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ORIGINAL ARTICLE

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Molecular analysis of TBL1Y, a Y-linked homologue of TBL1X related with X-linked late-onset sensorineural deafness

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Abstract Recent progress in sequencing the human Y chromosome has unveiled a series of X-Y homologous genes. In the present study, we focused on Transducin beta-like 1Y (TBL1Y), which is a Y-linked homologue of TBL1X that is related with X-linked late-onset sensorineural deafness. Recently, it has been shown that TBLR1, another homologue whose gene resides on chromosome 3, and TBL1X act as a corepressor/coactivator exchanger for several nuclear receptors and transcription factors. However, the expression pattern and function of TBL1Y remain unknown. The RT-PCR analysis of the TBL1 family revealed that TBL1Y was expressed in all 13 tissues examined but not in leukocytes. Among the cell lines tested, however, it was only expressed in NT2/D1 cells and in lymphoblasts transformed with Epstein Barr (EB) virus. To compare the functions of the TBL1 family, we generated a series of expression plasmids for GAL4DBD-fused proteins of the TBL1 family. We carried out dual luciferase assays using these plasmids in combination with a plasmid having a luciferase reporter gene harboring 5×GAL4 binding sites. Unlike the other constructs, GAL4DBD-fused TBL1Y did not repress the promoter activity. Moreover, we found three novel polymorphisms in the TBL1Y gene, IVS7 + 9G > A, G268C, and IVS7 + 1G > C, which is presumed to cause splicing error. These polymorphisms are found in males within Y-haplogroup O3 (XO3e), which is defined as the Y-haplogroup O3 excluding O3e, a branch of O3. The results show that TBL1Y differs from other members of the TBL1 family in expression and function, suggesting other roles in maleness.

Keywords Y chromosome · TBL1Y · TBL1X · TBLR1 · Y-haplogroup

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Introduction

The Y-linked genes often have homologues on the X chromosome (Page et al. 1987; Fisher et al. 1990; Nakahori et al. 1991; Lahn and Page 1997). It is widely believed that the X and Y chromosome have the same ancestry in spite of differences in size and shape since they partly share similar sequences (Ohno 1967). The human genome project has identified many X-Y homologous genes (Skaletsky et al. 2003). Here we focus on the Transducin beta-like 1 (TBL1Y) gene, which has a homologue on the X chromosome and is similar to the Ebi gene in Drosophila (Bassi et al. 1999; Dong et al. 1999). Partial deletion of the gene for TBL1X, the homologue of TBL1Y, is suggested to have a relationship with X-linked recessive late-onset sensorineural deafness although the TBL1X gene evades X-chromosome inactivation (Bassi et al. 1999). Therefore, TBL1Y, which is highly homologous to TBL1X, cannot completely compensate for a lack of TBL1X.

The TBL1 family has at least three members: TBL1X, TBL1Y, and an autosomal homologue of TBL1X,

TBLR1 (Bassi et al. 1999; Skaletsky et al. 2003; Zhang et al. 2002). Each member has an F box and six WD40 repeats, which are found in many adaptor proteins in its N-terminal and C-terminal portions, respectively (Bassi et al. 1999; Dong et al. 1999; Perissi et al. 2004). Recent studies have shown that TBL1X and TBLR1 are involved in transcriptional suppressor complexes containing histone deacetylase 3 (HDAC 3) together with SMRT and N-CoR (Guenther et al. 2000; Li et al. 2000; Yoon et al. 2003). It has been also shown that TBL1X and TBLR1 can interact with SMRT and N-CoR at their N terminal and WD40 repeats (Guenther et al. 2000; Yoon et al. 2003). Since TBL1Y is homologous to TBL1X and TBLR1, it is expected to have similar functions (Yoon et al. 2003; Perissi et al. 2004). However, when we analyzed its molecular properties, we found that TBL1Y differs from other members of the TBL1 family in expression and function.

Here, we show expressional and functional differences of TBL1Y compared with TBL1X and TBLR1. Moreover, we show novel single nucleotide polymorphisms (SNPs) in the TBL1Y gene and discuss the evolutionary significance of TBL1Y.

Materials and methods

Cell culture

The NT2/D1 cells and HTC116 cells, which were originally derived from human testicular embryonal cell carcinoma and human colon cancer, respectively, were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). For HUVECs (human umbilical vein endothelial cells) and lymphoblasts transformed with Epstein Barr (EB) virus, EGM-2 (Bio Whitter, Inc., MD, USA) and RPMI1640 medium were used with 10% FCS, respectively.

RT-PCR

The cDNA derived from MTC panels I and II (Clontech, Palo Alto, CA, USA), originating from various human tissues, was used for RT-PCR analysis according to the manufacturer's directions. Total RNA (2.5 μg) derived from NT2/D1 cells, fetal lung fibroblasts, HCT116 cells, HUVECs, and lymphoblasts transformed with EB virus were subjected to reverse transcription. The first-strand cDNA was synthesized using SuperScript RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according the manufacturer's manual. The PCR amplification was performed with specific primers for each member of the TBL1 family. The primers used were: for TBL1X, 5′-GGCCGAGATCAGTATCAACG-3′ and 5′-TGAGGTCGTGGTGGCTGCTGT-3′; for TBL1Y, 5′-GGCTGAGATAAGCATCAACA-3′and 5′-ATGGTTGCCGCCTTTGCCATT-3′; and for TBLR1, 5′-TAAGCAGTGATGAGGTCAAC-3′and 5′-GACTCTATTGGTCGACCATC-3′.

Preparation of genomic DNA

Blood samples were obtained from male volunteers who participated in a study on the spermatogenic effects of endocrine disruptors led by Dr. Iwamoto, one of the authors of this paper. Genomic DNA was extracted from peripheral white blood cells derived from fertile males who participated in the same study using a QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). The present study was approved by the Ethical Committee of The University of Tokushima.

Screening for novel SNPs on the Y chromosome

The PCR was performed using pooled genomic DNA derived from two to eight males who had the four different haplogroups previously reported by Shinka et al. (1999). The primer sets used for analysis of the TBL1Y gene are listed in Table 1. All PCR products were confirmed to derive from only the Y chromosome using genomic DNA obtained from females. After the reaction, the PCR products were denatured to form heteroduplex DNA according to a previous report (Shinka et al. 2001). After the heteroduplex had formed, the products were subjected to DHPLC using the WAVE system (transgenomic) according to the previous report (Shinka et al. 2001). For the PCR products that gave more than two specific peaks on the chromatogram, each genomic DNA was individually analyzed with DHPLC to unveil the samples with SNPs. The PCR products derived from genomic DNA with SNPs were subjected to direct sequencing.

The PCR products generated with the primer set for TBL1Y3F and TBL1Y3R were digested with Dra *III*, ApaL *I*, or Nla *III*. The digested products were sepa-

Table 1 The primers used for analyzing polymorphisms in the TBL1Y gene

Amplicon	Forward primer	Reverse primer
TBL1Y1	caaacagagggtggttcca	tcattctgctcgtaagatgc
TBL1Y2	aatttgggtctcactcaacg	ctcttgcaccttcccagggc
TBL1Y3	tcacccttgtgagctgatgg	taaagagcagctactaagac
TBL1Y4	aggtagtgttacttagtacg	acaatacgagacacgtatag
TBL1Y5	catgcctacaatgtcacctg	tacatgggaccacctgcctc
TBL1Y6	atggcaccaactattgcctg	attacttgaaccctgctgag
TBL1Y7	ccatacataacccttgattg	ctcttaatatatgaaagatc
TBL1Y8	tgtatgatacatgttactag	aggcactgagatgggctacg
TBL1Y9	cttgactctgctgttcattg	ataggtgtgagccactgttc
TBL1Y10	ctctttgatgtcaggctgag	gtgtagatctctttgctgtg
TBL1Y11	cgatctcacagtacaacttg	gattacaggtgtgagccatg
TBL1Y12	acagggtctcaccatgttgc	gaaggtatcgtgccatgagc
TBL1Y13	cagttagctgagattgcgcc	cacttaggcatcagctgcct
TBL1Y14	tgctgaggctgagaagtctg	tatacactcctgtggcctgg

rated using 2.5% agarose gel with electrophoresis and visualized with ethidium bromide.

Y-haplogroup typing

According to the guidelines provided by the Y Chromosome Consortium (YCC), the allele types of the binary DNA markers on the Y chromosome were determined with a PCR-based technique described elsewhere [Y Chromosome Consortium (YCC) 2002]. The DNA markers used in this study were as follows: YAP (Hammer and Horai 1995), 12f2 (Blanco et al. 2000), M213, M9, LLY22g, M122, M95, M134 (YCC 2002), SRY465, 47Z (Shinka et al. 1999), and RPS4Y711 (Tajima et al. 2002). Nomenclature system of the Y-haplogroup depended on the standard provided by the YCC. In this nomenclature system, when not all markers within a clade are typed, a bracketing system that encloses an "x" is used to show excluding branches. For example, the Y-haplogroup O3 excluding O3e, a branch of O3, is described as O3 (xO3e).

Plasmid construction

The entire coding regions of TBL1X, TBL1Y, and TBLR1 were obtained from cDNA derived from NT2/D1 cells. To introduce them downstream of GAL4DBD in pBIND inframe, SalI site-tagged primers were used for TBL1X and TBL1Y, and BamHI site-tagged and EcoRI site-tagged primers were used for TBLR1. The primers used for the TBL1 family were: for TBL1X, 5′-GTCGACTTATGACCGAGCTCGCTGGCGC-3′ and 5′-GTCGACACCCTACACATTCACGGCTG-3′; for TBL1Y, 5′-GTCGACTTATGAGCATAACCAGTGA-CGAG-3′ and 5′-GTCGACCCATGCATTCAAGG-CAGGTC-3′; and for TBLR1, 5′-CGCGGATCCG-TATGAGTATAAGCAGT-3′ and 5′-CCGGATATC-GCTATGTACACATTC-3′. The authenticity of each construct was confirmed by sequencing.

Western blot analysis

Proteins were extracted from the cells, with each plasmid expressing the GAL4DBD-fused TBL1 family 48 h after the transfection. They were separated by 10% SDS polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane using a semidry blotting system. Primary antibody raised against GAL4DBD (Santa Cruz Biotechnology, CA, USA), which is a monoclonal antibody derived from mice, and secondary antibody, an antimouse horseradish peroxidase conjugate (Amersham Biosciences Corporation, Piscataway, NJ, USA), were diluted 1:1,000 and 1:10,000, respectively. They were used for probing the blot, and GAL4DBD-fused proteins were detected with ECL plus (Amersham Biosciences) according to the manufacturer's directions.

Transfection of plasmid DNA and dual luciferase assays

The NT2/D1 cells were maintained in 24-well plates in DMEM supplemented with 10% FCS. One hundred twenty-five nanogram of pG5luc-reporter plasmid (Promega, Madison, WI, USA) and 75 ng of pBIND or pBIND-TBL1 were cotransfected into the cells with FuGENE6 transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's directions. The dual luciferase assay was carried out using a Pichagene luciferase kit (Toyoinki, Japan) according to the manufacturer's instructions. Assays were repeated at least three times.

Results

Expression analysis of the TBL1 family

To examine the expression pattern of the TBL1 family in various tissues, we carried out RT-PCR with primer sets

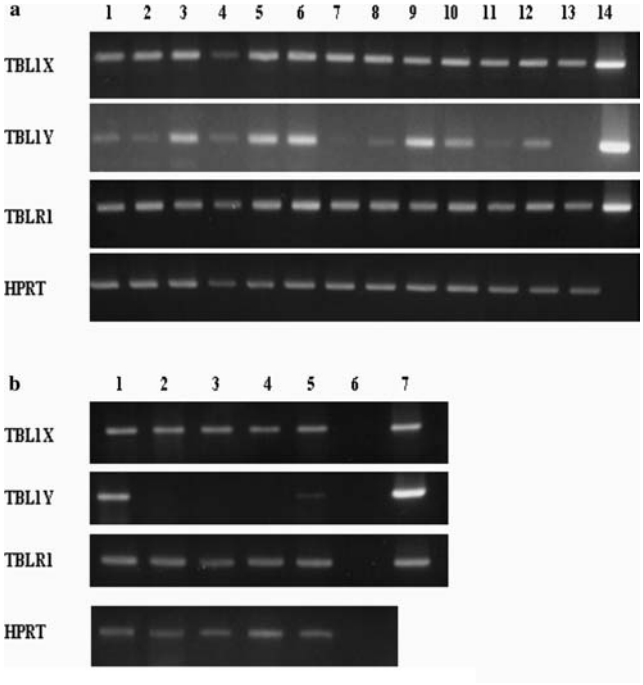

Fig. 1 The RT-PCR analysis of the TBL1 family in various human tissues (**a**) and in several human cell lines (**b**). **a** *Lane 1* heart, *lane 2* brain, *lane 3* liver, *lane 4* skeletal muscle, *lane 5* kidney, *lane 6* pancreas, *lane 7* spleen, *lane 8* thymus, *lane 9* prostate, *lane 10* testis, *lane 11* intestine, *lane 12* colon, *lane 13* leukocyte. *lane 14* cloned cDNA of each member of the TBL1 family. **b** *Lane 1* NT2/D1, *lane 2* fibroblast, *lane 3* HCT116, *lane 4* HUVEC, *lane 5* lymphoblast, *lane 6* water, *lane 7* cloned cDNA of each member of the TBL1 family. Specific primers for hypoxanthine guanine phosphoribosyl transferase (*HPRT*) were used to confirm the quantity of each sample of cDNA

specific for each member of the TBL1 family. TBL1Y was found to be widely expressed, though not in lymphocytes, while TBL1X and TBLR1 were expressed in all tissues tested (Fig.1a). Moreover, we performed RT-PCR using several cell lines and found that the TBL1Y gene was only expressed in the NT2/D1 cells, which were originally derived from human testicular embryonal cell carcinoma and lymphoblasts transformed with EB virus (Fig.1b).

Functional analysis of TBL1Y

The TBL1Y is highly homologous with TBL1X and TBLR1 in amino acid sequence (Fig.2a). However, since even slight differences between isoforms can result in dramatic differences in function, we examined whether there are functional differences between TBL1Y and other members of the TBL1 family. We generated expression vectors for GAL4 DNA-binding domain-fused proteins of the TBL1 family and introduced them into NT2/D1 cells together with a luciferase reporter plasmid that harbors 5×GAL4 binding elements in the promoter region. The GAL4DBD-fused TBL1X and TBLR1 repressed the promoter activity of the reporter gene by up to 20% while GAL4DBD-fused TBL1Y showed no repressive activity (Fig.2b). To confirm the expression of the GAL4-fused TBL1 family, we carried out a Western blot analysis with a monoclonal antibody against GAL4DBD. This monoclonal antibody detected specific bands for each GAL4DBD-fused TBL1 family (Fig.2c). GAL4DBD-fused TBL1X was detected, as expected. However, GAL4DBD-fused TBL1Y, which has higher calculated molecular weight than GAL4DBD-fused TBLR1, showed greater mobility than GAL4DBD-fused TBLR1 due to unknown reasons

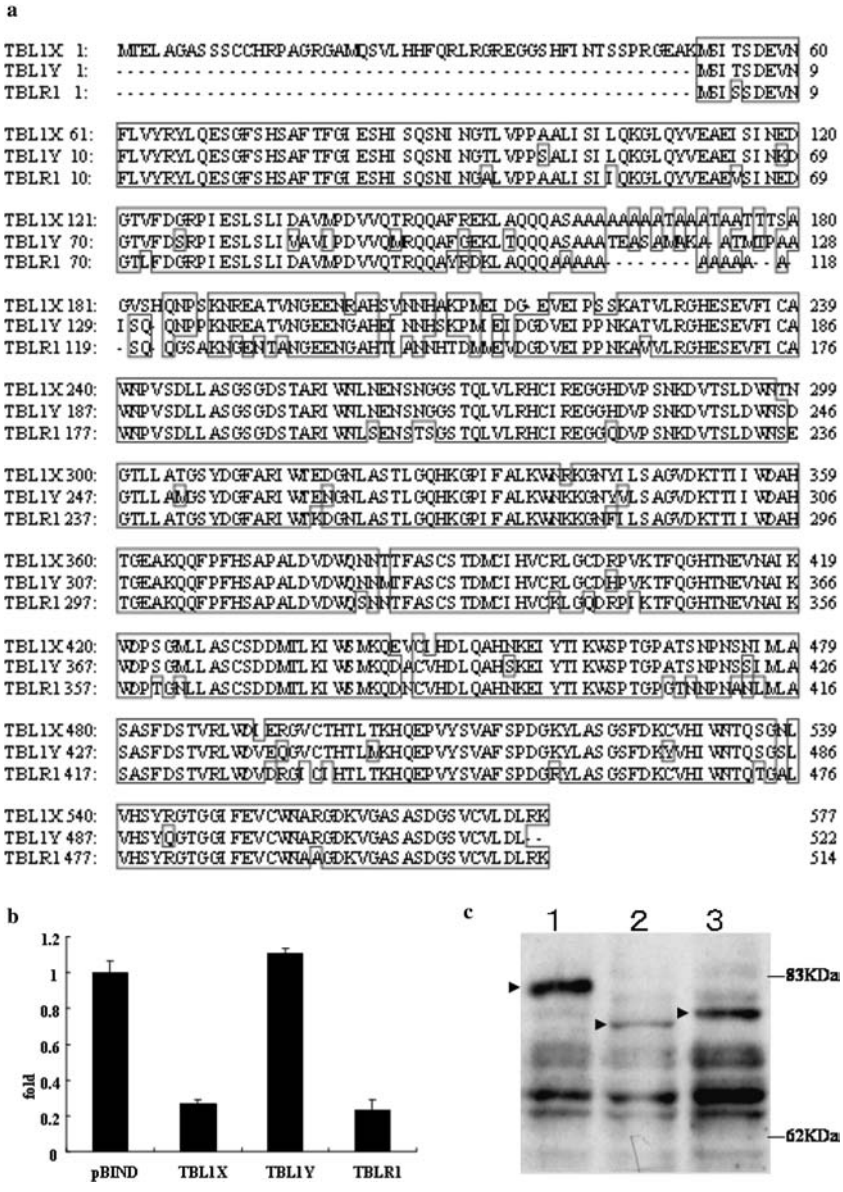

Fig. 2 Structural and functional differences between TBL1Y and other members of the TBL1 family. **a** Comparison of amino acid sequences for the TBL1 family. *Boxes* show amino acids conserved among at least two members of the TBL1 family. *Numbers* indicate position from the initial methionine. **b** Dual luciferase assay with GAL4DBD-fused TBL1 proteins. Relative firefly luciferase activity normalized with Renilla luciferase activity is shown with standard *error bars*. Each experiment was carried out at least three times. **c** Western blot analysis of GAL4DBD-fused TBL1 proteins with monoclonal antibody against GAL4DBD. *Lane 1* TBL1X, *lane 2* TBL1Y, *lane 3* TBLR1. *Arrows* show specific bands corresponding to each member of the TBL1 family. Several nonspecific bands were observed in addition to the specific ones. Those nonspecific bands were also detected for the NT2/D1 cells transfected with pBIND empty vector (data is not shown)

(Fig.2c). This deviation of mobility was observed in similar experiments with NT2/D1 cells and COS1 cells (data is not shown).

Screening for SNPs in the TBL1Y gene

To search for SNPs in the TBL1Y gene, we set 14 amplicons that cover all presumed coding exons. The primers used in this study are listed in Table 1. When we analyzed the PCR products generated with the primer set for TBL1Y3F and TBL1Y3R—which cover exon 7 with the exon–intron boundaries—using a DHPLC system, four different chromatograms were obtained. To identify the polymorphisms, we directly sequenced each PCR product that gave different chromatograms. As a result, three different SNPs were identified. The first polymorphism, IVS7 + 1G > C, changed gt to ct in the consensus sequence in the splicing donor site of intron 7 of the TBL1Y gene. The second polymorphism, IVS7 + 9G > A, was located 7 bp downstream from the splicing donor site within intron 7. The third polymorphism, which is a transversion of G-to-C (G268C) presumed to change aspartate to histidine at the 90th amino acid, was located 190 bp upstream of the splicing donor site within intron 7.

To confirm that three base substitutions exist in the TBL1Y gene, the PCR products were digested with the restriction enzymes, Dra *III*, ApaL *I* and Nla *III* for IVS7 + 1G > C, IVS7 + 9G > A and G268C, respectively. The patterns of digestion for each polymorphism were as expected (Fig.3). Furthermore, we revealed that lineage of the Y chromosome have the three novel polymorphisms in the TBL1Y gene. We have already classified the Y chromosomes in Japanese men into 11 haplogroups using 10 biallelic DNA markers and determined the frequencies of each haplogroup (unpublished data). The pooled PCR products of TBL1Y3, which were derived from different Y chromosome lineages, were subjected to a DHPLC analysis. The polymorphisms were all found in Y-haplogroup O3 (xO3e), which is defined as the Y-haplogroup O3 excluding O3e, a branch of O3. A total of 80 Y chromosomes harboring Y-haplogroup O3 (xO3e) were analyzed with PCR-RFLP. It became clear that the frequencies of IVS7 + 1G > C, IVS7 + 9G > A, and G268C in Y-haplogroup O3 (xO3e), roughly estimated to be 10% in the Japanese population in our unpublished study, were 6.3, 2.5 and 2.5%, respectively. Furthermore, it was postulated that those polymorphisms had occurred independently on the Y chromosome within Y-haplogroup O3 (xO3e) since we could find no males who simultaneously had two polymorphisms out of the three.

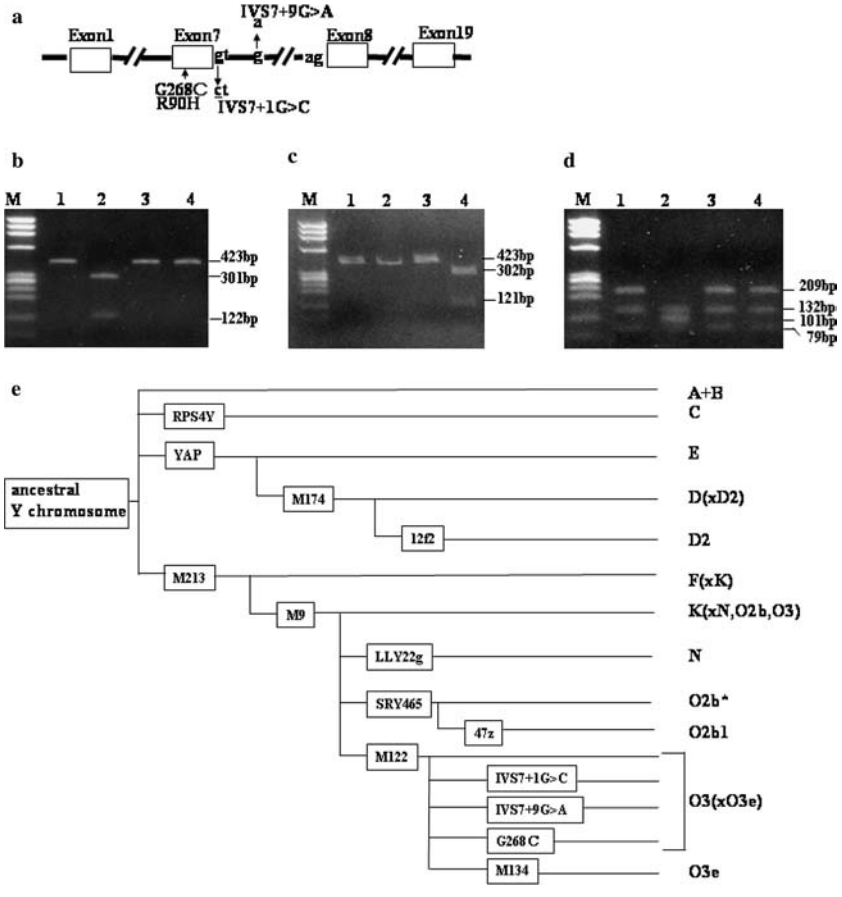

Fig. 3 Novel polymorphisms in the TBL1Y gene. **a** Schematic representation of the exon–intron organization of the TBL1Y gene and locations of the polymorphisms. **b–d**. Examples for PCR-RFLP analysis of the novel polymorphisms found in the amplicon TBL1Y3 in the TBL1Y gene. *b, c*, and *d* show Dra *III*, ApaL *I*, and Nla *III* digestion of the PCR products for IVS7 + 1G > C, IVS7 + 9G > A, and G268C, respectively. *M* DNA marker, *lanes 1–4* males with haplogroup O3 (xO3e). **e** Relationship between Y-haplogroup and the three novel polymorphisms. When not all biallelic DNA markers within a clade are typed, a bracketing system that encloses an "x" is used to show excluding branches (see "Materials and methods")

Discussion

TBL1Y differs from other members of the TBL1 family in its expression

The fact that partial deletion of the TBL1X gene is related with X-linked late-onset sensorineural deafness indicates that TBL1Y cannot compensate for a lack of TBL1X, at least for sensorineural hearing, suggesting that TBL1Y and TBL1X differs in their pattern of expression or function (Bassi et al. 1999). Although mouse Tbl1, which is located on the X chromosome and an ortholog of TBL1X, is expressed in the cochlear, we do not know whether TBL1Y is expressed in the cochlear (Bassi et al. 1999). In the present study, we showed that the expression pattern differed between TBL1Y and TBL1X in lymphocytes and several cell lines. The cis-elements in the promoter region that affect gene expression may be different between TBL1Y and TBL1X because these two genes have their own expression profile.

TBL1Y is functionally different from other members of the TBL1 family

It has been shown that TBL1X and TBLR1 are involved in N-CoR or SMRT corepressor complexes, suggesting that they contribute to transcriptional repression (Guenther et al. 2000; Li et al. 2000; Yoon et al. 2003). However, Perissi et al. (2004) recently showed that TBL1X and TBLR1 are required for not only transcriptional repression but transcriptional activation for some nuclear receptors and some transcription factors. They suggested that the TBL1 family is involved in corepressor/coactivator exchange for transcriptional regulation, at least in some cases. They also showed that the functions of TBL1X and TBLR1 are not the same in transcriptional regulation. In the present study with GAL4DBD-fused protein, we showed that, unlike other members of the TBL1 family, TBL1Y could not repress transcriptional activity for the major late promoter of adenovirus, suggesting that TBL1Y differs from other members of the TBL1 family in transcriptional function. Yoon et al. (2003) showed that the N-terminal portions of TBL1X and TBLR1 are crucial for transcriptional repression. Although most differences between TBL1X or TBLR1 and TBL1Y occur in an alanine-rich region of the N terminal (see Fig.2a), so far, it remains unclear what causes the functional difference observed among the TBL1 family.

In the present study, we analyzed the TBL1 family in the point of view of the transcriptional repression. The WD40 repeats in the C-terminal portion are highly conserved among the TBL1 family. It is known that some WD40 repeats can often interact with various proteins and can work as the adaptor domain (Neer et al. 1994; Thornton et al. 2004). Therefore, WD40 repeats of the TBL1 family may interact with proteins other than N-CoR and SMRT, and the TBL1 family involving TBL1Y may have other functions than transcriptional repression.

Functional relationships between the X and Y homologues

Genes that evade X inactivation often have homologues located on the Y chromosome. The RPS4X and RPS4Y as well as DDX3X (DBX) and DDY3Y (DBY) have been shown to be interchangeable with either protein in the pair, rescuing a mutant hamster cell line that was otherwise incapable of growth at modestly elevated temperatures (Watanabe et al. 1993; Sekiguchi et al. 2004). For other genes with homologues on the X and the Y chromosomes, there has been no direct attempt to assess functional consistency. For the TBL1 gene, as we show in this study, functions of the X and Y homologues may not be equal. It would be intriguing to determine whether transcription of the TBL1X gene per X chromosome is twofold greater in males than in females.

Significance of polymorphisms in the TBL1Y gene

In the present study, we found three novel polymorphisms in the TBL1Y gene and that they were on the Y chromosome with Y-haplogroup O3 (xO3e), which is defined as the Y-haplogroup O3 excluding O3e. Among those polymorphisms, IVS7 + 1G > C is presumed to cause a splicing error, probably leading to a nonfunctional protein. Santos et al. (1998) reported two males from Sri Lanka in whom recombination between TSPYA and TSPYB resulted in a deletion of AMELY and maybe TBL1Y although the phenotypical change was unclear. Deletion of TBL1Y does not directly lead to serious diseases. However, it may be that genetic variations in the TBL1Y gene contribute to a variety of phenotypes among maleness in combination with other genetic backgrounds. It is worth pursuing the relationships between male phenotype and those polymorphisms.

Now, it is difficult to assess whether TBL1Y is a pseudogene. However, there is a possibility that the TBL1Y gene may be becoming a pseudogene since it has the polymorphism disrupting the conserved splice donor site and loss of the activity for transcriptional repression found in other members of the TBL1 family. Intriguingly, the TBL1Y gene has its obvious pseudogene located on the Y chromosome (NG_002819). The TBL1Y gene contains all coding exons corresponding to ones of the TBL1X gene while the pseudogene does not.

In conclusion, we showed that TBL1Y differs from TBL1X and TBLR1 in expression pattern and function. Furthermore, we demonstrated genetic variation in the TBL1Y gene of Japanese males. The TBL1Y may contribute to the variation in male-specific phenotypes.

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