Assessment of Telomere Length and Molecular Characterization of *TERT* Gene Promoter in Periapillary Carcinomas

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Abstract

Genetic and epigenetic alterations of the telomere maintenance machinery like telomere length and telomerase reverse transcriptase (encoded by *TERT* gene) are reported in several human malignancies. However, there is limited knowledge on the status of the telomere machinery in periampullary carcinomas (PAC) that are rare and heterogeneous groups of cancers arising from different anatomic sites around the ampulla of Vater. In the current study, we investigated the relative telomere length (RTL) and the most frequent genetic and epigenetic alterations in the *TERT* promoter in PAC (n=20) and compared with tumor-adjacent nonpathological duodenum (NDu; n=16). We found shorter RTLs (1.27 vs 1.33, $P=0.01$) and lower TERT protein expression ($p=0.04$) in PAC tissues as compared to the NDu. Although we did not find any mutation at two reactivating hotspot mutation sites of the *TERT* promoter, we detected polymorphism in 55% (11/20) of the cases at rs2853669 (T>C). Also, we found a hypermethylated region in the *TERT* promoter of PACs consisting of four CpGs (cg10896616 with $\Delta\beta$ 7%; cg02545192 with $\Delta\beta$ 9%; cg03323598 with $\Delta\beta$ 19%; and cg07285213 with $\Delta\beta$ 15%). In conclusion, we identified shorter telomeres with DNA hypermethylation in the *TERT* promoter region and lower TERT protein expression in PAC tissues. Further studies with a larger sample size are necessary to substantiate these results.

Introduction:

Periampullary carcinomas (PACs) encompass a heterogeneous group of neoplasms (L. 2001) arising from four distinct anatomic sites proximal to the major duodenal papilla, namely - ampullary adenocarcinoma (AA), distal cholangiocarcinoma (DCC), duodenal adenocarcinoma (DA), and pancreatic head adenocarcinoma (PDAC) (Sarmiento, Nagomey et al. 2001, Albores-Saavedra, Schwartz et al. 2009, Kamarajah 2018, Hester, Dogeas et al. 2019). PACs are a rare malignancy whose incidence rate is difficult to establish as most reported studies only reflect the prevalence of surgically resected cancers (Yeo, Sohn et al. 1998, Riall, Cameron et al. 2006, He, Ahuja et al. 2014, Chandrasegaram, Chiam et al. 2015). However, on average, taken together, they represent 5% of all gastrointestinal malignancies (Sarmiento, Nagomey et al. 2001, Berberat, Künzli et al. 2009, Kamarajah 2018, Song, Kim et al. 2020). Due to their anatomical proximity, PACs share clinical characteristics and interventions, the most effective of which, regardless of the site of origin, is surgical resection by 'pancreaticoduodenectomy' (PD) (Ohtsuka and Miyazaki 2018). However, even after surgical resection, the long-term survival (≥ 5 years) rate in patients is unsatisfactory ($\sim 20\%$) (El Nakeeb, El Sorogy et al. 2018) and varies greatly among the subtypes of PACs (Bramhall, Allum et al. 1995, Neoptolemos, Russell et al. 1997, Poultides, Huang et al. 2012).

For better clinical outcomes and therapeutic management, it is important to distinguish the site of origin of cancer and thus the tumor identity before deciding on the required intervention. However, the precise site of origin of PACs is usually challenging to determine before the surgical intervention. Thus, the correct preliminary diagnosis is often difficult, as in the case of PAC patients with locally advanced or metastatic disease that impedes the curative surgical resection. For this reason, during the past years, many studies (Ching and Rhodes 1989, Steinberg 1990, Lundin, Roberts et al. 1994, Ritts, Nagorney et al. 1994, Satake and Takeuchi 1994, Kau, Shyr et al. 1999) have established distinct molecular markers of

PACs, specifically carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9), but also CA 242 (Haglund, Lundin et al. 1994), CA 50, CA 12-5, tissue polypeptide antigen (TPA) (Benini, Cavallini et al. 1988) and various mRNAs and miRNAs (Sandhu, Bowitz Lothe et al. 2015). These markers exhibit some diagnostic and prognostic value and can partially explain differing tumor type behavior and patient survival (Forsmark, Lambiase et al. 1994, Okusaka, Okada et al. 1998, Distler, Pilarsky et al. 2013, Park, Shin et al. 2021). However, this is far from enough to fully understand this malignancy and necessitates a more detailed molecular characterization of the disease.

Eukaryotic organisms exhibit highly specialized DNA-protein structures at the end of linear chromosomes, called telomeres; that help maintains genomic stability (Hernandez-Sanchez, Xu et al. 2016). Owing to the end-replication problem, telomeres are progressively shortened during successive cell divisions that cause cellular senescence, ultimately leading to cell mortality. Since cancer is a disease of cellular immortality, it is not surprising that cancer cells need to overcome this senescence by countering telomere shortening (Shay 1995). Cancer cells achieve this with the help of an enzyme called telomerase, which adds telomere repeat sequence to the 3' end of the telomere, giving them infinite replicative potential (Feng, Funk et al. 1995). The catalytic subunit of telomerase is telomerase reverse transcriptase (TERT) which has been implicated to have a role in human cancer (Kirkpatrick and Mokbel 2001). Approximately 90% of all human cancers exhibit transcriptional activation of TERT (Holt, Wright et al. 1997) (Shay and Bacchetti 1997). Hundreds of TERT polymorphisms have been found in human cancers, (Mocellin, Verdi et al. 2012) including hot-spot mutation of the gene promoter causing several sequence variations (Bell, Rube et al. 2016, Dratwa, Wysoczańska et al. 2020), which act as pivotal players in the activation of TERT transcription (Yuan, Larsson et al. 2019). In addition, epigenetic modification of the TERT promoter via methylation has been reported in about 50% of all human cancers (Lee, Leao et al. 2019, Dratwa, Wysoczańska et al. 2020). Despite the available detailed descriptions of the specific mechanism behind telomere maintenance of many cancers, it is still poorly understood in PAC. Thus, we reasoned that the information on the telomeric status and TERT gene features of PAC will help in a better understanding of its molecular landscapes. Following this rationale, we inspected and report the relative telomere length (RTL), as well as the most frequent genetic and epigenetic alterations in the *TERT* gene, using Sanger sequencing and 450K methylation array. In addition, we inspected *TERT* gene expression by immunohistochemistry in AA and DA. Lastly, we also explored whether these parameters were correlated with clinicopathological features and patient prognosis. This study constitutes a pilot study in the field of telomere biology in AA and DA and reveals new information about the telomere maintenance in these cancers.

Methods:

Study samples

Patients with PAC and DUOAC were recruited between March 2011 and September 2018 from the Hospital de Clínicas de Porto Alegre (HCPA/UFRGS), Brazil. The inclusion criteria were recent pathology-proven diagnosis and no history of previous or current chemo- or radiotherapy treatment. Samples from

tumor and nonpathological mucosae of the duodenum (NDu) were obtained during surgical pancreatoduodenectomy with curative intent without known residual disease and stored according to the biobank protocols from the hospital. A portion of each tissue sample was frozen and stored at -80°C, whereas the remaining tissue was fixed in 10% buffered formalin to prepare Formalin-Fixed Paraffin-Embedded (FFPE) blocks. The characteristics of the patients were obtained from medical records and the prognostic factors of these cases was published recently (Vilhordo, Gregorio et al. 2021). The study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre (Project number: 2014–0526) and conducted following the Declaration of Helsinki. All subjects gave their informed consent for inclusion before they participated in the study.

Pathology

Tissue sections derived from FFPE blocks were stained with hematoxylin & eosin to confirm the diagnosis and assess the sample quality. The tumoral and non-tumoral samples were independently examined by two experienced pathologists (R.C.R. and S.M.S.M.), in a blinded manner. The classification of malignant epithelial tumors of the exocrine pancreas recommended by The World Health Organization (WHO) was applied for the histological type (Nagtegaal, Odze et al. 2020). The histopathological grade was based on the extension of the glandular formation, being considered well-differentiated tumors with more than 95% of glands, moderately differentiated tumors with 50-95% of glands and poorly differentiated tumors with 49% or less of glands (Adsay, Basturk et al. 2005). The location of the neoplasia was defined according to the anatomical subdivisions recommended by the AJCC, as follows (Amin, Greene et al. 2017): Tumors of the head of the pancreas are those arising to the right of the left border of the superior mesenteric vein (the uncinate process is part of the head). Tumors of the body of the pancreas are those arising between the left border of the superior mesenteric vein and the left border of the aorta. Tumors of the tail of the pancreas are those arising between the left border of the aorta and the hilum of the spleen.

Sample preparation

DNA isolation was done from fresh frozen tumor tissues (n=20) and NDu (n=16) using the PureLink Genomic DNA Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The samples with pathologically >60% neoplastic cells were considered as tumor and 100% nonpathological mucosae of the duodenum was considered normal. The eluted DNA was quantified using Qubit V2.0 (Invitrogen, Carlsbad, USA).

Relative telomere length measurement

We performed a qRT-PCR based estimation on relative telomere length (RTL) assessment by comparing the abundance of the telomeric template (T) relative to a single-copy gene (S, here albumin) and expressed as the ratio between T/S (Hosen, Rachakonda et al. 2015). Each reaction was performed in triplicate, in an optical 96-well reaction plate compatible with the Bio-Rad Real-Time PCR Detection System using the SYBR Green Supermix (Bio-Rad, Hercules, CA). The thermal cycling was performed as per the modified Cawthon method (Cawthon 2009).

The signal acquisition at 74°C provided the Ct values for the amplification of the telomere template and signal acquisition at 88°C gave the Ct values for the albumin template. After the final incubation at 88°C, the specificity of all amplifications was determined by melting curve analysis. Reactions were then cooled to 55°C, and the signal was acquired from 55°C to 95°C, in increment 0.5°C steps, with a 30 s period per step. PCR conditions and the primers used for the telomere and the albumin are listed in **Supplementary Table T1**. The quality of PCR amplification was determined by standard curves using reference DNA in triplicates (serial dilutions in the range of 150–5.55 ng). Telomere and albumin reactions were added in different wells and Ct values were obtained based on the respective averages.

TERT promoter mutational detection

The tumor mutational status of the *TERT* core promoter and the surrounding region was determined by Sanger sequencing (Rachakonda, Hosen et al. 2013), using the Applied Biosystems 3730xl genetic analyzer. Sequencing data was analyzed using the CodonCode Aligner version 7.1 software referring to the sequences from the NCBI gene database, *TERT* (chr5: 1,295,071–1,295,521, hg19 GRCH37). The primer sequences and PCR conditions for Sanger sequencing are shown in **Supplementary Table T2**.

TERT promoter DNA methylation estimation

We retrieved beta β values from Illumina Infinium Human Methylation450K Bead-Chips HM450K data (Illumina, San Diego, CA, USA). To investigate the DNA methylation status *TERT* promoter, we retrieved the normalized β values of the probes from the genomic regions (chr5:1,291,235-1,295,737), which included probes in its promoter, gene body, and introns. The β values of four CpGs from this region are shown in Figure 4 with their genomic coordinates.

TERT protein expression

Tumor samples and their respective surrounding duodenal non-tumoral FFPE tissue were retrieved and included in tissue microarrays (TMAs). TMAs were prepared from formalin-fixed paraffin-embedded tissue sections using a 1-mm punch in triplicate. Immunohistochemistry (IHC) was carried out on 4- μ m sections. Briefly, slides were deparaffinized and then rehydrated before the staining procedure can be performed. For the antigen retrieval process, slides were placed in a preheated Epitope Retrieval Solution pH6 (95°C to 99°C) and maintained at this temperature for 10 minutes followed by a 20-minute cool-down period at room temperature. After a brief rinse in distilled water, slides were immersed in Wash Buffer in preparation for immunostaining.

Specimens were blocked with a peroxidase-blocking reagent for 5 minutes followed by 1h incubation with the polyclonal primary antibody anti-telomerase reverse transcriptase (600-401-252S, Rockland Inc., Limerick, PA, USA) at a dilution of 1:1000. Slides were then incubated for 30 minutes with the visualization reagent followed by five minutes incubation with DAB. Slides were subsequently counterstained with hematoxylin, dehydrated, mounted with permanent mounting medium, and coverslipped. All steps, except for the epitope retrieval, were performed at room temperature. Immunoreactivity was visualized by light microscopy and the gradation was done based on slides

displaying intensities “negative/no staining as 0,” “low/weak staining as 1,” “medium staining 2,” or “high staining as 3.” Also, the percentage of stained cells was noted for the final score determination. A final score was calculated by multiplying the staining intensity and percentage of positively stained cells, which is described in **Supplementary Table T3**.

Statistical Analysis:

The comparison between groups (PAC vs NDU) of the RTL and methylation profile was performed using the Wilcoxon test. Association between clinicopathological factors and RTL and rs2853669 was performed by Mann-Whitney and Fisher's Exact test, respectively. Correlation between RTL and methylation profile was performed by Spearman correlation. Five-year survival rates were calculated by the Kaplan–Meier method, and univariate survival analysis was performed by the Log-rank, Breslow, and Tarone-Ware tests. All statistical analysis was performed with SPSS version 24 (IBM). $p < 0.05$ was considered statistically significant.

Results:

Sample characteristics

Our study included 20 PAC cases among which 12 were males and 8 were females. Among these, half of our PAC cases (50%) were from intestinal-type adenocarcinoma. Details of all pathological sub-types are shown in Table 1. 30% ($n=6$) of the cases were ≥ 65 years of age and all the cases were from stage II to IV. The tumor grade was moderate or poor differentiation for 95% (19/20) of the cases (Table 1). We also included macroscopically examined tumor-adjacent nonpathological duodenum tissues (NDU, $n=16$) for comparison.

Relative Telomere Length (RTL)

We analyzed the RTL of PACs and NDU by comparing the abundance of the telomeric template (T) relative to a single-copy gene (S, here albumin) using qRT-PCR. The RTL is expressed as the ratio between T/S and the higher value will signify longer RTL. The average RTL for PAC tissue was 1.27 (range 1.10–1.42) and NDU tissue was 1.33 (range 1.2–1.38); $P = 0.01$ which signifies shorter telomere length in tumors than the adjacent nonpathological duodenum (Figure 1).

TERT protein expression

To investigate the TERT protein expression, we compared the immunohistochemistry (IHC) score between PAC and NDU tissues. Negative staining was considered as 0 and three categories of positive staining ($C1$: 1-3; $C2$: 4-6 and $C3$: 7-9) was generated by scoring the intensity and percentage of positively stained cells. The details of the score calculation are specified in **Supplementary Table T1**. PAC tissues exhibited a greater number of category $C1$ (55% vs 20%) than NDU tissues, which is associated with weak/poor staining. However, TERT positive expression rate was observed higher in NDU than the PAC for category

C2 (45% vs 30%) and C3 (15% vs 10%). Since scores for category C2 and C3 were derived from medium/high staining, it is apparent that TERT protein expression is more prominent in NDU (Table 2).

TERT promoter mutation

TERT promoter sequencing results revealed no mutation at the two reactivating hotspot mutation sites C228T (NG_009265.1: g.4935C>T) and C250T (NG_009265.1: g.4913C>T) in the PAC tumors (Figure 3 and **Supplementary Figure S1**). However, within the sequenced region, we observed 11/20 (55%) cases with polymorphism at rs2853669; T>C (NG_009265.1: g.4814T>C). The polymorphic site is present in the upstream region of the two hotspot mutation sites shown in Figure 3. This polymorphism at rs2853669 with the two hotspot mutations C228T and C250T is usually associated with an increased risk of developing cancer (Vinothkumar, Arun et al. 2020). Although in our PAC samples we could observe this polymorphism, we did not detect any sequence variation in the two-hotspot region. To investigate further if there is any clinical significance of the identified polymorphic site rs2853669, we performed survival analysis by stratifying cases based on the status of polymorphism (**Supplementary Figure S2**). Nevertheless, the differences in survival in all patients with and without the rs2853669 polymorphism were not significant ($P=0.80$).

TERT promoter methylation

We identified a differentially methylated region in the *TERT* promoter that was hypermethylated in PAC tissues in comparison with NDU tissues. Seven CpG sites showed this differential methylated trend with a $\Delta\beta$ (DNA methylation difference) of 9% among which, four CpGs were significantly hypermethylated in PAC samples. The identified differentially methylated region in the *TERT* promoter region constitutes multiple transcription factors and related protein binding sites that overlap with the four significantly hypermethylated CpG sites. In these CpGs $\Delta\beta$ at cg10896616 was 7%, $P=0.006$; at cg02545192 $\Delta\beta$ was 9%, $P=0.005$; at cg03323598 $\Delta\beta$ was 19%, $P=0.002$, and at cg07285213 $\Delta\beta$ was 15%, $P=0.04$ (Figure 4).

Discussion:

In this study, we report shorter RTL in PAC and aimed to investigate molecular features of the *TERT* gene and its protein expression to understand the possible link with alteration of telomere length (Figure 5). However, it should be noted that telomere lengthening can be independent of *TERT* gene alterations and alternative mechanisms can lead to variations in RTL (Yuan, Larsson et al. 2019). Here we observed lower TERT protein expression in tumors relative to NDU based on IHC analysis. Previous studies have shown that shorter telomeres are associated with a higher risk for several cancers (Broberg, Björk et al. 2005, Risques, Vaughan et al. 2007, Jang, Choi et al. 2008, Hou, Savage et al. 2009, Xing, Ajani et al. 2009, Mirabello, Garcia-Closas et al. 2010, Willeit, Willeit et al. 2010), including pancreatic cancers (Kobitsu, Tsutsumi et al. 1997, Skinner, Gangnon et al. 2012) (Hong, Heaphy et al. 2011) and hepatocellular carcinomas (Oh, Jo Chae et al. 2003). Truncated telomeres are considered a defining characteristic of most carcinomas (McGrath, Wong et al. 2007). Genetic instability associated with short telomeres is

described as an early event in tumorigenesis that acts as a predisposition factor for many cancers (Wu, Amos et al. 2003). Since telomere shortening is very unlikely to provide any direct growth advantage to the cells, it is presumed to promote cancer progression by causing telomere crisis (van Heek, Meeker et al. 2002), which can induce numerous changes relevant to cancer including chromothripsis, kataegis, and tetraploidization (Maciejowski and de Lange 2017). This is in line with our observation, and it would be interesting to inspect these tumors for chromosomal abnormalities.

While telomere dysfunction provokes chromosomal aberrations and aids initiation of carcinogenesis, telomerase-mediated telomere maintenance enables such predisposed cells to efficiently achieve a fully malignant endpoint, including metastasis (Chang, Khoo et al. 2003). Therefore, telomerase activation by various mechanisms including *TERT* promoter mutation is common in many cancers, including hepatocellular carcinomas (Huang, Wang et al. 2015). However, these are extremely rare in pancreatic cancers (Vinagre, Nabais et al. 2016, Posch, Hofer-Zeni et al. 2020), but no hotspot mutations were found in our samples. We also observed a polymorphism at rs2853669 in 55% of the cases but we could not find any significant correlation with prognosis. A recent study proposed the association of this polymorphism with the two hotspot mutations in increasing the risk of cervical cancer (Vinothkumar, Arun et al. 2020). Nevertheless, there are limited functional studies to establish the role of rs2853669 polymorphism in carcinogenesis.

Then, we aimed to examine if the *TERT* expression was altered by epigenetic mechanisms in these cancers instead. In previous studies, *TERT* Hypermethylated Oncological Region (THOR) located in promoter was found to be upregulating *TERT* expression and associated with poorer clinical outcomes (Castelo-Branco, Leao et al. 2016, Lee, Leao et al. 2019). In line with this, we observed hypermethylation of tumor tissues in a region downstream of THOR and near the transcription start site (TSS). The detected lower *TERT* protein expression in tumors might be due to the hypermethylated region near the TSS of the *TERT* promoter. Also, the presence of some transcription factor binding site at the hypermethylated region further substantiate that this could be the possible reason for the downregulation of *TERT* expression. One of the constraints of this study is that we did not have RNA samples to validate the association of *TERT* promoter hypermethylation with reduced *TERT* protein expression in PAC tissues.

In conclusion, we observed shorter RTL and lower *TERT* protein expression in PAC. We further report a hypermethylated region in *TERT* promoter and polymorphism at rs2853669 in 55% of the cases. The *TERT* promoter hypermethylation might be linked with shorter RTL and lower *TERT* protein expression in PAC. Additional studies with a larger sample size could validate these findings in PAC and associate with clinical parameters to improve patient outcomes.

Declarations:

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COMPETING INTERESTS

The authors declare no conflict of interest.

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CONTRIBUTION

CG: conceptualization, data curation, methodology, formal analysis, validation, writing—original draft preparation and editing; **ST:** data interpretation, validation, writing—original draft preparation and editing; **RCR:** methodology, investigation and formal analysis; **SMSM:** methodology, investigation and formal analysis; **CC:** methodology; validation; **ChC:** methodology; validation; **VW:** methodology, investigation and formal analysis; **IC:** methodology and investigation; **KV:** methodology, investigation and formal analysis; **MGD:** methodology, investigation and formal analysis; **ABO:** methodology, investigation and formal analysis; **PAP:** conceptualization, project administration, supervision, investigation, resources, fund acquisition; **ZH:** conceptualization, project administration, supervision, investigation, resources, fund acquisition; **FT:** conceptualization, project administration, supervision, investigation, data curation, methodology, formal analysis, validation, writing—original draft preparation and editing. All authors reviewed and edited the manuscript.

CONSENT TO PARTICIPATE

All patients provided informed consent to participate in this study. The study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre (Project number: 2014–0526) and conducted following the Declaration of Helsinki.

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Tables:

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Figures

Figure 1

Figure 1

Relative Telomere Length (RTL) estimation. (A) Figure illustrating telomere length measurement (B) Boxplots representing the RTL of nonpathological duodenum (NDu) and perampullary carcinomas (PAC).

Figure 2

Figure 2

TERT protein expression in perampullary carcinomas (PAC) and nonpathological duodenum (NDu). (A) High expression (C3) of TERT in PAC; (B) Moderate expression (C2) of TERT in PAC, (C) Low expression (C1) of TERT in PAC, (D) High expression (C3) of TERT in NDu; (E) Moderate expression (C2) of TERT in NDu, (F) Low expression (C1) of TERT in NDu. The histological sections stained are presented at x200 magnification and the left squares represent x40 magnification.

Figure 3

Figure 3

TERT promoter sequencing. Representative amplicon and sequencing electropherogram. Hotspot mutation site C228T (NG_009265.1:g.4935C>T) and C250T (NG_009265.1:g.4913C>T) was investigated. A polymorphic site rs2853669 (NG_009265.1: g.4814T>C) was also within the sequenced region. TSS: Transcription Start Site, reference genome: GRCh37/hg19.

Figure 4

Figure 4

DNA methylation status of TERT promoter. (A) Structure of the TERT gene; (B), DNA hypersensitivity and Transcription Factors binding site (GRCh37/hg19, ENCODE); (C), Differentially methylated region (DMR) of TERT promoter (D) Box plot for CpGs within the TERT DMR. DNA Methylation is expressed as Beta-values deduced from Illumina 450K arrays.

Figure 5

Figure 5

Result summary; showing compiled results of telomere length, TERT promoter hot-spot mutation status, TERT promoter DNA methylation status, rs2853669 SnP polymorphism in TERT promoter and TERT protein expression.

Supplementary Files

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