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ORIGINAL ARTICLE

Telomere length and TERT abnormalities in pituitary adenomas

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Abstract

OBJECTIVES: Pituitary adenomas (PAs) are among the most frequent intracranial tumors in humans. Abnormal telomerase activity and telomere lengthening are features of tumor cells. They may result from mutations in *TERT* promoter region, gene amplification or aberrant DNA methylation pattern. Such changes were found in variety of tumors including those of brain. Aim of the study was to evaluate the incidence of *TERT* abnormalities and to assess their role in telomere lengthening in PAs.

METHODS: Study involved 101 patients with PA including both nonfunctioning and functioning subtypes. Telomerase length as well as *TERT* mRNA level and gene amplification were estimated using quantitative PCR (qPCR). Promoter mutations were assessed using Sanger sequencing. The results from genome-wide DNA methylation profiling with HumanMethylation 450K (Illumina) were used for the analysis of *TERT* locus.

RESULTS: Variable telomere length was observed in patients, however no relationship with clinicopathological features was found. We observed a missense variant in *TERT* promoter in one patient only whereas increased *TERT* copy number were identified in 6 patients (5.6%). However no relationship between these results and telomere length or *TERT* expression was found. DNA methylation at *TERT* locus was not found to be changed when adenoma samples and normal tissue sections were compared.

CONCLUSION: The results indicate that telomerase abnormalities do not play a role in pathogenesis of pituitary tumors.

Abbreaviations:

PA	- pituitary adenoma
qPCR	- quantitative PCR
PBMC	- peripheral blood mononuclear cells
T/S ratio	- telomere/single copy gene
CNV	- copy number variation
GCR	- gene copy number ratio
HMK450	- HumanMethylation 450K (Illumina)
qRT-PCR	- quantitative reverse transcribed PCR

INTRODUCTION

Pituitary adenomas (PAs) are benign intracranial tumors occurring in approximately 20% of all intracranial neoplasms (Mete & Asa 2012). They are basically divided into functioning and non-functioning subtypes. About 30% newly diagnosed pituitary adenomas are endocrinologically inactive and other 70% are distinguished according to the secreted hormone (Dworakowska & Grossman 2009). The molecular background of pathogenesis of PAs is relatively poorly understood.

Telomeres are short repeated DNA sequences at the ends of chromosomes, which protect genetic information during DNA replication and prevent recognition of chromosome ends as double strand break and possible chromosome joining in repair process (Lu *et al.* 2013). Telomeres become shorter after each round of DNA replication and this limits the number of cells' divisions. In some specific types of normal dividing cell, including stem cells or activated lymphocytes, telomeres can be extended by telomerase enzyme. However, this enzyme is not present in vast majority of normal somatic cells (Shay & Wright 2005). Telomerase is a heterodimer composed of two main components: *TERT* being a catalytic subunit of enzyme, encoded by *TERT* gene as well as TERC – RNA molecule, that acts as a template for DNA synthesis (Cohen *et al.* 2007). It was shown in previous studies that *TERT* subunit is limiting for enzyme activity and *TERT* overexpression corresponds with telomere length (Newbold 2002).

Reactivation of telomeres plays a role in neoplastic transformation and allows cells to escape apoptosis and immortalize. Telomerase overexpression and telomere lengthening were observed in many types of tumors (Akincilar *et al.* 2016) including those of brain (Hiraga *et al.* 1998). It was estimated that telomerase is reactivated in 85% of all cancers (Akincilar *et al.* 2016).

The most common mechanism of telomerase activation in cancer is the point mutation in a hot-spot region located at the promoter of *TERT* gene. These mutations create binding site for the ETS transcription factors, allows their binding to the *TERT* promoter that results in forced gene expression as a consequence (Bell *et al.* 2016).

Alternatively, in some tumors *TERT* may be upregulated due to gene amplification (Piscuoglio *et al.* 2016; Xie *et al.* 2014; Diaz *et al.* 2014) as well as aberrant epigenetic regulation. However, the role of DNA methylation in telomerase activation is still unclear as tumor cells exhibit complex and heterogeneous methylation pattern at *TERT* 5' region (Akincilar *et al.* 2016).

The role of telomere lengthening in pathogenesis of PAs is unclear. Only few studies on telomerase activity and expression were published, with slightly inconsistent results. Mutations of *TERT* promoter were not found in pituitary tumors in two previous reports that compared the incidence of these mutations in different intracranial tumors, but these studies included very small series of PA samples (Chen *et al.* 2014; Koelsche *et al.* 2013). In turn, aberrant promoter methylation was observed as common in pituitary tumors, however its impact on telomerase gene expression was not investigated (Kochling *et al.* 2016).

In our study we determined the telomere length, *TERT* expression and incidence of abnormalities of *TERT*: promoter mutations, gene amplification and DNA methylation pattern in a series of pituitary adenomas.

MATERIALS AND METHODS

Patients and samples

Tissue samples from 101 patients were collected during transsphenoidal surgery and immediately frozen in liquid nitrogen, than stored in -80°C. Parts of all tumors were assessed histopathologically. Diagnoses were based on WHO 2004 criteria (DeLellis 2004). Approval was obtained from the Institutional Ethics

Tab. 1. Patients' characteristics.

Pituitary adenoma patients (number of patients)	**101**
Age (years)	
range	15-85
median	60
Gender (number of patients)	
male	64/101
female	37/101
Functional classification (number of patients)	
nonfunctioning PA	81/101
somatotrophic PA	12/101
lactotrophic PA	6/101
corticotrophic PA	2/101
Clinical classification (number of patients)	
newly diagnosed	94/101
recurrent	8/101
invasive	40/101
atypical	15/101
macroadenomas	59/101
microadenomas	42/101

Committee for experimenting on human patient samples. Patient profile characterization including clinical classification of pituitary tumors is presented in Table 1. Peripheral blood was obtained from 24 healthy donors and peripheral blood mononuclear cells (PBMC) were isolated by ficoll centrifugation. Six tissue sections of normal pituitary were collected during autopsies. DNA was isolated using QIAamp DNA Mini Kit [QIAGEN] and its quality was investigated spectrophotometrically using NanoDrop 2000 [ThermoScientific]. Afterwards, DNA was stored in -20°C till the moment of analysis. RNA was isolated using RNeasy Mini Kit [QIAGEN] and rotor-stator homogenizer Omni Tissue Master [Omni International]. Quality of RNA was assessed spectrophotometrically using NanoDrop 2000 [ThermoScientific]. Isolated RNA was stored in -80°C until the moment of analysis. RNA extracted from HepG2 cells served as positive control when evaluatig *TERT* expresson level.

Telomere length assessment

Telomere length was investigated with qPCR-based technique as described previously (O'Callaghan & Fenech 2011). DNA samples were amplified in qPCR reactions using Power SYBR Green PCR Master Mix [Applied Biosystems] and PCR primer used previously (O'Callaghan & Fenech 2011). Standard curves, based on the known concentrations of human PBMC DNA as well as serial dilutions 10^{-1}–10^{-6} M of artificial synthesized oligonucleotide with telomere sequence were used for calculations of the amount of DNA single copy gene (ACTB) and telomere DNA, respectively. Telomere length was determined as relative telomere/single copy gene (T/S ratio) for each sample (O'Callaghan & Fenech 2011). PCR primers' sequences are listed in Table 2.

Assessment of TERT expression

Expression of telomerase (*TERT*) was assessed using qRT-PCR. One μg of total RNA was subjected to reverse transcription with Transcriptor First Strand cDNA Synthesis Kit [Roche]. Gene expression assays [Applied Biosystems] was used for *TERT* (assay Hs00972651_m1), and *GAPDH* (Hs99999905_m1) amplification and detection with the use of TaqMan Gene Expression Master Mix [Applied Biosystems] and Applied Biosystems 7900HT Fast Real-Time PCR [Applied Biosystems instrument]. PCR primers' sequences together with probe sequence are listed in Table 2. RNA isolated from HepG2 cell line served as positive control for telomerase expression. GAPDH has been chosen as a reference gene based on previous validation (Bujko *et al.* 2016).

Mutations of TERT promoter region

The incidence of mutations at *TERT* core promoter was assessed using Sanger sequencing technique. DNA from PA samples was PCR amplified using FastStart Taq DNA Polymerase with a provided GC rich buffer [Roche] with previously reported primers that flank the mutation hot-spot region (Wang *et al.* 2014). The PCR product was purified using ExoStar [GE Healthcare Life Sciences, Pittsburgh, PA, USA], labeled with BigDye Terminator v.3.1 [Applied Biosytems] according to the manufacturer's instructions and analyzed by capillary electrophoresis with the ABI PRISM 3300 Genetic Ana-

Tab. 2. Sequences of the oligonucleotides used for PCR amplification.

PCR assay	PCR primer/oligonucleotide	Sequence 5′→3′
Telomere length		
	forward	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT
	reverse	GGCTTGCCTTACCCTTACCCTTACCC TTACCCTTACCCT
	control oligo	CAGCAAGTGGGAAGGTGTAATCCGTCTCCACAGACAA GGCCAGGACTCGTTTGTACCCGTTGATGATAGAATGGG
***TERT* promoter mutations**		
	forward	CACCCGTCCTGCCCCTTCACCTT
	reverse	GGCTTCCCACGTGCGCAGCAGGA
***TERT* copy number variation**		
TERT region 1	forward	GTGTTCGTTGATTGTGCCAG
	reverse	CATGTGGCCCTCTTCATACC
TERT region 2	forward	TGTTCAGAGGTCTCATCTGGT
	reverse	ATGCGTACACTCAAACCGAG
TBP	forward	TGAGAAGAGTGTGCTGGAGA
	reverse	CAGGCTGTTGTTCTGATCCA
ATP5J	forward	CTGTTGGAATGTGGTGGTGA
	reverse	TCAACAGGTCCTCCAGATGT

lyzer [Applied Biosystems]. Sequencing results from both – forward and reverse primers was evaluated for each sample using MutationSurveyor V 5.0 software.

Copy number variation analysis

Copy number variation (CNV) investigation was evaluated with qPCR technique by the comparison of the amplification level of region of interest and control regions, according to previous recommendations (D'haene *et al.* 2010). Two regions of *TERT* gene locus were amplified as well as two reference regions – TBP and ATP5J. 20 ng of each DNA sample was amplified using Power SYBR Green PCR Master Mix [Applied Biosystems] and 7900HT Fast Real-Time PCR System [Applied Biosystems]. PCR primers' sequences are listed in Table 2. The standard curves based on amplification of known concentrations of human DNA from mixed PBMC samples were used for quantification. DNA from PBMC from healthy donors served as control of normal copy number (n=2). To describe CNV for each sample gene copy number ratio (GCR) was calculated using the following formula:

$$GCR = \frac{\sqrt[2]{Ct_{TERTa} + Ct_{TERTb}}}{\sqrt[2]{Ct_{TBP} + Ct_{ATP5J}}}.$$

Bayesian Information Criterion was used for tumor samples classification using R package CNVtools (Barnes *et al.* 2008) based on the results GCR values for 24 samples of normal PBMCs from healthy donors.

Evaluation of TERT promoter DNA methylation

The Infinium HumanMethylation450 BeadChip [Illumina] was used to profile whole-genome DNA methylation in 41 nonfuctioning pituitary adenoma (NFPA) samples. Laboratory procedures were performed by the AROS Applied Biotechnology service. Genomic DNA was bisulfite-converted, using the EZ-96 DNA Methylation kit [Zymo Research, Orange, CA, USA]. Probes with missing intensity signals were discarded (9541 probes out of 485, 578). Probes were divided into design classes and annotated to genome location according to the lluminaHumanMethylation450K.db library. Intensities were normalized with the BMIQ package version 1.3; (Teschendorff *et al.* 2013) using default parameters. Probe mapping to differentially methylated sites were selected according to the P-value from the t-test (Welch variant) after correction for multiple hypothesis testing with the Benjamini–Hochberg algorithm.

Statistical analysis

Quantitative continuous variables were analyzed using the two-sided Mann–Whitney U test and the Kruskal–Wallis test when more than two groups of samples were compared. Normality of the distribution was assessed using the Shapiro-Wilk test. A significance threshold level p = 0.05 was used. Data were analyzed and visualized using GraphPad Prism (GraphPad Software).

RESULTS

Telomere length assessment

Variable telomere length was found among PA patients (median T/S ratio of 1,32; SD 0.78). ACTH-secreting tumors showed slightly higher telomere length, however, no significant difference was observed between particular pituitary subtypes (p=0.096) (Figure 1A). Notably, only two ACTH-secreting tumors were included. Similarly, we did not observe any significant relationship between telomere length and clinicopathological features: tumor size, invasive growth, Ki-67 labeling index.

Gene expression

The expression of *TERT* mRNA was under detection level in qRT-PCR in majority of pituitary adenoma patients while it was clearly detected in cDNA HepG2 cell line which served as positive control. PCR amplification with primers and probe specific to *TERT* mRNA was observed only in 5 PA samples including 2 somatotrophic adenomas, 2 NFPAs and 1 lactotrophic adenoma. PCR amplification for *GAPDH* reference gene was obtained for all the samples with median Ct value of 23,7. Good quality of the RNA samples was previ-

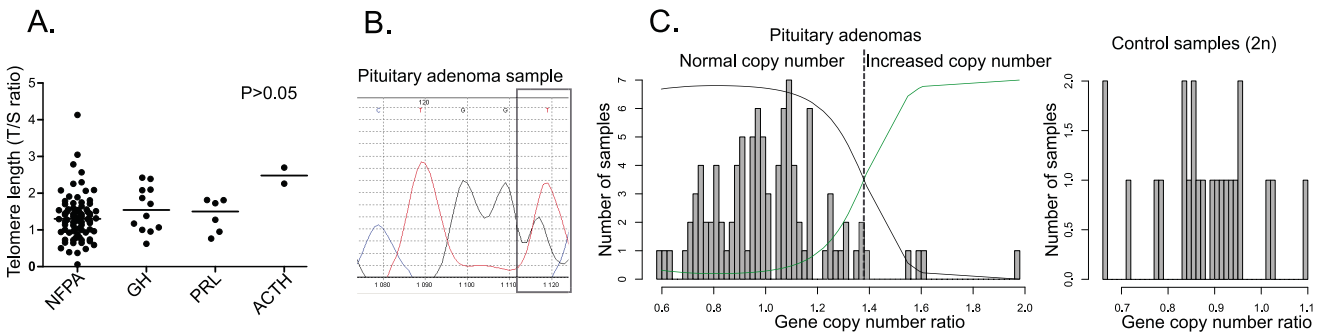

Fig. 1. Telomere length and TERT abnormalities in PAs. A) The comparison of telomere length in pituitary adenomas. B) The results of Sanger sequencing, of gene promoter region, the only sample with identified sequence variation. C) Copy number classifier based on series of assessment of normal diploid DNA. NFPA: nonfunctioning adenomas, GH: GH-secreting adenomas, PRL: PRL-secreting adenomas, ACTH: ACTH-secreting adenomas. Horizontal lines denote the mean value.

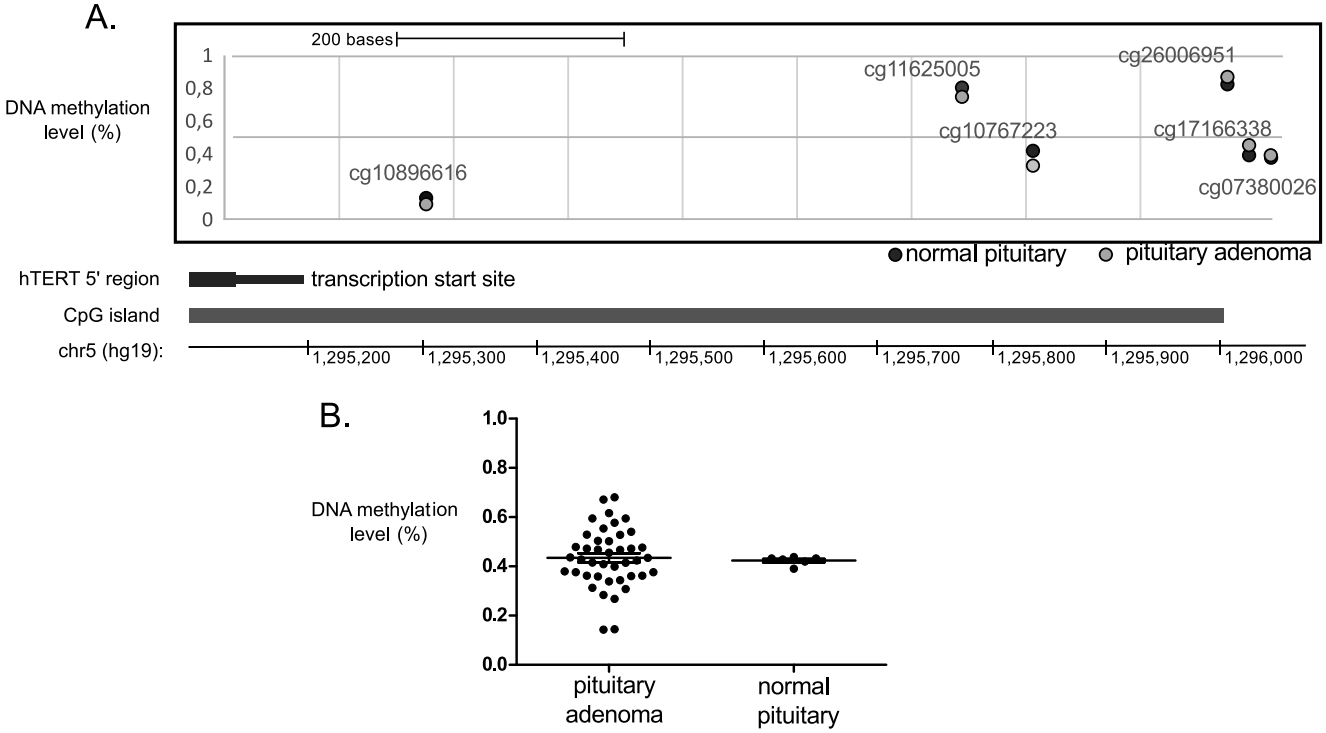

Fig. 2. A) DNA methylation level at TERT prmoter region measured by the HumanMethylation 450K array (Illumina) in PAs and normal pituitary Each blue/red dot represents an individual CpG. B) An avearage promoter DNA methylation levels in PAs and normal sections. (HumanMethylation 450K microarray data). Each dot represents individual sample and error bars represent standard deviation. Horizontal lines denote the mean value.

ously assessed with 2100 Bioanalyzer [Agilent Genomics] (Bujko *et al.* 2016).

TERT promoter mutations and copy number abnormalities

Sequencing of *TERT* promoter region revealed one single nucleotide substitution C>A (chr5:1,295,217; hg19) in one patient only (Figure 3A). Disappointingly, this variant does not represent the previously reported recurrent hotspot mutations (Bell *et al.* 2016).

The analysis of *TERT* copy number locus showed normal copy number in most PA samples. The increased copy number was identified in 6 tumor samples (5,6%), according to CNVtools algorithm (Barnes *et al.* 2008). Two of these 6 samples only slightly crossed the threshold value. Decreased gene copy number was not observed in any of PAs. (Figure 3B). We did not observe the correlation between telomere length and GCR determined in qPCR CNV assay or the difference in telomere length between patients with normal and increased *TERT* copy number.

DNA methylation analysis

The results of genome-wide DNA methylation profiling of 41 PA samples and 6 samples from normal pituitary with HM450 arrays [Illumina] were utilized. HM450 array covers 98 CpG sites at *TERT* locus, including 5 CpGs at gene promoter. The comparison of PA and normal samples did not reveal significant differences at any of the *TERT*-related CpGs, neither in gene body nor promoter regions. Heterogeneous DNA methylation pattern of *TERT* promoter was observed with low methylation near transcription start site and generally variable methylation level in promoter region (Fig. 2A). The average promoter DNA methylation in normal pituitary samples was 42,3%, whereas similar methylation level of 43,4% was observed in tumors. Some variety of promoter methylation in PA was found, ranging from 14,2% to 68%. (Figure 2B). Nevertheless, we did not observe any correlation between promoter methylation level and telomere length. Patients were stratified based on the results of normal pituitary testing as hypermethylated, normal and hypomethylated but we did not observe any significant relationship with DNA methylation status and clinicopathological features: PA subtype, invasiveness or Ki-67 labelling index.

DISCUSSION

Our results indicate that telomerase abnormalities do not play any special role in pathogenesis of pituitary tumors.

The measurement of telomere length can be easily achieved by qPCR but the interpretation of the results should be done cautiously. The length of telomeres depends on many different features: tissue type, the age of the patient, genetic background and lifestyle (Soerensen *et al.* 2011; Freitas-Simoes *et al.* 2016; Bojesen

2013). Therefore, the appropriate experimental design would be the comparison of the telomere length in tumor and normal tissue from the same individuals. Such matched samples would be hardly available when investigating tumors of pituitary gland. For this reason we focused on the relationship of telomere length in PA samples and the incidence of abnormalities of *TERT* gene, *TERT* expression and clinicopathological features. Unfortunately, no relationships were found.

This observation is consistent with recently published data where similar telomere length in PAs and samples of age adjusted normal pituitary from autopsies were observed (Lv *et al.* 2016).

The observed dispersion of relative telomere length in PA tumor samples is comparable to the results obtained for PBMCs from healthy donors (not shown) and also similar to the qPCR results of obtained previously in normal pituitary (Martins *et al.* 2015) and other normal control tissues (Lv *et al.* 2016). This suggests that the differences in telomere length between individual PA patients represent the diversity that is normally observed in humans and should not be linked to pituitary adenoma pathogenesis. Telomere length was reported to be shortened in PAs in one previous study but pituitary sections were compared to normal brain samples, which does not seem to be suitable according to the current knowledge (Hiraga *et al.* 1998).

TERT mRNA expression appears to be very low in PAs as it was undetectable in most of tumor samples. This result is in line with previous reports where telomerase mRNA and protein expression was undetectable or detected only in minor part of samples (Ortiz-Plata *et al.* 2007; Martins *et al.* 2015; Harada *et al.* 2000; Yoshino *et al.* 2003).

Single nucleotide DNA sequence variant in *TERT* promoter region was observed only in one PA sample. This variant is not reported as known single nucleotide polymorphism in SNPdb what may suggest its somatic nature. However, the acquired/ hereditary nature of this variant could not be assessed as reference DNA sample from this patient was not available. Nevertheless, the identified variant does not represent the previously reported recurrent *TERT* promoter mutations, which were validated as functionally relevant tumor drivers. It's rather more probable that it is a so-called "passenger" mutation that does not play a role in tumorigenic potential. Gene amplification does not seem to play a role. It was observed in very minor number of patients and was related neither to *TERT* expression nor telomere length.

The recently published data suggest that methylation of CpG sites at *TERT* promoter is clinically relevant in PA. In this study DNA methylation was assessed in a series of PA patients with the use of qualitative MSP method that allows for the stratification of the samples as methylation negative and positive (Kochling *et al.* 2016). Recently, we completed the result of genome-wide DNA methylation profiling of PA adenomas and normal pituitary sections. The analysis enrolled 42 PA patients that were also subjected to the assessment of telomere length and *TERT* abnormalities. HumanMethylation450 [Illumina] array which was used covers 92 individual CpGs at *TERT* locus. None of this CpGs revealed significantly aberrant DNA methylation level when PA and normal samples were compared. Importantly, DNA methylation of approximately 50% was identified in *TERT* promoter in normal pituitary sections. This implies that some degree of DNA methylation at this genomic region is normal in pituitary tissue and this methylation level generally is not altered in pituitary tumors. For this reason the qualitative methods like MSP, used in previous study, are probably not suitable for DNA methylation analysis at this locus. However, some associations with important clinical features were observed in the mentioned report (Kochling *et al.* 2016). We did not find any significant relationships between methylation levels and clinical features neither when DNA methylation was analyzed as continuous variable nor when it was converted to categorical data: low, normal and high. Moreover, no relationship between promoter methylation and *TERT* expression was found. Our data clearly do not support the role of DNA methylation in pathogenesis of pituitary tumors.

Adenomas of pituitary gland are generally slow-growing, benign tumors with relatively low mitotic activity. Unaffected telomere biology reflects this benign nature of PAs. Our results confirm that telomerase is not activated in PAs as it is in case of many other tumor types. Telomere length testing as well as screening for genetic/epigenetic abnormalities of telomerase gene does not seem to be useful in clinics.

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