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Validation of *CFTR* intronic variants identified during cystic fibrosis population screening by a minigene splicing assay

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Abstract

Background: Cystic fibrosis, caused by mutations of the *CFTR* gene, is the most common autosomal recessive condition in the European population and there are specific screening programs aimed at investigating healthy carriers. They are usually articulated in two steps: initially individuals are screened with a panel of the 20–50 most common *CFTR* mutations; the second step is offered to partners of carriers who were found negative at the first test and consists in the analysis of the entire *CFTR* gene. This strategy provides high sensitivity, however, it often identifies novel variants (especially in introns) of unknown significance. Establishing the pathogenicity of these variants of the *CFTR* gene is not a simple task.
Methods: We have examined five *CFTR* intronic variants of unclear significance (c.274-6T>C, c.744-6T>G, c.1117-64G>A, c.2620-26A>G, and c.3468+51C>A) using a functional splicing assay based on hybrid minigenes.
Results: Four out of five variants (including c.2620-26A>G which was previously reported as a possible splice-site mutation) did not alter the correct splicing of the minigene and are likely to be neutral polymorphisms, whereas c.744-6T>G caused complete skipping of *CFTR* exon 7 and should be therefore regarded as a pathogenic *CFTR* mutation.
Conclusions: Hybrid minigenes assay are a simple and rapid tool to evaluate the effects of intronic variants without the need of analyzing patient's mRNA, and are particularly suited to analyze variants identified during population screenings.

Keywords: carrier screening; cystic fibrosis; hybrid minigene; intronic variants; splicing.

Introduction

Cystic fibrosis (CF), caused by mutations in the *CFTR* gene, is the most common autosomal recessive condition in the Caucasian population, with an incidence of approximately 1:2500 [1]. The high carrier frequency of this disease has stimulated the development of population screening programs aimed at identifying couples at risk of having a child with CF. These programs screen the population for the most common disease causing mutations in the *CFTR* gene and have been highly successful in decreasing the incidence of CF [2].

However, especially in southern Europe, CF is characterized by high mutational heterogeneity. In Italy and Spain the common F508del mutation accounts only for 50% or less of all CF alleles. This fact has complicated the development of mutation panels in these countries. In fact it is necessary to include in the panels large numbers of mutations (over 50) in order to reach detection rates of 85%–90% [3].

Several programs offer a two-step screening. The first-line test is based on a panel of mutations with a detection rate of 70%–90% (depending on the mutations included and on the specific population). If only one of the members of the couple is found to harbor a *CFTR* mutation a second-line test, based on the analysis of the entire *CFTR* gene is offered to the individual who is found negative at the first-line test. This second-line test has a

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detection rate around 95%, and if no mutations are found the residual risk for the couple is estimated in 1:2000 (if the negative individual is of Caucasian origin) [4].

The major disadvantage of this approach is that it often detects previously unreported variants of unknown significance. In these situations it can be very difficult to provide genetic counseling to the couples.

We report the characterization of five *CFTR* intronic variants identified during population screening using a functional splicing assay based on hybrid minigenes.

Materials and methods

We studied five healthy couples who were referred to our clinic for genetic counseling because after one of the members had tested positive for a CF mutation in the first-line population screening test, the second-line test had identified a variant of unclear significance in the partner. All analyses have been performed with the informed consent of the individuals involved. The five variants studied are listed in Table 1. Of these only c.2620-26A>G had been previously published [5] as a possible pathogenic variant, but no validation had been performed.

Computational analysis of variants

To analyze the effect of variants, in silico analyses were performed. Three programs were used: NetGene2 (available at http://www.cbs.dtu.dk/services/NetGene2/), Human Splicing Finder (HSF)-Version 2.4.1 (available at http://www.umd.be/HSF/) and Neural Network SPLICE (NNSplice) 0.9 from the Berkeley Drosophila Genome Project (available at http://www.fruitfly.org/seq_tools/splice.html).

Construction of the minigenes

For each variant, a fragment containing the exon adjacent to the variant and approximately 100 nucleotides of the preceding and subsequent intron was amplified from genomic DNA of the patient and cloned within intron 2 of the β-globin gene previously cloned in the pcDNA3.1 expression vector. In case of the c.2620-26A>G variant the amplicon included *CFTR* exons 16 and 17. The generation of this minigene backbone has been previously described [6] and its structure is depicted in Figure 1A. Primers and PCR conditions are available from the authors upon request. Correctness of all plasmids was verified by direct sequencing.

Expression of minigenes

The constructs containing the wild type or the mutant hybrid minigene were employed to transfect HeLa cells. Cells were collected after 48 h and total RNA was extracted and retro-transcribed as described [6]. Transcripts were amplified using primers specific for β-globin exon 2 and β-globin exon 3, and visualized by agarose gel electrophoresis (for the c.744-6T>G we employed also polyacrylamide gel electrophoresis). RT-PCR products were also directly sequenced as described [7].

Table 1: *CFTR* variants included in the study.

Variant	Legacy name	Previous reports	Type of splice site	NetGene2	HSF	NNSplice	Minigene assay
c.274-6T>C	406-6 T>C	Only in CFTR mutation database as possibly neutral.	Acceptor	Score for the main acceptor site increases from 0.94 to 0.97	Score for the main acceptor site increases from 84.25 to 84.9	Score for the main acceptor site decreases from 0.99 to 0.98	Neutral
c.744-6T>G	876-6 T>G	NO	Acceptor	Score for the main acceptor site decreases from 0.15 to 0	Score for the main acceptor site decreases from 85.59 to 82.76	The WT consensus sequence is not recognized	Pathogenic (skipping of exon 7)
c.1117-64G>A	1249-64 G>A	NO	Acceptor	Score for the main acceptor site increases from 0.25 to 0.26	Possible activation of cryptic donor site	Possible activation of cryptic donor site	Neutral
c.2620-26A>G	2752-26 A>G	YES [5]. Pathogenic (but no experimental evidence provided)	Acceptor	Score for the main acceptor site decreases from 0.68 to 0.57	New acceptor site created	Neutral	Neutral
c.3468+51C>A	3600+51 C>A	NO	Donor	Neutral	Neutral	Neutral	Neutral

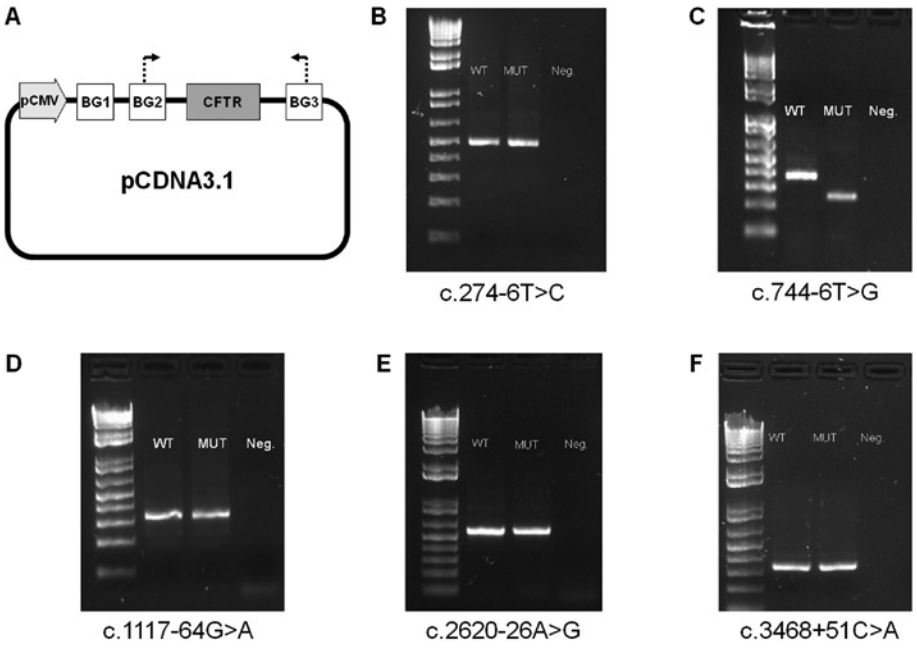

Figure 1: (A) Structure of the β-globin minigene employed in the study.
BG1,BG2,BG3, β-globin exon 1,2, and 3; CFTR, CFTR fragment containing exon(s) adjacent to the mutation studied and approximately 150 bp of the relative introns. Arrows indicate the position of the primers used for sequencing. (B, C, D, E, F) Total RNA extracted from HeLa cells expressing the different minigene constructs was retro-transcribed and PCR-amplified using primers specific for β-globin exon 2 and 3 and separated by agarose gel electrophoresis. For all gels the 1 kb marker (Invitrogen) was used.

Results

The results of the analysis with bioinformatic tools (*NetGene2*, *HSF* and *NNSplice*) are showed in Table 1. Both c.274-6T>C and c.3468+51C>A were predicted to be likely non-pathogenic, while the analyses for c.744-6T>G, c.1117-64G>A, and c.2620-26A>G gave inconclusive results.

We therefore constructed wild type and mutant minigenes for each variant, which were expressed in HeLa cells. After RNA isolation, retro-transcription, and PCR amplification, analysis of the PCR products by agarose gel electrophoresis revealed an identical pattern with the wild type and mutated constructs in the case of the c.274-6T>C, c.1117-64G>A, c.2620-26A>G, and c.3468+51C>A variants (Figure 1B, D, E, F). The correct processing of the mRNA was confirmed by direct sequencing of the PCR products (not shown). These data suggest that these variants represent neutral polymorphisms. Instead, in the case of the c.744-6T>G variant, the wild type construct was correctly spliced, while the transcripts expressed from the mutant minigene did not contain *CFTR* exon 7 (Figure 1C). This result was clear cut and there were virtually no correctly spliced transcripts originating from the mutant minigene (the assay is sufficiently sensitive to detect <1% of correctly spliced fragments [8]), indicating that this should be considered a pathogenic *CFTR* mutation. Skipping of exon 7 is predicted to cause a deletion of 42 amino acids in the CFTR polypeptide, however even more severe consequences on the transcript cannot be ruled out.

Discussion

Establishing the pathogenicity of *CFTR* intronic variants identified during carrier screening is not simple, but it is crucial to provide adequate genetic counseling to couples. Variants affecting the first two or last two nucleotides of the intron are almost always pathological [9], but variants in other positions of the intron cannot be qualified "a priori" as harmless or pathological. There are several online software programs that predict the consequences of both intronic and exonic variants but their reliability is limited [10, 11], especially when dealing with variants identified in healthy carriers (where no correlation with a specific phenotype can be made). Evaluation of allelic frequencies may be helpful [12], but since most of these variants are rare (with a frequency <1:500) the analysis may not yield informative results.

The most logical experimental approach to characterize these variants would be to study *CFTR* transcripts in cells from the individual harboring the variant. However, the *CFTR* gene is not expressed in peripheral blood cells and it is necessary to isolate RNA from other cell types, e.g., cells of the nasal mucosa [13], a task which can be relatively complex. Moreover, the presence of transcripts originating from the normal allele in trans may cause the misinterpretation of data [12], especially in the case of alleles which cause only a partial splicing defect. For these reasons hybrid minigenes have been used to study *CFTR* variants [14]: they do not rely on patient's RNA, and the analysis is not affected by the presence of a wild type allele.

In this work we have demonstrated that four out of five variants studied do not affect the processing of the transcript and are thus very likely to represent neutral polymorphisms. Three out of four of these variants were relatively far from the exon, however both c.274-6T>C and c.744-6T>G affect nucleotide -6 within the polypyrimidine tract at the end of the intron. c.274-6T>C does not seem to affect splicing, probably because a pyrimidine is substituted with another pyrimidine, whereas c.744-6T>G completely abolishes inclusion of exon in the mature transcript probably because the inclusion of a purine is detrimental for recognition by the pyrimidine-tract binding proteins and other components of the splicing machinery [9].

Two of these variants had been previously reported. c.274-6T>C is mentioned in the Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca/Home.html) as a possible neutral variant. Our results confirm its benign nature. The other, c.2620-26A>G (legacy name 2752-26 A>G), had been previously published as a disease causing mutation [5]. It had been detected (in the heterozygous state) by DGGE screening in a 2.5-year-old boy of Greek origin, without respiratory symptoms and borderline sweat chloride (43–56 mmol/L), who underwent CF testing because of failure to thrive. No other mutation was detected. The authors had hypothesized that the mutation could create an alternative splice site that competes with the normal acceptor site but no further experimental or bioinformatic work was performed. Our results argue against the pathogenicity of this variant which should be instead considered a neutral polymorphism. It should be noted that the pathogenicity of c.2620-26A>G is considered dubious also in the Cystic Fibrosis Mutation Database and it is not mentioned in the CFTR2 database (www.cftr2.org). The information gained through the minigene experiments was essential to provide correct counseling to these couples.

Additionally, our results confirm the limitations of the in silico analysis of intronic variants with bioinformatic tools in a diagnostic setting. In fact, for the c.744-6T>G, c.1117-64G>A, and c.2620-26A>G variants the analysis was inconclusive (and possibly even misleading).

In conclusion, the minigene assay is a simple and relatively fast method providing functional data on the consequences of specific intronic (and also of exonic) variants on transcript processing. It does not require patient's RNA or particularly sophisticated equipment. The utility of this tool is not restricted to the validation of *CFTR* variants, but it can be conveniently employed for any gene characterized by high mutational heterogeneity (e.g., *BRCA1*) [15].

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