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The testis-specific apoptosis related gene *TTL.6* underwent adaptive evolution in the lineage leading to humans☆

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Abstract

The *TTL.6* gene is a member of the tubulin-tyrosine ligase (TTL) family involved in apoptosis and preferentially expressed in the testis. We sequenced the coding region and part of the introns of *TTL.6* in world wide human populations and five representative nonhuman primate species covering great apes, lesser ape and Old World monkey. The sequence substitution patterns of *TTL.6* in primates demonstrated a sharp difference in evolutionary rates among different primate lineages. Our results indicated an accelerated evolution of *TTL.6* in the human lineage, which was caused by Darwinian positive selection. Further analysis on sequence variations in human populations demonstrated an excess of derived common alleles, which was likely caused by genetic hitchhiking, an implication of recent positive selection on *TTL.6* in human populations.

Keywords: Positive selection; Sexual selection; Human evolution; Male reproductive genes; Tubulin-tyrosine ligase

1. Introduction

TTL (tubulin-tyrosine ligase) is a gene family involved in apoptosis. It works together with carboxypeptidase, and acts in the reversible detyrosination/tyrosination modification of the α tubulin. It catalyses the ATP-dependent post-translational addition of a tyrosine to the carboxyl terminal of the α tubulin, where translational tyrosine was detyrosinated by carboxypeptidase (Argarana et al., 1977; Barra et al., 1973a,b, 1988, 1972). It was suggested that TTL can irreversibly incorporate nitrotyrosine onto α tubulin which may lead to disruption of the microtubule network, ultimately resulting in apoptosis (Eiserich et al., 1999). Hence, TTL is an important player in the apoptosis process (Idriss, 2004) and was also shown to be involved in cancers (Banerjee, 2002; Idriss, 2001; Lafanechere et al., 1998; Mialhe et al., 2001).

TTL.6, also known as *sarslee* is a member of the TTL family. *TTL.6* is located on human chromosome 17q21.32, spanning about 55 kb with 16 exons. It contains the TTL domain which is conserved in the TTL family. There are five potential alternative splice forms for *TTL.6* (Ota et al., 2004; Strausberg et al., 2002). According to the NCBI EST database (http://www.ncbi.nlm.nih.gov/UniGene/), *TTL.6* is preferentially expressed in testis, and low expressions were also detected in stomach and brain. Since both testis and germ cells are subject to spontaneous or regulatory stimuli initiated cell apoptosis which are involved in sexual organ differentiation, seasonal reproduction, spermatogenesis, and male fertility, *TTL.6* might be involved in these processes (Dohle et al., 2003; McClusky, 2005; Sawhney et al., 2005; Sinha Hikim et al., 2003; Spierings et al., 2003; Xavier and Allard, 2003).

It has been shown that sexual selection plays a crucial role in driving the rapid evolution of male reproductive genes in primates (Dorus et al., 2004; Wang and Zhang, 2004; Wyckoff et al., 2000). In this study, we intend to delineate the molecular evolution of *TTL.6* in humans and nonhuman

Abbreviations: TTL, tubulin tyrosine ligase.

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primates and test whether selection acted on the evolution of *TTL.6* in primates. Our results showed that the human lineage underwent rapid evolution due to Darwinian positive selection.

2. Materials and methods

2.1. Samples

We sampled a total of 35 human individuals (70 chromosomes) from the major continental populations, including 10 Africans, 10 Europeans, 11 East Asians and 4 Melanesians. We also sampled five representative nonhuman primate species reflecting a 25 million-year history of primate evolution (Goodman et al., 1998). The nonhuman primates studied include three great ape species (three chimpanzees, *Pan troglodytes*; one gorilla, *Gorilla gorilla* and one orangutan, *Pango pygmaeus*), one lesser ape species (white-browed gibbon, *Hylobates hoolock*), and one Old World monkey species (rhesus monkey, *Macaca mulatta).* We sequenced the last 8 exons of *TTL.6* which encode one of the five potential splice forms of *TTL.6* which was expressed in testis (http://www.ncbi.nlm.nih.gov/UniGene/). We also sequenced a 289 bp intron fragment in the human and chimpanzee samples (the intron region between exon 6 and exon 7). All the DNA samples were from collections in Kunming Cell Bank of CAS. The primers used for PCR and sequencing were shown in Table 1.

2.2. Data analysis

The sequences were aligned and edited with DNASTAR (DNASTAR, Inc.), then checked manually. The putative haplotypes for the human and chimpanzee sequences were generated using PHASE (Stephens et al., 2001). Based on the phylogenetic relationship of primates by Goodman et al. (1998), we inferred the ancestral sequences of the internal nodes using PAML (Yang, 1997; Yang et al., 1995). We used the Pamilo–Bianchi–Li's method (Li, 1993; Pamilo and Bianchi, 1993) in MEGA2 (Kumar et al., 2001) and Yang–Nielsen's method (Yang and Nielsen, 2000) in PAML (Yang, 1997; Yang et al., 1995) in estimating the synonymous and nonsynonymous substitution rates (Ks and Ka). The *Z* test and Fisher's exact test were employed for the statistical inference of whether the Ka/Ks ratios (denoted as ω in Yang–Nielsen's method) are deviated from the neutral expectation (Ka/Ks=1 or $\omega=1$). The likelihood ratio test in the Codeml package of PAML was used to detect rate variations among different evolutionary lineages (Yang, 1998). The individual codon-based substitution pattern was also analyzed with the Codeml package in PAML program with continuous ω distribution (M11) to identify positively selected amino acid sites (Yang, 1997; Yang et al., 2000). The DnaSP3.99 program (Rozas and Rozas, 1999) was used to analyze within species polymorphisms and between species divergence, and a sliding window based analysis was conducted to reveal the Ka/Ks ratio distribution across the gene coding region (window length, 100 codons; step size, 1 codon). DnaSP3.99 was also used to conduct neutrality tests, including the Tajima's *D* test (Tajima, 1989) and the MacDonald–Kreitman's (M–K) test (McDonald and Kreitman, 1991). The Fay and Wu's *H* test and Fu's Fs test were performed using the Neutrality Test program (Fay and Wu, 2000; Fu, 1997).

Table 1
The primer sequences in this study

Exon	Primer sequence
Exon1F	5′>CGTGGCACTCTGGGAGGTCTA<3′
Exon1R	5′>GAAGTACAGTGGGGTGAGAGGGA<3′
Exon2F	5′>AAGGCGTTCATCCTCGCTG<3′
Exon2R	5′>CGGAATCCTTGAAGCGGG<3′
Exon3F	5′>CACTGTGGCTCTAGTCCAGATGC<3′
Exon3R	5′>GCCCAGGACAGATCATTCTTATTG<3′
Exon4F	5′>GACGACCTCCAGCTTGGACATA<3′
Exon4R	5′>CACAATCCCCAGAACCCCTC<3′
Exon5F	5′>ACTTTTCGCCCAGTCATGTCC<3′
Exon5R	5′>CACCGCCCCGTCGTTG<3′
Exon6F	5′>CCCACATTAGAGAGACTTTACTATCG<3′
Exon6R	5′>CAAGGGGAGACACCAAGGC<3′
Exon7–8F	5′>TTAACCAATTCATGGTGAGG<3′
Exon7–8R	5′>GTCTACTTACCTGTATGTCTTTATCA<3′
Exon7_seq	5′>AGACTTCCTAATGGTATGGACTGTG<3′
Exon8_seq	5′>AAACTATTTGGAGAAAAGGAGCA<3′

3. Results

3.1. Substitution patterns of TTL.6 in primates

We sequenced the coding region (1710 bp, exon 9–16) of *TTL.6* in 35 human samples and five nonhuman primate species. In addition, 289 bp intron sequences were also obtained in the human and chimpanzee samples. The aligned protein sequences were shown in Fig. 1. One early-stop substitution in the 3′ end of *TTL.6* in rhesus monkey was observed, which might not have functional consequence because only three amino acids are lost at the 3′ end.

We calculated the nonsynonymous/synonymous substitution ratios (ω) following Yang and Nielsen (2000). The ω values of different evolutionary lineages in the phylogenetic tree were shown in Fig. 2. A strong functional constraint was observed in the gibbon lineage with its ω value significantly smaller than 1 ($p=0.004$, two-tailed Fisher's exact test) and also in the internal branch B–C ($p=0.029$, two-tailed Fisher's exact test; Fig. 2). The orangutan and rhesus monkey lineages also showed signatures of functional constraints ($\omega<1$) although they are not as intense as in the gibbon lineage (Fig. 2). The gorilla and chimpanzee lineages have ω values larger than one, but statistically indifferent from the neutral expectation ($\omega=1$) ($p>0.05$, two-tailed Fisher exact test). The human lineage showed an extremely different pattern from the nonhuman primate lineages. All the seven substitutions observed in the human lineage are nonsynonymous and the *Z* test showed a significant deviation from neutral expectation ($p=0.004$, two-tailed *Z* test), an implication of Darwinian positive selection. Considering the small number of substitutions in the human lineage (Zhang et al., 1997), to be conservative, we also conducted the Fisher's exact test for the human lineage and a

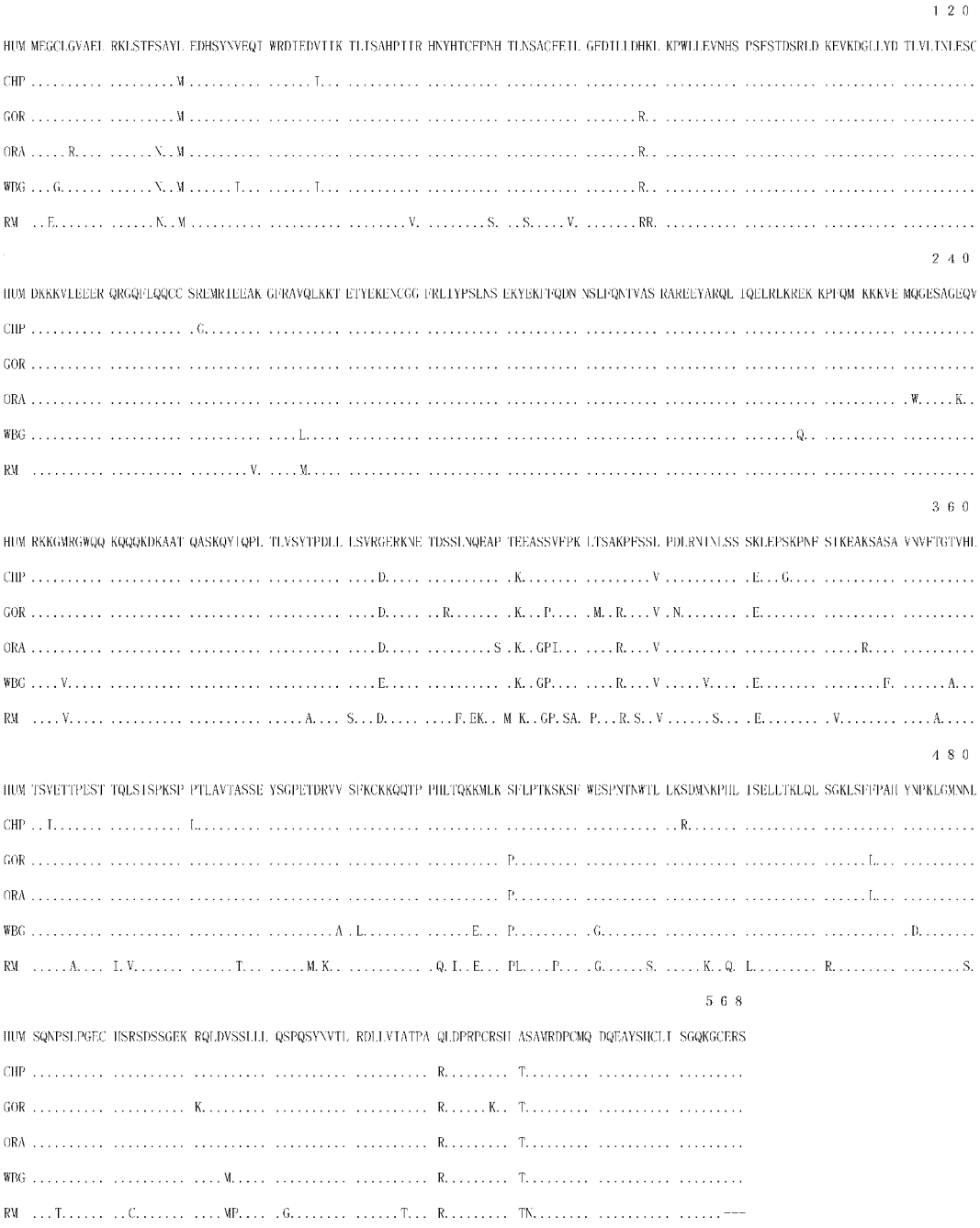

Fig. 1. Protein sequence alignment of *TTL.6* in human and nonhuman primates. Both the human and chimpanzee protein sequences were inferred from their consensus DNA sequences, respectively.

marginal significance was observed ($p=0.08$, Fisher's one-tailed exact test). We also calculated the Ka/Ks ratios using Pamilo–Bianchi–Li's method which showed the same pattern (Pamilo and Bianchi, 1993; Li, 1993).

To test whether the human lineage showed an accelerated evolution than the nonhuman primates, we conducted the model-based tests (Yang, 1998). The likelihood values of the one ratio (M0) and two ratios (M2, assuming that the human lineage has a different rate from the nonhuman primate lineages) models were calculated and a significant difference was detected between the human and nonhuman primate lineages ($2\Delta\ln L=6.957$, $p<0.01$, $df=1$). Hence, the human lineage evolved significantly faster than the nonhuman primate lineages, which is consistent with the proposed positive selection in the human lineage. The internal branch C–D (Fig. 1) also showed an elevated ω value ($2\Delta\ln L=4.347$, $p<0.05$), indicating that positive selection might also act during the evolution of the common ancestor of humans and chimpanzees.

We next conducted the MacDonald–Kreitman's (M–K) neutrality test, which was developed to compare the between-species divergence and within-species polymorphisms of synonymous and nonsynonymous substitution patterns. In the coding region, a total of 15 fixed substitutions were

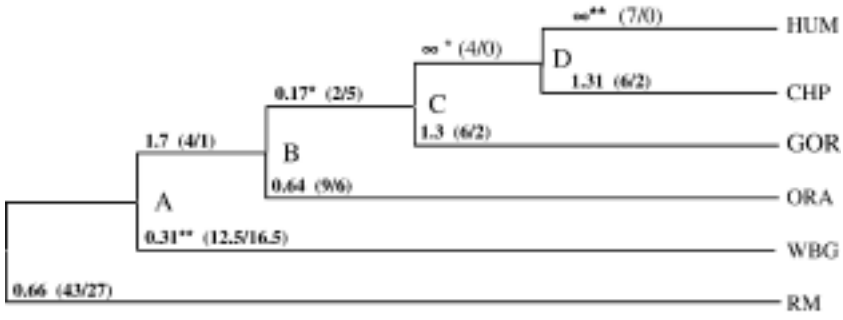

Fig. 2. The Ka/Ks ratios (ω) on different evolutionary lineages in primates. The coding sequences were used for calculation based on Yang and Nielson (2000a). The Z test was used in detecting deviation of the ω values from neutral expectation ($\omega=1$), where "**" and "*" refer to $p<0.01$ and $p<0.05$, respectively. The numbers in parentheses indicate the numbers of nonsynonymous and synonymous substitutions of each lineage. The labels A–D are inferred ancestral nodes. HUM—human, CHP—chimpanzee, GOR—gorilla, ORA—orangutan, WBG—white-browed gibbon, RM—rhesus monkey.

observed between human and chimpanzee, and 13 of them are nonsynonymous substitutions. In the human populations, we observed 4 polymorphic sites with two of them nonsynonymous, while in the three chimpanzee samples sequenced, we identified 5 polymorphic sites and 3 of them are nonsynonymous substitutions. The Fisher's exact test did not show significant between-species and within-species difference in substitution patterns ($p=0.150$, two-tailed Fisher's exact test). However, to increase the sample size, we added the 289 bp intron sequences (treated as synonymous sites) for the M–K test, and a significant deviation from neutral expectation was detected ($p=0.005$, two-tailed Fisher's exact test; 7 and 1 polymorphic sites in humans and chimpanzees, respectively, and one fixed substitutions between humans and chimpanzees). The GC content and the substitution rate of the intron sequences used are similar with the synonymous sites in the *TTL.6* coding region, therefore, would not bring much bias in the M–K test. Hence, the M–K test also supports the proposed positive selection in the human lineage. The within-species sequence polymorphisms were listed in Table 2.

We further sought to detect individual sites under positive selection by using the codon-based neutrality test proposed by Yang et al. (2000). A total of seven sites showed deviations from neutral expectation and two of them have nonsynonymous substitutions in the human lineage. The two human-lineage nonsynonymous substitutions are the G/D substitution ($p=0.002$) at site 285 and the K/E substitution ($p=0.005$) at site 332. In addition, the sliding window analysis between human and chimpanzee revealed two peaks of high Ka/Ks values located in the non-TTL domains, consistent with the locations of the two human-specific sites in the codon-based neutrality test (Fig. 3).

3.2. Sequence variations in human populations

In human populations, a total of 11 sequence variations were identified in the coding and non-coding regions of *TTL.6*. The distribution of the 11 polymorphic sites in world populations indicated an excess of derived common alleles, which was supported by the Fay and Wu's H test ($p=0.036$). The Fu's Fs test also indicated the deviation from neutrality ($Fs=-5.41$, $p=0.020$). Therefore, our data implied a strong signature of genetic hitchhiking caused by recent positive selection of *TTL.6* in human populations.

4. Discussion

4.1. Varied evolutionary rates of TTL.6 in primates

The sequence substitution patterns of *TTL.6* in primates demonstrated a sharp difference in evolutionary rates among different primate lineages. Functional constraint due to negative selection was observed in the orangutan, gibbon and rhesus monkey lineages, and also the common ancestor of human, chimpanzee and gorilla, while the constraint seems to be relaxed in gorilla and chimpanzee, and the pattern became opposite in the human lineage. The rapid evolution of the human *TTL.6* was likely caused by functional adaptation as confirmed by the neutrality tests. The varied evolutionary patterns of *TTL.6* in primates are likely correlated with the differences in mating

Table 2
Sequence polymorphisms of *TTL.6* in human populations

	CDS				Intron						
Site position	1062	1170	1230	1517	1740	1754	1755	1756	1868	1919	1996
Derived/Ancestral	T/C	T/G(Asp/Glu)	G/T	A/C(Tyr/Ser)	A/G	C/T	A/G	T/A	G/A	A/T	T/C
Asian (22)	1/21	6/16	0/22	2/20	5/17	22/0	0/22	16/6	17/5	17/5	0/22
African (20)	0/20	5/15	5/15	0/20	7/15	20/0	2/18	5/15	14/6	14/6	1/19
Caucasian (20)	0/20	6/14	0/20	0/20	5/15	19/1	3/17	4/16	15/5	15/5	0/20
Melanesian (8)	0/8	0/8	0/8	0/8	0/8	8/0	1/7	5/3	8/0	8/0	0/8
Frequency (70)	1.43%	24.30%	7.14%	2.86%	24.30%	98.60%	8.57%	42.86%	77.14%	77.14%	1.43%

The numbers in parentheses are chromosome numbers.

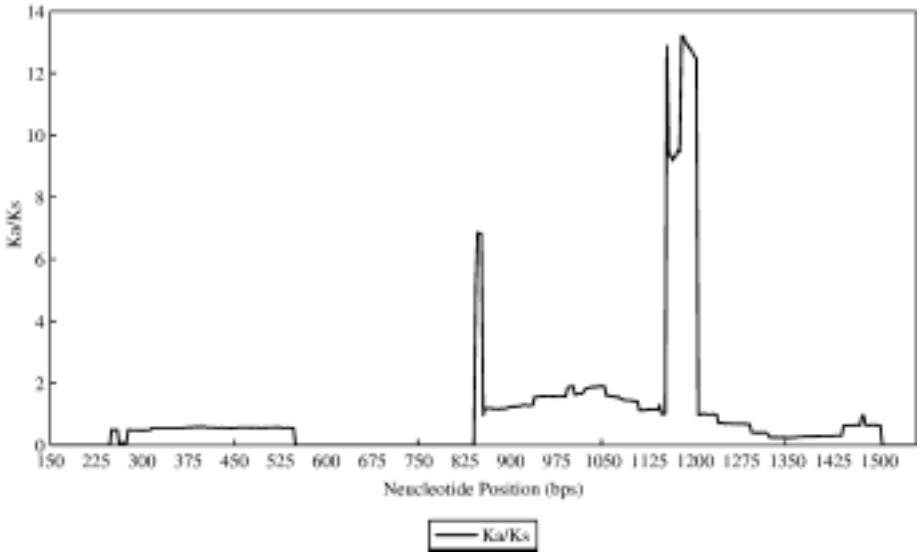

Fig. 3. The sliding-window analysis of the Ka / Ks ratios between human and chimpanzee across the coding sequences of *TTL.6*. The window size used was 100 codons and the step size was one codon. The DnaSP3.99 program was used and the calculation of the Ka / Ks ratios was based on Nei and Gojobori (1986). The gaps refer to the regions where the denominator was zero (Ks = 0).

behavior among different species as revealed in previous studies (Dorus et al., 2004; Wyckoff et al., 2000).

4.2. Positive selection and functional consequence of adaptive evolution of TTL.6

Tubulin-tyrosine ligase is ATP dependent. The ATP/Mg^{2+} binding sites and flanking hydrophobic sites are necessary for ATP usage (Artymiuk et al., 1996; Dideberg and Bertrand, 1998; Fan et al., 1994; Matsuda et al., 1996). We compared the thirteen ATP/Mg^{2+}-binding sites between the human *TTL.6* and the human tubulin tyrosine ligase gene (one of the members of the TTL family, Erck et al., 2003), and only three substitutions were detected between them. The 8 exons of *TTL.6* we sequenced include 6 ATP/Mg^{2+}-binding sites and all of them are conserved. Considering all these critical binding sites are generally conserved in *TTL.6*, and the sites under positive selection were outside the TTL domain, the adaptive evolution of *TTL.6* may lead to the modification of its function, e.g. enzyme activity, tissue specificity and/or level of gene expression.

TTL.6 is mainly expressed in testis and involved in apoptosis (Eiserich et al., 1999), it may play a role in sexual organ differentiation, seasonal reproduction, spermatogenesis, and male fertility (Dohle et al., 2003; McClusky, 2005; Sawhney et al., 2005; Sinha Hikim et al., 2003; Spierings et al., 2003; Xavier and Allard, 2003). Previous studies demonstrated that the male reproductive genes in primates tend to evolve rapidly probably due to sexual selection (Dorus et al., 2004; Wang and Zhang, 2004; Wyckoff et al., 2000). Wang and Zhang suggested that it is a general pattern for mammalian X-linked testis-expressed homeobox genes to evolve rapidly (Wang and Zhang, 2004). A recent study proposed that a point mutation in *FGFR2* involved in human spermatogonia is under positive selection (Goriely et al., 2005). The adaptive evolution of *TTL.6* seems to occur only in the human lineage, which is different from the other genes reported; therefore, further studies in understanding the biological functions of *TTL.6* may reveal the mechanism of positive selection.

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