Yun Joong Kim participated in study design and coordination, and data analysis; Nan Young Kim carried out the experiments and data acquisition; Ho Suk Kang, Min Jeong Kim, Kyueng-Whan Min, Kyung Chan Choi, Eun Sook Nam, Seong Jin Cho, Hye-Rim Park, Soo Kee Min, Jinwon Seo, and Ji-Young Choe collected clinical samples and analyzed clinical data.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2019.02.007.

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