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A novel *CDKN2A* in-frame deletion associated with pancreatic cancer-melanoma syndrome

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Abstract

Pancreatic cancer-melanoma syndrome (PCMS) is an inherited condition in which mutation carriers have an increased risk of malignant melanoma and/or pancreatic cancer. About 30% of PCMS cases carry mutations in *CDKN2A*. This gene encodes several protein isoforms, one of which, known as p16, regulates the cell-cycle by interacting with CDK4/CDK6 kinases and with several non-CDK proteins. Herein, we report on a novel *CDKN2A* germline in-frame deletion (c.52_57delACGGCC) found in an Italian family with PCMS. By segregation analysis, the c.52_57delACGGCC was proven to segregate in kindred with cutaneous melanoma (CM), in kindred with CM and pancreatic cancer, and in a single case presenting only with pancreatic cancer. In the literature, duplication mapping in the same genic region has been already reported at the germline level in several unrelated CM cases as a variant of unknown clinical significance. A computational approach for studying the effect of mutational changes over p16 protein structure showed that both the deletion and the duplication of the c.52_57 nucleotides result in protein misfolding and loss of interactors' binding. In conclusion, the present results argue that the quantitative alteration of nucleotides c.52_57 has a pathogenic role in p16 function and that the c.52_57delACGGCC is associated with PCMS.

Keywords: PCMS, cutaneous melanoma, p16, pancreatic cancer, germline mutation

Introduction

Pancreatic cancer-melanoma syndrome (PCMS), (OMIM # 606719) is an inherited condition in which mutation carriers have an increased risk of developing malignant melanoma and/or pancreatic cancer. Mutations of *CDKN2A* gene, responsible for most cases of familial cutaneous melanoma (CM), have been found in about 30% of patients with PCMS [1]. *CDKN2A* is a tumor suppressor gene encoding a cyclin-dependent kinase inhibitor that promotes the arrest of the cell cycle at the G1 checkpoint. Translation of this gene leads to several protein isoforms, one of which, known as p16, acts as inhibitor of two kinases, namely CDK4 and CDK6 [2]. The protein p16 is composed of 148 residues arranged in four ankyrin repeats, amino acid motifs consisting of two alpha helices separated by one loop (i.e. helix-turn-helix conformation), [3]. The ankyrin repeats mediate the binding between p16 and its interactors; the second and the third ankyrin domains interact with CDK4 and CDK6, whereas the first and fourth ankyrin repeats stabilize the structure of p16 and bind several non-CDK proteins including GRIM-19, JNKs, and TFIIH [4]. By these interactions, p16 coordinates cell cycle progression through alternative and independent regulatory pathways (Figure1).

The present study reports on the novel *CDKN2A* c.52_57delACGGCC germline deletion found in an Italian kindred affected by PCMS and mapping in the

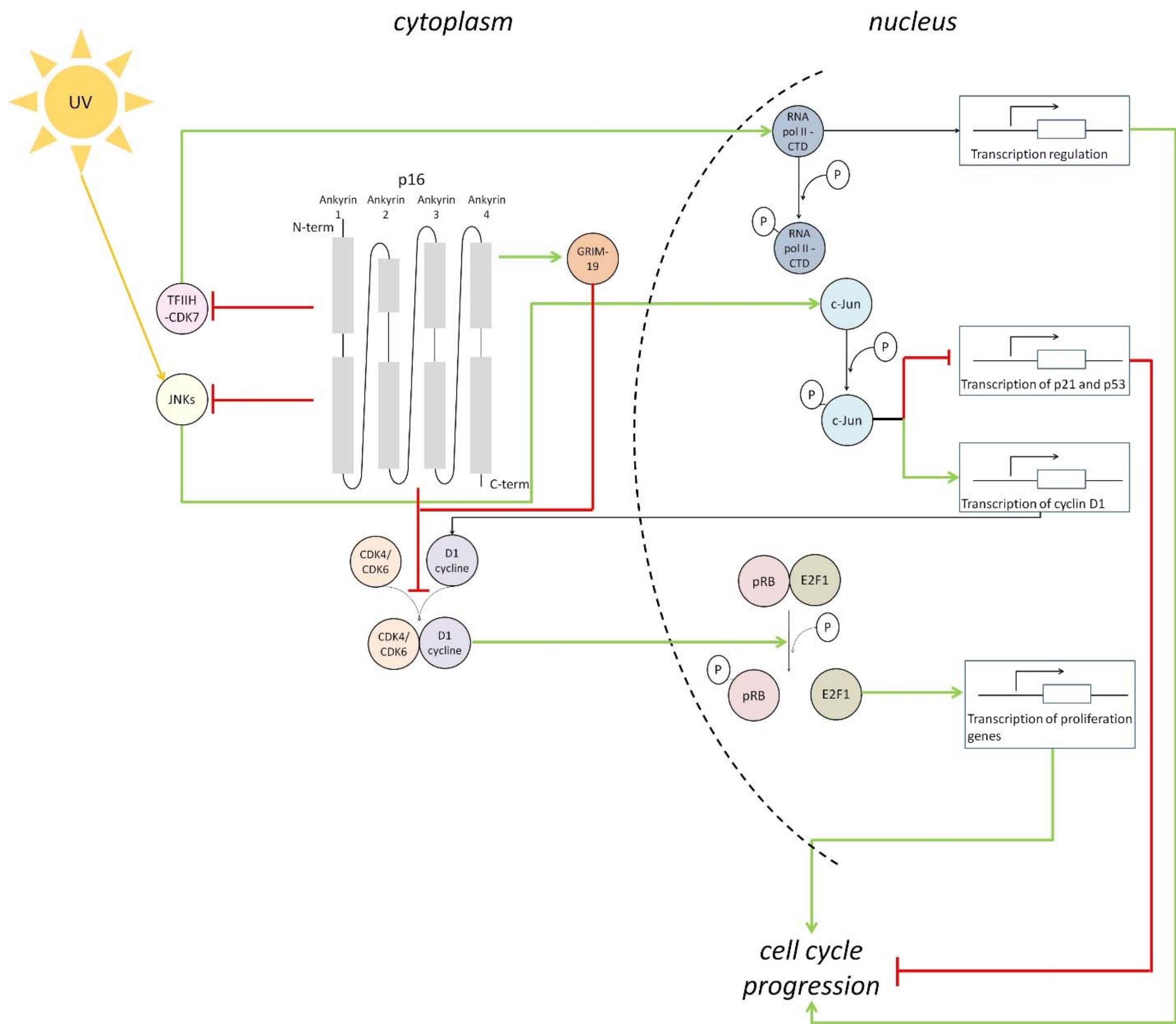

Figure 1. *Molecular pathways regulated by p16 protein. The structure of p16, arranged in four ankyrin domains, is given in grey. p16 regulates the cell cycle by interacting with both CDK and non-CDK proteins including:*

CDK4 and CDK6, acting in the retinoblastoma protein (pRB) pathway. Phosphorylation of pRB by the CDK4/CDK6-D1 cyclin complex releases the E2F1 transcription factor from the pRB/E2F1 complex, which activates the transcription of proliferation-associated genes. p16 interacts with the CDK4/CDK6-D1 cyclin complex to inhibit the phosphorylation of pRB, thus preventing the cell cycle progression [15]; (ii) GRIM-19, that binds p16 preventing the association of CDK4 with cyclin D1 [15]; (iii) TFIIH, a multifunctional complex that links transcription to DNA repair and cell cycle regulation. TFIIH is composed of two sub-complexes: the core and the cyclin activating kinases (CAK) complex including the CDK7 subunit. CDK7 phosphorylates, and thus activates, the CDK4/CDK6 complex [16]. Also, CDK7 phosphorylates the C-terminal domain (CTD) of RNA polymerase II [17]. p16 inhibits the CAK activity by interacting with TFIIH, thus creating a link between the basal transcription apparatus and the regulation of cell cycle progression [18]; (iv) JNK kinases, that, after activation by UV-radiation or other genotoxic stress, translocate to the nucleus. Here, they phosphorylate c-Jun transcription factor. c-Jun can stimulate cell cycle progression through two mechanisms: induction of cyclin D1 transcription and repression of p21 and p53 gene expression transcription [19]. p16 inhibits the JNK activities.

Alterations of p16 protein can then enhance cell cycle progression by impairing the inhibitory activity over the CDK4/CDK6-D1 cyclin complex, over the CDK7 subunit of TFIIH and over the JNKs, while promoting the function of GRIM-19.

first ankyrin domain of p16. Genetic analyses were performed by Next Generation Sequencing (NGS), a technique that enables the simultaneous analysis of many genes/genomic regions by analyzing, in one reaction, millions of DNA fragments. The pathogenic role of the c.52_57delACGGCC over the p16 structure

and ability of binding interactors was investigated by molecular modeling, a computational technique used to represent and to simulate the behavior of molecules. By this approach, the three-dimensional atomic structure of a protein can be constructed starting from its amino acid sequence. Hence, amino acid alterations can be modeled in the obtained protein structure to study their impact over the molecule's stability and ability of binding interactors [5].

Case Synopsis

A 53-year-old man (**Figure 2**, case III:5) was referred to genetic counseling because of a personal and familial history of cancer. At the age of 41, he developed a primary cutaneous melanoma on his right leg. The melanoma was characterized by a Breslow index of 0.72mm, a Clark Level III and a thickness <1mm without ulceration (pT1a). During genetic counseling, a family history of CM emerged. Patient III:5 reported that one of his sons (IV:4) and a paternal uncle (II:2) had developed CM, respectively on the right hand at 27 years old and on the thoracic region at 45 years old. Moreover, about 11 years before our evaluation of patient III:5, case II:2 underwent *CDKN2A* testing and was found to carry the NM_000077.4:c.52_57delACGGCC variant in heterozygosity. At that time, this alteration was reported as a variant of unknown significance and no other familial studies were conducted. The family history was also remarkable for the presence of other cancer types: pancreatic cancer (cases II:2 and II:3), lung cancer (case I:1), and head and neck cancer (case I:1).

To verify the familial segregation of the *CDKN2A* c.52_57delACGGCC and of other possible cancer-susceptibility variants, other kindred were recruited. After obtaining informed consent which was approved by local ethic committees in accordance

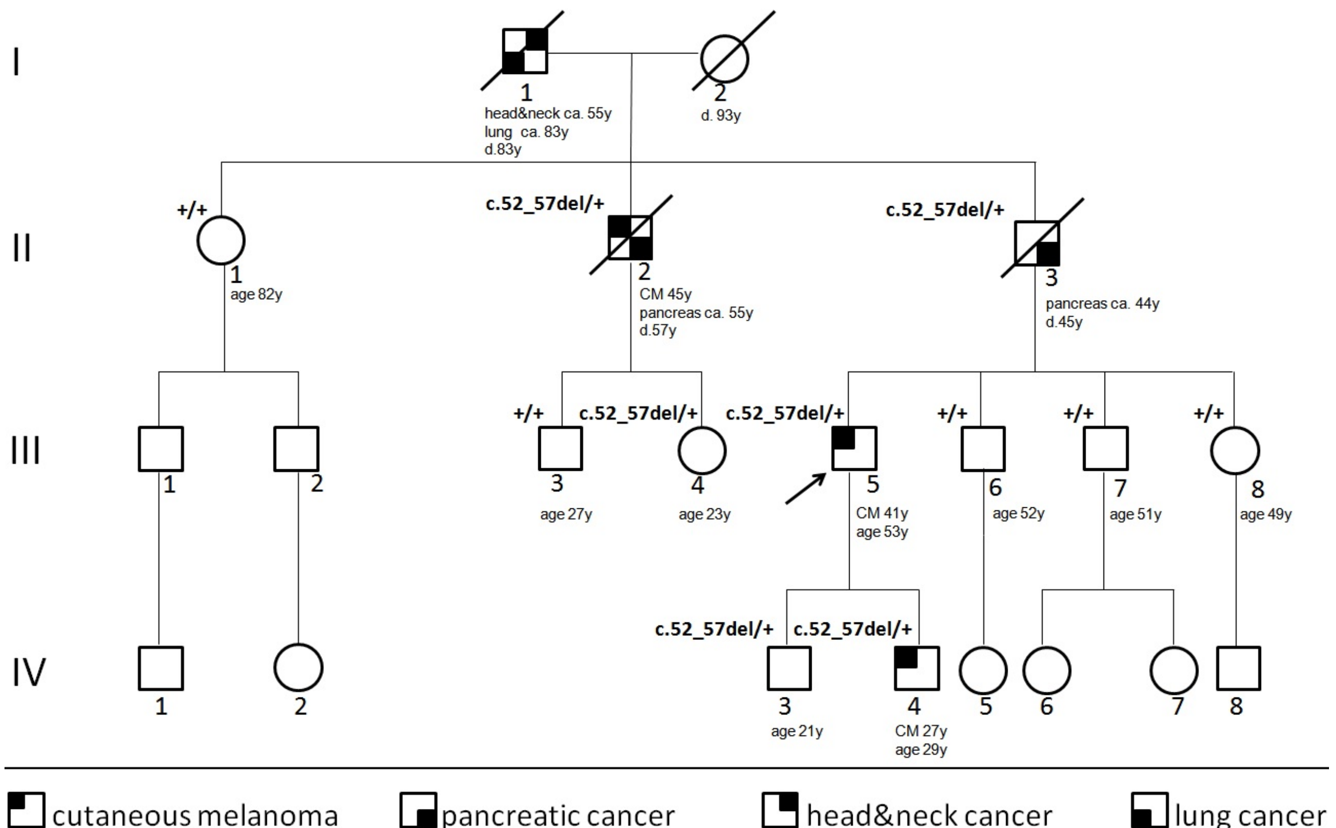

Figure 2. *Family pedigree showing the segregation of the CDKN2A c.52_57delACGGCC. The clinical legend is given under the pedigree. Generation numbers (i.e. I, II, III and IV) are shown on the left. For each generation, individuals are numbered consecutively. Genomic DNA was not available for individual II:3, and consequently, for this patient, the presence of the CDKN2A change was inferred.*

+, CDKN2A wild type allele; d, age at death; CM, cutaneous melanoma; ca, cancer; y, years.

with the principles of the Declaration of Helsinki, DNA of cases II:1, III:3, III:4, III:5, III:6, III:7, III:8, IV:3 and IV:4 were collected. Firstly, patient III:5 was analyzed for five melanoma susceptibility genes (*CDKN2A*, *CDK4*, *BAP1*, *MITF* and *POT1*), [6] by NGS on the Ion S5XL System (ThermoFisher, Carlsbad, CA, USA). The sequencing reads produced by the NGS approach were firstly aligned over the reference human genome sequence (assembly GRCh37, hg19). Then, these mapped reads were analyzed by the Variant Caller v5.4.0.46 plugin (ThermoFisher) for identifying nucleotide variants with respect to the hg19. The identified DNA variants were classified according to the ACMG/AMP 2015 guidelines that recommends a five-tier classification system based on typical types of variant evidence (e.g. population data, computational data, functional data, segregation data), [7]. According to this system, a variant can be indeed classified as: pathogenic, likely pathogenic, uncertain significance (VUS), likely benign, and benign. Finally, to rule out "false positives" variants, the genetic changes found in case III:5 were confirmed by another sequencing technique (i.e. Sanger sequencing) and their segregation was tested in the other family members. Sanger method is required for validation of NGS results because, even if limited to determine the sequence of one fragment of DNA per reaction, it is an accurate sequencing technique.

Following the genetic analyses, the role of *CDKN2A* variants over p16 structure and its ability of binding interactors was investigated by molecular modeling obtained with PyMOL 2.0 software.

Apart from benign changes, the *CDKN2A* c.52_57delACGGCC was the only variant filtered in patient III:5 (**Figure 3A)**. This alteration was found to segregate in heterozygosity in cases affected by CM (III:5 and IV:4), in a patient with CM and pancreatic cancer (II:2), but also in a patient who developed only pancreatic cancer (II:3). This patient, even if not analyzed, was indeed inferred to be a heterozygous obligate carrier. The c.52_57delACGGCC showed incomplete penetrance in cases III:4 and IV:3, respectively 23 and 21 years old, not affected by any cancer type. The five kindred that resulted as wild type for nucleotides c.52_57 (II:1, III:3, III:6, III:7, III:8), did not show the presence of any cancer type. Their age at the last clinical evaluation ranged from 27 years old to 82 years old. The c.52_57delACGGCC removes 6 nucleotides in exon 1α of the p16 mRNA but otherwise preserves the integrity of the reading frame. Structural protein analyses showed that the c.52_57 nucleotides are located in the first ankyrin domain of *CDKN2A*, at the alpha-helix comprising residues 15-22, which is directly involved in the binding with each one of p16 interactors (i.e. CDK6, CDK4, JNKs and TFIIH), (**Figure 3B)**. The deletion and the duplication of nucleotides c.52_57 are responsible respectively for the deletion of amino acids threonine-18 and alanine-19 (p.T18_A19del) and for the insertion of aminoacids threonine and alanine between alanine-19 and alanine-20 (p.A19_A20insTA), (**Figure 3B)**. Modeling analysis showed that both these alterations result in the complete misfolding of the alpha-helix and in the loss of key polar contacts with all the p16 interactors (**Figure 3C)**.

Case Discussion

Based on the collected data and according to American College of Medical Genetics and Genomics and the Association for Molecular Pathology 2015 criteria [7], the *CDKN2A* c.52_57delACGGCC variant can be classified as "likely pathogenic" and was responsible for PCMS in the present family.

The *CDKN2A* c.52_57delACGGCC indeed met two pathogenic moderate (PM), two pathogenic supporting (PP) and one benign supporting (BP) ACMG/AMP 2015 criteria [7]. These included: (i) PM1: the c.52_57delACGGCC is located in a *CDKN2A* mutational hot spot region belonging to the ankyrin-1 domain; (ii) PM2: the c.52_57delACGGCC is a rare variant never reported in literature, not recorded in the ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and also absent from databases of large-scale sequencing projects (i.e. Exome Sequencing Project, 1000 Genomes Project, Exome Aggregation Consortium); (iii) PP1: in the present family, the c.52_57delACGGCC segregates in patients with melanoma and/or pancreatic cancer, while absent in five unaffected cases; (iv) PP3: computational

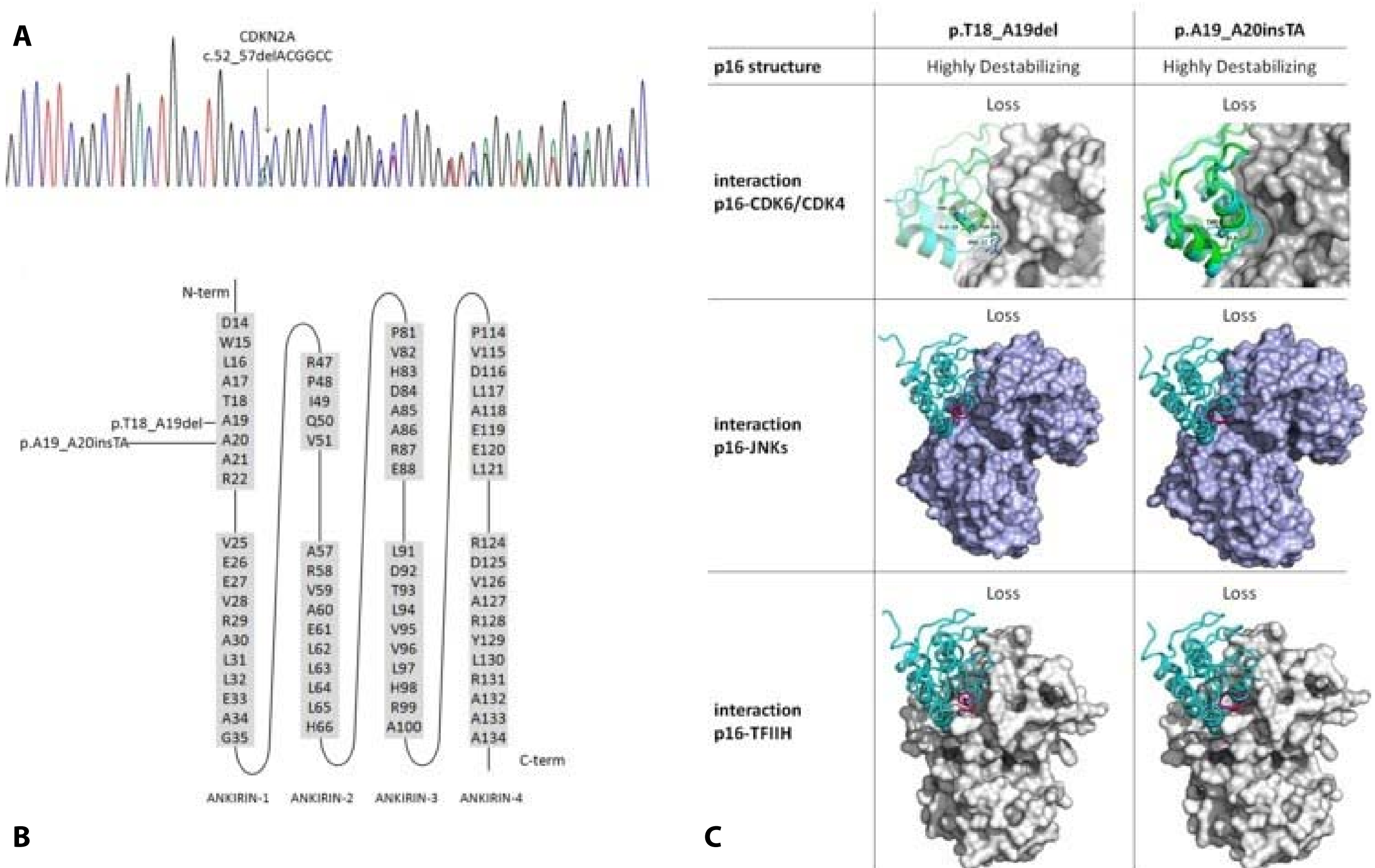

Figure 3. *Molecular characterization of the CDKN2A c.52_57delACGGCC mutation.* ***A)*** *Sanger sequencing electropherogram of CDKN2A exon 1α in patient III:5, showing the presence of the c.52_57delACGGCC in heterozygosity.* ***B)*** *Topology diagram of the p16 protein structure. Helices are shown as rectangles, with the residue numbers forming the helices indicated. Structural turns and loops are shown as lines. The four ankyrin domains are indicated below their corresponding residues. The p.T18_A19del and p.A19_A20insTA alterations are mapped over the protein diagram.* ***C)*** *Computational modeling predictions of p16 protein, obtained with PyMOL 2.0 software by in silico mutagenesis of the crystal structure of p16 in complex with CDK6 (PDB:1BI7), [20]. PDB 1BI7 was used also as a structural template to model the interaction between p16 and CDK6 (PDB:1BLX), [21], JNK (PDB:4YR8), [22] and CDK7-TFIIH (PDB:1UA2), [23].The table reports the effects of the p.T18_A19del and of the p.A19_A20insTA mutations of p16 structure and on the binding of CDK and non-CDK interactors. The view of the crystal structure of p16 is given for each prediction. The p16 wild type and mutant protein are shown respectively as green and cyan ribbons. The α-helix in which the mutation is located is colored in purple. CDK4 and CDK6 kinases are represented by a grey surface, while JNKs and CDK7-TFIIH are represented respectively as pale blue surface and grey surface.*

evidence (i.e. molecular modeling) supports the deleterious effect of the c.52_57delACGGCC on the p16 gene product; (v) BP3: the c.52_57delACGGCC is an in-frame deletion in a repetitive region.

As proposed by Walsh et al., 2018 [8], the pathogenic role of the c.52_57delACGGCC is also strengthened by the observation that it is a somatic mutation hotspot, since present in samples of adenocarcinoma, cholangiocarcinoma, squamous cell carcinoma, esophageal cancer, and pancreatic cancer (data obtained from PMKB, ICGC Somatic, and COSMIC databases). In addition, a six-nucleotide duplication mapping in the same genic region, namely the c.52_57dupACGGCC, had been already included in ClinVar as a VUS since it is a rare variant already reported at germline level in several melanoma-prone families [9-14]. Experimental studies have shown that it alters the binding with CDK4 [13]. However, being an insertion of two aminoacids to the p16 protein (p.Thr18_Ala19dup) that does not alter the reading frame, the evidence had been considered, in ClinVar, insufficient to determine the role of CDKN2A c.52_57dupACGGCC in disease.

Our results demonstrated that both the c.52_57delACGGCC and the c.52_57dupACGGCC affect the folding of p16 protein and also its ability of binding interactors.

Conclusion

The present results support the pathogenic role of the quantitative alteration of nucleotides *CDKN2A* c.52_57 in disease onset. In particular, the *CDKN2A* c.52_57delACGGCC was responsible for pancreatic cancer-melanoma syndrome in the described family.

Potential conflicts of interest

The authors declare no conflicts of interests.

References

1. Ghiorzo P, Fornarini G, Sciallero S, et al. CDKN2A is the main susceptibility gene in Italian pancreatic cancer families. *J Med Genet*. 2012;49:164-70. [PMID: 22368299].
2. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*. 1999;13:1501-12. [PMID: 10385618].
3. Byeon IJ, Li J, Ericson K, et al. Tumor suppressor p16INK4A: determination of solution structure and analyses of its interaction with cyclin-dependent kinase 4. *Mol Cell*. 1998;1:421-31. [PMID: 9660926].
4. Li J, Poi MJ, Tsai MD. Regulatory mechanisms of tumor suppressor P16(INK**4A)** and their relevance to cancer. *Biochemistry*. 2011;50:5566-82. [PMID: 21619050].
5. Feyfant E, Sali A, Fiser A. Modeling mutations in protein structures. *Protein Sci*. 2007;16:2030-41. [PMID: 17766392].
6. Leachman SA, Lucero OM, Sampson JE, et al. Identification, genetic testing, and management of hereditary melanoma. *Cancer Metastasis Rev*. 2017;36:77-90. [PMID: 28283772].
7. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17:405-24. [PMID: 25741868].
8. Walsh MF, Ritter DI, Kesserwan C, et al. Integrating somatic variant data and biomarkers for germline variant classification in cancer predisposition genes. *Hum Mutat*. 2018;39:1542-52. [PMID: 30311369].
9. Soufir N, Avril MF, Chompret A, et al. Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. *Hum Mol Genet*. 1998;7:209-16. [PMID: 9425228].
10. Goldstein AM, Chan M, Harland M, et al. High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. *Cancer Res*. 2006;66:9818-28. [PMID: 17047042].
11. Goldstein AM, Chaudru V, Ghiorzo P, et al. Cutaneous phenotype and MC1R variants as modifying factors for the development of melanoma in CDKN2A G101W mutation carriers from four countries. *Int J Cancer*. 2007;121:825-31. [PMID: 17397031].
12. Harland M, Cust AE, Badenas C, et al. Prevalence and predictors of germline CDKN2A mutations for melanoma cases from Australia, Spain and the United Kingdom. *Hered Cancer Clin Pract*. 2014;12:20. [PMID: 25780468].
13. Kannengiesser C, Brookes S, del Arroyo AG, et al. Functional, structural, and genetic evaluation of 20 CDKN2A germ line mutations identified in melanoma-prone families or patients. *Hum Mutat*. 2009;30:564-74. [PMID: 19260062].
14. Maubec E, Chaudru V, Mohamdi H, et al. Familial melanoma: clinical factors associated with germline CDKN2A mutations according to the number of patients affected by melanoma in a family. *J Am Acad Dermatol*. 2012;67:1257-64. [PMID: 22841127].
15. Sun P, Nallar SC, Raha A, et al. GRIM-19 and p16(INK4a) synergistically regulate cell cycle progression and E2F1-responsive gene expression. *J Biol Chem*. 2010;285:27545-52. [PMID: 20522552].
16. Lolli G, Johnson LN. Recognition of Cdk2 by Cdk7. *Proteins*. 2007;67:1048-59. [PMID: 17373709].
17. Matsuno M, Kose H, Okabe M, Hiromi Y. TFIIH controls developmentally-regulated cell cycle progression as a holocomplex. *Genes Cells*. 2007;12:1289-300. [PMID: 17986012].
18. Nishiwaki E, Turner SL, Harju S, et al. Regulation of CDK7-carboxyl-terminal domain kinase activity by the tumor suppressor p16(INK**4A)** contributes to cell cycle regulation. *Mol Cell Biol*. 2000;20:7726-34. [PMID: 11003668].
19. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol*. 2002;4:E131-6. [PMID: 11988758].
20. Russo AA, Tong L, Lee JO, et al. Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumour suppressor p16INK4a. *Nature*. 1998;395:237-43. [PMID: 9751050].
21. Brotherton DH, Dhanaraj V, Wick S, et al. Crystal structure of the complex of the cyclin D-dependent kinase Cdk6 bound to the cell-cycle inhibitor p19INK4d. *Nature*. 1998;395:244-50. [PMID: 9751051].
22. Liu X, Zhang CS, Lu C, et al. A conserved motif in JNK/p38-specific MAPK phosphatases as a determinant for JNK1 recognition and inactivation. *Nat Commun*. 2016;7:10879. [PMID: 26988444].
23. Lolli G, Lowe ED, Brown NR, Johnson LN. The crystal structure of human CDK7 and its protein recognition properties. *Structure*. 2004;12:2067-79. [PMID: 15530371].