Telomere Length Abnormalities and Telomerase RNA Component Expression in Gastroenteropancreatic Neuroendocrine Tumors

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Abstract. *Telomere lengths in normal human cells are tightly regulated within a narrow range. Telomere length abnormalities are prevalent genetic alterations in malignant transformation. We studied telomere length abnormalities, telomerase RNA component (TERC) expression, alpha-thalassemia X-linked mental retardation (ATRX) expression, and death domain-associated protein (DAXX) expression in gastroenteropancreatic neuroendocrine tumors (GEP-NETs). We used tissue microarrays to perform telomere fluorescent in situ hybridization (FISH) and TERC in situ hybridization in 327 formalin-fixed paraffin-embedded tissues of GEP-NETs. Telomere length abnormalities were detected in 35% of 253 informative cases by using telomere FISH. Ten cases had altered lengthening of telomeres (ALT), an ALT-positive phenotype (4%), and 79 cases had telomere shortening (31%). The ALT-positive phenotype was significantly associated with tumors of pancreatic origin (7/10) and loss of ATRX or DAXX protein (8/10). Telomere shortening was significantly associated with low TERC expression. In the survival analysis, loss of ATRX or DAXX protein was associated with a decreased overall survival. Multivariate regression analysis showed that lymph node metastasis and high TERC expression were independent prognostic factors of reduced overall survival (OS) for patients with GEP-NETs. Our results showed that telomere lengthening (the ALT-positive phenotype) and telomere shortening accompanied by low TERC levels are two types of clinically significant telomere abnormalities in GEP-NETs.*

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Gastrointestinal neuroendocrine tumors (GI-NETs) occur at a rate of approximately 1.95-2.50 per 100,000 in Western populations and pancreatic NETs occur at approximately one per 100,000 (1). In Japan, the prevalence of pancreatic NETs is estimated at 2.23 per 100,000, with an annual onset incidence of 1.01 per 100,000; the prevalence of GI-NETs is estimated at 3.45 per 100,000 with an annual onset incidence of 2.10 per 100,000 (13). In Korea, a continuous increase in the incidence of gastroenteropancreatic (GEP)-NETs has been observed, with an incidence in 2009 that was nine times that reported in 2000 (5).

Consisting of non-coding TTAGGG repeats and telomere-binding proteins, telomeres protect chromosomal ends from degradation, aberrant recombination, and end-to-end fusion. Telomerase catalyzes the addition of telomere repeats to chromosome ends. Post-neonatal human somatic cells repress telomerase expression (15, 27); however, most neoplastic cells de-repress telomerase expression to support immortalization and tumor formation (15). Telomerase has been detected in germline and stem cells, as well as in 60% of immortal cells and 85% of carcinomas (3).

The two essential components of human telomerase are i) the integral human telomerase RNA component (TERC), encoded on chromosome 3q26.3, which serves as a template for the synthesis of telomeric repeats; and ii) a protein subunit, human telomerase reverse transcriptase (TERT), encoded on 5p15.33, which provides catalytic activity (8, 21). TERT is thought to be the major determinant of human telomerase activity (20). However, overexpression of TERC is a known biological marker for cervical cancer (28).

Telomere shortening is concomitant with aging and is associated with diseases such as dyskeratosis congenita, idiopathic pulmonary fibrosis, and aplastic anemia. Telomere

length assessment in human clinical specimens through the use of fluorescence *in situ* hybridization (FISH) has shown evidence of marked telomere shortening in >90% of precursor lesions of the prostate and the pancreas (18, 19, 24, 25). However, telomere shortening in NETs has not yet been reported as far as we are aware of.

In the absence of telomerase activity, both yeast and mammals can maintain or elongate telomeric or sub-telomeric DNA through an alternative mechanism for lengthening of telomeres (ALT) (2). Some neoplastic cells use ALT to add telomere repeats by telomere recombination in the absence of telomerase activity. In humans, ALT-positive (ALT^+) cells are characterized by heterogeneous telomere length, with the presence of both unusually short and long telomeres (3). ALT is relatively common in sarcomas, certain endocrine tumors, a subset of nervous system tumors, small-cell bladder carcinoma, and non-seminomatous germ cell tumors (10-12).

ALT is known to be responsible for tumorigenesis of pancreatic endocrine tumors and mutations in alpha-thalassemia X-linked mental retardation (*ATRX*) or death domain associated protein (*DAXX*) are associated with ALT (14). The ATRX–DAXX complex is required for chromatin deposition of histone H3.3, a histone variant associated with transcriptionally active open chromatin, transcription factor-binding sites, and telomeres. Loss of ATRX or DAXX limits H3.3 incorporation into telomeric chromatin, disrupting telomeric heterochromatin and facilitating telomere recombination (16). Mutations in *DAXX* or *ATRX* correlate with loss of protein expression (17).

Few comprehensive large-scale studies are available that have examined the association of telomere length abnormalities with TERC changes and ALT with loss of ATRX or DAXX in GEP-NETs. To study the prevalence and clinical significance of telomere shortening and ALT in GEP-NETs, we examined telomere length by using fluorescent *in situ* hybridization (FISH), TERC expression by using *in situ* hybridization (ISH), and ATRX, DAXX and TERT expression by using immunohistochemistry (IHC). We prepared formalin-fixed paraffin-embedded (FFPE) samples for visual evaluation of transcription under light microscopy, and we performed a novel RNA ISH technique using paired DNA oligoprobes and pre-amplifier, amplifier, and label probes for visualization (23).

Materials and Methods

Patients. The medical records of 327 patients (mean age=53 years; range=11-91 years) with histopathologically-confirmed NETs of the gastroenteropancreatic tract who were treated at Seoul National University Hospital (n=230) or Seoul National University Bundang Hospital (n=97) between 1989 and 2009 were analyzed retrospectively. Histopathological confirmation of a GEP-NET diagnosis was required for study inclusion. Patient files were reviewed systematically from the date of initial diagnosis, regarding location of the primary tumor, histopathological diagnosis, tissue site from which samples were taken for histopathological diagnosis, clinical staging at initial diagnosis, and presence of a functional syndrome. Of the 327 patients, 145 were women and 182 were men; family history of disease was present in seven patients and 26 had symptoms at presentation, including five patients with endocrine symptoms. Standard histopathological examinations included assessment of the pathological tumor stage according to the criteria of the seventh edition of the American Joint Committee on Cancer (AJCC) Staging Manual (6). Histological classifications were performed as recommended by the World Health Organization (WHO) 2010 classification (4).

FFPE tumor tissues of patients treated from 1989 to 2008 were retrieved from the archives at the Departments of Pathology at Seoul National University Hospitals in Seoul and Bundang, Korea. Inclusion criteria were as follows: patients diagnosed with primary GEP-NETs (with or without metastases) who were evaluated and treated *via* endoscopic biopsy and surgical resection.

Among the 327 patients, follow-up and survival information was obtained for 262. The mean follow-up period was 53 months (range=1-243 months). Patient survival data, including dates and causes of death, were obtained from the Korean Central Cancer Registry at the Ministry of Health and Welfare.

Adjuvant treatment given to 52 patients included postoperative chemotherapy (n=25); postoperative chemotherapy/radio frequency ablation (RFA)/sandostatin long-acting release (LAR) (n=1); preoperative chemotherapy (n=2); preoperative and postoperative chemotherapy (n=1); postoperative radiotherapy (RT) and chemotherapy (n=7); RT (n=2); RFA (n=4); transarterial chemoembolization (TACE) (n=4); transarterial embolization (TAE) and RT (n=1); TACE and chemotherapy (n=1); TACE and everlorimus trial (n=1); TACE plus RFA with RAD001 trial (n=1); TAE plus interferon, streptomycin, adriamycin, sandostatin, LAR, thalidomide, Avastin and RAD001 (n=1); and TACE, RFA, radiotherapy, sandostatin and chemotherapy (n=1).

Ethical statement. All human specimens were obtained during therapeutic surgery or endoscopic resection. The retrospective study was performed using the pathology specimens after diagnosis, and all samples were anonymized before the study. The participants did not provide their written consent to participate in this study. However, the Institutional Review Board committee of Seoul National University Hospital approved this retrospective study under the condition of anonymization (Reference: C-1012-027-343).

Tissue-array preparation. Tissues obtained from patients were routinely fixed in 10% buffered formalin and embedded in paraffin blocks. After screening the available samples for each case, we selected a well-fixed paraffin block containing a representative tumor section. A single tissue column (2.0 mm diameter) was obtained from each selected paraffin block and samples were arranged in separate, new, 60-hole paraffin blocks by using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). Microarray blocks were sectioned at 4 μm and processed for immunohistochemical staining. After removing the paraffin with xylene, sections were rehydrated with graded ethanol and immersed in Tris-buffered saline.

Telomere FISH. Locked nucleic acid (LNA)-substituted oligonucleotides of either the 23 bp human satellite-2 repeat sequence (ATTCCATTCGATTCCATTCGATC) or a 24 bp sequence of four blocks of the 6 bp telomere repeat (TTAGGG) were used. A DNA control and the telomere-specific LNA probe was made with an LNA-2 design that substitutes LNA at every second position (Exiqon, Vedbaek, Denmark). FISH was performed as described previously (22) with the following modifications. Denaturation of the target DNA and the probe was performed at 75˚C for 5 minutes under a coverslip with a hybridization mixture of 8:2 hybridization buffer from Macrogen (Seoul, Korea) and LNA probe. Hybridization was performed overnight at 37˚C. Post washing was performed with 0.3% NP40 (Abbott, Des Plaines, IL, USA). Hybridization signals with biotin-labeled LNA/DNA mixmers were visualized indirectly by using two layers of fluorescein-labeled avidin (Vector Laboratories, Burlingame, CA, USA) linked by a biotinylated anti-avidin molecule, which amplified signals 8-64 times. Hybridization of Cy3-labeled molecules was visualized directly after a short washing procedure. Slides were mounted in 4',6-diamidino-2-phenylindole (DAPI) mixture by mixing equal volumes of DAPI (Abbott) and antifade solution (Abbott). All procedures were performed in a darkroom.

Tumors were classified as ALT+ using the following criteria: (i) presence of ultrabright, intranuclear foci of telomere FISH signals with integrated total signal intensities for individual foci >10-fold compared to the per-cell mean integrated signal intensities for all telomeric signals in individual benign stromal cells from the same case; and (ii) ≥1% of neoplastic cells with ALT-associated telomeric DNA foci. Tumor samples lacking ALT-associated telomeric foci in which at least 5,000 cells were assessed were considered ALT-negative (ALT−) (9).

Telomere fluorescence intensities are linearly related to telomere length (18). Intensities in GEP-NETs were compared with adjacent normal epithelium or stromal fibroblasts. Telomere fluorescence results for tumors were classified as preserved or reduced compared to normal structures.

RNA ISH. *In situ* detection of TERC was performed by a manual method using RNAscope kits (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, 4 μm FFPE tissue sections were pretreated by heating and protease application prior to hybridization with a probe targeting TERC as described previously (23). TERC expression was categorized into five grades based on the number of dots per cell. Expression was graded as 0 for no stain or fewer than 1 dot/cell (visible at 40×); 1 for 1-3 dots/cell (visible at ×20-40); 2 for 4-10 dots/cell with no or very few dot clusters (visible at ×20-40); 3 for >10 dots/cell with fewer than 10% of positive cells having dot clusters (visible at ×20); 4 for >10 dots/cell with more than 10% of positive cells having dot clusters (visible at ×20) (26). Cases were then dichotomized into those with low (score 0 or 1) or high TERC expression (score 2, 3 or 4) for statistical analyses.

Immunohistochemistry. From the tissue microarray, 4-μm sections were stained with antibodies against TERT (EST21A) (anti-hEst2/telomerase, polyclonal rabbit; Alpha Diagnostic I International, San Antonio, TX, USA), DAXX (anti-DAXX, polyclonal rabbit; Sigma, St. Gallen, Switzerland), ATRX (anti-ATRX, polyclonal rabbit; Sigma), or Ki-67 (mouse monoclonal, MIB-1; DAKO, Glostrup, Denmark). Antigen retrieval for DAXX was performed by heating samples in citrate buffer at 100˚C for 30 min and for ATRX by heating at 95˚C in Tris buffer for 40 min. For TERT, heat pretreatment in Bond epitope retrieval solution 2 was performed at 100˚C for 20 min in pH 9.0 for antigen retrieval and a Bond polymer detection kit (Leica Biosystems, Newcastle, UK) with Leica Bond-max autostainer was used. Incubation with primary antibody was performed for 30 minutes at 1:40 for DAXX, 1:400 for ATRX, and 1:300 for Ki-67. Tissue samples were stained with a labeled avidin-biotin-peroxidase complex using Vectastain Elite ABC kits (Vector Laboratories) after antigen retrieval. Diaminobenzidine was used for color development. Only nuclear protein staining was considered as positive for both DAXX and ATRX scoring. Samples with both negative tumor nuclei and non-neoplastic stromal and endothelial cells were scored as non-informative and excluded from further analysis. The nuclear Ki-67 labeling index was expressed as the percentage of positively stained cells.

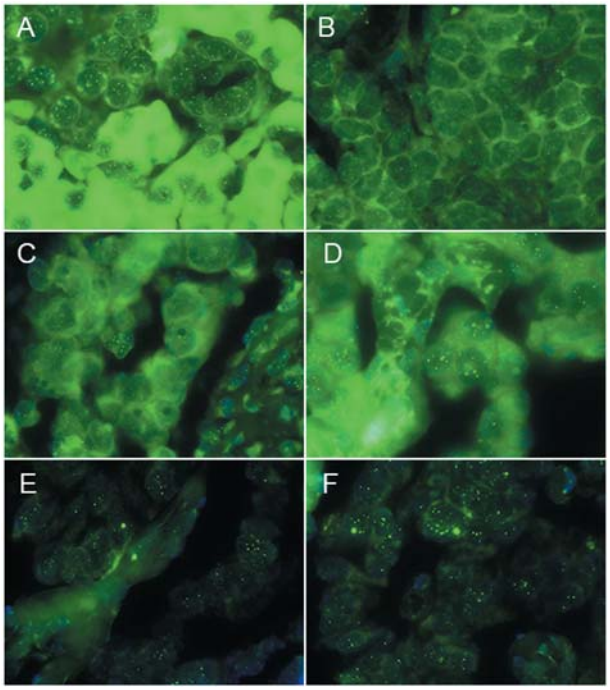

Figure 1. *Telomere fluorescence in situ hybridization in gastro-enteropancreatic neuroendocrine tumors (GEP-NETs). A, B: Normally preserved telomere. C, D: Reduced signal in telomere shortening. E, F: Altered lengthening of telomere-postive (ALT+) cases.*

Statistical analyses. Survival rates were calculated using the Kaplan–Meier method and groups were compared using the log rank test. Kaplan–Meier curves were plotted using overall survival data. Multivariate Cox regression analysis was performed with the variables Ki-67 grade 3, lymph node metastasis, ATRX loss, DAXX loss, high TERC expression, and telomere shortening. A value of $p<0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA).

Table I. *Clinicopathological analyses for ALT+, TERC and TERT expression in GEP-NETs.*

				Telomere length			TERC*					
	ALT− N (%)	ALT+ N (%)	*p*-Value	Normal N (%)	Short N (%)	*p*-Value	Low (n=208) N (%)	High (n=61) N (%)	*p*-Value	TERT− (n=142) N (%)	TERT+ (n=142) N (%)	*p*-Value
Total	243	10		164	79		208	61		142	142	
Gender												
Female	104 (94)	7 (6)	0.089	74 (71)	30 (29)	0.292	102 (85)	18 (15)	0.007	58 (46)	68 (54)	0.232
Male	139 (98)	3 (2)		90 (65)	49 (35)		106 (71)	43 (29)		84 (53)	74 (47)	
Age (years)												
<55	125 (99)	1 (1)	0.01	90 (72)	35 (28)	0.122	115 (84)	22 (16)	0.008	69 (48)	76 (52)	0.406
≥55	118 (93)	9 (7)		74 (63)	44 (37)		93 (70)	39 (30)		73 (53)	66 (47)	
Primary organ												
Stomach	41 (95)	2 (5)	0.003	22 (54)	19 (46)	0.003	24 (56)	19 (44)	<0.001	26 (59)	18 (41)	0.002
Duodenum	17 (100)	0 (0)		13 (76)	4 (24)		15 (94)	1 (6)		14 (78)	4 (22)	
Pancreas	40 (85)	7 (15)		27 (68)	13 (33)		37 (76)	12 (24)		14 (29)	35 (71)	
Hepatobiliary	12 (100)	0 (0)		4 (33)	8 (67)		3 (27)	8 (73)		4 (40)	6 (60)	
Appendix	14 (100)	0 (0)		13 (93)	1 (7)		27 (100)	0 (0)		11 (37)	19 (63)	
Midgut colon	4 (100)	0 (0)		1 (25)	3 (75)		1 (25)	3 (75)		3 (75)	1 (25)	
Hindgut colon	115 (99)	1 (1)		84 (73)	31 (27)		101 (85)	18 (15)		70 (54)	59 (46)	
Tumor size (cm)												
<1.0 cm	119 (99)	1 (1)	0.021	94 (79)	25 (21)	<0.001	125 (95)	7 (5)	<0.001	78 (53)	68 (47)	0.144
≥1.0 cm	118 (94)	8 (6)		68 (58)	50 (42)		78 (59)	54 (41)		58 (45)	72 (55)	
Mitosis grade												
1	156 (98)	4 (3)	0.067	122 (78)	34 (22)	<0.001	154 (90)	18 (10)	<0.001	95 (52)	88 (48)	0.54
2	33 (89)	4 (11)		17 (52)	16 (48)		33 (87)	5 (13)		17 (43)	23 (58)	
3	51 (96)	2 (4)		23 (45)	28 (55)		19 (34)	37 (66)		28 (48)	30 (52)	
Ki-67 Grade												
1	149 (96)	7 (4)	0.881	111 (74)	38 (26)	0.003	155 (91)	16 (9)	<0.001	99 (54)	85 (46)	0.276
2	64 (97)	2 (3)		36 (56)	28 (44)		46 (70)	20 (30)		30 (45)	36 (55)	
3	24 (96)	1 (4)		11 (46)	13 (54)		2 (8)	22 (92)		10 (40)	15 (60)	
WHO 2010												
NET G1	125 (97)	4 (3)	0.799	97 (78)	28 (22)	<0.001	128 (91)	13 (9)	<0.001	81 (53)	71 (47)	0.457
NET G2	63 (94)	4 (6)		42 (67)	21 (33)		59 (87)	9 (13)		31 (44)	40 (56)	
NEC	30 (97)	1 (3)		14 (47)	16 (53)		10 (31)	22 (69)		15 (44)	19 (56)	
MANC	25 (96)	1 (4)		11 (44)	14 (56)		11 (39)	17 (61)		15 (56)	12 (44)	
Lymph node metastasis												
No	194 (98)	4 (2)	0.005	138 (71)	56 (29)	0.018	182 (85)	31 (15)	<0.001	117 (52)	110 (48)	0.327
Yes	42 (89)	5 (11)		22 (52)	20 (48)		21 (44)	27 (56)		21 (44)	27 (56)	
AJCC stage												
I	181 (98)	4 (2)	0.01	138 (76)	43 (24)	<0.001	173 (87)	26 (13)	<0.001	114 (54)	99 (46)	0.055
II	23 (88)	3 (12)		8 (35)	15 (65)		14 (56)	11 (44)		8 (33)	16 (67)	
III	19 (100)	0 (0)		9 (47)	10 (53)		5 (24)	16 (76)		6 (29)	15 (71)	
IV	20 (87)	3 (13)		9 (45)	11 (55)		16 (70)	7 (30)		13 (52)	12 (48)	
Recurrence/metastasis												
No	209 (97)	6 (3)	0.024	148 (71)	61 (29)	0.006	179 (78)	50 (22)	0.430	125 (52)	116 (48)	0.136
Yes	34 (89)	4 (11)		16 (47)	18 (53)		29 (73)	11 (28)		17 (40)	26 (60)	
ATRX												
Negative	70 (93)	5 (7)	0.184	43 (61)	27 (39)	0.181	58 (72)	23 (28)	0.121	56 (65)	30 (35)	0.001
Positive	162 (97)	5 (3)		114 (70)	48 (30)		150 (80)	37 (20)		85 (43)	111 (57)	
DAXX												
Negative	56 (90)	6 (10)	0.010	39 (70)	17 (30)	0.697	47 (72)	18 (28)	0.238	42 (61)	27 (39)	0.041
Positive	178 (98)	4 (2)		119 (67)	59 (33)		161 (79)	42 (21)		100 (47)	114 (53)	
TERC												
Low	156 (94)	10 (6)	0.056	116 (74)	40 (26)	0.003	208 (100)	0 (0)		107 (52)	99 (48)	0.014
High	58 (100)	0 (0)		31 (53)	27 (47)		0 (0)	61 (100)		20 (34)	39 (66)	
TERT												
Negative	108 (97)	3 (3)	0.388	71 (66)	37 (34)	0.674	107 (84)	20 (16)	0.01	142 (100)	0 (0)	
Positive	117 (95)	6 (5)		80 (68)	37 (32)		98 (71)	40 (29)		0 (0)	142 (100)	

*Low score: 0, 1; high score: 2-4. ALT, Altered lengthening of telomere; TERC, telomerase RNA component; TERT, telomerase reverse transcriptase; GEP-NET, gastroenteropancreatic neuroendocrine tumor; WHO, World Health Organization; NET, neuroendocrine tumor; MANEC, mixed adenoneuroendocrine carcinoma; AJCC, American Joint Committee on Cancer; ATRX, alpha-thalassemia X-linked mental retardation; N, negative; P, positive; DAXX, death domain associated.

Table II. *Clinicopathological profiles of the 10 ALT+ GEP-NETs*

Gender /Age	Primary site	WHO 2010	Size (cm)	Mitosis grade	Ki-67 grade	ATRX	DAXX	ATRX or DAXX loss	TERT	TERC score	Recurrence or metastasis	AJCC stage	Symptoms	Functionality	Adjuvant Treatment
M/58	Pancreas	NEC	21	3	2	N	P	Loss	P	0	Liver	IV	VitB12 deficiency, gallbladder stone	NF	TAE, IFN, streptomycin +adriamycin, sandostatin LAR, thalidomide, bevacizumab, everolimus
M/58	Pancreas	NET grade 1	3	1	1	P	N	Loss	P	0	No	IV	No	NF	NA
F/36	Pancreas	NET grade 2	NA	2	2	N	N	Loss	N	1	Stomach	IV	Zollinger Ellison syndrome	Gastrinoma	No
F/56	Pancreas	NET grade 2	4	2	1	N	N	Loss	P	1	Liver	IB	No	NF	RFA
M/64	Pancreas	NET grade 2	1.6	2	1	P	N	Loss	P	1	No	IA	No	NF	No
F/71	Pancreas	NET grade 1	2	1	1	P	N	Loss	P	1	No	2B	No	NF	No
F/63	Colon	NET grade 2	0.8	2	1	P	P	No loss	N	0	No	I	FGID, indigestion, multiple athralgia	NF	No
F/70	Stomach	NET grade 1	2	1	1	P	P	No loss	NA	0	No	IIA	Palpitation, hematemesis, melena	NF	No
F/56	Stomach	MANEC	4.8	3	3	N	P	Loss	N	0	No	IIA	No	NF	NA
F/60	Pancreas	NET grade 1	3	1	1	N	N	Loss	P	1	No	I	No	NF	No

ALT, altered lengthening of telomere; GEP-NET, gastroenteropancreatic neuroendocrine tumor; WHO, World Health Organization; NET, neuroendocrine tumor; NEC, neuroendocrine carcinoma; MANEC, mixed adenoneuroendocrine carcinoma; ATRX, alpha-thalassemia X-linked mental retardation; N, negative; P, positive; DAXX, death domain associated; TERT, telomerase reverse transcriptase; TERC, telomerase RNA component; AJCC, American Joint Committee on Cancer; FGID, functional gastrointestinal disturbance; NF, non-functional; TAE, transarterial embolization; IFN, interferon; LAR, long-acting repeatable; NA, not available.

Results

Telomere length abnormalities, ALT status, and TERC and TERT expression in GEP-NETs. In 253 cases, telomere FISH yielded informative results. ALT$^+$ status was scored in 10 cases (4%) and telomere shortening was observed in 79 cases (31%). Among the 253 cases, 194 were gastrointestinal in origin, 12 were hepatobiliary, and 47 were pancreatic. Telomere shortening was seen in 28% (13/47) of pancreatic NET cases and 32% (66/206) of GI-NETs. Among the 10 ALT$^+$ cases, 7 were of pancreatic origin. ALT$^+$ frequency was 15% (7/47) for pancreatic NETs and 1% (3/206) for GI-NETs. Clinicopathological comparisons were performed for the following sub-groups: ALT$^+$ *vs*. ALT$^-$ phenotypes, normal telomere length *vs*. telomere shortening, low TERC *vs*. high TERC expression, and negative TERT *vs*. positive TERT expression. ALT positivity was significantly associated with increased patient age; tumors that were pancreatic in origin, large, grade 2 (based on mitotic rate), and stage IV; presence of recurrence or metastasis; and loss of either ATRX or DAXX. Telomere shortening was significantly associated with tumors of the stomach, hepatobiliary, or midcolon; tumors that were large, grade 3 (based on mitotic rate and Ki-67 expression), WHO grade 3 or 4, or AJCC stage II; and recurrence or metastasis.

High TERC expression was noted in 23% of cases (61/269) and was associated with positive TERT expression (p=0.010). Among 67 cases with telomere shortening, 40 cases (60%) had low TERC expression (p=0.003). However, the ALT$^+$ phenotype was not associated with TERC or TERT

Table III. *Clinicopathological analyses of ATRX and DAXX in GEP-NETs by immunohistochemistry.*

	ATRX			DAXX			ATRX or DAXX		
Factor	Negative N (%)	Positive N (%)	*p*	Negative N (%)	Positive N (%)	*p*	Loss N (%)	No loss N (%)	*p*
Total	87	210		72	229		119	180	
Gender									
Female	34 (25)	100 (75)	0.178	29 (21)	106 (79)	0.371	45 (34)	89 (66)	0.048
Male	53 (33)	110 (67)		43 (26)	123 (74)		74 (45)	91 (55)	
Age (years)									
<55	35 (23)	119 (77)	0.01	39 (25)	116 (75)	0.603	55 (35)	100 (65)	0.114
≥55	52 (36)	91 (64)		33 (23)	113 (77)		64 (44)	80 (56)	
Primary Organ									
Stomach	17 (36)	30 (64)	0.028	16 (34)	31 (66)	<0.001	22 (47)	25 (53)	<0.001
Duodenum	4 (21)	15 (79)		6 (29)	15 (71)		8 (40)	12 (60)	
Pancreas	19 (39)	30 (61)		28 (57)	21 (43)		31 (63)	18 (37)	
Hepatobiliary	6 (60)	4 (40)		3 (30)	7 (70)		6 (60)	4 (40)	
Appendix	4 (13)	27 (87)		5 (16)	27 (84)		8 (25)	24 (75)	
Midgut Colon	2 (50)	2 (50)		1 (25)	3 (75)		3 (75)	1 (25)	
Hindgut Colon	35 (26)	102 (74)		13 (9)	125 (91)		41 (30)	96 (70)	
Tumor Size (cm)									
<1.0 cm	27 (17)	131 (83)	<0.001	19 (12)	142 (88)	<0.001	41 (26)	119 (74)	<0.001
≥1.0 cm	57 (43)	75 (57)		52 (39)	80 (61)		75 (57)	57 (43)	
Mitosis Grade									
1	47 (24)	149 (76)	0.002	42 (21)	157 (79)	0.048	69 (35)	129 (65)	0.05
2	10 (24)	31 (76)		16 (39)	25 (61)		17 (41)	24 (59)	
3	27 (47)	30 (53)		13 (22)	45 (78)		30 (53)	27 (47)	
Ki-67 Grade									
1	52 (27)	141 (73)	0.023	53 (27)	142 (73)	0.151	78 (40)	116 (60)	0.088
2	21 (30)	49 (70)		12 (17)	58 (83)		23 (33)	47 (67)	
3	13 (54)	11 (46)		4 (16)	21 (84)		14 (58)	10 (42)	
WHO 2010									
NET G1	39 (24)	123 (76)	<0.001	38 (23)	127 (77)	0.885	61 (37)	103 (63)	0.008
NET G2	19 (25)	56 (75)		20 (27)	55 (73)		26 (35)	49 (65)	
NEC	11 (33)	22 (67)		7 (21)	27 (79)		13 (39)	20 (61)	
MANEC	18 (67)	9 (33)		7 (26)	20 (74)		19 (70)	8 (30)	
Lymph Node Metastasis									
No	60 (25)	180 (75)	<0.001	54 (22)	189 (78)	0.513	86 (36)	156 (64)	0.005
Yes	25 (51)	24 (49)		13 (27)	36 (73)		28 (57)	21 (43)	
AJCC Stage									
I	54 (24)	172 (76)	0.002	49 (21)	180 (79)	0.135	79 (35)	149 (65)	0.012
II	11 (44)	14 (56)		10 (40)	15 (60)		14 (56)	11 (44)	
III	9 (43)	12 (57)		4 (19)	17 (81)		10 (48)	11 (52)	
IV	13 (54)	11 (46)		8 (32)	17 (68)		15 (63)	9 (38)	
Recurrence or Metastasis									
No	67 (26)	188 (74)	0.005	58 (22)	200 (78)	0.152	96 (37)	161 (63)	0.033
Yes	20 (48)	22 (52)		14 (33)	29 (67)		23 (55)	19 (45)	

ATRX, Alpha-thalassemia X-linked mental retardation; DAXX, death domain associated ; GEP-NET, gastroenteropancreatic neuroendocrine tumor; WHO, World Health Organization; NET, neuroendocrine tumor; NEC, neuroendocrine carcinoma; MANEC, mixed adenoneuroendocrine carcinoma; AJCC, American Joint Committee on Cancer.

expression. High TERC expression was significantly associated with male gender, age, hepatobiliary or midcolon origin, large tumor size, high WHO grade, advanced stage, and lymph node metastasis.

TERT expression was positive in 50% (142/284) of cases. Positive expression of TERT was more common in NETs located in the stomach, pancreas, hepatobiliary tract, or appendix. A significant association was noted between negative TERT and loss of ATRX or DAXX and low TERC expression (RNAscope score 0 or 1) (Table I).

Of the 10 ALT^+ cases, 8 showed immunohistochemical loss of ATRX or DAXX. Three ALT^+ cases were TERT

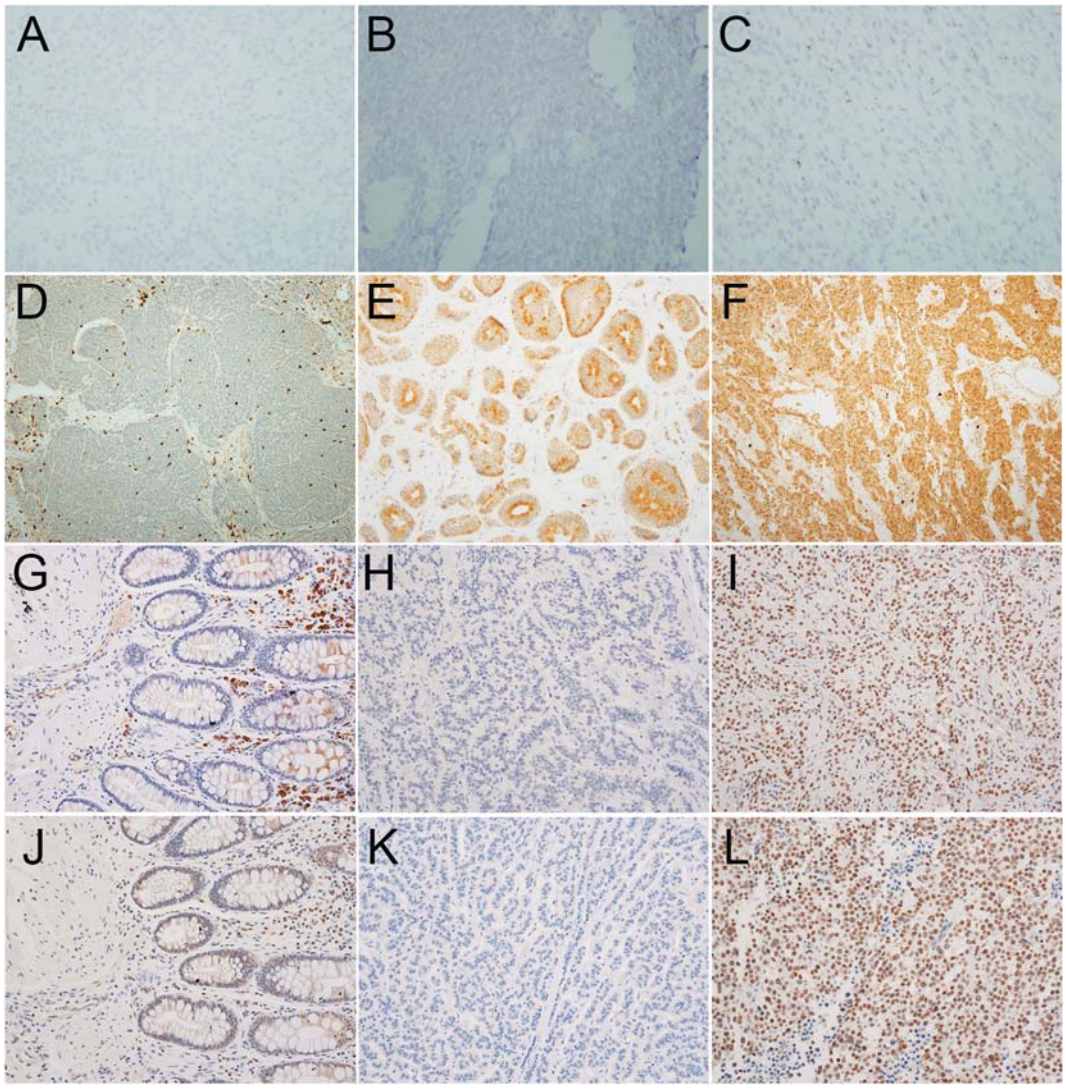

Figure 2. *In situ visualization of telomerase RNA component (TERC), telomerase reverse transcriptase (TERT), alpha-thalassemia X-linked mental retardation (ATRX), and death-associated protein 6 (DAXX) in GEP-NETs. TERC in situ hybridization: A: score 0, B: score 2, C: score 4. TERT immunohistochemistry (IHC): D: Negative in normal mucosa, E: negative but cytoplasmic positive, F: positive. ATRX IHC: G: Negative in normal mucosa, H: negative in neuroendocrine tumor (NET), I: positive in NET. DAXX IHC: J: Negative in normal mucosa, K: negative in NET, L: positive in NET.*

negative and 6 were TERT positive. The clinicopathological profiles of the 10 ALT^+ cases are shown in Table II.

TERC ISH showed low expression in normal epithelial and stromal cells and variable expression in lymphocytes. In 269 cases, TERC ISH yielded informative results, with a score of 0 in 114 cases (42%), 1 in 95 cases (35%), 2 in 50 cases (19%), 3 in eight cases (3%), and 4 in two cases (1%). Dichotomization of informative cases resulted in 209 cases (78%) with a low TERC score (0 or 1) and 60 cases (22%) with a high TERC score (2, 3, or 4).

Only nuclear localization of TERT was counted as positive staining. In the normal epithelium, the majority of cells were negative except for a few colon crypts that appeared as regenerative crypts. Thus, 284 cases were informative for TERT IHC with 142 negative (50%) and 142 positive (50%).

Loss of ATRX or DAXX protein expression in GEP-NETs. ATRX loss was significantly associated with increased age, hepatobiliary or midgut colon origin, large tumor size, grade 3 (based on mitotic rate and Ki-67 expression), WHO grade 3 or 4, higher AJCC stage, or recurrence or metastasis. DAXX loss was significantly associated with grade 2 tumors (based on mitotic rate) that were pancreatic in origin and large in size. Together, loss of ATRX or DAXX was

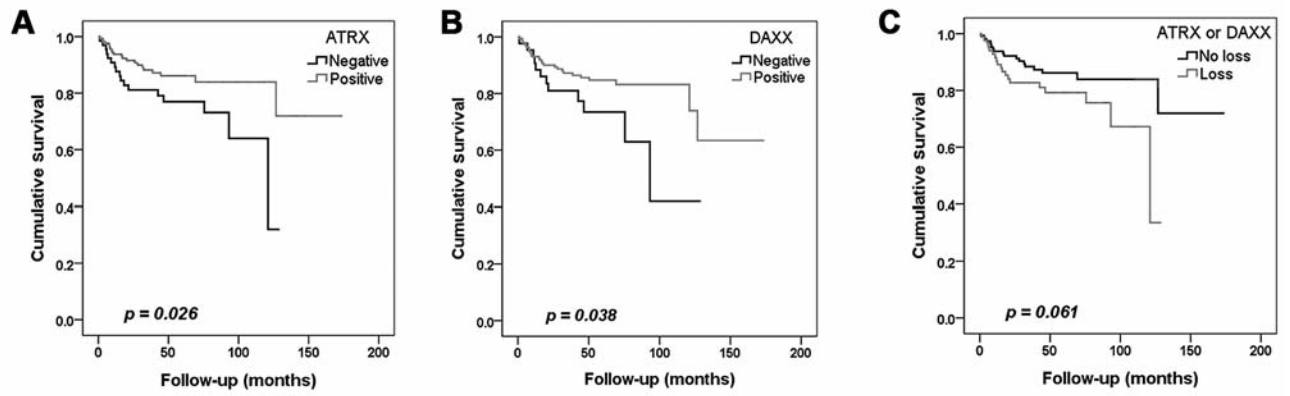

Figure 3. *Kaplan–Meier analyses of overall survival in patients with gastroenteropancreatic neuroendocrine tumors (GEP-NETs) according to alpha-thalassemia X-linked mental retardation (ATRX) immunohistochemistry (IHC) (A), DAXX IHC (B), and loss of ATRX or DAXX protein expression (C).*

Table IV. *Univariate and multivariate Cox regression analyses for overall survival of patients with GEP-NETs*

		Univariate analysis					Multivariate analysis				
					95% Confidence Interval					95% Confidence Interval	
Factor		N	*p*-Value	Hazard ratio	Lower	Upper	N	*p*-Value	Hazard ratio	Lower	Upper
Ki-67	G1 or G2	228					141				
	G3	19	0.001	4.256	1.87	9.70	17	0.339	0.557	0.17	1.85
Lymph node metastasis	No	214					125				
	Yes	41	<0.001	9.782	5.03	19.04	33	<0.001	9.597	3.52	26.17
ATRX	Positive	169					113				
	Negative	66	0.030	2.040	1.07	3.88	45	0.735	1.176	0.46	3.01
DAXX	Positive	196					131				
	Negative	43	0.043	2.019	1.02	3.98	27	0.545	1.400	0.47	4.16
TERC*	Low	166					121				
	High	42	<0.001	5.347	2.72	10.51	37	0.036	2.897	1.07	7.83
Telomere FISH	Preserved	135					112				
	Reduced	57	0.020	2.334	1.14	4.77	46	0.957	0.976	0.40	2.36

*Low score: 0, 1; high score: 2-4. GEP-NET, Gastroenteropancreatic neuroendocrine tumor; ATRX, alpha-thalassemia X-linked mental retardation; DAXX, death domain associated; TERC, telomerase RNA component; FISH, fluorescent *in situ* hybridization

associated with pancreatic, hepatobiliary, or midgut colon origin; large tumor size; grade 3 (based on mitotic rate); WHO grade 4, advanced AJCC stage, or recurrence or metastasis (Table III). Kaplan–Meier analysis showed that the overall survival of patients with GEP-NETs with loss of ATRX loss (p=0.026) or DAXX (p=0.038) was significantly lower compared to other groups (Figure 3).

Regression analysis for overall survival in GEP-NETs. The variables of grade 3 (based on Ki-67 expression), lymph node metastasis, ATRX loss, DAXX loss, high TERC expression, and telomere shortening were analyzed by regression analysis for overall survival. All six variables were significantly associated with lower rates of overall survival. In our multivariate analysis, lymph node metastasis and high TERC expression were independent variables for decreased overall survival (Table IV).

Discussion

In previous reports, 61% (25/41) of pancreatic NET samples exhibited ALT and 76% (19/25) of ALT$^+$ cases had *ATRX* or

DAXX gene mutations (9). Mutations in the *DAXX* or *ATRX* genes and loss of ARTX/DAXX protein is associated with ALT in 43% of pancreatic NETs (14).

Although the ALT frequency in our study was lower than in previous reports, more pancreatic NET samples than GI-NET samples were ALT$^+$. At the time of writing, no reports were available on ALT in non-pancreatic GI-NETs, hence our study results suggest that ALT$^+$ status is a unique feature of NETs of pancreatic origin and indicate the existence of a difference from non-pancreatic GI-NETs.

The correlation between the ALT$^+$ phenotype and loss of ATRX or DAXX was strong, similarly to previous reports (14, 17). No significant association between ALT$^+$ status and TERT negativity was found in our study, which might reflect the limitations of IHC for TERT evaluation (10, 14, 17).

In our results, 8 out of 10 ALT$^+$ cases lost either ATRX or DAXX protein. Loss of ATRX or DAXX is associated with aggressive pathological factors. In the multivariate analysis, lymph node metastasis and high TERC expression were independent prognostic factors for overall survival. Our results of the Kaplan–Meier analysis also showed that both ATRX loss and DAXX loss were associated with lower rates of overall survival.

Our results suggest a relationship between telomere dysfunction and TERC expression. ALT$^+$ status was associated with low TERC expression and telomere shortening was associated with high TERC expression. Immunohistochemically, TERT+ samples were associated with higher TERC expression. TERC is the template for the addition of telomeric repeats in a reverse transcriptase reaction at chromosome ends. TERC levels increase with tumor progression, (21) and genomic amplification of *hTERC* is a biological marker for cervical cancer and cervical intraepithelial neoplasm (1). In a study of telomerase hTERT through the use of IHC and the RNA TERC component *via* ISH, the distribution of TERC correlated with the localization of hTERT in high-grade squamous intraepithelial lesions and squamous cell carcinomas (7). In pulmonary NETs, TERC expression was detected in 59% of typical carcinoids and as high as 98% in small cell lung cancer using ISH (22).

No previous reports are available on telomere shortening in NETs. Our results detected telomere shortening in 31% of GEP-NETs. These results showed that telomere shortening, as well as ALT positivity, contributed to tumorigenesis in a subset of GEP-NETs. In a previous report on pancreatic ductal intraepithelial neoplasia, telomere signals were reduced in 79 (96%) out of 82 pancreatic intraepithelial neoplasia compared to adjacent normal structures (24). This difference in the frequency of telomere shortening indicates a fundamental difference in the mechanisms of tumorigenesis of ductal neoplasia and pancreatic NETs.

Conclusion

Our results show that two types of telomere length abnormalities exist in a subset of GEP-NETs. The ALT$^+$ phenotype was associated with loss of ATRX or DAXX protein, whereas telomere shortening was associated with low TERC expression. Lymph node metastasis and high TERC expression were independent prognostic factors in patients with GEP-NETs.

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