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# Detection of a rare *CDKN2A* intronic mutation in a Hungarian melanoma-prone family and its role in splicing regulation

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**Conflicts of interest**
None declared.

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## Summary

*Background* The major locus for melanoma predisposition is the cell cycle regulatory *CDKN2A* gene on chromosome 9p21. However, the frequency of germline coding mutations of the *CDKN2A* gene is lower than expected in melanoma-prone families linked to chromosome 9p21.

*Objectives* To investigate whether the rare IVS1+37 G/C intronic mutation of the *CDKN2A* gene, recently identified in a Hungarian melanoma-prone family, influences mRNA splicing regulation.

*Methods* *CDKN2A* minigenes containing the wild-type and the mutant intronic sequence were created and transfected into HeLa cells with the aim of studying the mRNA transcripts.

*Results* The results revealed the emergence of a differential splicing pattern from the wild-type and the mutant minigene, suggesting that this mutation may alter the splicing of *CDKN2A* primary mRNA and therefore might have a pathogenetic role in familial melanoma.

*Conclusions* We believe that these results confirm the importance of the identification and characterization of *CDKN2A* intronic mutations with a view to improving our understanding of the pathogenesis, and explain why the frequency of germline coding mutations of the *CDKN2A* gene is lower than expected in melanoma-prone families linked to chromosome 9p21.

The major locus for melanoma predisposition is the cell cycle regulatory *CDKN2A* gene on chromosome 9p21. In the last two decades we have gained much information on the melanoma-predisposing coding mutations of the gene;[1,2] however, we know much less about the role of its intronic mutations.[3–7] The frequency of germline coding mutations of the *CDKN2A* gene is lower than expected in melanoma-prone families linked to chromosome 9p21; therefore identification and functional characterization of the intronic variants may provide further insight into the genetic determinants of malignant melanoma.

We report the detection and functional data on the possible pathogenetic role of a rare intronic mutation of the *CDKN2A* gene found in an extensive Hungarian family with familial melanoma and atypical multiple mole (FAMMM) syndrome.

## Patients and methods

Detailed analysis of the family tree revealed other types of malignancies besides melanoma (Fig. 1). We recently detected the IVS1+37 G/C heterozygote intronic mutation of the *CDKN2A* gene in the above-mentioned Hungarian melanoma-prone family. Regarding the occurrence of atypical naevi and/or melanoma in nearly all family members carrying the IVS1+37 G/C mutation, along with the family history of manifestation of other malignant tumours, we hypothesized that the mutation may result in aberrant splicing and that the aberrant mRNA may play a pathogenetic role in the development of melanoma. Unfortunately, the members of the melanoma-prone Hungarian family declined to provide skin specimens to facilitate the *in vivo* identification of the alternative *CDKN2A* splice variants. We therefore conducted *in vitro* functional analysis to investigate whether the IVS1+37 G/C intronic mutation had any effect on splicing regulation. Two minigenes were constructed: one that harboured the wild-type and one that harboured the mutant allele of *CDKN2A* (Fig. 2a).

For investigation of the effects of the mutation on splicing, HeLa cells were transfected with the wild-type and mutant minigenes. Transfection was carried out with the TurboFect reagent (Fermentas, Vilnius, Lithuania), the cells were cultured for 24 h and total RNA was isolated through the use of TRI Reagent Solution (Applied Biosystems, Foster City, CA, U.S.A.). Reverse transcription was performed with the iScript

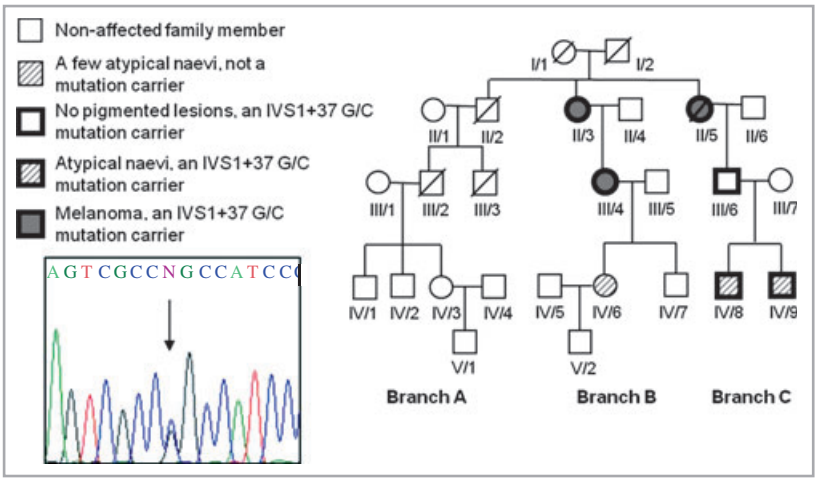


**Fig 1.** Family tree of the extensive melanoma-prone Hungarian family and detection of the IVS1+37 G/C *CDKN2A* intronic mutation. Direct sequencing of the *CDKN2A* gene was performed on specimens from seven members of the family (II/3, II/5, III/4, III/6, IV/6, IV/8 and IV/9) in branches B and C. Members in branch A were not available for genetic screening. Most of the family members carrying the IVS1+37 G/C mutation developed atypical naevi and/or melanoma. However, collateral history revealed that of family members of branch A, individual III/2 died from lung carcinoma and individual IV/1 developed prostate carcinoma at a young age.

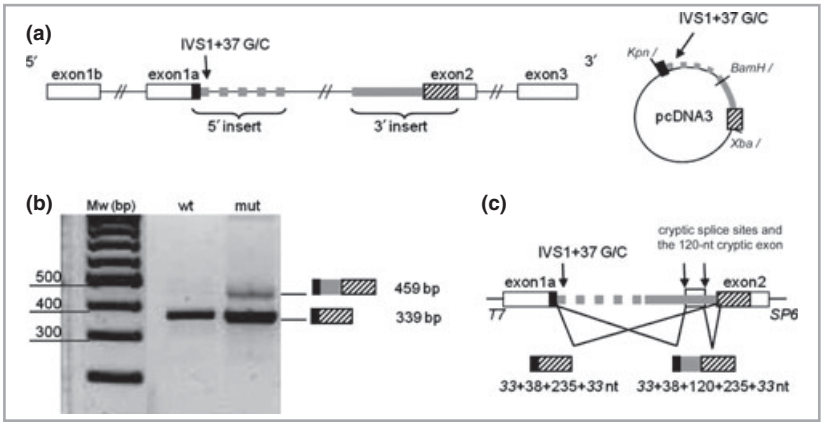


**Fig 2.** *In vitro* minigene approach for studying the effects of the IVS1+37 G/C *CDKN2A* intronic mutation on splicing regulation. (a) Two regions of the *CDKN2A* gene (Acc. No. AL449423.14; 5′ insert: nt 65456–64678 and 3′ insert: nt 62359–61715) were amplified from the genomic DNA of patient II/5, who carried the IVS1+37 G/C *CDKN2A* intronic mutation in a heterozygous form, and were cloned consequently into the pcDNA3 vector (Life Technologies, Carlsbad, CA, U.S.A.). The first insert of the minigene construct included a 38-nt sequence of exon 1a and a 741-nt sequence of the downstream intron (wild-type and mutant versions), while the second insert consisted of a 410-nt sequence of intron 1 and a 236-nt sequence of exon 2. The entire length of the insert was 1424 nt, lacking a 2318-nt deep intronic sequence of the *CDKN2A* gene (Acc. No. AL449423.14; nt 64677–62360). (b) Reverse transcription–polymerase chain reaction, in which the mRNA arising from the wild-type minigene (wt) was compared with that from the mutant (mut), revealed a differential splicing pattern. (c) Sequence analysis demonstrated that the IVS1+37 G/C mutation resulted in the recognition of cryptic splice sites in intron 1 and thus a 120-nt extension of the mRNA product.

cDNA Synthesis Kit (BioRad, Hercules, CA, U.S.A.) and the splice variants were detected with polymerase chain reaction (PCR), T7 and Sp6 primers being used for the pcDNA3 vector: in this way, the amplification of internal *CDKN2A* transcripts could be avoided. PCR reactions were performed in GoTaq Hot Start mix (Promega, Madison, WI, U.S.A.). The PCR products were run on 2% agarose gel and photographed, and the bands were purified from the gel and sequenced (Fig. 2b). Sequences were analysed with the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

## Results

The mutation is situated in intron 1 (IVS1+37 G/C) and has so far been mentioned twice in case reports: in a FAMMM pedigree and in a single patient with primary melanoma, both from Italy,[5,6] and summarized in a review paper by Orlow *et al.*[8] *In silico* assays reported in one of these papers did not reveal any predicted defects in mRNA processing caused by the mutation and it was therefore qualified as a mutation of unknown significance.

Our sequence analysis involving the comparison of the mRNA arising from the wild-type and that from the mutant minigene revealed a differential splicing pattern (Fig. 2b): the shorter band corresponds to a 339-nt *CDKN2A* sequence as a result of normal splicing, while the 459-nt upper band relates to an extended alternative splice product formed by the addition of a 120-nt sequence of intron 1 as an exon (Fig. 2c). This result was identically obtained in three independent transfection experiments on HeLa cells, suggesting that the

*in vitro* minigene approach applied was suitable for studying the effects of the identified intronic IVS1+37 G/C mutation on splicing.

## Discussion

Whether the aberrant mRNA is stable and translated or not, our results indicate that it may play a pathogenetic role in familial melanoma. If the aberrant mRNA were translated, the inclusion of the cryptic exon would result in a frameshift and an early stop codon would change the structure of the p16 protein; if the aberrant mRNA were not stable, it would reduce the quantity of functional p16 indirectly.

In conclusion, the segregation of the IVS1+37 G/C intronic *CDKN2A* mutation with FAMMM in the extensive melanoma-prone family and the results of our *in vitro* minigene experiments suggest that this mutation may have a pathogenetic role, most likely involving alteration of the splicing of the *CDKN2A* primary mRNA. Besides *CDKN2A* coding mutations, intronic mutations of the loci may contribute to melanoma susceptibility and identification of these mutations would facilitate our understanding of why the frequency of germline coding mutations of the *CDKN2A* gene is lower than expected in melanoma-prone families linked to chromosome 9p21.

### What's already known about this topic?

- The major locus for melanoma predisposition is the cell cycle regulatory *CDKN2A* gene on chromosome 9p21.
- However, the frequency of germline coding mutations of the *CDKN2A* gene is lower than expected in melanoma-prone families linked to chromosome 9p21.

### What does this study add?

- We report the detection and functional data on the possible pathogenetic role of a rare IVS1+37 G/C intronic mutation of the *CDKN2A* gene found in a Hungarian melanoma-prone family.
- This mutation most likely involves alteration of the splicing of the *CDKN2A* primary mRNA.

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