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Evolution of peroxisome proliferator-activated receptor gamma alternative splicing

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1. ABSTRACT

Peroxisome proliferator-activated receptor gamma (PPAR gamma) plays an important role in the control of energy balance and lipid and glucose homeostasis. Different transcript variants were investigated not only in human but also in other vertebrates. To look into the evolutionary changes of these variants, we analyzed the genomic sequences of PPAR gamma genes from several vertebrate species, as well as their mRNA and EST data. Several potential alternative splicing exons at the 5'-end of the PPAR gamma gene were identified. The 5'-end of the PPAR gamma gene is discovered to be evolutionarily active and recruits new exons via different strategies. Moreover, it is shown that the only coding alternative exon (exon B) processes much higher Ka/Ks compared with its constitutive counterparts. In addition, its Ka/Ks is greater than 1 in the rat, mouse, and rabbit, indicating adaptive evolution and possible energy storage related gain-of-function for the exon.

2 INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) comprise an ancient subfamily of the nuclear hormone receptor, and likely appeared before the arthropod/vertebrate divergence (1). PPARs regulate important physiological processes related to lipid homeostasis, inflammation, adipogenesis, reproduction, wound healing, and carcinogenesis (2-6). Gene duplication events in vertebrates may contribute to the diversification of subfamily, resulting in three distinct members, PPAR alpha, PPAR beta and PPAR gamma [1], with distinct expression patterns and functions (Table 1) (7-13).

Like many other nuclear receptors, PPAR gamma is characterized by four major domains: an N-terminal A/B domain, a DNA-binding domain with two zinc fingers (C domain), a D domain, and a C-terminal ligand-binding domain (LBD, E/F domain) (14). PPAR gamma is thought to be critical in the control of energy balance and lipid and glucose homeostasis. Previous studies

Table 1. The expression patterns and functions of PPAR family members

PPAR member	PPAR alpha	PPAR beta	PPAR gamma
Expression	Highly expressed in livers, cardiac myocytes and kidneys	Less studied, believed to be widely expressed in various organs	Highly expressed in spleen, adrenal, heart, and adipose tissues
Functions	Peroxisome proliferation, lipid catabolism, inflammatory responses, skin wound healing, inhibition of arachidonic acid	Cell proliferation, myelination, embryonic implantation, adipocyte differentiation, skeletal muscle fatty acid catabolism, glucose metabolism, inflammation, and skin wound healing	Lipid storage, macrophage maturation, embryonic implantation, adipocyte differentiation and inflammation control

have indicated that PPAR gamma (coupled to RXR) involved in the process of cell growth arrest and progression into the fully differentiated adipocyte phenotype. (15, 16). Recently, the relationships between PPAR gamma and a series of metabolic diseases such as obesity and type 2 diabetes, especially insulin resistance, have also been investigated (17-19). Meanwhile, mutations and single nucleotide polymorphisms (SNPs) in PPAR gamma have become a hot topic for research, and the replacement of proline with alanine in codon 12 (Pro12Ala) in the human PPAR gamma gene is associated with reduced body mass index (BMI) and insulin sensitivity (20). Studies also indicate that polymorphisms of PPAR gamma can often be associated with cognitive impairment (21) and thyroid-associated orbitopathy (22)

Other than physiological studies, some researches have investigated the alternative splicing of PPAR gamma. So far, four human PPAR gamma isoforms of mRNA have been identified, namely PPAR gamma 1, PPAR gamma 2, PPAR gamma 3 and PPAR gamma 4. The protein translated from PPAR gamma 2 contains an additional NH2-terminal region, composed of 28 amino acids, while proteins derived from PPAR gamma 1, gamma 2, and gamma 3 mRNA are identical, with a peptide comprising 477 amino acids (23).

In all four isoforms, six constitutive exons and four alternative splicing exons (exon A1, A', A2 and B) have been identified. Exons A1 and A2 are reported in PPAR gamma 1; exon B is seen in PPAR gamma 2; exon A' and A2 are part of PPAR gamma 3; and PPAR gamma 4 has exon A1 as the only alternative exon. These four isoforms have different expression levels. RT-competitive PCR assays have demonstrated that hPPAR gamma 1 is expressed at significantly higher levels compared with hPPAR gamma 2, whereas the other isoforms expressed at even lower levels are found in adipose tissue (PPAR gamma 4) and white adipose tissue/large intestine/macrophages (PPAR gamma 3). In terms of the expression of two protein isoforms, one study has indicated that hPPAR gamma 2 is predominantly expressed in adipose tissues, whereas hPPAR gamma 1 is more widely expressed (13).

In addition to the human genome, PPAR gamma has been reported among other vertebrates, including mouse (24, 25), hamster (26), human (27-29), cattle (30), rat (31), monkey (32) and pig (33-35). Alternative splicing has also been observed in some species such as the cow and domestic pig. In some species (e.g. domestic pig), these alternative splicing variants resemble the four corresponding human variants, whereas others have their own species-specific exons.

Although alternative splicing events of PPAR gamma have been reported in various species, most studies have focused on the biological functions and their relationship with clinical diseases (such as type 2 diabetes). The evolutionary story of these variants, in particular the four alternative exons, the species-specific exons remains unknown. Therefore, a study analyzing the alternative splicing of PPAR gamma from a comparative genomic perspective is needed to determine the expression characteristics of PPAR gamma and their evolutionary implications.

3 MATERIAL AND METHODS

3.1. Sequence collection

Genomic, mRNA and EST sequences were downloaded from the Genbank and Ensembl databases. (Genbank Release No. 167, http://www.ncbi.nlm.nih.gov/Genbank/index.html and Ensembl, release No.50 http://www.ensembl.org/index.html) Nine vertebrate species were studied: human, chimpanzee, cow, domestic pig, mouse, rat, sheep, chicken and dog.
The mRNA sequences collected are shown in Table 2.

3.2. Isoform identification

EST/cDNA sequences were aligned to corresponding genome sequences by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with parameters adjusted for short and gapped alignments. All exon boundaries were confirmed by consensus acceptor and donor sites. Splicing isoforms were predicted by various EST/cDNA-to-genome alignments. Predicted splicing isoforms were verified by BLAST analysis against the EST database. Multiple sequence alignments were performed using ClustalX. Repeat sequences of human and mouse genomes were compared uisng Repeatmarsker (http://repeatmasker.org) (36).

3.3. Ka/Ks

The Ka/Ks ratio was calculated using K-Estimator 6.1 developed by Josep M. Comeron (free software download available at http://www.biology.uiowa.edu/comeron/index_files/Page432.htm), and confirmed using PAML4 (free software download available at http://abacus.gene.ucl.ac.uk/software/paml.html) (37).

3.4. SNP

All SNPs within the genome region of hPPAR gamma were collected from dbSNP (build 129, http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?chooseRs=all&go=Go&locusId=5468). As the SNP database only provides SNP information for this isoform, the SNPs in

Table 2. The PPAR gamma mRNA sequences used in the analysis

AS Name	Genbank ID	AS Name	Genbank ID
hPPAR1	NM_138712	mPPAR1	NM_001127330
hPPAR2	NM_015869	mPPAR2	NM_011146
hPPAR3	NM_138711	bPPAR1	Y12419
hPPAR4	NM_005037	bPPAR2	NM_181024
sPPAR1a	AB097926	rPPAR2	NM_013124
sPPAR1b	AB097927	gPPAR	NM_001001460
sPPAR1c	AB097928	cPPAR2	NM_001024632
sPPAR1d	AB097929	oPPAR1	U84893
sPPAR2	NM_214379	oPPAR3	NM_001082148

Table 3. The exons combination of different PPAR gamma transcript variants in respective species

Human	A1+A2+Latter[1]	B+Latter[1]	A2+Latter[1]	A1+Latter[1]	A'+A2+Latter[1]
Chimpanzee	A1+A2+Latter[2]	B+Latter[2]	A2+Latter[2]	A1+Latter[2]	A'+A2+Latter[2]
Dog	---	B+Latter[1]	A2+Latter[2]	------	A'+A2+Latter[2]
Cow	A1+A2+Latter[2]	B+Latter[1]	A2+Latter[2]	A1+Latter[2]	A'+A2+Latter[1]
Pig	A1+A2+Latter[1]	B+Latter[1]	A'+A2+Latter[1]	A1+Latter[1]	A'+Latter[1]
Rabbit	-----	B+Latter[2]	A2+Latter[2]	-----	OS1+OS2+Latter[1]; A'+A2+Latter[1]
Mouse	-----	B+Latter[1]	------	-----	RS1+RS2+Latter[1]; A'+RS3+RS2+Latter[3]; A'+Latter[3] A'+ RS2+Latter[2]
Rat	-----	B+Latter[1]	------	-----	RS1+RS2+Latter[1]; A'+ RS2+Latter[1] A'+Latter[2]
Chicken	------	-------	-------	------	Latter

[1] Report mRNA sequence support, [2] Prediction by genomics analysis, [3] EST sequence support

other isoforms were determined by manual sequence comparison.

4 RESULTS

4.1. Genomic Comparison

The neighboring genes of PPAR gamma on the chromosome are the same in various vertebrates. However, the intergenic region of PPAR gamma and the 5'-upstream gene SYN2 in mammals was nearly twice as long as that in birds (Figure 1). Repeatmarsker (36) analysis shows that insertion of repeat sequences make important contributions to this phenomenon; 50% of this region comprises repeat sequences in humans, 25% in mice, but only 2% in chickens.

4.2. Exon Profile

Exon Profile results show that various AS forms of PPAR gamma are present. In chickens, there is only one transcription product that contains constitutive exons (Figure 2). However, in mammals, various numbers of alternative splicing forms are observed (Table3 and Figure 2).

In total, four common alternative splice exons, namely A1, A', A2 and the coding exon B, together with some specific alternative splicing exons in respective species, have been identified (Figure 2). Different alternative splicing forms of these exons are listed in Table3. Figure2 shows the distribution of alternative splicing exons in different species, all of which are located at the 5' end of the gene. Exon A' (or its genomic homolog) exists in virtually all species examined. Moreover, some species-specific exons or mRNA fragments are also identified through inter-species sequence alignments.

4.3. SNP frequency

We recorded a total number of 737 SNPs in the human PPAR gamma gene region with 7 found in alternative splice exons, 9 in constitutive exons, and 721 in introns. Correspondingly, the SNP frequencies for alternative splicing exons, constitutive exons, and introns are 11.4, 5.5 and 4.9 SNP/1,000bp respectively.

4.4. Ka/Ks in coding alternative splicing exon (exon B)

After determining the evolutionary features of alternative exons in PPAR gamma at the nucleotide level, we further investigated the evolutionary characteristics of its open reading frame (ORF). In fact, the alternative splicing exon (exon B, encoding 28 amono acids) carries some interesting characteristics in terms of the Ka/Ks ratio, a crucial indicator of selective pressure acting on a protein-coding gene, which shows a significant difference compared with the peptide coded by constitutive exons (Table 4a, 4b).

The average value for the Ka/Ks ratio in exon B is 0.960, significantly higher than the average Ka/Ks ratio for the constitutive exons, which is 0.0214 (P value = $4.09*10^{-6}$).

To illustrate that the Ka/Ks difference is more possibly due to alternative splicing, we performed a sliding window based Ka/Ks analysis of the human and mouse PPAR gamma sequences. The full-length mRNA PPAR gamma sequence is divided into 14 segments, each of about 100 base pairs (about the same size as exon B), and the ratio is calculated respectively with the same method. Results demonstrate that the ratio of all 14 segments are significantly lower than that of the coding alternative splice exon B, and the lowest ratios are seen for the segments that encode conserved functional domains of PPAR gamma

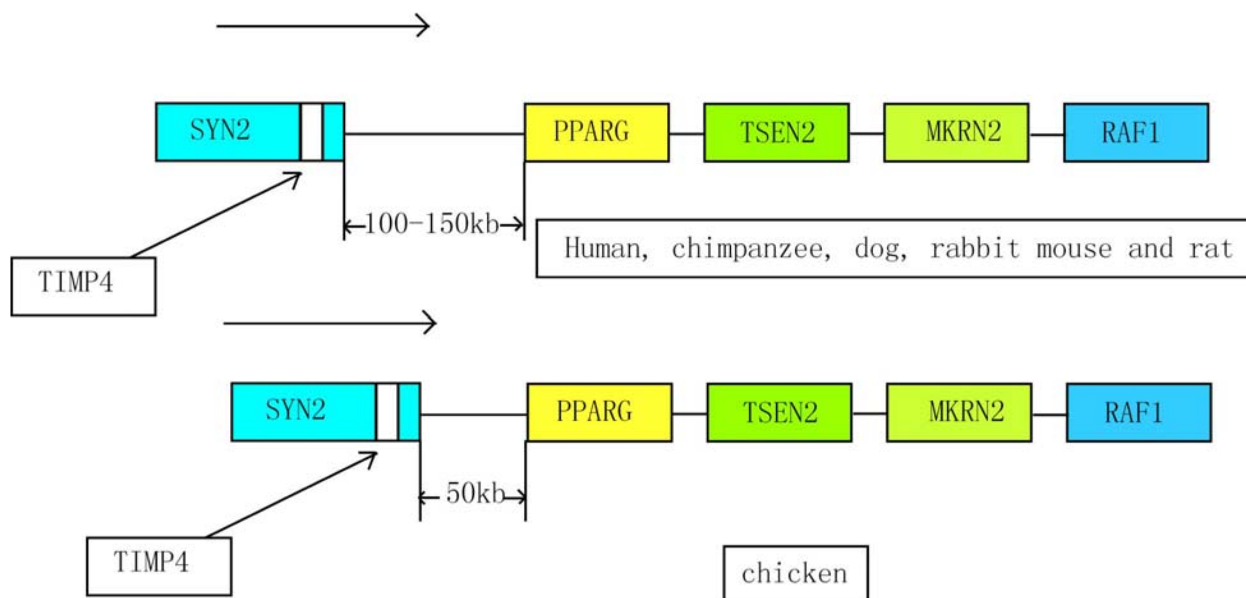

Figure 1. Genome structure of the PPAR gamma gene in human, chimpanzee, dog, cow, rabbit, mouse, rat and chicken. Nearby genes located in the 5'- and 3'-region of PPAR gamma are highlighted by boxes with different colors. Black arrow shows the transcript direction. Data were based on NCBI genomic viewer and Ensembl genomic data.

Table 4. The Ka/Ks ratios for PPAR gammas in different species

A. The Ka/Ks ratios for coding alternative splice exon B of PPAR gamma in six species						
	Human	Mouse	Rat	Pig	Dog	Cow
Human						
Mouse	1.32					
Rat	1.484	n/a (Ks=0)				
Pig	0.218	1.149	1.291			
Dog	0.288	0.752	0.852	0.225		
Cow	0.727	2.076	2.076	0.689	0.294	
B. The Ka/Ks ratios for other constitutive coding exons						
	Human	Mouse	Rat	Pig	Dog	Cow
Homo						
Mouse	0.0198					
Rat	0.0214	0.00746				
Pig	0.0206	0.0213	0.0187			
Dog	0.0151	0.0174	0.0146	0.0172		
Cow	0.0348	0.0282	0.0252	0.0259	0.0336	
C. The Ka/Ks ratios for coding alternative splice exon B of PPAR gamma in rabbit						
	Human	Mouse	Rat	Pig	Dog	Cow
Rabbit	1.484	n/a (Ks=0)	n/a (Ks=0)	1.291	0.852	2.076

(Figure 3). Therefore, we can see a clear Ka/Ks change – the alternative splice PPAR gamma exons have higher Ka/Ks ratios. This is in agreement with the results of previous studies indicating that alternative splicing exons have a higher Ka/Ks ratio compared with constitutive exons, and show more rapid functional evolution due to weaker selective constraints. (38-43)

In addition, analysis of the Ka/Ks in certain species has shown interesting results. The average Ka/Ks ratio for the mouse is 1.32425 and for the rat is 1.4275 (Table 4a). Both values are greater than 1, significantly higher than those for other species (P value = 0.000871).

To confirm our assumption that the high ratio is not an accidental event but an evolutionary phenomenon related to rodents, we investigated whether similar high Ka/Ks values are seen in specie phylogenetically close to rodents. We chose rabbit for Ka/Ks analysis and found that the average ratio is also greater than 1 (Table 4c). The extremely high Ka/Ks ratio in rodents and rabbit indicates that exon B has a stronger adaptive (rather than pro-selective) evolutionary trend in these species.

5 DISCUSSION

5.1. Evolutionary history of the alternative splicing exons

PPAR gamma is an antique gene, which can be traced back to ancient vertebrates (zebrafish). Its existence and basic gene structure remain stable in all higher vertebrates (mammals), possibly owing to its metabolic importance. Genomic comparison shows that all the PPAR 5'-alternative splicing exons are involved in corresponding transcripts after divergence of mammals and birds. Among

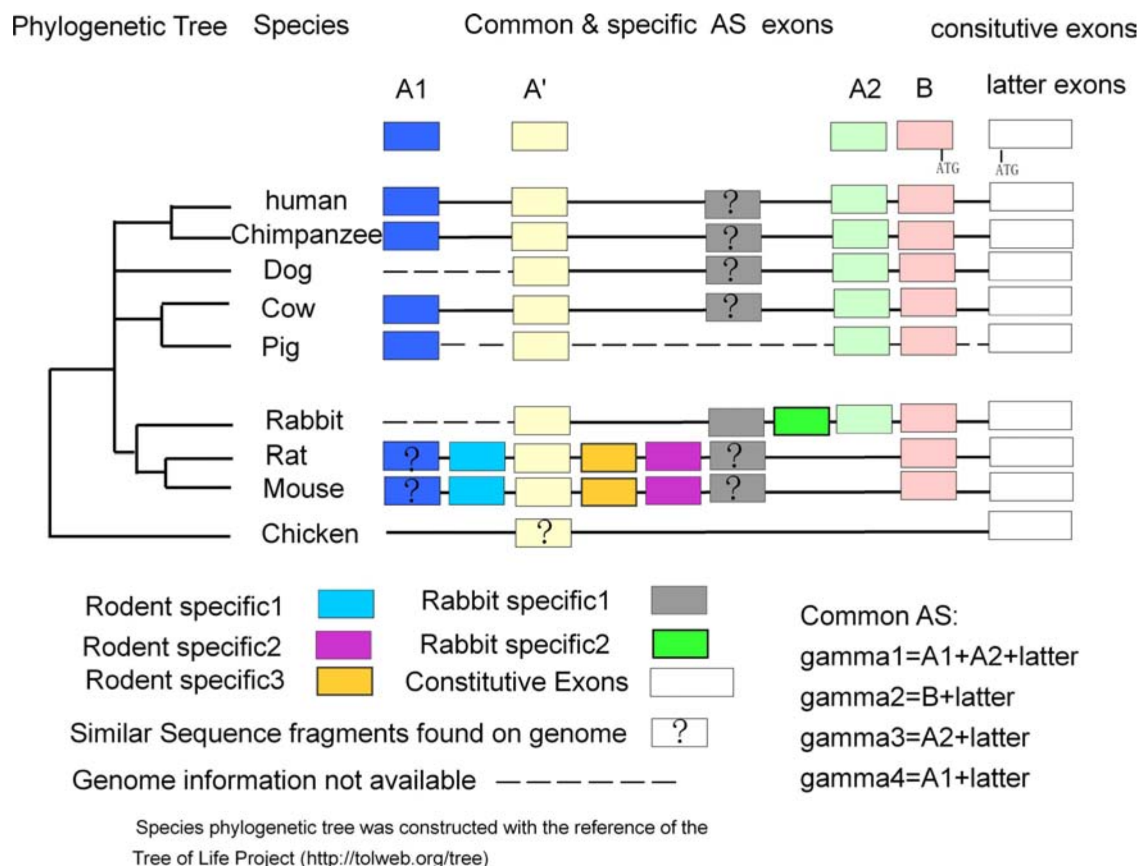

Figure 2. Alternative spliced and constituted exon distribution in PPAR gamma in different species. Phylogenetic tree of analyzed species was based on tree-of-life data. PPAR gamma exon distribution and genome location order in human, chimpanzee, dog, cow, pig, rabbit, mouse, rat and chicken were shown as lines and boxes. Boxes of the same color represent corresponding exons among species. Exons with mRNA, ESTs or high genomic similarity results are shown in direct edge boxes. Putative exon fragments or ruins are shown in boxes with question marks.

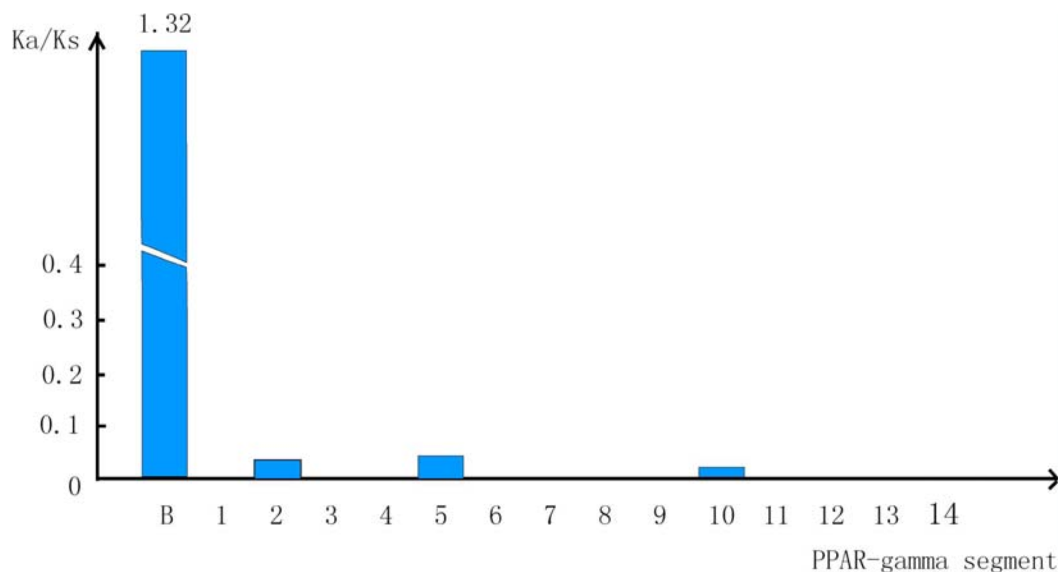

Figure 3. The Ka/Ks ratios for different segments of PPAR gamma between mouse and human. Y axis represents Ka/Ks ratio and X axis represents different segments.

all alternative splicing exons, only the similar fragment of exon A' is found in both bird's (gallus) and mammalian genomes. As the others are all absent from chickens, it can be assumed that the sequence structure of exon A' appeared before the bird–mammal divergence, and that the other three exons, together with other species-specific exons, were introduced into the genome during mammalian evolution.

Previous genome studies have indicated that repeat sequences could have important roles in the appearance of new exons in mammals (44-46). In our study, we believe that the rise of exon A1 may relate to the GC-repeats ((CGGGGG)n or (CCGGG)n), because they coexist in all examined mammal genomes However, the evolutionary details of exon A2 seem more complicated. Comparison of phylogenetic trees of different species indicates that exon A2 may have been introduced early in mammalian evolution, and undergone exon loss in certain species, because A2 is not detected in rodents but is present in all other species studied (Figure 2). Inter-species sequence alignment shows that exon B exists in all mammalian genomes (the existence of exon B in rabbit and chimpanzee is supported by Ensembl genomic data) and is the only coding alternative splicing exon in the PPAR gamma family. In addition, exon B has a higher Ka/Ks value compared with constitutive exons, indicating a possible adaptive evolution and gain of function.

Apart from the alternative splicing exons mentioned above (A1, A', A2, and B), certain exons like rabbit specific exon1 may evolve from the orginal intron sequences since similar intron region sequences are found in other species(Fig.2). However, BLAST search did not detect any fragments similar to rodent-specific exon1, 2 or rabbit specific exon2 in genomic regions of other species analyzed (From the last exon of SYN2 to the first constitutive exon of PPAR-gamma). And the evolutionary details of these species specific exons remain uncovered. By combining genomic comparison and EST analyses, we predicted some possible new alternative splicing forms in corresponding species (Table 3).

All the abovementioned alternative splicing exons or their similar fragments are located in the 5'-region of the PPAR gamma gene. Similar results were found in previous study, as novel exons are usually developed in the 5'-UTR region, later they will become incorporated into protein-coding region and may gain new biological functions (38). Two different alternative splicing exons obtaining strategies of PPAR gamma gene could be observed. One is the 'exonization' of intron region, such as the appearance of certain alternative splicing exons (for example, rabbit-specific exon 1 and exon A') as these exons (or intronic sequences similar to these exons) are present in most genomes studied. The other strategy is the 'de-novo' approach. Certain species-specific exons (such as rodent-specific exons 1, 2 and rabbit specific exon2) have no similar genomic fragments in the genomes of other species. This strategy is more common in smaller mammals (such as rodents). These mammals might experience greater evolutionary pressure than their larger counterparts owing to their diverse and rapidly changing environment and may therefore require more diversified functions and gene expression patterns.

5.2. Implication of high SNP frequency

Recent research has shown that evolutionary rates can also be represented by SNP frequency which measures the intraspecies polymorphism.SNP density is 2-fold greater in recently acquired exons than in ancient ones. Therefore, a newly acquired alternative splicing exon is likely to have higher SNP frequency compared with their constitutive counterparts, making SNP a useful tool to measure the evolution rate of an exon (47).

Our data show that alternative exons in PPAR gamma have much higher SNP frequencies (11.4 SNPs/kb), whereas the frequencies for constitutive exons (5.5 SNPs/kb) and introns (4.9 SNPs/kb) are similar, indicating increased nucleotide polymorphisms and more rapid evolution rate for alternative spliced exons compared with constitutive ones in PPAR gamma.

5.3. The Ka/Ks story

In most cases, functional exons, especially conserved domains are under selective pressure. Previous studies have indicated that alternative splicing can reduce the Ka/Ks selective pressure by up to 7-fold. In addition, this effect is localized to the alternatively spliced exons, which may help to create evolutionary 'hotspots' within a protein sequence, particularly for evolution of higher vertebrates(47). By alternative splicing, new exons with evolutionary potential can be introduced into the gene without disturbing its original bio-function. Thus, alternative splicing substantially reduces negative selection pressure and contributes to the creation of new gene functions (48-52).

In our Ka/Ks analysis, the high Ka/Ks value of exon B demonstrates the evolutionary potential of alternative splicing exons, particularly at the 5'-end (N-terminal of PPAR gamma). The high Ka/Ks ratio indicates that exon B is still evolutionarily active and has the potential to obtain biological functions. Although no previous research has precisely determined the function of exon B, previous studies have suggested that it may increase the DNA-binding activity of the receptor and thereby contribute to white adipose tissue (WAT) differentiation (2, 53).

As the rodent and rabbit Ka/Ks ratios are greater than 1, it can be assumed that exon B has a particularly important biological function in these small mammals and has probably undergone adaptive evolution and gain-of-function during evolutionary process. Previous experiments have shown that knockout of the exon in the mouse can result in decreased levels of long-chain triacylglycerol in WAT and insensitivity to insulin. The phenomenon indicates that the new function of this exon may contribute to energy conservation. Furthermore, compared with other mammals, small mammals have different evolutionary characteristics such as more rapid reproduction, larger offspring number and higher basal metabolic rate. These all require increased intake of energy and efficient conservation. Thus, exon B may benefit fat storage and

energy metabolism in small mammals, resulting in an extremely rapid mutation rate. And to discover the exact function of exon B in small mammals (such as rodents and rabbits) would be an interesting topic, particularly in terms of its functions in PPAR gamma expression and regulation.

6. CONCLUSION

In this study on the alternative splicing of PPAR gamma, we analyzed the distribution of alternative splicing exons among various species and investigated the possible evolutionary process of exons A', A1, A2 and B. Inter-species genomic sequence comparison and SNP frequency analyses indicate that PPAR gamma has an evolutionarily active 5'-end, which recruits new alternative splicing exons during evolutionary process via two different strategies (changing intron sequences into exons or *de-novo* exon recruitment. This evolutionary potential can also be seen at both nucleotide and amino acid levels, as the coding AS exon (exon B) has greater Ka/Ks than constitutive exons. Exon B also showed adaptive evolution in small mammals because the average Ka/Ks, compared with other mammals, was greater than 1 in rat, mouse and rabbit. Considering with previous results, this might be due to the need for various biological functions and different evolutionary strategies. One possibility is that exon B may have more important biological functions in energy conservation and metabolism, in rodents than in other species. Moreover, we believe that, with the increasing amount of genomic data and gene expression data, comparative genomic approaches, as used in this study, would provide more detailed expressional, functional and evolutionary clues for our understanding of the human and mammalian genome.

7. ACKNOWLEDGMENTS

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