Uncovering the heterogeneous genetic variations in two insulin-expressing tumors in a patient with MEN1

ZAI WANG[1*], LIGUO LIU[2*], JIE LUO[3], JING GUO[1], MIN ZHAI[1], WENJIAN ZHANG[1] and ZHIYING YANG[2]

[1]Institute of Clinical Medical Sciences; Departments of [2]General Surgery and [3]Pathology, China-Japan Friendship Hospital, Beijing 100029, P.R. China

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Abstract. Multiple endocrine neoplasia type 1 (MEN1) is associated with a heterozygous inherited mutation of the menin 1 (*MEN1*) gene; however, the molecular pathogenesis remains to be fully elucidated. In the present study, whole exome sequencing was performed on two pancreatic neuroendocrine tumors (PNETs), termed T1 and T2, peri-tumoral tissue (PT) and a blood sample obtained from a patient with MEN1. The cells in T1 and T2, but not PT, showed loss of chromosome 11 where *MEN1* was located, confirming that the loss of heterozygosity (LOH) of *MEN1* was a crucial event in tumorigenesis. PT exhibited chromosome copy number variations (CNVs), suggesting that CNVs may occur ahead of MEN1-associated tumorigenesis. The ploidy, CNVs and somatic point mutations were completely different in T1 and T2, showing the first evidence that multiple PNETs in patients with MEN1 are heterogeneous and arise from polyclonal origins. With the except of one recurrent and possibly benign mutation, no other suspicious driver mutations were identified in the tumors. By contrast, accompanying several chromosome losses, germline heterozygous mutations in the tumor suppressor genes, mucin 6, oligomeric mucus/gel-forming (*MUC6*), and G protein-coupled receptor 17 (*GPR17*) showed loss of heterozygosity in the two tumors, or in T2, respectively. These data demonstrated that chromosome instability may aggravate inherited mutations other than *MEN1*, thus contributing to the tumorigenesis in MEN1-associated PNETs.

Correspondence to: Dr Zhiying Yang, Department of General Surgery, China-Japan Friendship Hospital, 2 Yinghua East Road, Chaoyang, Beijing 100029, P.R. China
E-mail: yangzhy@aliyun.com

*Contributed equally

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Introduction

The menin 1 (*MEN1*) inherited mutation is associated with multiple endocrine neoplasia type 1 (MEN1), an autosomal dominant disorder characterized by the occurrence of multiple endocrine tumors in the parathyroid, anterior pituitary, pancreatic islets and duodenal endocrine cells (1). Pancreatic neuroendocrine tumors (PNETs) occur in ~60% of patients with MEN1, and are the leading cause of mortality from this disease. Numerous MEN1 tumors lead to clinical manifestations due to abnormalities in hormone secretion, including insulinoma or gastrinoma (2).

The *MEN1* gene locates in chromosomal 11q13. The *MEN1*-encoded protein, menin, is a nuclear scaffold protein, which coordinates chromatin remodeling and functions in the maintenance of genomic integrity (3). It acts as a tumor repressor via its involvement in transcriptional and cell signaling regulation (4), telomere maintenance (5) and homologous recombination-directed DNA repair (6). The inherited mutation of *MEN1* is heterozygous. Loss of heterozygosity (LOH) or somatic mutation in the other allele to completely inactivate menin is detected in the majority of MEN1 tumors (7) and even microadenoma (8), indicating it as an early event of tumorigenesis. *MEN1* is also the most common somatic mutation in patients with sporadic PNET, and LOH has been frequently detected (9,10).

Since the identification of the *MEN1* gene in 1997, ~1,544 germline and somatic mutations in this gene have been identified (11,12). However, somatic mutations in other genes accumulated in the latent period and during the tumor progression remain to be fully elucidated. In *MEN1* heterozygous mice, it takes ~12 months to develop pancreatic endocrine tumors (13); whereas, in the conditional β-cell null mutant, it takes ~6 months for the development of insulinoma (14). This indicates that, following the functional inactivation of menin, it takes time to develop clinically identifiable tumors, and other somatic alterations may be involved.

Another feature of MEN1-associated PNET is that the tumors are often multicentric, and secrete different hormones that lead to different clinical manifestations (15). However, as the molecular pathogenesis of MEN1 tumors remains to be elucidated, whether these multifocal tumors are polyclonal lacks genetic evidence; and whether the tumors with

the same hormone expression in one patient share common genetic variations remains to be fully elucidated.

Whole exome sequencing (WES) has been used to identify somatic mutations in non-functional PNETs (10), sporadic insulinomas (16-18) and MEN1-associated hyperparathyroidism (19), but not in MEN1-associated PNETs, particularly multiple tumors in the same patient. The present study aimed to improve current understanding of the genetic progression of MEN1-associated PNETs by performing WES on two insulin-expressing tumors and peri-tumoral tissue (PT) in one patient with MEN1.

Materials and methods

Clinical samples. Following partial pancreatectomy in a patient with MEN1 (36-year-old male, diagnosed with hypoglycemia and Whipple triad and multiple tumors in the pancreas) in China-Japan Friendship Hospital on April 8, 2015 (date of surgery and tissue/blood collection), paraffin section-based pathological analysis was regularly performed. In addition, regions of two pancreatic tumors (T1 and T2), a section of PT of T2, and 1 ml of peripheral blood were preserved at 80˚C for further analysis. For the present study, informed consent was obtained from the patient. The investigation described was performed in accordance with The Code of Ethics of the World Medical Association (20), and all protocols were approved by the Ethics Committee in China-Japan Friendship Hospital (Beijing, China).

WES. Genomic DNA was extracted from the T1, T2, PT and blood samples using Gentra Puregene Tissue or Blood kits (Qiagen, Inc., Valencia, CA, USA). DNA fragment size selection was performed to remove smaller DNA fragments using AxyPrep Fragment Select I beads (Corning Costar, Corning, NY, USA). DNA was quantified by Qubit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and examined by agarose gel eletrophoresis for integrity. Whole exome capture was performed on ≥1.0 μg of genomic DNA per sample, based on KAPA Hyper Prep kits (KapaBiosystems, Inc., Wilmington, MA, UA) using the IDT xGen Exome Research Panel v1.0+ IDT xGen® Lockdown® (Integrated DNA Technologies, Inc., Skokie, IL, USA) according to the manufacturer's protocol. Paired-end multiplex sequencing of the samples was performed on the Illumina HiSeq X10 sequencing platform with a 350-bp insert size. WES was performed at a mean target region depth of 150X for the blood sample, and 400X for the T1, T2 and PT samples (Geneseeq Technology, Inc., Nanjing, China).

Variant calling and pathway analysis. The raw reads were subsequently mapped to the human reference genome [University of California, Santa Cruz (UCSC) Genome Browserhg19 (ftp://hgdownload.soe.ucsc.edu/goldenPath/hg19) using Burrows-Wheeler Aligner (version:0.7.15-r1140, http://bio-bwa.sourceforge.net/) software. The aligned reads were further processed following the GATK Best Practices of duplicate removal, indel realignment and base recalibration. Somatic single-nucleotide substitutions were detected using MuTect in High Confidence mode (21). Small indels were identified using Strelka (https://sites.google.com/site/strelkasomaticvariantcaller/home) (22). The mutational consequences were annotated by ANNOVAR (http://annovar.openbioinformatics.org/) (23) based on UCSC RefGene. Two calling results were combined, and filtering was performed using an in-house pipeline implemented in Perl. The following filtering criteria were applied: i) Total read count in tumor DNA ≥20; ii) variant allele frequency (VAF)=0 in normal; iii) variants in positions listed in 1,000 G with MAF>0.01 were removed. Tumor mutational burden was calculated as the number of only somatic nonsynonymous missense, nonsense and frame shift indel mutations with VAF ≥5% divided by the coding region of a tumor genome (45 Mb). Pathway analysis was performed for mutated genes via Ingenuity Pathway Analysis web software (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/). Signal pathway enrichment analysis was used to derive the related pathways, using novel missense mutations, pathogenic mutations and previously reported changes to derive regulated genes and P<0.01 to define significantly enriched pathways. Copy number data were derived from the WES reads using the probabilistic model of Sequenza (version:2.1.2, https://cran.r-project.org/web/packages/sequenza/index.html) (24).

Variant validation by Sanger sequencing. The candidate mutations were validated and verified by amplification of the targeted genomic region by polymerase chain reaction (PCR) followed by Sanger sequencing (Sangon Biotech Co., Ltd., Shanghai, China).

Immunofluorescence. The samples were embedded in OCT and cut into 5-μm frozen sections. The slides were fixed with 4% PFA/PBS and then permeablized with 0.2% Triton X-100. The staining performed was according to a standard procedure. The primary antibodies used were as follows: mouse anti-insulin (Sigma; Merck Millipore) and rabbit anti-glucagon (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Fluorescence images were captured using a Zeiss LSM 800 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Reverse transcription-PCR (RT-PCR) analysis. Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following extraction, 1 μg of total RNA was used for RT using a FastQuant RT kit with gDNase (Tiangen Biotech Co., Ltd., Beijing, China) in a 20 μl system. The PCR was performed on an Applied Biosystems instrument, (ABI 7500 system; Thermo Fisher Scientific, Inc.), using 1 μl template cDNA, 0.2 μM primers, SYBR® Green Realtime PCR master mix (Toyobo Co., Ltd., Osaka, Japan) for 40 cycles. The thermocycling steps were as follows: 95˚C for 10 min, and 40 cycles at 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. The primers used were as follows: *GAPDH*, forward 3'-CTGCACCACCAACTGCTTAG-5' and reverse 3'-GAGCTTCCCGTTCAGCTCAG-5'; Insulin, forward 3'-CTCACACCTGGTGGAAGCTC-5' and reverse 3'-AGAGGGAGCAGATGCTGGTA-5'.

Results

Characterization of two pancreatic tumors and PT in a patient with MEN1. As reported in our previous study (25),

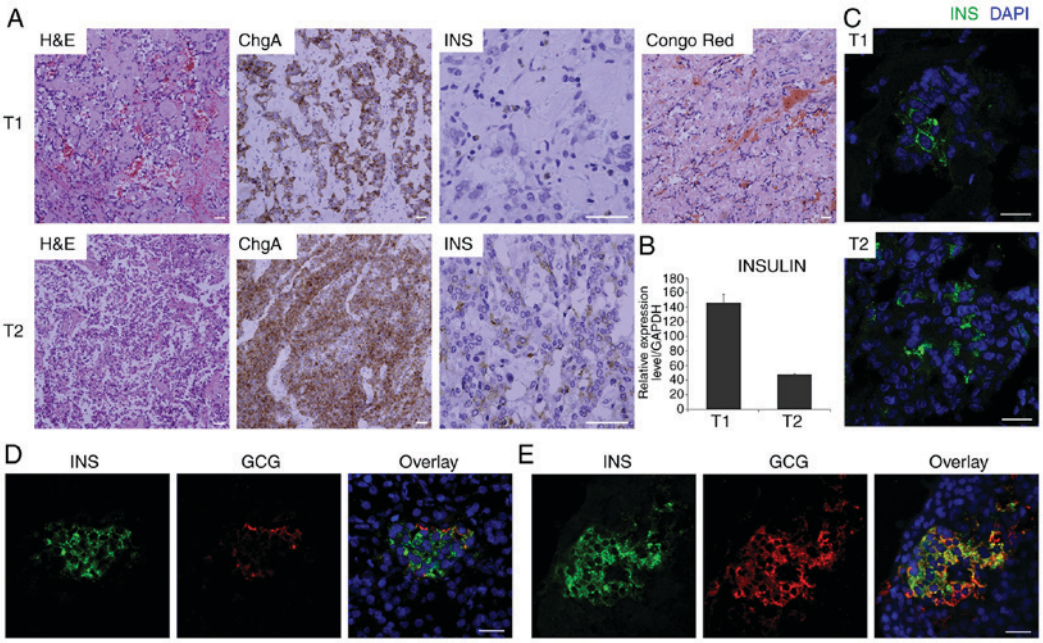

Figure 1. Characterization of two multiple endocrine neoplasia type 1-associated tumors and PT sample for WES. (A) Characterization of T1 and T2. Pathological analysis of T1 and T2, including H&E staining, ChgA, INS and Congo Red staining, as indicated, are shown. Scale bar=50 μm (B) Reverse transcription-polymerase chain reaction analysis was performed to examine the gene expression of *INS* in T1 and T2. (C) Immunofluorescence of INS was performed on freshly prepared T1 and T2 frozen samples. Scale bar=20 μm. Hormone expression in the PT sample. Coimmunofluorescence of INS and GCG were performed on the PT sample. (D) One normal islet without hormone-coexpressing cells and (E) one islet with multiple hormone-coexpressing cells are shown. Scale bar=20 μm. PT, peri-tumoral tissue; ChgA, chromogranin A; INS, insulin; GCG, glucagon.

a 36-year-old male patient with multiple pancreatic tumors presented with seizures associated with hypoglycemia (blood glucose, 1.6-1.8 mmol/l; serum insulin levels, 18.6-27.6 μ IU/ml; Whipple triad) for 3 months. The patient had a history of pituitary tumor, parathyroid tumor and verrucous nodules on the skin. Together with the multiple pancreatic tumors, the patient was diagnosed as MEN1. As recorded, the patient's father was also a MEN1 syndrome patient. The patient underwent laparotomy at the China-Japan Friendship Hospital. The tumor (T1) in the neck was first enucleated, which resulted in the normalization of blood glucose. However, the insulin level in the peripheral and portal venous blood increased, suggesting that further insulin-secreting tumors may be present. Therefore, the body and tail of the pancreas were then excised, and seven additional tumors, including two insulin-positive, three somatostatin-positive and two hormone-negative tumors, were identified (25). One of the insulin-positive tumors, termed T2, and its PT were collected. Pathological analysis based on immunohistochemistry was regularly performed (Fig. 1A and data not shown). Whereas other tumors were of G1 grade, the T1 and T2 tumors were G2 tumors according to the Ki67 index, which was generally 3% and focally 18% in T1, and generally 5% and focally 18% in T2. Chromogranin A was positive in T1 and T2. Insulin was positive in T2 and was sparsely positive in T1. Congo Red Staining, which detects IAPP-associated amyloidosis, showed a positive result in the T1 tumor, which has been reported to be a common observation in insulinomas (26,27). RT-PCR analysis using frozen samples confirmed that insulin was expressed in the T1 and T2 tumors (Fig. 1B). As frozen samples were used for sequencing in the present study, insulin immunostaining (Fig. 1C) was also re-examined in the T1 and T2 frozen samples. For the PT sample, coimmunofluorescence of insulin and glucagon was performed. In addition to normal islets (Fig. 1D), islets with glucagon and insulin coexpressing cells were observed (Fig. 1E), which has also been reported as a common occurrence in insulinomas (28).

MEN1 mutation analysis in T1, T2, PT and blood samples. Although the patient presented with typical MEN1 syndrome (25), and the patient's father was also an MEN1 patient, the *MEN1* germline mutation has not been analyzed previously. In the present study, WES was performed on T1, T2 and PT, controlled by the blood sample of the patient. The *MEN1* gene status was first characterized in the four samples. The results revealed a G-A mutation, leading to an R420X germline mutation (Table I; Fig. 2A), which was further verified by Sanger sequencing (Fig. 2B). This mutation is a rare event, which specifically occurs in patients with MEN1 (11), suggesting that it was the driver mutation in this patient. Although the reads of the reference allele and altered allele were almost equal in the blood and PT samples, indicating heterogenicity of the mutation, the VAF was increased to 78% in T1, and 84% in T2 (Table I), suggesting a loss of the chromosomal region harboring the wild-type allele in the two tumors. This was consistent with the *MEN1* LOH detected in the majority of MEN1 tumors (7), and was further verified by the chromosomal CNV analysis.

Chromosomal CNV analysis in T1, T2 and PT samples. The results of the chromosomal CNV analysis showed that, whereas T2 was a diploid, T1 was generally a tetraploid. LOH of chromosome 11 occurred in the T1 and T2 tumors. No *MEN1* LOH was found in the PT samples (Fig. 3), indicating

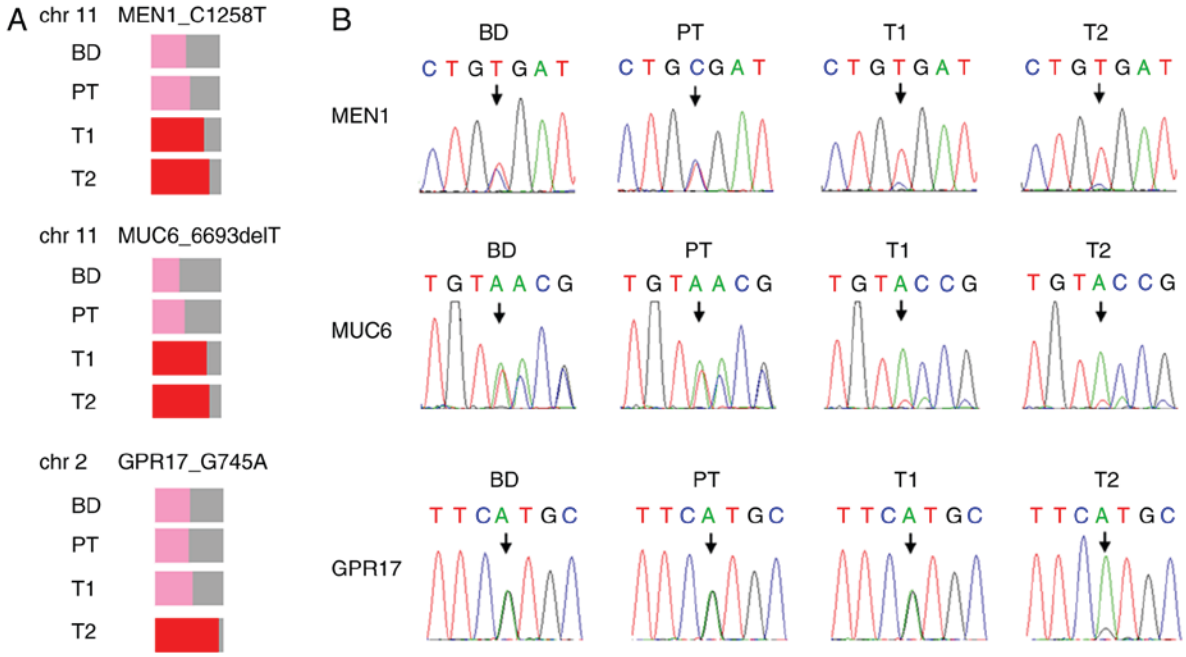

Figure 2. Germline mutations in BD, T1 and T2, and PT samples. (A) *MEN1* C1258T, *MUC6* 6693delT and *GPR17* G745A were identified in the BD, PT, T1 and T2 tumor samples. Variant allele frequency is shown as the percentage of the colored region. Mutations without LOH are shown in pink, and mutations with LOH are shown in red. (B) Verification of the mutations in the BD, PT, T1 and T2 tumor samples by Sanger sequencing are shown. *MEN1*, menin 1; *MUC6*, mucin 6, oligomeric mucus/gel-forming; *GPR17*, G protein-coupled receptor 17; BD, blood; PT, peri-tumoral tissue; LOH, loss of heterozygosity; chr, chromosome.

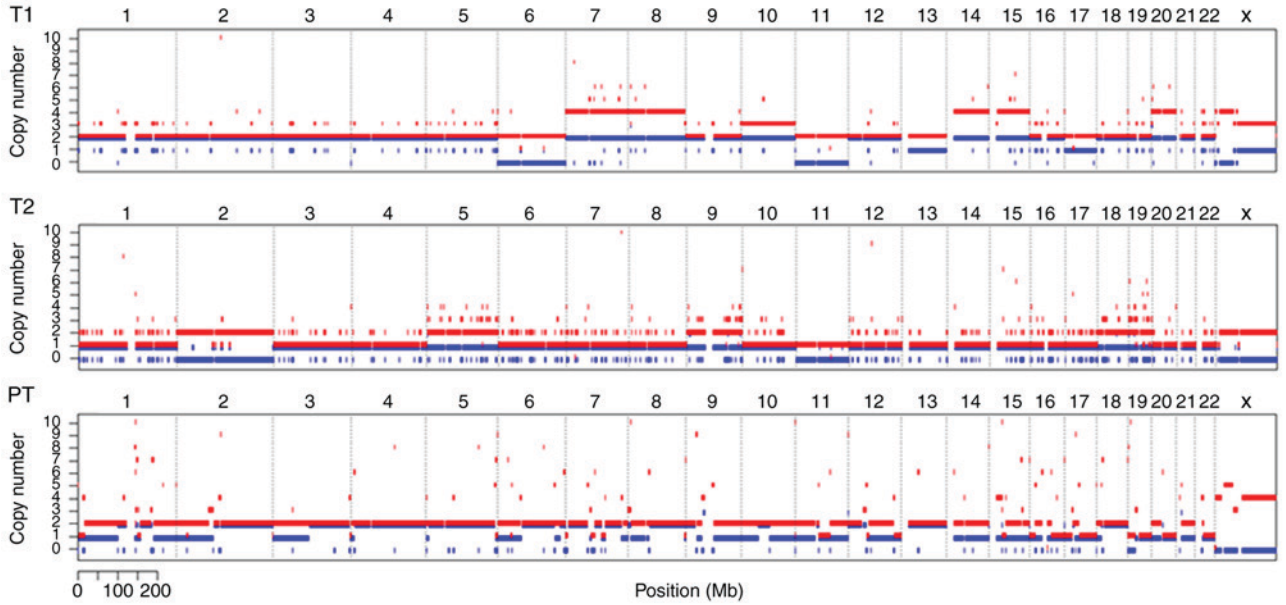

Figure 3. Chromosome copy number variations in T1, T2 and PT samples. Copy number of A and B alleles in T1, T2 and PT tissue samples are shown. PT, peri-tumoral tissue.

that LOH may be an important event for MEN1 tumorigenesis. LOH of chromosome 6 in T1 was also present. The B allele frequency of chromosomes 7, 8, 10, 13, 14, 15, 17 and 20 were also altered in T1 (Fig. 3). In addition to the LOH of chromosome 11, LOH of chromosome 2 was detected in T2, presenting as loss of one allele and duplication of the other allele. CNV with a gain in one allele was detected in chromosomes 5, 9, 18 and 19 in T2 (Fig. 3). Of note, CNV was also detected in ~12% of the PT cells, and the estimated ploidy of PT was 2.2 (Fig. 3), indicating that alterations at the chromosome level caused by a heterozygous *MEN1* mutation had already occurred in the tissues without tumorigenesis.

Somatic single nucleotide variants (SNVs) in T1 and T2 tumors. The present study analyzed somatic SNV status in the tumor samples. In total, eight and seven missense mutations with VAF>10% were identified in T1 and T2, respectively (Table II; Fig. 4A). The mutation burden was low in the two tumors (0.16

Table I. Germline mutations in T1, T2, peri-tumoral tissue and blood samples.

Chr.	Position	Base change	Mutation[a] (ref. allele, non-ref. allele) VAF % T1	T2	PT	Blood	Gene	AA change	refGene	1000g 2015 aug_all
11	64572613	C1258T	1 (36,126) 78	1 (39,211) 84	1 (46,61) 57	1 (50,50) 50	*MEN1*	R420X	NM_000244	NAN
11	1016108	6693delT	1 (22,84) 79	1 (34,174) 84	1 (65,61) 48	1 (38,26) 41	MUC6	fs	NM_005961	NAN
2	128409054	G745A	1 (110,140) 56	1 (17,233) 93	1 (88,87) 50	1 (82,88) 52	GPR17	V249M	NM_001161417	NAN

[a]Mutations are indicated as '1', otherwise are indicated as '0'. Chr., chromosome; ref. allele, reads of reference allele; non-ref. allele, reads of non-reference allele; VAF, variant allele frequency; fs, frameshift; PT, peri-tumoral tissue; 1000g2015 aug_all, 1,000 reference genomes sequencing database from August 2015.

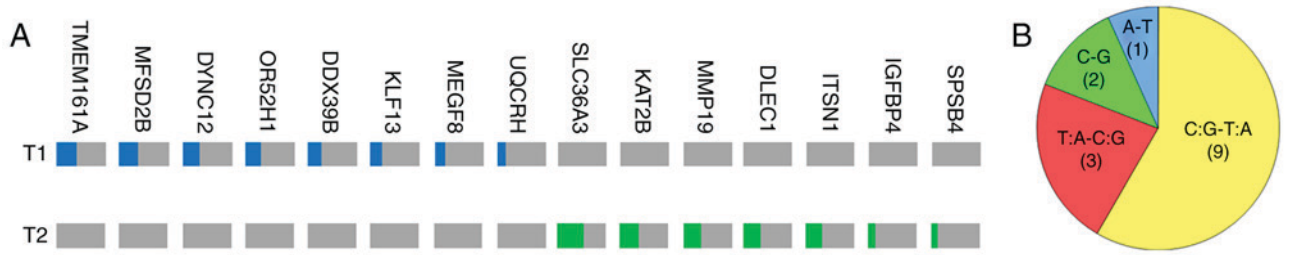

Figure 4. Somatic mutations in T1 and T2 samples. (A) Eight and seven different somatic mutations with VAF>10% were identified in T1 and T2 samples. VAF is shown as the percentage of the colored region. (B) Pie chart of the mutation spectrum of the somatic mutations identified. VAF, variant allele frequency.

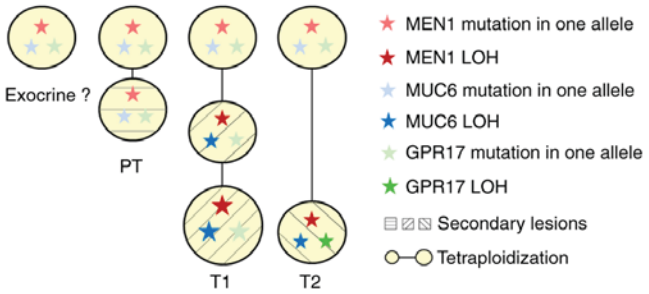

Figure 5. Schematic diagram showing the deduced molecular evolutionary processes of T1 and T2 tumors and other pancreatic cells. Mutations in *MEN1*, *MUC6* and *GPR17* were the common genetic background of the pancreatic cells. In the endocrine cells without tumorigenesis, mild CNV without loss of chromosome 11 was observed. *MEN1* LOH and *MUC6* LOH accompanying the loss of chromosome 11 occurred in T1 and T2 tumorigenesis. T1 underwent further tetraploidization. *GPR17* LOH accompanying loss of chromosome 2 occurred in T2. T1 and T2 underwent distinct molecular evolutionary processes, as suggested by the different CNV and SNV spectra. PT, peri-tumoral tissue; *MEN1*, menin 1; *MUC6*, mucin 6, oligomeric mucus/gel-forming; *GPR17*, G protein-coupled receptor 17; CNV, copy number variation; SNV, single nucleotide variant; LOH, loss of heterozygosity.

and 0.18 per megabase). The majority of the mutations in T1 and T2 were verified by Sanger sequencing, with the exception of *KAT2B* and *IGFBP4* due to difficulties in amplifying the particular DNA fragments (data not shown). These mutations were completely different in T1 and T2, further demonstrating their high level of heterogeneity and polyclonal origin.

For the mutation spectrum, 9/15 (60%) SNVs in T1 and T2 were C/G-T/A mutations, which was the absolute dominant form. This frequency was higher than in previously reported sporadic PNETs (41.8%) (10) and sporadic insulinomas (29.2 or 39.18%) (16,17), which may represent a characteristic for MEN1-associated PNETs. In the other six mutations, three T/A-C/G, two C-G and one A-T mutations were present (Fig. 4B).

The present study then analyzed whether these mutations were tumor-associated mutations. No mutations in frequently mutated genes identified in sporadic insulinoma or non-functional PNET were present, for example *YY1*, *DAXX*, *ATRX*, *MUTYH* or genes in the mammalian target of rapamycin pathway (9,10,16-18). Only one mutation in *TMEM161A* was a recurrent mutation in the Catalogue of Somatic Mutations in Cancer (COSMIC) database, but this was predicted to be benign by several software programs used for protein function prediction, including SIFT, Polyphen2 and LRT (29). As shown by pathway analysis, only two mutated genes in T1 and one mutated gene in T2 were associated with other diseases or dysfunctions: *DYNC1I2* in Huntington's disease signaling, *UQCRH* in mitochondrial dysfunction, and *IGFBP4* in hepatic fibrosis and hepatic stellate cell activation. However, no therapeutic implications were drawn based on these SNVs.

Tumor suppressor genes mucin 6, oligomeric mucus/gel-forming (MUC6) and G protein-coupled receptor 17 (GPR17) show germline mutations and tumor-specific LOH. As no other suspicious driver mutations were identified in the somatic SNVs, it was hypothesized that the consequence of chromosome alterations, including LOH of other tumor suppressor genes, gene amplification and gene fusion, may

Table II. Somatic mutations in MEN1-pancreatic neuroendocrine tumors identified by whole exome sequencing.

Chr.	Position	Base change	Mutation[a] (ref. allele, non-ref. allele) VAF %				Gene	AA change	refGene	1000g 2015aug_all
			T1	T2	PT	Blood				
19	19240997	G563A	1 (177,109) 41	0 (665,1) 0	0 (101,0) 0	0 (87,0) 0	TMEM161A	R188Q	NM_017814	NAN
2	24247120	C1469T	1 (162,101) 40	0 (365,0) 0	0 (110,0) 0	0 (78,1) 0	MFSD2B	S490F	NM_001080473	NAN
2	172583350	A896T	1 (136,70) 34	0 (328,0) 0	0 (128,0) 0	0 (70,0) 0	DYNC1I2	H299L	NM_001271786	NAN
11	5566506	C248T	1 (143,70) 33	0 (351,0) 0	0 (190,0) 0	0 (111,0) 0	OR52H1	T83I	NM_001005289	NAN
6	31506952	T311C	1 (161,66) 28	0 (596,0) 0	0 (150,0) 0	0 (99,0) 0	DDX39B	L104P	NM_004640	NAN
15	31664484	C849G	1 (410,137) 25	0 (717,0) 0	0 (151,0) 0	0 (99,0) 0	KLF13	S283R	NM_015995	NAN
19	42872733	C6199T	1 (285,79) 21	0 (858,0) 0	0 (151,0) 0	0 (111,0) 0	MEGF8	P2067S	NM_001410	NAN
1	46775912	G140A	1 (495,103) 17	0 (860,0) 0	0 (246,1) 0	0 (158,0) 0	UQCRH	R47H	NM_001297566	NAN
5	150663732	T847C	0 (288,0) 0	1 (266,301) 54	0 (146,1) 0	0 (61,0) 0	SLC36A3	F283L	NM_181774	NAN
3	20082151	C182T	0 (166,0) 0	1 (140,90) 39	0 (67,0) 0	0 (61,0) 0	KAT2B	A61V	NM_003884	NAN
12	56233474	A572G	0 (431,0) 0	1 (422,236) 36	0 (147,0) 0	0 (122,0) 0	MMP19	D191G	NM_002429	NAN
3	38104084	C886T	0 (278,0) 0	1 (271,145) 35	0 (137,0) 0	0 (74,1) 0	DLEC1	P296S	NM_007335	NAN
21	35237565	G4001A	0 (332,0) 0	1 (269,149) 34	0 (126,1) 0	0 (94,0) 0	ITSN1	S1334N	NM_003024	NAN
17	38600109	C122G	0 (208,0) 0	1 (282,53) 15	0 (86,1) 0	0 (58,1) 0	IGFBP4	P41R	NM_001552	NAN
3	140785241	G295A	0 (632,0) 0	1 (758,126) 14	0 (282,0) 0	0 (232,0) 0	SPSB4	A99T	NM_080862	NAN

Mutations with VAF>10% are listed and were verified by Sanger sequencing. None of the mutations were documented in the 1000g2015aug_all database. [a]Mutations are indicated as '1', otherwise are indicated as '0'. Chr., chromosome; ref. allele, reads of reference allele; non-ref. allele, reads of non-reference allele; PT, peri-tumoral tissue; 1000g2015aug_all, 1,000 reference genomes sequencing database from August 2015.

contribute to the frequent tumorigenesis in this patient. The germline mutations in chromosome 11 were examined first, which showed LOH in T1 and T2, and chromosome 6 and 2, which showed LOH in T1 and T2, respectively. In addition to *MEN1*, a frame shift deletion was identified in the C-terminus of *MUC6* located in chromosome 11, which showed evidence of LOH in T1 and T2; and a recurrent missense mutation in *GPR17* located in chromosome 2, predicted to be deleterious by several software programs, (29) showed evidence of LOH in T2 only (Table I; Fig. 2A and B). Taking the ploidy change into consideration, while *MUC6* and *GPR17* showed one normal allele and one altered allele in the blood and PT samples, *MUC6* had one and two copies of the altered allele in T2 and T1, respectively, with no normal alleles; and *GPR17* had two normal and two altered alleles in T1, and only two altered alleles in T2. *MUC6* is a tumor suppressor gene, which may inhibit tumor invasion. The C-terminal domain is critical for its function (30,31). There is experimental evidence that *GPR17* has an anti-proliferative role in glioma cells (32). Based on these data, the present study demonstrated that tumor suppressor genes other than *MEN1* may undergo LOH upon chromosome alterations and contribute to tumorigenesis.

As WES is not suitable for the detection of specific gene amplifications, it is only possible to estimate the gene copy number changes based on the information of chromosome CNVs. For example, the present study found that chromosomes harboring pro-tumor genes, including *EGFR*, *MYC*, *MET* and *YY1*, amplified to six copies, whereas chromosomes harboring *TP53* had three copies in T1, demonstrating that chromosome instability may have an effect on the copy number and expression levels of tumor-associated genes, thus promoting tumorigenesis in MEN1-associated PNETs.

Discussion

MEN1-associated PNETs are always multiple, with an average of three or four per patient (15). Although the inherited mutation in the *MEN1* gene and the secondary LOH of *MEN1* is known to be associated with tumorigenesis, the molecular pathogenesis remains to be fully elucidated. In the present study, in a case of MEN1 with multiple PNETs, WES was performed on two insulin-expressing tumors and a PT sample, in order to provide an initial understanding of the molecular tumorigenesis in MEN1-associatedpancreatic tumors (Fig. 5).

The results of the present study demonstrated that the PT region already had chromosome number variations without LOH of *MEN1*, indicating that the genetic alterations may occur independently of *MEN1* LOH. Multiple low frequent SNVs (VAF<10%) were also present in the PT sample, for which Sanger sequencing-based verification was difficult. However, considering that the pancreatic islets accounts for <5% of the pancreatic volume, the frequency of CNVs and SNVs detected in the PT may not be low if the variations are concentrated in the endocrine compartments. Therefore, the separation of endocrine and exocrine cells prior to WES is required for the identification of early events prior to tumorigenesis in the islets; it is also useful to examine the genetic changes in exocrine cells, which are presumably considered to be unaffected by *MEN1* mutations. In the present study, frozen samples were used for WES. The technical limitation of isolating endocrine compartments from the pancreas made it impossible to achieve the above. In addition, abnormal insulin and glucagon coexpression were detected in the islet cells in the PT samples, which are commonly observed in pancreatic endocrine tumors (28). Whether this is one of the phenotypic clues for the genetic variations requires further investigation.

For the two tumors showing insulin expression, they were considered to be from different origins based on the high genetic heterogeneity. With the exception of the loss of chromosome 11, the ploidy and CNVs in T1 and T2 were entirely different. As loss of chromosome 11 has been reported in several cases of MEN1 (19,33), it was considered to be a non-small probability event. Therefore, the loss of chromosome 11 may occur independently in these two tumors and may not be considered as co-evolutionary evidence. The SNVs with VAF>10% were also completely different in T1 and T2, further confirming their polyclonal origins.

With the exception of one recurrent but possibly benign mutation, no other suspicious tumor-associated mutations were identified in the somatic mutations; the relevance of these somatic mutations with tumor formation was unclear. By contrast, accompanying the chromosome alterations, germline mutations in tumor suppressor genes *MUC6* and *GPR17* showed LOH in the two tumors, or in T2, respectively; chromosomes harboring pro-tumor genes, including *EGFR*, *MYC*, *MET* and *YY1*, amplified to six copies, whereas chromosome harboring *TP53* had three copies in T1. These data demonstrated that chromosome instability may aggravate inherited mutations other than *MEN1*, affecting the copy number and expression levels of tumor-associated genes, and thus contributing to the tumorigenesis in MEN1-associated PNETs.

From the therapeutic viewpoint, the low tumor mutation burden in these two MEN1 tumors suggested that they may not be suitable for immune checkpoint therapy (34). Although menin is involved in DNA repair (6), the low mutation burden suggests a different genomic signature from *BRCA1/2* mutant tumors (35). This is also consistent with a previous finding that *MEN1*-mutant spontaneous PNETs exhibited a longer telomere and reduced gene fusion event, suggesting that *MEN1*-mutant tumors do not show significant DNA repair deficiency (9). The high level of heterogeneity of the pancreatic tumors in the same MEN1 patient, but with the same hormone-expression, suggested that personalized treatment for patients with MEN1 should be based on the alterations in the specific tumors of clinical concern. Rather than genetic mutations, which may not provide sufficient therapeutic clues in certain cases, alterations at the epigenetic, transcriptional and translational level, in addition to the activation status of key signaling pathways, requires systematic investigation in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZW and LL were the major contributors in the experimental design, sequencing data analysis and manuscript writing; JL interpreted the histological data; JG, MZ and WZ contributed to the real time PCR and immunofluorescence, ZY was responsible for the clinical samples and the whole project.

Ethics approval and consent to participate

Informed consent was obtained from the patient. The investigation was performed in accordance with The Code of Ethics of the World Medical Association, and all protocols were approved by the Ethics Committee in China-Japan Friendship Hospital.

Consent for publication

Informed consent was obtained from the patient.

Competing interests

The authors declare that they have no competing interests.

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