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Polymorphisms in DNA polymerase γ affect the mtDNA stability and the NRTI-induced mitochondrial toxicity in *Saccharomyces cerevisiae*

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ABSTRACT

Several pathological mutations have been identified in human POLG gene, encoding for the catalytic subunit of Pol γ, the solely mitochondrial replicase in animals and fungi. However, little is known regarding non-pathological polymorphisms found in this gene. Here we studied, in the yeast model *Saccharomyces cerevisiae*, eight human polymorphisms. We found that most of them are not neutral but enhanced both mtDNA extended mutability and the accumulation of mtDNA point mutations, either alone or in combination with a pathological mutation. In addition, we found that the presence of some SNPs increased the stavudine and/or zalcitabine-induced mtDNA mutability and instability.

1. Introduction

Mitochondrial DNA (mtDNA) is replicated by the DNA polymerase γ, or Pol γ, which in animals is the only DNA polymerase present in mitochondria. The catalytic subunit of Pol γ belongs to a subfamily of the prokaryotic PolA family and its amino acid sequence is well conserved from yeast to humans. Structurally, human Pol γ is a heterotrimer composed of one catalytic subunit, which is encoded by POLG(1), and two accessory subunits, which are encoded by POLG2 (Yakubovskaya et al., 2006). The catalytic subunit contains an N-terminal exonuclease domain (residues 170–440), a C-terminal polymerase domain (residues 440–475 and 785–1239) and a spacer region encompassing residues 475–785 (Lee et al., 2009). To date, about 250 pathological mutations associated with severe mitochondrial disorders have been identified in POLG (http://tools.niehs.nih.gov/Polg/). Among them, a few mutations have been recently described to be involved in stavudine-induced toxicity (Chiappini et al., 2009; Yamanaka et al., 2007), in valproate-induced hepatotoxicity (Saneto et al., 2010; Stewart et al., 2010), in testicular cancer (Blomberg Jensen et al., 2008), in breast tumorigenesis (Azrak et al., 2012; Popanda et al., 2013; Singh et al., 2009) and in idiopathic sporadic parkinsonism (Luoma et al., 2007; Gui et al., 2012). Besides these pathological mutations, several single nucleotide polymorphisms (SNPs) have been identified in Pol γ, most of which appear to be, or are considered, neutral polymorphisms. Traditionally, a SNP is defined as a DNA sequence variation occurring within a population with an allelic frequency higher than 1%. Using next generation sequencing techniques applied to the exome, and to full genome sequencing, such as the 1000 Genomes Project (http://www.1000genomes.org/), a great number of new polymorphisms have been identified with a frequency much lower than 1%, so that now any nucleotide variation is generally considered as a SNP. A polymorphism differs from a pathological mutation since it is not associated with any pathology. However, several SNPs/polymorphisms behave as phenotypic modifiers of other mutations, or may alter the response to certain drugs or the susceptibility to environmental factors such as toxins, making them the subject of pharmacogenetic and toxicogenetic research.

In Pol γ, more than 1000 polymorphisms have been identified, most of which are non-coding or synonymous. Coding and non-synonymous SNPs have not yet been characterized so it is not known if they really are neutral polymorphisms. An exception is the E1143G mutation, which appears with a frequency of 3–4% in the European population (GeneSNps) and its effects on human Pol γ have been the subject of several studies. However, *in vitro* studies led to contradictory conclusions on the role of this mutation as a modulator of mutations or neutral polymorphism (Chan et al., 2006; Palin et al., 2010).

Abbreviations: d4T, 3′-deoxy-2′,3′-didehydrothymidine or stavudine; ddC, 2′,3′-dideoxycytidine or zalcitabine; Ed4T, 2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine; Ery^R, resistant to erythromycin; exo, exonuclease; FLT, 3′-fluoro-3′-deoxythymidine; HAART, highly active antiretroviral therapy; mtDNA, mitochondrial DNA; NRTI, nucleoside reverse transcriptase inhibitor; PEO, progressive external ophthalmoplegia; Pol γ, DNA polymerase γ; SNP, single nucleotide polymorphism; TP, triphosphate; wt, wild type.

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The *Saccharomyces cerevisiae* DNA polymerase γ, encoded by the *MIP1* gene, shows 43% similarity with the human Pol γ catalytic subunit. Thanks to this similarity, yeast has been largely used to validate the role of human putative pathological mutations, to understand biochemical consequences associated with these mutations, to find molecules able to rescue their detrimental effects and to study the pharmacogenetics of drugs such as valproate and stavudine (Baile and Claypool, 2013; Baruffini and Lodi, 2010; Baruffini et al., 2006, 2007, 2011; Qian et al., 2014; Spinazzola et al., 2009; Stewart et al., 2010; Stricker et al., 2009; Stuart et al., 2006; Stumpf and Copeland, 2013; Stumpf et al., 2010; Szczepanowska and Foury, 2010). Yeast is a suitable model organism for the study of the effects of Pol γ mutations on mtDNA stability, thanks to its ability to survive in the absence of a functional mitochondrial genome. Yeast cells containing deletions-carrying mtDNA, called *rho*$^{-}$, or cells which have completely lost mtDNA, called *rho*^{0}, are respiratory-deficient and produce small-sized colonies, called *petite*. *Petite* mutants arise spontaneously with high frequency (approximately 10^{-2}) (Dujon, 1981), which is increased in the case of Mip1 mutant strains with reduced polymerase activity. *Petite* mutability can be easily measured and provides an estimate of the mtDNA extended mutability, *i.e.* loss or rearrangements in mtDNA. mtDNA point mutability can also be easily measured as the frequency of spontaneous mutants which are resistant to erythromycin (Ery^{R} mutants), an antibiotic that inhibits mitochondrial but not cytoplasmic translation. In fact, resistance to erythromycin is acquired through specific transversions or transitions in the mitochondrial gene encoding the 21 S rRNA (Cui and Mason, 1989; Sor and Fukuhara, 1982, 1984; Vanderstraeten et al., 1998). Ery^{R} mutability is increased by Mip1 mutations which reduce the fidelity of replication.

Yeast has also been used to evaluate the correlation between specific mutations in Mip1, corresponding to human mutations, and mtDNA mutability induced by treatment with stavudine (3′-deoxy-2′,3′-didehydrothymidine, or d4T) (Baruffini and Lodi, 2010), a nucleoside reverse transcriptase inhibitor (NRTI) which has been successfully used in the highly active antiretroviral therapy (HAART). Although HAART has significantly increased the life expectancy of HIV patients, prolonged treatment can induce side effects in some patients, most of which are due to the interference of the NRTIs with mitochondrial metabolism. Several observations suggest that NRTIs mitochondrial toxicity depends on their interference with Pol γ activity (Koczor and Lewis, 2010; Kohler and Lewis, 2007; Lee et al., 2003; Lewis et al., 2006), at least in part and especially for pyrimidine analogs such as stavudine and zalcitabine (2′,3′-dideoxycytidine, or ddC). It has been also demonstrated that several triphosphorylated NRTIs can inhibit the activity of human Pol γ *in vitro* (Johnson et al., 2001; Lim and Copeland, 2001; Martin et al., 1994). According to the "Pol γ hypothesis", NRTI toxicity could be due to direct inhibition of polymerase catalytic activity, incorporation of NRTI in the nascent strand with subsequent chain termination or persistence of the analog in mitochondrial DNA because of inefficient excision (Lewis, 2007). Consequently, some mutations in Pol γ can result in different biochemical properties towards the NRTI-triphosphate (NRTI-TP), such as a greater K_i by the NRTI-TP, a lower NRTI-TP discrimination or a lower excision efficiency of the NRTI from the mtDNA and can increase the susceptibility to NRTIs toxicity, as was shown for patients carrying the mutation R964C (Bailey et al., 2009; Yamanaka et al., 2007). Thus, a pharmacogenetic approach in the NRTI treatment requires to establish whether polymorphisms are neutral or if they affect mtDNA polymerase activity.

The aim of this study is to characterize, by the use of specific *ad hoc* yeast models, some polymorphisms in Pol γ, in order to assess whether they are neutral nucleotide variations or not. Specifically, we will try to answer the following questions: (i) Is the mutation neutral, *i.e.* does the mutant Mip1 behave *in vivo* like the wt Mip1 concerning mtDNA extended and point mutability? (ii) Is the mutation a phenotypic modifier or mutation modulator, *i.e.* can the mutation worsen the phenotype of an *in cis* pathological mutation? (iii) Does the mutation increase the toxicity induced by stavudine or zalcitabine treatment? An answer to the last question is of particular relevance in view of possible pharmacogenetic applications.

We found that most of the considered polymorphisms are not neutral but rather behave as phenotypic modifiers, and that three polymorphisms, besides the R964C and E1143G mutations previously studied (Baruffini and Lodi, 2010), showed an altered sensitivity to stavudine and/or zalcitabine toxicity.

2. Materials and methods

2.1. Strains, plasmids and media

The yeast strains used in this work are reported in Table 1. The primers used are reported in Supplementary Table 1. Yeast media are YP (1% yeast extract (Formedium), 2% peptone (Formedium)) or SC (0.69% yeast nitrogen base without amino acids (Formedium), 0.1% drop out mix according to Kaiser et al., 1994). Appropriate carbon sources were added to the medium at a final concentration of 2%. 5-FOA plates contained 0.69% yeast nitrogen base without amino acids, 1% fluoroorotic acid (Formedium), 40 mg/l of each amino acid or base necessary to complement the auxotrophy, 40 mg/l uracil and 2% glucose. YPGen and YPHyg were YP supplemented with 200 mg/l G418 sulfate (Gibco) or 250 mg/l hygromycin B (Formedium). YPAEG-Ery medium contained 1% yeast extract (Difco), 2% peptone (Difco), 100 mg/l adenine, 3% [v/v] glycerol, 3% [v/v] ethanol, 3 g/l erythromycin (Sigma). If necessary, the medium was solidified by adding 2% agar (Formedium).

Human *ENT1* was disrupted by using the one step gene disruption technique, with the *HIS5* gene from *Schizosaccharomyces pombe* cassette. The *SpHIS5* cassette, flanked by 40–45 bp of *hENT1*, was amplified by using pUG27 (Güldener et al., 2002) as a template and oligonucleotides hENT1DFw and hENT1D2Rv as primers. The cassette was introduced in strain YLV3t3m3 and the disruption was confirmed by PCR, thus obtaining the YLV3t3m3ΔH strain.

For construction of the strain W1BCK1, a fragment of pFL61 containing the PGK promoter and the PGK terminator was digested with *Bam*HI and *Bgl*II from plasmid pFL61, and subcloned in *Bam*HI-digested pFL26 integrative plasmid (Bonneaud et al., 1991; Minet et al., 1992). Human *DCK1* cDNA was amplified with primers DCKcFw and DCKcRv, digested with *Not*I and subcloned in pFL26PGK under the control of the PGK promoter. The plasmid was introduced into the strain W303-1B and transformants in which the plasmid was integrated at the *leu2* locus were selected on SC medium without leucine. Integration was verified by PCR.

For construction of the strain W1BCK1-10B, the *mip1*::*HphMX4* cassette was amplified from DWM-9A genomic DNA (Baruffini et al., 2009) with primers MIP1DFw and MIP1Drv, and introduced into the W1BCK strain. Disruption was verified by phenotypic analysis and PCR.

Human *CNT3* cDNA was amplified with primers hCNT3Fw and hCNT3Rv, and cloned in the centromeric plasmid pUSG-E12 (a personal gift of A. Inga) after digestion with *Xho*I and *Not*I, and in the episomal plasmid pYES2 (Life Technologies) after digestion with *Hind*III and *Not*I. In both plasmids, the *hCNT3* ORF was cloned under the *GAL1-10* promoter.

2.2. Construction of mutant mip1 strains

mip1 mutant alleles were constructed through mutagenic overlap PCR (Ho et al., 1989). After overlap PCR, the *MIP1* fragments containing mutations E166Q, E166R, P207L or L340V were digested with *Not*I and *Avr*II and subcloned in pFL39*MIP1* (Baruffini et al., 2006), whereas *MIP1* fragments containing mutations Y753F, S889R, S899W, K903R or K903C were digested with *Avr*II and *Bsr*GI and subcloned in the same plasmid. *mip1* mutant alleles harboring G224A, A692T, E698G, Q766R, Q766C and E900G were constructed previously (Baruffini et al., 2006, 2007; Spinazzola et al., 2009; Stricker et al., 2009). *mip1* double mutant