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Role of nonsense-mediated decay and nonsense-associated altered splicing in the mRNA pattern of two new α-thalassemia mutants

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ABSTRACT

α-thalassemia is a common disease characterized mainly by deletion mutants. We identified two new α-thalassemia pointform mutants: α1cod22 GGC > GGT Gly > Gly creating a 5′ splicing sequence and α1cod23 GAG > TAG Glu > stop. We performed qualitative and semi-quantitative analysis of the mRNA molecules, from carriers' blood, to define the molecular mechanisms giving rise to the thalassemia phenotype. *In vitro* analysis using α-globin constructs and cycloheximide was performed to evaluate if the mutants are substrates of nonsense-mediated mRNA decay (NMD). In the α1cod22 GGC > GGT the new 5′ splicing site in exon 1 completely substitutes the normal one. We demonstrated the presence of mRNA decay as the abnormally spliced mRNA was consistent in the nucleus, partially degraded in the cytoplasm of cultured cells, but only 2.8% in the reticulocytes. The analysis of the αcod23 transcript showed an escape from the NMD as for the human β-globin transcript with nonsense mutations in the first exon: the anomalous mRNA was reduced in the nucleus, followed by only a slight lowering from 32% to 27% of the normal α1 mRNA in the reticulocytes. In both the mutants we showed a moderate sensitivity to the NMD assay and we speculate the activation of other RNA surveillance mechanisms for the αcod22 mutant. No activation of cryptic splice sites was detected and no role could be assigned to the nonsense-associated altered splicing.

Studies on transcripts from patient cells represent a very useful approach providing considerable information about the processes occuring *in vivo*.

1. Introduction

Messenger RNAs (mRNA) are monitored for errors that arise during gene expression by a mechanism called RNA surveillance, with the result that most mRNAs that cannot be translated along their full length are rapidly degraded (Culbertson, 1999).

Nonsense-mediated mRNA decay (NMD) (Neu-Yilik et al., 2011) is a well characterized RNA surveillance mechanism leading to a down modulation of anomalous mRNAs carrying premature translation-termination codons (PTC).

Another mechanism, with a minor impact on the modulation of the disease phenotype, is represented by nonsense-associated altered splicing (NAS) (Liu et al., 2001) in which the PTC or missense mutation causes a disruption of the exonic splicing enhancer (ESE) with an up-regulation of alternatively spliced transcripts that skip the mutation and have the potential to rescue the protein function.

Other mRNA surveillance mechanisms remain to be determined like those responsible for the disappearance of mRNAs in mutations at the 3′ end of the mature mRNAs or in the case of splicing mutants (Rawa et al., 2017; Grosso et al., 2008; Musollino et al., 2012).

Abbreviations: NMD, nonsense-mediated decay; NAS, nonsense-associated altered splicing; PTC, premature termination codon; DG-DGGE, double gradient-denaturing gradient gel electrophoresis; PBSC, peripheral blood stem cell; HPLC, high performance liquid chromatography; ARMS, amplification refractory mutation system; RFLP, restriction fragments length polymorphisms; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; TEA buffer, tris-acetate-EDTA; LDH, lactate dehydrogenase; BFU/CFU, burst forming unit/colony forming unit

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NMD has a large spectrum of substrates, including abnormally spliced mRNAs, and functions as a multi-purpose tool in the modulation of gene expression (Neu-Yilik et al., 2011). Essentially, NMD limits the production of truncated peptides that could have a dominant negative effect on the organism (Pereira et al., 2006).

Characterization of the NMD pathway has defined a number of parameters in the decay process. In particular, in mammalian cells, a termination codon is ordinarily recognized as "premature" if it is located greater than 50–54 nucleotides 5′ to the final exon-exon junction. It has also been demonstrated that both splicing and translation are crucial for the recognition of the translation event as premature (Thermann et al., 1998; Zhang et al., 1998).

β-globin mRNA has been widely used as a model system for the study of NMD mechanisms and exceptions to the "50–54 nt boundary rule" have been reported for mutant human β-globin mRNAs. In fact, it has been shown that β-globin transcripts, containing naturally occurring nonsense mutations in the 5′ region of exon 1, accumulate to levels similar to those of normal β-globin transcripts, these being, unexpectedly in relation to their position, NMD-resistant (Romão et al., 2000; Inácio et al., 2004), whereas nonsense mutations in the 3′ region of the exon 1 and within the 2/3 of exon 2 elicit NMD (Zhang et al., 1998). To explain this behavior it has been suggested that: a) the β-globin exon 1 is bisected by a sharp border that separates NMD-bypassing from NMD-activating nonsense mutations; b) the bypassing of the NMD depends on the ability to reinitiate translation at a downstream start codon (Peixeiro et al., 2011; Neu-Yilik et al., 2011).

α-thalassemia is the most common worldwide disorder showing a variable phenotype depending on the number of inactive α-globin genes which are duplicated. Sequence variations account for about 25% of all α-thalassemia defects in southern Italy and the Mediterranean area, while long deletions account for about 75% (Lacerra et al., 2004). The role of NMD and/or NAS in the modulation of α-globin gene expression in erythroid cells has been poorly investigated principally because of the unavailability of appropriate mutants. During a study of the molecular epidemiology of α-thalassemia and hemoglobin variants in southern Italy, we found several new α-globin gene mutants. To verify if the puzzling behavior of the PTC mutants of the β-globin gene is present also in the α-globin genes, we highlighted the possibility that NMD and NAS processes could be associated with two new α-globin thalassemia mutants generating anomalous mRNAs: α1-globin cod22 GGC > GGT Gly > Gly (Fig. 1B) creating a new 5′ splicing sequence generating a PTC at codon 48/49 and α1-globin cod23 GAG > TAG generating a PTC at codon 23 (Fig. 1E) (Lacerra et al., 2005).

2. Materials and methods

Probands and their families were selected by the thalassemia centers collaborating in this study among those referred to them for hematological diagnosis. A special committee of the Ministry for Research approved the study (Decreto n. 250 of 22 June 1999) and two scientists were appointed as supervisors. We obtained written informed consent from the participants for the use of blood samples. Hematological parameters, reticulocyte counts, and isopropanol and heat Hb stability tests were performed according to standard methods (Huisman and Jonxis, 1977). Hemoglobin analysis was carried out by cation exchange high performance liquid chromatography (HPLC) (Variant II System; Bio-Rad Laboratories, USA) and by Mass Spectrometry.

2.1. Deletion and mutational analysis

DNA was purified from white blood cells by the salting out method (Miller et al., 1988). α-thalassemia deletions were tested by gap-PCR (Lacerra et al., 2004); point mutations were analyzed by multiplex ARMS and DG-DGGE (Lacerra et al., 2007; Lacerra et al., 2004). The α1- or α2-globin genes were sequenced from −181 to +884 (α2) and +894 (α1), as previously reported (Lacerra et al., 2003). Two ARMS-PCR assays for the definition of the heterozygous α1-globin cod22 G-GC > GGT and for the α1-globin cod23 GAG > TAG genotype were performed. Analysis of the RFLP RsaI at 5′ of the α2-globin gene (rs2541669) was carried out on PCR fragments. The SNPs α2 +14 (HBA2:c.-24C > G, rs772829778) and α2 +861 (HBA2:c.565G > A, rs2685121) were determined by DNA sequencing or DG-DGGE analysis (Lacerra et al., 2004; Lacerra et al., 2003). The RFLP and SNP association with the mutated α-globin allele was assessed by family segregation studies. The primers used in the different applications are reported in Table 1.

2.2. PBSC differentiated in vitro

In order to study the molecular alterations of the mRNA due to the new mutants in the transcriptionally active cells, peripheral blood stem cells (PBSC) were differentiated *in vitro*. The cultures showed a new mRNA synthesis from the fifth day and were incubated for the subsequent 9–16 days. The blood samples were from one carrier of α1-globin cod22 GGC > GGT, one carrier of α1-globin cod23 GAG > TAG and one normal subject.

The separation and differentiation *in vitro* of the PBSCs and the separation and extraction of the RNA from the nuclei and cytoplasm were performed as previously reported (Bisconte et al., 2015; Lacerra et al., 2000).

2.2.1. mRNA analysis

The analysis of mRNA molecules in the reticulocytes and erythroid cells at different stages of differentiation gave information about the production rate and degradation profile of the anomalous mRNAs produced by the two mutants under study. Total RNA from reticulocyte-enriched peripheral blood cells and from PBSCs differentiated *in vitro* was isolated with Triazol (Life Technologies, New York, NY, USA) (Bisconte et al., 2015; Lacerra et al., 2000). The α-globin gene cDNA was sequenced by RT-PCR and Automated Cycle Sequencing (Lacerra et al., 2008).

2.2.2. Detection of anomalous mRNAs

cDNAs of an anomalous length were separated by acrylamide gel. The RT-PCR of the α-globin gene −at a low number of cycles (22–24 cycles) and containing 0.2 μl of P32 α-dCTP- was carried out using the primers H and E reported in Table 1 generating a cDNA amplicon of 265 bp. Ten μl of the RT-PCR was separated on a 6.5% (37.5:1) acrylamide gel in TEA 1 X Buffer run at 300 V for 6 h. The gel was dried on a gel drier, and the intensity of each band was quantified by using a phosphor imager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA) (Musollino et al., 2012).

2.2.3. Semi-quantitative analysis of mRNA by restriction enzyme analysis

A semi-quantitative analysis of the mutated/normal cDNA was carried out by MaeI restriction enzyme analysis for which the mutation cod23 GAG > TAG created a new site 5′-CˆTAG-3′. The DNA and cDNA PCR amplification was carried out at 22–24 cycles, twice on each DNA or cDNA sample respectively with the primers B and G, generating a DNA amplicon of 251 bp, and with the primers H and E, generating a cDNA amplicon of 265 bp. The amplified fragments were restricted according to the manufacturer's recommendations with 20 U of MaeI. 100–200 ng of the digested products were size fractionated and the ratio of undigested/digested bands (that is mutated/normal mRNAs or DNA) was obtained as previously reported (Bisconte et al., 2015; Lacerra et al., 2008). After the separation on NuSieve gel a semi-quantitative analysis of the bands was performed with the Kodak software Carestream MI.

2.3. Construction of expression vectors

The α1-globin gene was specifically amplified by PCR from genomic