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Isolation and Characterization of a New Peroxisome Deficient CHO Mutant Cell Belonging to Complementation Group 12

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Abstract

We searched for novel Chinese hamster ovary (CHO) cell mutants defective in peroxisome biogenesis by an improved method using peroxisome targeting sequence (PTS) of Pex3p (amino acid residues 1–40)-fused enhanced green fluorescent protein (EGFP). From mutagenized TKaEG3(1–40) cells, the wild-type CHO-K1 stably expressing rat Pex2p and of rat Pex3p(1–40)-EGFP, numerous cell colonies resistant to the 9-(10-pyrene) nonanol/ultraviolet treatment were grown. These colonies were examined for intracellular location of Pex3p(1–40)-EGFP. By this method, we have isolated one CHO cell mutant, ZPEG403, which was found to belong to complementation group G (CG-G). Expression of the human peroxin, Pex3p cDNA encoding a 373-amino-acid peroxisomal membrane protein morphologically and biochemically restored peroxisome biogenesis, including peroxisomal membrane assembly, in ZPEG403 cells. Mutation and genomic DNA PCR analyses showed that, the dysfunction of Pex3p in ZPEG403, was due to one base (A) substitution in place of (G) in the first base of splicing site at the boundary of exon 6 and intron 6 of PEX3 gene, giving rise to remaining of all of intron 6, thereby inducing 81 bp insertion between positions 523–524 of PEX3 ORF, resulting in deletion of 200 amino acid residues from the C-terminus of Pex3p and a frame shift inducing both 18-amino-acid substitution and an early termination codon.

Keywords: CHO cell mutant; Peroxisome biogenesis; Peroxin; PEX3

Introduction

Peroxisome is a single membrane-bounded organelle present in eukaryotes and functions in various metabolic pathways, such as β-oxidation of very-long-chain fatty acids and the synthesis of ether lipids [1]. Peroxisomal

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matrix and membrane proteins are synthesized on free polyribosomes in the cytoplasm and post-translationally imported into peroxisomes [2,3]. Two distinct topogenic sequences, peroxisomal targeting signal types, 1 (PTS1) and 2 (PTS2) [3], direct newly synthesized proteins to the peroxisomal matrix. PTS1 is the C-terminal tripeptide SKL motif [4,5] and PTS2 comprises N-terminal cleavable presequence containing a nonapeptide with the conserved sequence, (R/K)(L/V/I)X5(H/Q)(L/A) [6,7]. A number of PEX genes encoding peroxins are essential for peroxisome biogenesis [8-10]. Peroxisomal functions are highlighted by human fatal genetic, peroxisomal biogenesis disorders (PBDs) are autosomal recessive and include thirteen different known genotypes with three distinct phenotypes, Zellweger syndrome (ZS), neonatal adrenoleukodystrophy, and infantile Refsum disease [8,10,11]. The pathogenic PEX genes responsible for all CGs of PBDs were recently cloned and characterized [3,8,9]. To delineate the mechanism of peroxisome biogenesis, a potential approach using mammalian somatic cell mutants has been successful [12]. Peroxisome assembly-defective Chinese hamster ovary (CHO) cell mutants are indeed proven to be useful for investigating molecular and cellular mechanisms involved in peroxisome biogenesis and for delineation of the primary defects of PBD. Thus CHO cell mutants defective in peroxisomal biogenesis were isolated by colony autoradiographic screening [13] and the 9-(10-pyrene)nonanol (P9OH)/UV selection method [14]. Here we attempted to isolate more CGs of peroxisome biogenesis-defective animal cell mutants, by a modified method using ‘‘enhanced’’ green fluorescent protein (EGFP). We report here a pex3 mutant, ZPEG403, isolation with a novel mutation.

Abbreviations: CG, complementation group; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein; MDH, malate dehydrogenase; MNNG, N-methyl-N’-nitro-N-nitrosoguanidine; PBD, peroxisome biogenesis disorder; PMP70, Peroxisomal membrane protein 70kDa; P9OH/UV, 9-(19-pyrene) nonanol/ultraviolet; PTS1 and PTS2, peroxisome-targeting signal 1 and 2; RT, reverse transcription; thiolase, 3-ketoacyl-CoA thiolase.

Materials and Methods

Cell lines and Selection of Peroxisome-Deficient CHO Cell Mutants

CHO cells were grown in Ham’s F12 medium (Gibco BRL, USA), supplemented with 10% fetal calf serum. CHO-TKa cells, wild-type CHO-K1 cells transfected with rat PEX2 cDNA [15,16], were transfected with pUcD2Hyg/(1-40aa)Pex3-EGFP [17]. Stable transformant, termed TKaEG3(1–40) cells [17], was selected in the presence of 200 μg/ml of hygromycin B (Sigma, USA), and transfectants highly expressing EGFP were cloned by the limiting dilution method. (1-40aa) Pex3-EGFP [17], which were superimposable on those stained with anti-catalase antibody in respective cells (data not shown), was indicative of localization of (1-40aa)Pex3-EGFP in peroxisomes [17]. TKaEG3 (1–40) cells were mutagenized with N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) (Nacalai Tesque, Kyoto, Japan) and selected for cell mutants resistant to the P9OH/UV treatment, as described [18,19]. P9OH is incorporated into plasmalogens at an early step of synthesis and produces active oxygen on UV irradiation [14]. Cell culture in the presence of P9OH, followed by a short exposure to UV, kills wild-type CHO cells, but not peroxisome-defective mutants. Viable cell colonies examined for peroxisome morphology by virtue of localization of Pex3p(1–40)-EGFP as described (Fig. 1) [17,18].

Morphological Analysis

(1-40aa)Pex3-EGFP in cells grown on cover glass was observed without cell fixation, under a Carl Zeiss Axioskop FL microscope using a No.17 filter (Oberkochen, Germany) [17,18]. Peroxisomes in CHO cells were also visualized by indirect immunofluorescence light microscopy. For indirect immunofluorescence staining, cells were fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 30 min, and then washed with 1% bovine serum albumin in PBS for 10 min. Primary and secondary antibodies were diluted in PBS solutions,

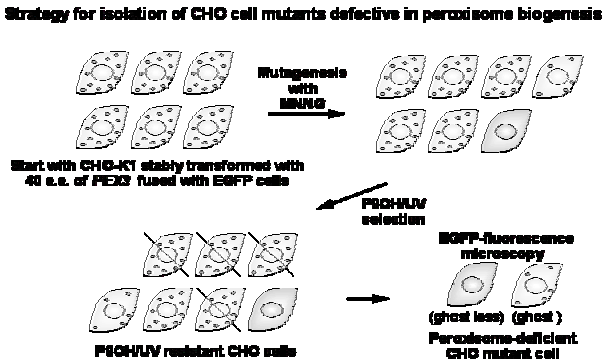

Figure 1. Strategy for isolation of CHO cell mutants defective in peroxisome biogenesis.

incubated with cells for 60 and 90 min respectively at room temperature [20]. Antigen–antibody complex was detected under a Carl Zeiss Axioskop FL microscope. Primary antibodies used were rabbit antibodies to 3-ketoacyl-CoA thiolase [21], PTS1 peptide [15], rat 70-kDa peroxisomal integral membrane protein (PMP70) [16] and malate dehydrogenase (MDH) [22]. Secondary antibody used for immunofluorescence staining was fluorescein Texas Red-conjugated goat antibody against rabbit IgG (Leinco Technologies, USA).

DNA Transfection and Cell Fusion

Transfection of *PEX* cDNAs to CHO mutant cells was done with 1 µg of cDNA, using 12 µg of Lipofectamine (Invitrogen, Sweden), as described [6]. The cells were cultured for 2 days and then incubated overnight in 2 ml of serum-free F12 medium before immunostaining. For cell fusion, two types of CHO cells to be examined were cocultured for 24 h and then fused using polyethylene glycol, as described [20]. Complementation of cell mutants was assessed by peroxisomal punctuate localization of (1-40aa)Pex3-EGFP [17].

Mutation Analysis

Total RNA was obtained from CHO-K1 and ZPEG403cells with an RNeasy Mini-kit (Qiagen, USA). Reverse transcription (RT)-PCR was done with Superscript reverse transcriptase (Gibco-BRL, USA), 6 □g of total RNA and 18-mer oligo-dT primer (Amersham Biosciences, USA). To abtain ZPEG403-derived PEX3 cDNA, PCR was performed using Ex Taq DNA polymerase (Takara, Japan) and a pair of specific primers used, were ClPEX3 Full F: 5'CAGAGTCTGAAGATGCTGAGATCAATG3' and ClPEX3 Full R: 5'TCTTCTTGAAGGAAGAAAGTC ATTTCTCCAGTTGTTG3' [20]. The RT-PCR products were cloned into the pGEM-T Easy vector (Promega, USA) and sequenced in both strands by the dideoxy-chain termination method using a Big Dye-terminator DNA sequence kit (Applied Biosystems, USA). To search for both a mutation site in the intron and zygosity of ZPEG 403 derived PEX3 mutant allele, genomic PCR with a pair of PEX3-specific primers-forward primer ClPEX3 Exon6F (5'GCTCCACCAGAT GTACAGCAGCAGTAT3') and reverse primer ClPEX3 Exon7R (5'TCCTAAAATCCTCTGTACAGC TTG3') were used to amplify the sequence between residues 465 and 575, encompassing the entire length of intron 6.

Other Methods

Western blot analysis was carried out with primary antibodies, including anti-AOx [15], anti thiolase and anti PMP70 antiserum, and a second antibody, donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, USA), using ECL Western blotting detection reagent (Amersham Pharmacia Biotech, USA).

Results

Isolation and Morphological Analysis of ZPEG403

TkaEG3(1-40) cells, CHO-K1 cells stably expressing Pex2p and (1-40aa)Pex3-EGFP, were mutagenized with MNNG. Cell colonies resistant to the P9OH/UV treatment were examined for intracellular localization of EGFP (Fig. 1). By this method, we isolated one peroxisome-deficient mutant, named ZPEG403 (Fig. 3a). (1-40aa)Pex3-EGFP was mainly traced to be mislocalized in mitochondrial structures as colocalized with MDH, as a mitochondrial marker in a super-imposable manner (Fig. 2). To assess the deficiency of peroxisome biogenesis, cell mutants were stained with several antibodies to peroxisomal matrix and membrane proteins. In ZPEG403 cells, PTS1 containing matrix proteins and thiolase (a PTS2 protein), were detected as diffused staining patterns at (Fig. 3b, c respectively), indicating the cytosolic localization of peroxisomal matrix proteins. ZPEG403 cells were then stained with antiserum against PMP70, an abundant protein in peroxisomal membranes [23], which showed a diffuse pattern in the cytoplasm (Fig. 3d), thus suggesting a defect in transport of peroxisomal membrane protein. A phenotype similar to previously isolated CHO mutants, ZP119 [16] and ZPG208 [20], was seen, due to a defect in transport of peroxisomal membrane protein in this mutant. Thus ZPEG403 devoid of peroxisomal remnant structures so called "ghost vesicles" that were found in the other CHO mutants (Table 1).

Complementation Group (CG) Analysis

To classify CHO mutant, ZPRG403, isolated in this study, CG analysis was performed by PEX cDNA transfection. Thirteen different PEX cDNAs, including PEX1, PEX2, PEX3, PEX5, PEX6, PEX7, PEX10, PEX12, PEX13, PEX14, PEX16, PEX19, and PEX26 were separately transfected into cell mutants. Peroxisomal restoration of EGFP, was shown upon Human PEX3 cDNA [20] transfection (Fig. 3, e), as ZPG208 [20], there by suggesting that they are the same CG. In transient HsPEX3-transformant of ZPEG403,

Table 1. Complementation Group and Complementing Genes of Peroxisome Deficiency[1]

Human Fibroblast			Phenotype	CHO Mutants	Complementing
Japan	USA	Europe			Gene
A	VIII		ZS, NALD, IRD	ZP124[2]	*PEX26*
B	VII, (V)		ZS, NALD		*PEX10*
C	IV	3	ZS	ZP92	*PEX6*
D	IX	3	ZS		*PEX16*
E	I	2	ZS, NALD, IRD	Z24	*PEX1*
F	X	5	ZS, IRD	Z65	*PEX2*
	II	4	ZS, NALD	ZP105, ZP139, ZPG231	*PEX5*
	III		ZS, NALD, IRD	ZP109	*PEX12*
	VI		ZS, NALD		
G[3]	XII		ZS	ZPG208, ZPEG403	*PEX3*
H	XIII		ZS, NALD	ZP128	*PEX13*
J			ZS	ZP119	*PEX19*
R	XI	1	RCDP	ZPG207[4], ZPEG237[5], ZPEG231[5]	*PEX7*
I[6]			ZS	ZP110	*PEX14*
				ZP114	
				ZP126	
					PEX11

ZS: Zellweger Syndrome; NALD: Neonatal Adrenoleukodystrophy;
IRD: Infantile Refsum Disease; RCDP: Rhizomelic Chondrodysplasia Punctata.
[1]Ref. No. 8, [2]Ref. No. 9, [3]Ref. No. 28, [4]Ref. No. 29, [5]Ref. No. 30, [6]Ref. No. 31

PTS1 proteins were noted in numerous vesicular structures, presumably peroxisomes, when stained with antibodies to PTS1 (Fig. 3, f), and 3-ketoacyl-CoA thiolase, a PTS2 protein (Fig. 3, g). Numerous PMP70-positive particles were detected in transiently HsPEX3 transfected ZPEG403 cells (Fig. 3, h), strongly suggested that these cells had morphologically normal peroxisomes. We next verified the biogenesis of peroxisomal proteins in ZPEG403 cells. PMP70 was not discernible in ZPEG403 cells (Fig. 4, bottom panel, lane 2), presumably because of a rapid degradation, as seen in the CHO pex19 mutant ZP119 [16], although PMP70 was detectable in normal CHO-K1 cells (lane 1). When ZPEG403 cells were transiently transfected with HsPEX3, PMP70 was detected (lane 3), indicating that the impaired biogenesis of PMP70 was restored. Peroxisomal 3-ketoacyl-CoA thiolase of fatty acid β-oxidation system is synthesized as a larger, 44-kDa precursor with an amino-terminal presequence cleavable PTS2 [6,7] and is processed to 41-kDa mature form in peroxisomes [24]. In CHO-K1 cells, only the matured thiolase was detected (Fig. 4, middle panel, lane 1), thereby providing evidence for a rapid processing of the precursor form. In ZPEG403 cells, only the larger precursor was detectable (Fig. 4, middle panel, lane 2). When ZPEG403 cells were transiently transfected with HsPEX3, the mature thiolase was clearly discerned (Fig. 4, middle panel, lane 3). A PTS1 protein, acyl-CoA oxidase (AOx), is synthesized as a 75-kDa polypeptide (A component) and is proteolytically converted into 53- and 22-kDa polypeptides (B and C components, respectively) in peroxisomes [21,25,26]. All three polypeptide components were evident in normal control cells (Fig. 4, top panel, lane 1), whereas A component

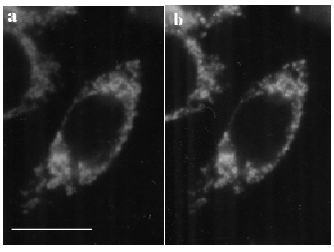

Figure 2. Mislocalization of Pex3p(1–40)-EGFP chimera to mitochondria in ZPEG403. (a) Fluorescence microscopy of Pex3p(1–40)-EGFP. (b) ZPEG403 cells stained with antibody to malate dehydrogenase. Bar, 20 nm.

was only detectable in ZPEG403 cells, indicating cytosolic retention of AOx (Fig. 4, top panel, lane 2). When ZPEG403 cells were transfected with HsPEX3, the three components of AOx were detected at a distinct level, indicative of proper import and proteolytic conversion of AOx (Fig. 4, top panel, lane 3). These results demonstrate that HsPEX3 can complement the impaired biogenesis of peroxisomal proteins in ZPEG403 cells. Moreover mutant cell classification was examined by cell fusion analysis with combination of two CHO cell lines. When resulted hybrid cells produced EGFP positive particles indicating that the respective pair of mutants belonging to the different CG as was the case for ZPEG403 cell fusion with, pex2 mutant (Table 1), Z65, (Fig. 5, a). Conversely, no EGFP containing structures, peroxisomes, were noted in hybrid cells of mutants belonging to the same CG in good agreement with the results seen by cDNA transfections in corresponding CG CHO mutants as seen in fused cell of ZPEG403 with ZPG208 (Data not shown). Likewise peroxisomes were not complemented in homologous hybrids of ZPEG403 cells (Fig. 5, b).

Mutation Analysis of ZPEG403 Cells

To investigate the genetic cause of the dysfunction of PEX3 in ZPEG403 cells, we performed RT-PCR using total RNA and PEX3-specific primers was detected in all seven cDNA clones isolated, indicating a homozygous mutation. Inactivating mutation was 81 residues insertion between positions 523–524 resulting in a frame shift inducing both 18-amino-acid substitution and a termination codon (Fig. 6A). To search for a mutation site in the intron, genomic PCR with a pair of PEX3-specific primers was used to amplify the sequence of intron 6 in the ClPEX3 gene which was suggested to be resembled with HsPEX3 gene (GenBank accession number AJ009873) [27,28]. In ten independent clones isolated from PCR products, we identified a single type of nucleotide sequence, which, apparently, gave rise to an insertion of intron 6: G was mutated to A at first base of the splicing site at the boundary of exon 6 and intron 6 (Fig. 6B, right). These observations probably mean that ZPEG403 cells, were a homozygote for the G to A mutation, causing the remaining of intron 6 and inactivation of PEX3. This mutation inactivated the function of PEX3.

Discussion

We have already shown that 1-40 amino acid residues from the N-terminus of Pex3p are sufficient for localization of Pex3p to peroxisomal membranes as

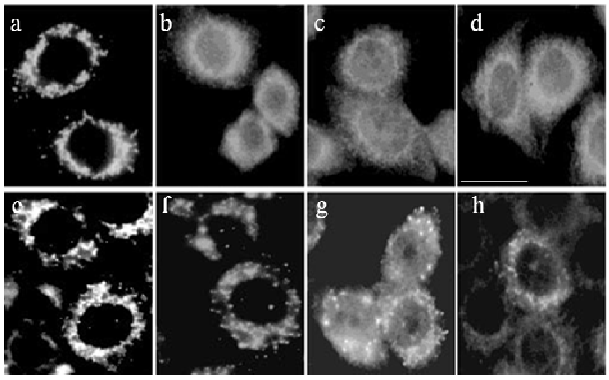

Figure 3. Immunostaining of ZPEG403 and *HsPEX3*-transfected ZPEG403. (a, e) Fluorescent micrograph of EGFP-expressing ZPEG403 and *HsPEX3*-transfected ZPEG403 cells respectively. (b, c, d) ZPEG403cells stained with rabbit antisera to PTS1, thiolase and PMP70 respectively. (f, g, h) *HsPEX3*-transfected ZPEG403cells stained with antibodies against PTS1, thiolase and PMP70 respectively. Bar, 20 nm.

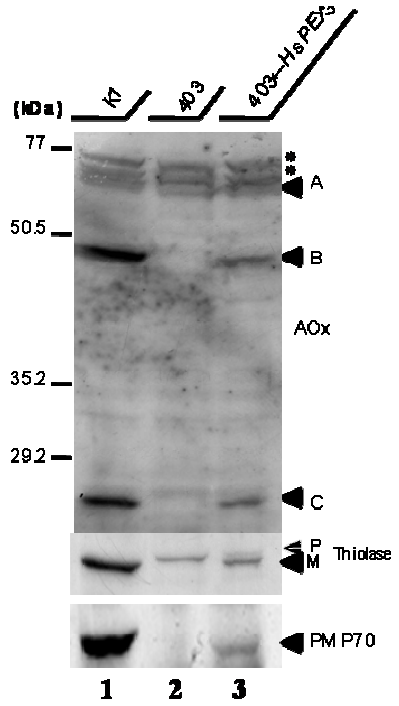

Figure 4. Complementation of biogenesis of peroxisomal proteins. Total cell lysates from CHO-K1, ZPEG403, and *HsPEX3*-transfected ZPEG403 cells were subjected to SDSPAGE and transferred to a polyvinylidene difluoride membrane. Immunoblot analysis was done using rabbit antibodies to peroxisomal AOx (upper panel), thiolase (middle panel), and PMP70 (lower panel). Cell types are indicated at the top. Arrows show AOx components A–C; P and M designate a larger precursor and mature protein of thiolase, respectively. Open arrowheads indicate the thiolase precursor; the arrowhead shows PMP70.

Pex3p (1–40)-GFP was targeted to peroxisomes in the wild-type CHO-K1 cells [20]. In present work, by introduction of Pex3p(1–40)-EGFP that would make its intracellular location readily visible under fluorescent microscope without cell fixation, thereby highly accelerating the mutant screening step [17,18], we isolated and characterized a peroxisome-deficient CHO cell mutant, ZPEG403. ZPEG403 cells showed a morphological phenotype manifesting a defect in import of PTS1-proteins and thiolase. Moreover (1-40aa)Pex3p-EGFP was mainly mislocalized in mitochondrial structures in ZPEG403 cells implying that peroxisomal remnants, ''ghosts,'' were absent in this kind of CHO cell mutants [29,30], and thus membrane protein transport was also affected. ZPEG403 cells were restored for peroxisome assembly, by both morphological and biochemical criteria after *PEX3* transfection. We delineated that the mutated form of *PEX3* in the CHO mutant, ZPEG403, was 81 residues insertion between positions 523–524 of *PEX3*. Therefore, the method modified in the present work was indeed proven to be highly efficient and useful for isolating peroxisome biogenesis-defective mutant cells. In addition to a single, currently used pex3 mutant ZPG208 expressing PTS2-GFP [20], ZPEG403 may serve as a model mammalian cell system where a cargo Pex3p (1–40)-EGFP is more readily detectable. Moreover, this pex3 mutant also will be useful to study the function and dysfunction of Pex3p, a factor for peroxisome membrane integrity at molecular and cellular levels.

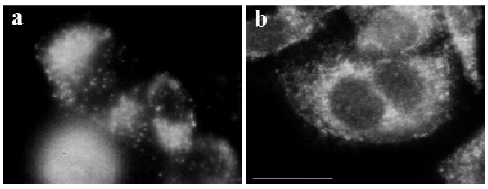

Figure 5. Complementation group analysis of ZPEG403 cells by Cell fusion. Cell mutants were pairwise fused: (a) Z65 cells (*pex2* CG) fused *pex3* CG mutant cells, ZPEG 403. (b) cell hybrids of ZPEG403 (*pex3* CG) with ZPEG403 (*pex3* CG). Fused cells were assessed by EGFP fluorescence. Bar, 20 mm.

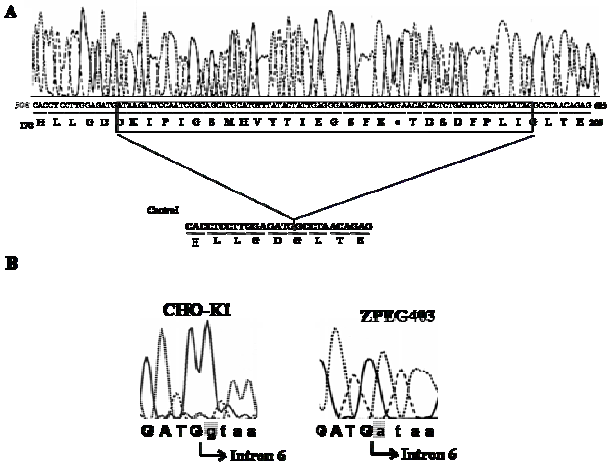

Figure 6. Impaired *PEX3* in ZPEG403 cells. A) Mutation analysis of *PEX3* in ZPEG403. Partial sequence and deduced amino acid sequence of *PEX3* cDNA isolated from CHO-K1 (lower panel) and a mutant ZPEG403 (upper panel) are shown. 81 distinct bp insertion between positions 523–524 of *PEX3*cDNA (boxed) was identified. B) Genomic PCR of *PEX3* in ZPEG403. PCR was of also done for DNA from a normal control (left panel) and from ZPEG403 cells (right panel); the nucleotide sequence of PCR products was determined. Only partial sequence at the boundary of exon 6 and intron 6 is shown; one base (A) in place of (G) is substituted in the first base of splicing site at the boundary of exon 6 and intron 6 of *PEX3* gene in ZPEG403 cells.

Acknowledgments

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