Gene 455 (2010) 1–7

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Analysis of relative gene dosage and expression differences of the paralogs *RABL2A* and *RABL2B* by Pyrosequencing

Marcel Kramer [a,1], Oliver Backhaus [a,1], Philip Rosenstiel [b], Denise Horn [c], Eva Klopocki [c], Gerd Birkenmeier [d], Stefan Schreiber [b], Matthias Platzer [a], Jochen Hampe [e], Klaus Huse [a,*]

[a] *Leibniz Institute for Age Research—Fritz Lipmann Institute, Genome Analysis, Beutenbergstr. 11, 07745 Jena, Germany*
[b] *Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Christian Albrechts University, Kiel, Germany*
[c] *Institute of Medical Genetics, Charite, University Medicine of Berlin, Germany*
[d] *Institute of Biochemistry, Medical Faculty, University of Leipzig, Leipzig, Germany*
[e] *Department of Internal Medicine I, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany*

ARTICLE INFO

Article history:
Received 30 October 2009
Received in revised form 25 January 2010
Accepted 25 January 2010
Available online 4 February 2010

Received by J.G. Zhang

Keywords:
Paralogs
RAB-like
Gene expression
Paralog ratio test
Pyrosequencing

ABSTRACT

The paralogous genes *RABL2A* (chr2) and *RABL2B* (chr22) emerged by duplication of a single gene in the human–chimpanzee ancestor and share a high degree of sequence similarity. In Phelan–McDermid-Syndrome microdeletions of 22q13 often also affecting*RABL2B* are of clinical importance but their incidence is still unknown. We analyzed a German population (190 individuals) for such aneuploidies and the paralogs' expression in cell lines by RABL2 paralogous sequence quantification. For determination of the genomic and transcriptional ratios of *RABL2A* and *RABL2B* a Pyrosequencing protocol was introduced as a high-throughput method. During PCR the 3′ end of the biotinylated strand is engineered by a backfolding oligonucleotide to hybridize in the Pyrosequencing reaction to an internal site near the sequence to be analyzed. In human samples no deviations of the euploid genomic state could be detected indicating that 22q13 microdeletions involving *RABL2B* are rare. However, despite equal gene dosage a preferential expression of *RABL2B* in human tissues and lymphoblastoid cell lines was detected which is most pronounced in brain and placenta. This renders a complete functional complementation of one paralog by the respective other unlikely and hints to a functional and clinical importance, in particular with respect to the 22q13 chromosomal deletion syndrome. Remarkably and in contrast to human, expression levels of the two paralogs in a chimpanzee cell line are equal. This finding is discussed in view of the relocation of *RABL2A* from its ancestral telomeric to its pericentromeric location in human.

1. Introduction

Two closely related human paralogous genes, *RABL2A* and *RABL2B* (RAB-LIKE 2A and 2B), have been identified at chromosomal locations 2q13 and 22q13.3 (Wong et al., 1999). They result from a very recent duplication (Wong et al., 1999; Martin et al., 2002) and share a high degree of sequence identity (>98%). The putatively coded proteins exhibit similarity with the RAS oncogene family (RAB). Although this large protein family of small GTPases attracts considerable interest for their manifold of functions and involvement in disease (Cheng et al., 2005; Wu et al., 2008) the *RABL2* genes and their putative products have not been studied in detail since their original description by Wong et al. (1999). This is the more surprising for *RABL2B* often falls within microdeletions associated with complex phenotypes such as mental retardation, impairment of speech and language development and autistic behavior (chromosome 22q13 deletion syndrome, Phelan–McDermid-Syndrome, OMIM #606232). The incidence of 22q13 microdeletions in the general population is still unknown (Phelan, 2008) mainly due to technical inconvenience of laborious methods such as FISH applied for their detection. A genetic screening for this cryptic subtelomeric chromosome rearrangement is routinely done for patients with mental retardation only. In a large population of 11,688 individuals with developmental inabilities Ravnan et al. (2006) detected 15 patients carrying 22q deletions. Two of them (13%) were de novo deletions underscroring the need for a routine screening independent and ahead of clinical conspicuousness. Although *RABL2B* is often affected in reported deletions showing phenotypic consequences, its contribution to patients' suffering has been questioned. Wong et al. (1999) argued that as both *RABL2A* and *RABL2B* are expressed and very close in sequence it seems to be unlikely that *RABL2B* is dosage sensitive. Also, Anderlid et al. (2002) consider *RABL2A* expression sufficient to compensate for a monosomic *RABL2B* deficiency. However, in their view, the high expression in fetal brain and the identification of a

Abbreviations: EBV, Epstein-Barr-Virus; ECACC, European Collection of Cell Cultures; FCS, fetal calf serum; IUPAC, International Union of Pure and Applied Chemistry; SD, standard deviation; TAE buffer, Tris-acetat-EDTA buffer.

* Corresponding author. Tel.: +49 3641 656254; fax: +49 3641 656255.
E-mail address: khuse@fli-leibniz.de (K. Huse).

[1] Both authors contributed equally to this work.

0378-1119/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2010.01.005

muscle-specific splice isoform could implicate a connection to the cognitive deficits and the muscular hypotonia found in patients with 22q13 deletions. A comparison of the relative expression of *RABL2A* and *RABL2B* in five tissues (Wong et al., 1999) revealed expression of both genes across all tissues tested. However, not all reported differences in expression levels of the two genes could be replicated in that study using paralog discrimination by restriction digest.

Another interesting aspect of the genetics of the *RABL2* paralogs is their evolutionary history. The duplication of the ancestral gene on chromosome 22 took place during hominid evolution only recently. Orangutan (*Pongo pygmaeus*) still represents the original situation with a single *RABL2* gene in its haploid genome (Martin et al., 2002). Thus, the gene duplication must have occurred after divergence of orangutan but before the split of human (*Homo sapiens*) and chimpanzee (*Pan troglodytes*). Chimpanzee retained the ancestral situation with two paralogs in the subtelomeric region of chromosomes 2b and 22. In human, the fusion of ancestral chromosomes 2a and 2b formed chromosome 2, finally placing *RABL2A* to its current pericentromeric position at 2q13. It is unknown whether the different localization of the human and chimpanzee paralogs affect their function and if so, contribute to phenotypic differences among the two primates.

The close relationship of the two paralogs offers the opportunity to use a paralog ratio test to evaluate both relative gene dosage as well as their relative expression. Gene dosage will be informative for unbalanced structural changes affecting the *RABL2B* locus within the subtelomeric region of the long arm of chromosome 22 or the *RABL2A* locus at chromosome 2, respectively. We aimed to develop a convenient high-throughput method for determination of RABL2A/B ratios by means of Pyrosequencing. Pyrosequencing has been widely used for sequencing short stretches of DNA and for the quantitative determination of nucleic acids (Ronaghi et al., 2007). On a genomic level it allows the evaluation of allele dosage/genotypes determined by single nucleotide polymorphisms or structural variations such as copy number variants (Langaee and Ronaghi, 2005; Huse et al., 2008) and it is also used to characterize gene expression (Wittkopp et al., 2008). In Pyrosequencing pyrophosphate is stoichiometrically split off from the deoxynucleoside triphosphates during polymerase reaction and initiates a reaction cascade leading to quantifiable light emission. Recently, Pyrosequencing was exploited for next-generation sequencing, too (Margulies et al., 2005).

For Pyrosequencing, templates must be single-stranded. After PCR amplification this is routinely achieved by avidin/streptavidin-mediated purification of the biotinylated strand by means of a biotin-labeled primer oligonucleotide. Subsequently, a sequencing primer is hybridized close to the sequence to be analyzed. The iterative addition of deoxynucleoside triphosphates then allows sequence detection.

Pyrosequencing is relatively straightforward but is also a laborious methodology. Purification of the single-stranded template and annealing of the sequencing primer are consecutive steps which, because intermittent washing is necessary each time, decelerate the assay. We therefore made an attempt to simplify the procedure of sample preparation by a specially designed backfolding oligonucleotide as PCR primer, thereby including the sequencing primer already during PCR amplification. Such a simplification is especially valuable for high-throughput assays for the evaluation of many samples, typically in biomedical screenings.

The ameliorated Pyrosequencing-based paralog ratio test was applied to the *RABL2* paralogs by exploiting sequence variations in order to determine genomic dosage and relative expression in human and chimpanzee lymphoblastoid cell lines and human tissues.

2. Materials and methods

2.1. Cell culture

Human EBV transformed lymphoblastoid cell lines GM10847, GM12760, GM12864, GM12870, GM12871, GM15215, GM15324, GM15386, GM18502, GM18552, GM18858, GM18972, GM19140, and GM19204 were obtained from the Coriell Cell Repository (Camden, USA). The chimpanzee cell line EB176(JC) was from ECACC (Salisbury, UK).

Cells were cultured at 37 °C in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 15% FCS (Gibco) and 2 mM L-glutamine (Gibco) in a 5% CO_2 atmosphere at 95% humidity.

2.2. Nucleic acids

Genomic DNA was isolated from blood of healthy, unrelated Caucasian volunteers (99 female and 91 male subjects) or from the indicated cell lines using the Blood & Cell Culture DNA Mini Kit (Qiagen, Hilden, Germany) according to standard protocols. Blood was collected at the University Hospital Kiel (Krawczak et al., 2006), and written consent was obtained from each participant. A genomic DNA reference sample of a patient with established 22q13 micro-deletion (22q13del) was obtained from the University of Berlin (patient characteristics as Supplementary material). Pooled genomic DNA originating from human blood of about 100 individuals was purchased from Roche (Mannheim, Germany). The 1 kb Plus DNA Ladder™ was purchased from Invitrogen (Karlsruhe, Germany).

Total RNA was isolated from the cell lines using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA first strand synthesis was performed with "Sprint RT Complete-Random Hexamer" cDNA synthesis kit (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) following the manufacturer's recommendations. Five milligram total RNA was used for reverse transcription.

Human tissue cDNAs were from Clontech (MTC Multiple Tissue cDNA Panels).

2.3. PCR amplification

Primers were designed using genomic reference sequences from the human (NCBI Build 36.1) and chimpanzee (Build 2 Version 1, Oct. 2005) assemblies to match completely both sequences.

Primer used for the classical Pyrosequencing approach: btRABL2ex89r (biotin-CTCAGCTGTGGGGAGAGG), and RABL2ex89f (CAATTCGATTAGCTGTGTC) for PCR, RABL2ex9fs (CAGCAGCATCGAGACCCCA) as sequencing primer.

Primer (bfOligo) used for the simplified Pyrosequencing approach: RABL2ex89f+s (TGGGGTCTCGATGCTGCAATTCGATTAGCTGTGTC) instead of RABL2ex89f.

Primers were from Metabion (Martinsried, Germany). Amplification was performed in 96-well PCR plates (ABgene, Epsom, UK) containing 25-µl reaction/well using the complete ready-to-use 2× reaction mix BioMix (Bioline, Taunton, USA) according to the manufacturer's recommendation. Template amounts were in the range of 10–20 ng. One hundred pmol of biotinylated primer (btRABL2ex89r) were always used as was RABL2ex89f in the classical Pyrosequencing approach. The simplified protocol was tested with 100–500 pmol of RABL2ex89f+s and 300 pmol was routinely used. Initial denaturation was at 95 °C for 1 min, cycling was done 25 times at 95 °C (30 s)/59 °C (30 s)/72 °C (1 min).

2.4. Amplicon verification

Amplicon sizes were characterized by 1% (w/v) agarose gel electrophoresis using Ultra Pure™ agarose (Invitrogen) in TAE buffer. Sequence integrity was checked by BigDye Terminator v3.1 chemistry and 3730xl DNA Analyzer (ABI, Foster City, USA) according to the manufacturer's instructions.

2.5. Pyrosequencing

Biotin-labeled PCR products were immobilized on Streptavidin Sepharose™ (Qiagen/Biotage, Uppsala, Sweden) by mixing 20 µl of a