Use of Splicing Reporter Minigene Assay to Evaluate the Effect on Splicing of Unclassified Genetic Variants

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Abstract

The interpretation of the numerous sequence variants of unknown biological and clinical significance (UV for “unclassified variant”) found in genetic screenings represents a major challenge in the molecular diagnosis of genetic disease, including cancer susceptibility. A fraction of UVs may be deleterious because they affect mRNA splicing. Here, we describe a functional splicing assay based on a minigene construct that assesses the impact of sequence variants on splicing. A genomic segment encompassing the variant sequence of interest along with flanking intronic sequences is PCR-amplified from patient genomic DNA and is cloned into a minigene vector. After transient transfection into cultured cells, the splicing patterns of the transcripts generated from the wild-type and from the variant constructs are compared by reverse transcription-PCR analysis and sequencing. This method represents a complementary approach to reverse transcription-PCR analyses of patient RNA, for the identification of pathogenic splicing mutations.

Key words: Cancer molecular diagnosis, Minigene construct, Splicing mutations, Unclassified genetic variants

1. Introduction

Functional splicing reporter minigene assays represent a powerful tool to assess the impact of sequence variants on splicing (1, 2). These assays are very useful to diagnostic laboratories for determining the biological and the pathological significance of certain sequence variations detected in genetic screenings of disease-predisposing genes. The protocol provided here is used routinely in our laboratory to evaluate whether unclassified variants (UVs) identified in genes associated with predisposition to Lynch syndrome (MLH1/MSH2) or to hereditary breast-ovarian cancer (BRCA1/BRCA2) lead to splicing defects (3, 4).
The assay relies on the use of a minigene vector, which contains a fragment of the C1 inhibitor gene (SERPING1/C1NH), with two exonic regions separated by an intron, cloned into the mammalian expression vector pcDNA3.1(−), downstream of the cytomegalovirus (CMV) promoter (3, 4) (see Fig. 1). The genomic segment encompassing the variant sequence of interest along with ~150 bp of the corresponding flanking intronic sequences is amplified by PCR from the genomic DNA of the patient and is cloned into the minigene expression plasmid by insertion into the SERPING1/C1NH intron (see Fig. 1). The resulting minigene constructs (variant and wild-type) are expressed by transient transfection into cultured cells. The splicing pattern of the chimeric transcripts generated from both constructs is then compared by RT-PCR analysis and sequencing (see Fig. 1).

The splicing reporter minigene assay presents many advantages. Patient blood RNA samples or lymphoblastoid cell lines are not always available to the diagnostic laboratories and the splicing reporter minigene assay, which is based on genomic DNA, circumvents this problem. In addition, the minigene approach facilitates the interpretation of the effect of sequence variant on splicing especially because it is monoallelic. Another significant advantage is the parallel analysis of the variant and wild-type construct in an identical cellular background. Moreover, each UV is tested within a defined sequence segment of the natural gene. This feature often allows one to rule out the alternative hypothesis that the defect observed in vivo may be due to a sequence alteration affecting, e.g., intronic regions that have not been sequenced. The splicing reporter minigene assay can nevertheless present some limitations. In the minigene assay, the expression of an artificial transcript is analyzed, whereas the RT-PCR analysis from patient blood RNA presents the advantage of assessing the natural endogenous expression of the gene of interest. In addition, the heterologous cellular system used in the minigene assay may not fully reflect the splicing regulatory process involved in the affected tissue. However, one should also note that even the observation of a splicing defect by RT-PCR analysis of patient blood RNA does not necessarily reflect its magnitude in the relevant tissue.

Fig. 1. (continued) the stretch of 114 bp derived from intron 1 of the SERPING1/C1NH gene has been removed, and the translation initiation site within exon A has been inactivated in order to prevent the induction of the NMD pathway. Genomic segments are inserted using BamHI and MluI cloning sites located within the intron 2. In this construct, the transcription is driven by the human CMV immediate-early promoter/enhancer (P CMV) from the pcDNA3.1(−) vector. (3) and (4) After transfection of the pCAS constructs into HeLa cells, total RNAs are extracted and the transcripts are analyzed by RT-PCR, using forward (F) and reverse (R) primers complementary to exon A and exon B of the minigene, respectively. The RT-PCR products are analyzed by electrophoresis on an agarose gel stained with ethidium bromide followed by direct sequencing of the different gel-excised bands. The effect of each variant is evaluated by comparison to the effect of the corresponding wild-type sequence. In this example, the variant sequence tested induces the complete skipping of the exon, while the wild-type exon is fully included in the mature transcript. Several other examples are presented in [3, 4].
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Patient genomic DNA (Peripheral blood)

1) Amplification by PCR of the genomic segment of the
cancer predisposition gene bearing the variant sequence

Wild-type sequence
Exon

150 bp of flanking
intrinsic sequence

Variant sequence
Exon

2) Cloning into the intron of splicing reporter minigene pCAS

3) Transfection into HeLa cells

4) RT-PCR analysis and DNA sequencing

Fig. 1. Schematic representation of the functional splicing reporter minigene assay. (1) The wild-type and the variant exonic sequences of interest are PCR-amplified from patient genomic DNA together with ~150 bp of their 5' and 3' intrinsic flanking sequences, using specific primers carrying 5' tails with BamHI and MluI restriction sites. (2) The ampli-
cons are cloned into the pCAS1 reporter vector, which is based on the pcDNA3.1 plasmid and contains a minigene composed of two exons (here, named A and B). The minigene contains 114 bp of intron 1, exon 2, the entire intron 2, and exon 3, fused to partial exon 4 of the SERPING1/C1NH gene. In a recent version of the minigene construct, named pCAS2,
2. Materials

2.1. Splicing Reporter Minigene pCAS

The splicing reporter minigene pCAS (pCAS1) was constructed using the pcDNA3.1(−) vector (Invitrogen), by cloning into the EcoRI and BamHI sites an EcoRI-BglII fragment of the C1 inhibitor gene (SERPING1/C1NH, GenBank NM_000062), as described in (3–5) (see Fig. 1). It contains the last 114 bp of intron 1, exon 2, intron 2, and exon 3 fused to 122 bp of exon 4 of the SERPING1/C1NH gene. A MluI site was generated in the intron 2 of this construct, 118 bp downstream of a natural intronic BamHI site (see Fig. 1). In addition, the sequence of exon 3 was modified by site directed mutagenesis at three positions (underlined) resulting in the sequence AGCCAAGATCCAGA (5). These modifications inactivate an internal BamHI site and allow the discrimination between the endogenous SERPING1/C1NH mRNA and the one expressed from the transfected construct. The MluI site at position 229 bp of the pcDNA3.1(−) vector was also inactivated by site directed mutagenesis. In this construct, transcription is driven by the human CMV immediate-early promoter/enhancer from the pcDNA3.1(−) vector (see Fig. 1). A modified version of the pCAS1, named pCAS2, has recently been constructed in our laboratory and is currently under evaluation (unpublished). This new version differs from pCAS1 in that the stretch of 114 bp derived from intron 1 of the SERPING1/C1NH gene has been deleted, and the protein initiation codon in exon 2 of the SERPING1/C1NH gene has been inactivated.

2.2. Cloning Variant Sequences into the pCAS Vector

1. Amplification by PCR. Thermoprime plus DNA polymerase (A&gene) or a high fidelity DNA polymerase, such as the AccuPrime Taq DNA Polymerase High Fidelity, 100 mM dNTP Set PCR Grade (Invitrogen).
2. Electrophoresis. SeaKem LE Agarose (Tebu-bio), Tris Borate EDTA (TBE) buffer: 89 mM Tris-HCl, 89 mM Boric Acid, 2 mM EDTA (Euromedex), Ethidium bromide (Qbiogene), DNA size marker (New England Biolabs).
3. DNA purification from agarose gels. NucleoSpin Extract II kit (Macherey-Nagel).
6. Transformation and bacterial culture. XL1B E. Coli competent cells (Stratagene), Bacterial growth Luria Bertani (LB) medium (MP Biomedicals), LB Agar (Invitrogen), Carbenicillin (Sigma).

2.3. Transfection of the pCAS Vector into HeLa Cells

1. Cell Culture. HeLa cells (ATCC), Dulbecco’s Modified Eagle’s Medium (D-MEM) with l-Glutamine, 4,500 mg/L d-Glucose, without Sodium Pyruvate (Gibco), Fetal bovine serum (FBS) (Biowest).

2. Transfection. FuGENE 6 Transfection Reagent (Roche Applied Science).

3. Treatment by puromycin (Sigma-Aldrich).

2.4. RT-PCR Analysis

1. RNA extraction. TriPure Isolation Reagent (Roche Applied Science).

2. DNase treatment. Deoxyribonuclease Amplification Grade RNase-free (Sigma-Aldrich).

3. First-strand cDNA synthesis. SuperScript II Reverse Transcriptase (Invitrogen), Oligo(dT)18 mRNA primer (New England Biolabs), 100 mM dNTP Set PCR Grade (Invitrogen), RNase OUT (Invitrogen).

4. Amplification by PCR. Thermoprime plus DNA polymerase (ABgene), 100 mM dNTP Set PCR Grade (Invitrogen).

5. Electrophoresis. SeaKem LE Agarose (Tebu-Bio), TBE buffer: 89 mM Tris-HCl, 89 mM Boric Acid, 2 mM EDTA (Euromedex), Ethidium bromide (Qbiogene), DNA size marker (New England Biolabs).

6. DNA purification from agarose gel: NucleoSpin Extract II kit (Macherey-Nagel).


3. Methods

3.1. Cloning of the Variant Sequence into the pCAS Vector

1. Amplification of the genomic fragments by PCR: The wild-type and the variant exonic sequences of interest are PCR-amplified from patient genomic DNA together with approximately 150 bp of their 5' and 3' intronic flanking sequences (see Notes 1 and 2). Specific forward and reverse primers carrying 5' tails that contain sites for BamHI and MluI restriction enzymes are used (see Note 3). To a 0.2 mL Eppendorf tube, add 5 μL of 10× reaction buffer, 0.4 μM of each forward and reverse primers, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1.25 U Thermoprime plus DNA Polymerase (ABgene) (see Note 4), and 100 ng genomic DNA, and add
sterile distilled water to a final volume of 50 μL. Place Eppendorf tubes in the thermal cycler. The PCR program is divided into four steps: (1) initial denaturation step at 94°C for 5 min, (2) 30 cycles of 94°C for 20 s, 57°C for 30 s and 72°C for 30 s, and (3) final elongation step at 72°C for 10 min (see Note 5).

2. Separation of the PCR products, alongside the DNA size marker, by electrophoresis through an agarose gel containing ethidium bromide (0.5 μg/mL) in 1× TBE buffer (see Note 6). Purify the DNA bands from the agarose gel using NucleoSpin Extract II kit, according to the manufacturer’s instructions (see Note 7).

3. Digestion of the purified PCR product by adding 10 U of the BamHI restriction enzyme and 10 U of the MluI restriction enzyme in NEBuffer 3 in the presence of BSA (1 μg/mL), add sterile distilled water to a final volume of 50 μL. Incubate at 37°C for 2 h for digestion. The double digestion reaction product is directly purified using NucleoSpin Extract II kit, according to the manufacturer’s instructions.

4. Ligation of the digested and purified fragments (wild-type and variant sequences) into the BamHI and MluI restriction sites of the splicing reporter pCAS minigene (see Fig. 1) (see Note 8). Bacterial transformations are performed following standard protocols. Each transformed bacterial culture is plated onto an LB agar plate supplemented with carbenicillin (50 μg/mL). After incubation overnight at 37°C, isolated colonies are inoculated into 3 mL LB supplemented with carbenicillin (50 μg/mL) and grown at 37°C overnight under shaking. Plasmid DNA is prepared from colonies using a NucleoSpin plasmid kit, according to the manufacturer’s instructions (see Note 9). Wild-type and mutant constructs are identified by sequencing. The sequence of the entire insert is verified to exclude the presence of unwanted PCR-generated mutations.

3.2. Cell Culture and Transfection

1. HeLa cells (see Note 10) are cultured as a monolayer at 37°C (95% air, 5% CO2) in DMEM supplemented with 10% fetal calf serum. The cells are plated 1 day before transfection onto six-well plates at a density of approximately 5 × 10⁵ cells/well.

2. Transfection is performed using FuGENE 6 transfection reagent, according to the manufacturer’s instructions, using 1 μg/well of plasmid DNA and 3 μL of FuGENE 6 transfection reagent/well in OptiMEM medium. Wild-type and mutant constructs are transiently transfected into HeLa cells alongside each other. Two transfections are performed for each construct, one in the absence and the other in the presence of puromycin. For puromycin treatment, 10 μg/mL puromycin
is added to the culture 5.5 h before harvesting. Cells are collected 24 h post-transfection. Puromycin treatment inhibits translation, thus preventing the degradation of transcripts containing a premature stop codon that are targets of the nonsense-mediated mRNA decay (NMD).

### 3.3. RT-PCR Analysis

1. Total RNAs are isolated from transfected cells using the TriPure Isolation Reagent (Roche), according to the manufacturer’s instructions. Total RNAs are quantified by spectrophotometry (optical density at 260 nm, OD$_{260}$). In order to eliminate contaminating DNA, RNAs are treated with Amplification Grade RNase-free DNase I (Sigma-Aldrich), as described by the manufacturer.

2. First-strand cDNAs are synthesized from 1 to 2 µg of each DNase-treated total RNA sample using oligo(dT)$_{18}$ mRNA primer and the SuperScript™ II Reverse Transcriptase in a 20 µL reaction volume, as described by the manufacturer (see Note 11).

3. PCR amplifications are performed from 6 µL of the first-strand cDNA reaction mixture using primers F and R located, respectively, in exon A and exon B of the minigene pCAS1 (see Fig. 1). Thermoprime plus DNA Polymerase (ABgene) is used for the PCR reaction in a 50 µL volume, under the same conditions as described in step 1 of Subheading 3.1. PCRs are performed as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 10 s, 57°C for 20 s, and 72°C for 50 s, with a final elongation step at 72°C for 10 min (see Note 12).

4. RT-PCR products are separated, alongside the DNA size marker, by electrophoresis through an agarose gel containing ethidium bromide (0.5 µg/mL) in 1× TBE buffer and visualized by the exposure to ultraviolet light (see Note 6). Each DNA band is gel-purified using Nucleospin Extract II kit and sequenced using Big Dye Terminator cycle sequencing kit and ABI Prism 3100 automated sequencer, as described by the manufacturers (see Note 13).

### 4. Notes

1. In most cases studied, the sequence variant to be tested is present in the heterozygous state. Therefore, the wild-type and the variant exonic sequences are coamplified by PCR from patient genomic DNA and subsequently selected after molecular cloning and sequencing.
2. A genomic segment containing up to 150 nucleotides of flanking intronic sequences is a reasonable starting point. When one of the flanking intronic sequences is smaller than 150 nucleotides, a genomic DNA fragment containing two exons can be amplified and cloned into the pCAS vector. For example, we use this strategy to study UVs in the exons 17 and 18 of the MLH1 gene.

3. When the region to be amplified contains one of these restriction sites, the restriction sites BglII or AscI can be included into the 5' tails of the primers, in lieu of BamHI and MluI, respectively. These sites are compatible with BamHI and MluI sites, respectively, present in the splicing reporter minigene pCAS.

4. For genomic fragments smaller than 500 bp in size, standard thermophilic DNA polymerases can be used. For genomic fragments greater than 500 bp in size, we recommend using a high fidelity DNA polymerase, such as the Pfu Ultra High-Fidelity DNA polymerase, in order to ensure error-free amplification.

5. In our hands, these PCR reaction conditions work well for most of the amplicons tested. However, for some specific DNA target sequence, the PCR conditions could require optimization (see Ref. 6).

6. The concentration of agarose used, the voltage applied and the time of electrophoresis will vary according to the size of the PCR product to be resolved.

7. It is important to minimize ultraviolet light (UV) exposure in order to protect the DNA bands from UV-induced mutations.

8. The preparation of the splicing reporter minigene vector involves the following steps: double digestion by BamHI and MluI restriction enzymes, phosphatase treatment, and agarose gel purification. The ligation of the PCR product into the minigene vector is then performed according to a standard protocol. Typically, we use the T4 DNA ligase (New England Biolabs), according to the manufacturer’s indications.

9. Typically, six plasmid DNAs are prepared and sequenced for each construct.

10. HeLa immortal cell line derived from human cervical cancer cells presents high transfection efficiency. Most in vitro studies on splicing are carried out using extracts from HeLa cells. Other cell lines can be used, taking into consideration that splicing regulation can be cell-type specific.

11. Alternatively, both reverse transcription and PCR amplification can be combined in a one-step reaction using commercial kit such as the “OneStep RT-PCR Kit” from Qiagen.
12. Typically, we use 30 cycles for PCR amplification in order to detect potential minor bands and to generate enough product for sequencing. In some cases, a semi-quantitative RT-PCR analysis can be useful to determine the ratio of the different mRNA splice variants. This requires using PCR conditions within the linear range of amplification, determined experimentally by using different number of cycles.

13. All wild-type MLH1, MSH2, BRCA1, and BRCA2 exons that we have tested in the pCAS assay were predominantly included in the mature transcript (see (3, 4)). A sequence variant can induce different effects on splicing such as exon skipping, cryptic splice site activation, cryptic splice site disruption, or generation of a new splice site. The impact of the variants on splicing is determined by comparison of the splicing patterns obtained from the wild-type and variant minigene constructs (see Fig. 1). The RT-PCR products are first analyzed by electrophoresis to detect size differences, but the real identity of each band is determined by sequencing.

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References
