Research Paper

Accelerated telomere shortening independent of LRRK2 variants in Chinese patients with Parkinson's disease

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Keywords: Parkinson's disease, telomere length, aging, LRRK2 variants
Received: May 12, 2020 **Accepted:** July 25, 2020 **Published:** October 29, 2020

ABSTRACT

Oxidative stress and inflammation play vital roles in Parkinson's disease (PD) development. Thus, telomere length is expected to be shortened in this disease, but current data are inconclusive. We performed a case-control study of 261 patients with PD and 270 sex and age-matched healthy controls treated at the Peking Union Medical College Hospital. We found leucocyte telomere length (LTL) was significantly shortened in PD as compared with controls [1.02 (0.84-1.39) *vs*. 1.48 (1.08-1.94), P<0.001] and shorter LTL was associated with a dramatically increased risk of PD (lowest *vs*. highest quartile odds ratio (OR) =9.54, 95% CI: 5.33-17.06, P<0.001). We also investigated the roles of six LRRK2 variants in the susceptibility to PD. R1441C/G/H, G2019S, and I2020T variations were not detected in our study. No significant differences were found in the presence of variants R1398H (15.4% *vs*. 17.0%, P=0.619) and R1628P (2.3% *vs*. 0.7%, P=0.159) in PD and controls, while the G2385R variant was found to be a risk factor associated with increased PD susceptibility (OR=2.14, 95% CI: 1.12-4.10, P=0.021). No significant association was found between different LRRK2 variants and telomere length. These findings suggest that shorter LTL might be associated with PD in a manner independent of LRRK2 variants.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease affecting about 2% of the population aged over 60 years [1, 2]. A meta-analysis of available worldwide data showed a rising prevalence of PD with aging [3]. To face the social and economic burden along with the increasing number of PD patients, uncovering PD genetic biology and developing neuroprotective interventions are essential.

Telomeres are the repeated sequences that protect the ends of chromosomes and avoid cellular senescence and apoptosis induced by genomic instability [4]. The shortening of telomere length is regarded as an indicator of cellular aging, which is accelerated by oxidative stress and inflammation [5]. Mitochondrial dysfunction produces reactive oxygen species that can lead to oxidative damage, contributing to telomere shortening [6]. Many lines of evidence suggest that mitochondrial dysfunction plays a central role in the pathogenesis of PD [7]. In this pathological condition, telomere erosion may be accelerated. However, data on telomere shortening in Parkinson's disease are inconsistent among various studies.

Leucine-rich repeat kinase 2 (LRRK2) is a multifunctional protein implicated in the regulation of various cellular functions [8]. Variants in LRRK2 have been identified as the most common candidate gene

linked to both familial and sporadic Parkinson's disease (PD) [9]. Several LRRK2 variants have been reported that affect the risk of PD, but data in Chinese patients are not always consistent. G2019S is the most common LRRK2 mutation with a high incidence in North African Arabic (39%) and Ashkenazi Jewish patients with PD (18.3%) [10, 11]. R1441C/G/H, three variations of the same codon, have been detected in several populations [12]. I2020T is identified in Japanese PD families, and is associated with increased kinase activity [13, 14]. G2385R and R1628P are two variants that are prevalent in Asian populations, also correlated with an increased risk of PD [15, 16]. However, the LRRK2 R1398H polymorphism is associated with a decreased risk of PD in a Han Chinese population [17]. Furthermore, LRRK2 variants are known to be associated with mitochondrial dysfunction. A single mutation in LRRK2 results in increased susceptibility to oxidative stressors, even though the mechanism behind it is not well understood [18].

We conducted this case-control study to investigate differences in the telomere length in a Chinese population. We also investigated the prevalence of three well-known pathogenic variants (R1441C/G/H, G2019S, I2020T) and three Asian-prevalent (R1398H, G2385R, R1628P) variants and assessed their roles in the susceptibility to PD. Furthermore, we analyzed the possible relationship between telomere length and LRRK2 variants.

RESULTS

General characteristics of subjects

Table 1 depicts the baseline characteristics of all participants. Significant shorter leukocyte telomere lengths (LTLs) were found in PD patients when compared with controls [1.02 (0.84-1.39) *vs*. 1.48 (1.08-1.94), P<0.001]. Moreover, the PD group had significantly lower levels of total protein (TP), albumin(Alb), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), higher level of homocysteine (Hcy). Telomere length was negatively correlated with age in controls, with a shorter LTL at higher age (r=-0.507, P<0.001), a relationship we did not find in the PD group (r=-0.073, P=0.239) (Figure 1). LTL did not differ significantly between males and females in any of the groups (data not shown).

Short LTL increases the risk of PD

Using age and sex-adjusted logistic regression analyses, we investigated the relationship between PD and biochemical parameters which were significantly different between the groups, as shown in Table 1. Univariate logistic regression showed that higher TP, Alb, TC, HDL-C, and LDL-C were associated with a decreased risk of PD (Table 2). For LTL, we divided the patients into four groups based on the quartiles. A shorter LTL was associated with an increased risk of PD (lowest *vs*. highest quartile odds ratio (OR) =9.54, 95% CI: 5.33-17.06, P<0.001; P-value for the trend over quartiles: <0.001, Table 3). The results became more pronounced after multivariable-adjustment accounting for TP, Alb, TC, and HDL-C (lowest *vs*. highest quartile OR=75.23, 95% CI: 22.65-249.90, P<0.001; P-value for the trend over quartiles: <0.001, Table 3). Considering the high correlation between TC and LDL-C, LDL-C was not included in the multi-adjusted model.

Detecting LRRK2 variants in PD

Among six Asian-prevalent LRRK2 variants, the R1441C/G/H, G2019S, and I2020T variations were not detected in our study, indicating they may not be common pathogenic SNPs in the Chinese population. PD patients carried a higher frequency of variant G2385R than control subjects (11.2% *vs*. 5.5%; AA+AG *vs*. GG OR=2.14, 95% CI 1.12-4.10, P=0.021; A *vs*. G OR=2.05, 95% CI 1.11-3.82, P=0.023). However, no significant differences were found in the prevalence of variant R1398H (15.4% *vs*. 17.0%, P=0.619) and R1628P (2.3% *vs*. 0.7%, P=0.159) in patients with PD and healthy controls. The detailed information of this analysis is displayed in Table 4.

LRRK2 variants and telomere length

To investigate whether LRRK2 variants affect telomere length, we divided PD patients into four groups: R1398H-positive, G2385R-positive, R1628P-positive, and triple-negative (indicating none of the three SNPs were positive). We excluded a patient carrying both R1398H and R1628P variants from further analysis. No significant differences were found among the four groups in age, sex, and LTL (Figure 2). The detailed information of this analysis is shown in Table 5.

DISCUSSION

Our study found that patients with PD displayed shortened LTL, and shorter LTL increased the risk of PD dramatically. This was the first study investigating the relationship between telomere length and PD in Chinese patients. We also analyzed the six LRRK2 variants in PD. We demonstrated that G2385R is a risk factor associated with increased PD susceptibility in Chinese patients. However, we did not find any evidence that R1398H is a protective factor for PD, as other studies have reported. Furthermore, no significant

Table 1. Clinical characteristics of patients with PD and control subjects.

Clinical characteristics	Control subjects (n=270)	PD patients (n=261)	P value
Age (years)	65 (55-74)	63 (54-74)	0.461
Male	148 (54.8%)	134 (51.3%)	0.423
LTL (T/S ratio)	1.48 (1.08-1.94)	1.02 (0.84-1.39)	**<0.001**
TP	72 (70-75)	71 (67-74)	**<0.001**
Alb	45 (44-46)	43 (41-46)	**<0.001**
TC (mmol/L)	4.78 ± 0.90	4.40 ± 1.02	**<0.001**
TG (mmol/L)	1.21 (0.87-1.56)	1.07 (0.75-1.45)	**0.02**
HDL-C (mmol/L)	1.35 (1.11-1.59)	1.17 (1.02-1.44)	**<0.001**
LDL-C (mmol/L)	3.13 ± 0.85	2.47 ± 0.79	**<0.001**
FBG (mmol/L)	5.3 (4.9-5.9)	5.4 (5.0-5.9)	0.182
hs-CRP (ng/mL)	0.67 (0.35-1.15)	0.79 (0.42-1.77)	0.277
Hcy (μmol/l)	13.5 (11.3-15.3)	15.3 (13.3-19.9)	**0.002**

Abbreviations: PD, Parkinson's disease; LTL (T/S ratio), leukocyte telomere length (ratio of telomere repeat copy number (T) to single-copy gene copy number (S)); TP, Total protein; TC, Total cholesterol; TG, Triglyceride; HDL-C, High density lipoprotein cholesterol; LDL-C, Low density lipoprotein cholesterol; FBG, fasting blood glucose; hs-CRP, high-sensitivity C-reactive protein; Hcy, homocysteine.

Data are presented as n (%), mean ± standard deviation (SD), median (interquartile range). Significant P-values (P<0.05) are indicated in bold print.

relationship was found between LRRK2 variants and telomere length.

Both environmental factors and genetic predisposition affect LTL. The average telomere lengths of peripheral leukocytes are reported to become shorter with aging [19–21], while Hudson et al. [22] showed no correlation between telomere length and age in both PD cases and controls. In our study, a strong correlation was found between age and telomere shortening in all participants and controls, but not in the PD group. This may reflect other mechanisms beyond age that may be involved in telomere regulation, or some confounders such as mutation status, disease duration, and other biological markers in patients with PD may affect this relationship.

The shortening of telomeres is accelerated in various diseases characterized by oxidative stress and inflammation [23, 24]. Studies on telomere length in patients with PD were inconclusive. A meta-analysis,

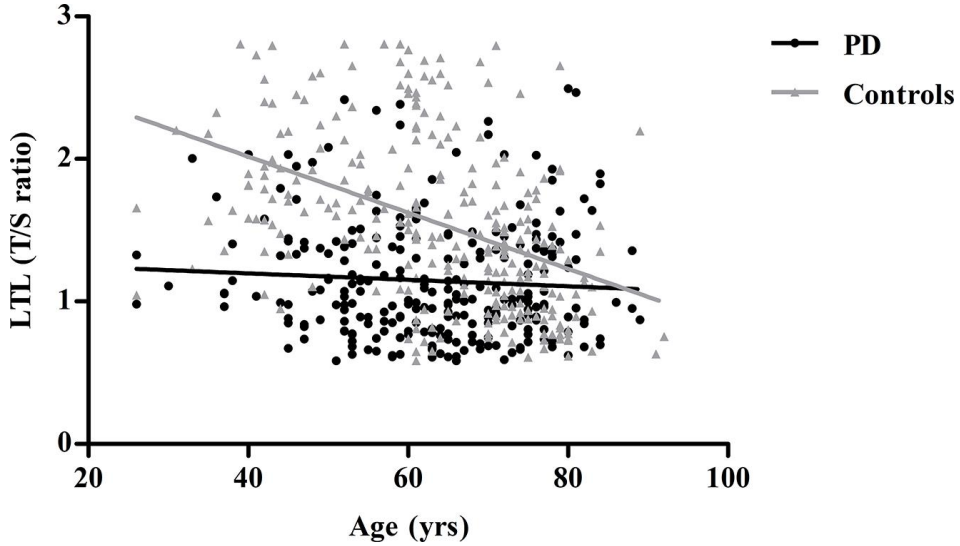

Figure 1. Linear regression analysis of the association between leukocyte telomere length (LTL) and age in controls and PD patients. Controls are shown as gray triangles (n=270) and PD patients as black dots (n=261).

Table 2. Logistic regression analyses of the association between clinical characteristics and PD adjusted for age and sex in all participants.

Clinical characteristics	Age and sex adjusted	
	OR (95% CI)	P
TG (mmol/L)	0.701 (0.484,1.016)	0.061
TP	0.924 (0.882,0.968)	**0.001**
Alb	0.842 (0.778,0.910)	**<0.001**
TC (mmol/L)	0.614 (0.480,0.787)	**<0.001**
HDL-C (mmol/L)	0.134 (0.062,0.293)	**<0.001**
LDL-C (mmol/L)	0.363 (0.268,0.492)	**<0.001**
Hcy (μmol/l)	1.021 (0.988,1.055)	0.21

Abbreviations: PD, Parkinson's disease; TG, Triglyceride; TP, Total protein; Alb, Albumin; TC, Total cholesterol; HDL-C, High density lipoprotein cholesterol; LDL-C, Low density lipoprotein cholesterol; Hcy, homocysteine.
P-values and their ORs for the clinical parameters and PD were calculated by univariate logistic regression analysis. Significant P-values (P<0.05) are indicated in bold print.

Table 3. Logistic regression analyses of the association between LTL and PD in all participants.

Clinical characteristics	Age and sex-adjusted model			Multivariable-adjusted model[a]		
	OR (95% CI)	P	P_{trend}	OR (95% CI)	P	P_{trend}
LTL (T/S ratio)						
Q1	9.54 (5.33,17.06)	**<0.001**		75.23 (22.65,249.90)	**<0.001**	
Q2	5.53 (3.18,9.62)	**<0.001**		5.61 (2.44,12.91)	**<0.001**	
Q3	3.26 (1.89,5.64)	**<0.001**		3.40 (1.57,7.35)	**0.002**	
Q4	Reference	-	**<0.001**	Reference	-	**<0.001**

Abbreviations: PD, Parkinson's disease; LTL (T/S ratio), ratio of telomere repeat copy number (T) to single-copy gene copy number (S).
[a] Adjusted for age, sex, total protein, albumin, total cholesterol, high density lipoprotein cholesterol. Significant P-values (P<0.05) are indicated in bold print.

Table 4. Genotype and allele distribution of Asian-prevalent variants and the association with Parkinson's disease.

Genetic variants	Genotypes	Dominant model				Alleles	Allele model			
		Patients n=260	Controls n=271	OR (95% CI)	P value		Patients n=520	Controls n=542	OR (95% CI)	P value
R1398H	AA	1	2			A	41	48	0.88 (0.57-1.36)	0.568
	AG	39	44	0.89 (0.56-1.41)	0.619	G	479	494		
	GG	220	225			-	-	-	-	-
G2385R	AA	1	1			A	30	16	2.05 (1.11-3.82)	**0.023**
	AG	28	14	2.14 (1.12-4.10)	**0.021**	G	490	526		
	GG	231	256			-	-	-	-	-
R1628P	CC	0	0			C	6	2	3.15 (0.63-15.69)	0.161
	CG	6	2	3.17 (0.64-15.89)	0.159	G	514	540		
	GG	254	269			-	-	-	-	-

including eight primary studies, did not find consistent evidence of shorter telomeres comparing 959 patients and 1,284 controls. However, in our study, a significant shorter telomere length was found in patients with PD, and shorter LTL significantly increased the risk of PD. The discrepancy between different studies may be explained by variations in the study design, ethnicity, sample size, and age of the participants. Experimental data have shown that mice with short telomeres are characterized by a declined motor performance and an increased formation of α-synuclein aggregates that accelerate the progress of PD [25]. Additionally, CRISPR-Cas9-mediated telomere removal leads to mitochondrial dysfunction and PD-associated protein aggregation [26]. Thus, telomere shortening resulting from the inability to fully replicate the ends of linear chromosomes might also contribute to PD pathology. Another explanation is the decreased canonical Wnt signaling during PD development [27, 28]. Telomere protection is enhanced by the upregulation of Wnt/β-Catenin signaling [29, 30]. β-Catenin regulates Tert expression, thereby maintaining telomere length. Telomeres are also protected by the Wnt/β-catenin signaling pathway, which maintains TRF2 levels [29].

Multiple studies investigated the role of LRRK2 in the etiology of PD. LRRK2 has been implicated in mitochondrial dysfunction, Wnt signaling transduction, and protein translation control [31]. Over a hundred variants of the LRRK2 gene have been reported to date. Of these, G2019S, R1628P, G2385R, and R1398H have received much attention [32]. In our study, three pathogenic variants, R1441C/G/H, G2019S, and I2020T, which are common in western populations, were not identified. R1628P is a variant usually found in Asians, and although we detected it in some participants, no significant association was found with PD risk. A meta-analysis showed that east Asian individuals who harbored the R1398H variant had a 20% reduced risk of PD [33]. Our data found a similar but not significant trend, which may be due to the limited sample size. G2385R roughly doubled the risk of PD, which is consistent with other studies [33–35].

Considering the distinctive effects of these variants, we further compared the telomere length among the PD patients with different variants. Berwick et al. reported G2385R weakened Wnt signaling, while R1398H produced the opposite result [36]. This was also supported by a study by Jonathon et al. [37], who found that G2385R and R1398H play opposite roles regarding the effect of Wnt signaling. Thus, the idea that different variants may result in changes in telomere length is an attractive hypothesis. However, our data did not support this assumption. The limited sample size in our study may account for the negative result if the effect is weak. More extensive cohort studies and experimental data are needed to clarify the relationship between different LRRK2 variants and telomere length.

In conclusion, this is the first study investigating the relationship between telomere shortening and LRRK2 variants in patients with PD. Our findings indicate that a shorter LTL is associated with a dramatically increased risk of PD. G2385R is a risk factor associated with increased PD susceptibility in a Chinese population. No association was found between different LRRK2 variants and telomere length. These findings suggest that shorter LTL might be associated with PD in a manner independent of LRRK2 variants.

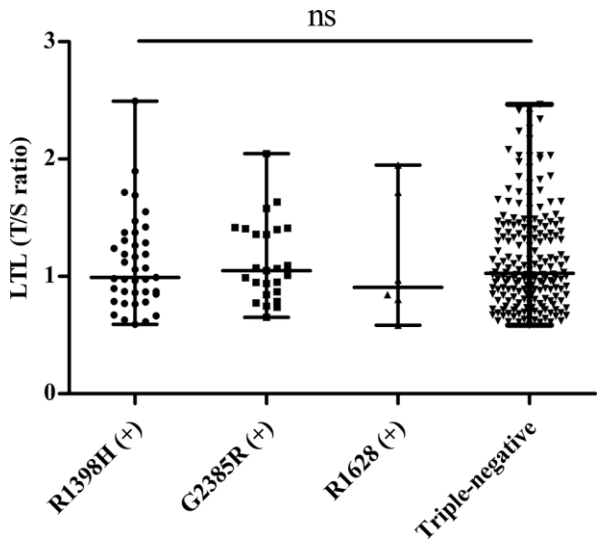

Figure 2. Distribution of telomere length in PD patients with different LRRK2 variants. "ns" means "not significant".

Table 5. Clinical characteristics of PD patients with different LRRK2 variants.

Clinical characteristics	G2385R- positive (n=25)	R1398H- positive (n=39)	R1628P- positive (n=6)	Triple-negative (n=190)	P value
Age (years)	68 (63-77)	63 (52-74)	57 (46-69)	64 (54-73)	0.196
Male	18 (46.2%)	15 (60.0%)	4 (66.7%)	91 (47.9%)	0.574
LTL (T/S ratio)	1.05 (0.86-1.40)	0.99 (0.85-1.31)	0.91 (0.75-1.77)	1.03 (0.83-1.42)	0.943

Abbreviations: PD, Parkinson's disease; LTL (T/S ratio), ratio of telomere repeat copy number (T) to single-copy gene copy number (S).
Data are presented as n (%), median (interquartile range).

MATERIALS AND METHODS

Study design

We randomly recruited 261 PD patients (127 women, 134 men) from the Peking Union Medical College Hospital, China. All patients were diagnosed according to the UK PD Society Brain Bank criteria for clinical PD [38]. We recruited 270 sex and age-matched healthy controls (122 women, 148 men) who were visiting the hospital for a general health examination and did not have any neurological disorders. Participants with cancer, cardiovascular diseases, diabetes, hypertension, stroke, and current infections (defined as having a serum high-sensitivity C-reactive protein (hs-CRP) value of >10 mg/L) were excluded. All the participants were from the Chinese Han population. The study was approved by the Peking Union Medical College Hospital Ethics Committee and conformed to the Declaration of Helsinki principles. The requirement for written informed consent was waived by the institutional review board.

Measurement of telomere length

Circulating leukocytes were collected, and DNA was extracted using the TIANamp Genomic DNA kit (Beijing). Telomere length was measured by a quantitative PCR method described by Cawthon et al. [39], which is based on the ratio of the telomere repeat copy number (T) to the single-copy gene copy number (S) expressed as the telomere length ratio (T/S ratio). The primers for telomere sequences used were: forward 5′-GGTTTTTGAGGGTGAGGG TGAGGGTGAGGG TGAGGGT-3′ and reverse 5′-TCCCGACTATCCCT ATCCCTATCCCTATCCCTATCCCTA-3′. We used 36B4 as a single-copy reference gene, and the primers for that were: forward 5′-CAGCAAGT GGGAAGGTG TAATCC-3′ and reverse 5′-CCCATTCTATCATCAA CGGGTACAA-3′. T/S ratios were expressed as LTL and were determined using the formula $T/S = 2^{-\Delta Ct}$, where ΔCt = average $Ct_{Telomere}$ - average Ct_{36B4}. 293T cells were used for reference DNA samples, and we measured telomere length ratios by using a dilution series from 1.56 to 100.00 ng (2-fold dilution; 7 points) [40]. All samples were analyzed on the LightCycler 480 (Roche, Switzerland) and measured in triplicate.

Clinical laboratory tests

Biochemical variables, including serum levels of TC, TG, HDL-C, and LDL-C were measured on a Beckman AU Series Automatic Biochemical Analyzer (Japan), using Sekisui Medical (Japan) reagents. Fasting blood glucose (FBG), high-sensitivity C-reactive protein (hs-CRP), TP, and Alb were measured with the same instrument, using Beckman AU reagents, and Hcy was examined using Beijing Leadman reagents.

Genotyping

DNA samples were extracted from peripheral blood and amplified for sequence analysis. Three well-known pathogenic variants (R1441C/G/H, G2019S, I2020T) and three Asian-prevalent (R1398H, G2385R, R1628P) variants were genotyped to assess their roles in the susceptibility to PD. We applied sequence analysis to identify the six LRRK2 variants. The primers used for this method are shown in Supplementary Table 1.

Statistics analysis

Continuous variables with a normal distribution were described as the mean ± standard deviation (SD) and analyzed by the unpaired t-test. In contrast, variables with a non-normal distribution are provided as the median (interquartile range) and were compared by the Mann-Whitney U or Kruskal Wallis Test. Sex was analyzed as percentages in the PD and control groups compared with the chi-square test. Linear regression was used to analyze the relationship between age and LTL. We used logistic regression to determine the risk of PD associated with each factor. The chi-square test was performed to compare the frequency distribution of genotypes and alleles, and the Hardy-Weinberg equilibrium was verified. Statistical significance was

assumed at $P < 0.05$, and all analyses were conducted using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

AUTHOR CONTRIBUTIONS

W.Y., Y.Z. and C.W. conceived and designed the project. P.Y. collected all the clinical blood, W.Y. and P.Y. acquired research data. Y.Z., L.K. and L.X. analyzed all the research data. W.Y. wrote the manuscript. C.W., P.Y. and Y.Z. contributed to helpful discussion and reviewed the manuscript.

ACKNOWLEDGMENTS

We are indebted to all volunteers (patients and controls) who participated in the present study.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FUNDING

This work was supported by grants from the CAMS Innovation Fund for Medical Sciences (CIFMS) [Grant No. 2017-I2M-3-005].

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SUPPLEMENTARY MATERIALS

Supplementary Table

Supplementary Table 1. Primers for LRRK2 variants sequencing.

Names	Primer sequences 5'-3'
LRRK2-1628F	CTTCTAGGCCACATGGTTG
LRRK2-1628R	TCCTATTGGCAAAGCAATCT
LRRK2-2385F	AGCCCTGTTGTGGAAGTGT
LRRK2-2385R	AGAGGCAGAAAGGAAGAA
LRRK2-1398F	TAGGTACTTTGATCGGTTGCTGAC
LRRK2-1398R	GACTTCATTACTCGGAAAGTTTCCC
LRRK2-1441F	TCAACAGGAATGTGAGCAGG
LRRK2-1441R	CCCACAATTTTAAGTGAGTTGC
LRRK2-2019F	GATTTCCTGTGCATTTTCTGG
LRRK2-2019R	ACCTACCTGGTGTGCCCTCT
LRRK2-2020F	CAGATACCTCCACTCAGCC
LRRK2-2020R	TTTGACTCTTCTGAACTCACATC