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The Human *CDKN2A* Gene: Analyzing How Mutations of a Section of the Gene Can Be Associated with Malignant Melanoma

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Abstract: **In this study, a section of the human *CDKN2A* gene was obtained from unknown individuals and analyzed for the purpose of detecting any probable single nucleotide polymorphisms (SNP). The exon-2 of this gene was chosen because its mutation was previously reported to be associated with malignant melanoma. At first, suitable primers were designed for a specific region of the CDKN2A gene including exon-2 and later the area was amplified several times at different temperatures through polymerase chain reactions (PCR). The PCR products of 60°C with 250ng human placental DNA was extracted for quantification and sequencing. After the sequencing, the three CDKN2A gene transcripts were found to be a 100% match with the database in NCBI and. no SNP was detected. However, in the genomic sequences, an insertion of nucleotide was detected in the intron of this gene. Furthermore, protein translation in 5'-3' open reading frame was significant.**

Keywords: **CDKN2A, SNP, malignant melanoma, PCR.**

I. INTRODUCTION

The CDKN2A gene is the major one whose mutation or any other abnormalities may lead to several types of genetic disorders particularly cancer. Among these, malignant melanoma is the most reported case[1]. The full name of CDKN2A gene is Cyclin-Dependent Kinase Inhibitor 2A. It is also known as ARF; MLM; p14; p16; p19; CMM2; INK4; MTS1; TP16; CDK4I; CDKN2; INK4a; p14ARF; p19Arf; p16INK4; p16INK4a. It is a human protein coding gene [2] which is responsible for encoding proteins that work as tumor suppressors. The CDKN2A gene is about 7282 base pairs (UCSC genome bioinformatics) with three exons. Exon-1 (E1) contains 125 basepairs, Exon-2 (E2) contains 307 basepairs, and Exon-3 (E3) contains 12 basepairs. The CDKN2A gene is responsible for encoding two key proteins i.e. p16(INK4) and p14(ARF) which regulate some vital cell cycle regulation pathways[3]. The p16 acts as cyclin-dependent kinase inhibitor (CDK4 and CDK6), whereas the p14 stabilizes p53 [4]. These proteins give a brake in the cell division cycle by halting the synthesis of DNA before the cell divides [1]. So, if there is any mutation in CDKN2A gene, these proteins cannot work properly which suddenly causes an alteration in skin growth and appearance. This may eventually lead to malignant melanoma. Although several types of cancers may develop from the abnormalities or malfunctioning of these protein products of CDKN2A gene, melanoma is the most reported disorder associated with them. The CDKN2A gene has been found to have roles in several types of disease such as pancreatic cancer, esophageal and gastric cancer, leukemia, bladder cancer, melanomas and so on. In pancreatic adenocarcinoma, allelic loss at 9p, containing 9p21 as well was observed at high frequency and came up with a decision that CDKN2A gene mutations are the main reason behind this [5]. Most common disorder due to CDKN2A mutation is malignant melanoma. About 75% of melanoma cell lines were found to have mutations or homozygous deletions in this gene [6]. In the same year, researchers also detected 6 probable disease-associated mutations at CDKN2A gene [7]. However, next year, another group did some biochemical analysis on the melanoma families and found a single somatic mutation [7],[8]. After reviewing the significance of CDKN2A mutation

on cutaneous melanoma development, Dracopoli and Fountain reported seven independent studies in which 34 of 76 families were found with CDKN2A germline mutation [9]. However, they found 14 distinct germline mutations of CDKN2A among which there were 7 missense, 1 nonsense and a splice donor mutation, 1 insertion as well as 4 deletions. In the next year, Harand et al. studied 27 families in UK who were with melanoma predisposition and found five distinct germline mutations of CDKN2 in 6 families[10]. In the same year, another group found that in case of lung squamous cell carcinomas, there is 100% heterozygosity loss in the 9p region [11]. Then in 1998, Monzon et al. tried to identify CDKN2A germline mutations in multiple primary melanoma patients who did not have any familial history of this disorder [12]. If expression of CDKN2A gene is enhanced rather than its inactivation, it may lead to the primary colorectal carcinomas [13].

After reviewing several journals, the exon 2 of the CDKN2A gene was chosen for PCR amplification. Mutation of this part of the gene was found in a number of studies[14],[15],[16]. We know that, CDKN2A gene is mainly responsible for encoding p16 and p14ARF proteins. These two proteins usually share exon-2 and part of exon-3 [4]. Overlapping open reading frames may cause some mutations in exon 2 and thus both p16 and p14ARF can be affected.

II. MATERIALS AND METHODS

Selection Of The Area Of Interest

A number of journal articles were reviewed to select a region within the gene and the part of the sequence selected was exon-2 with some introns upstream and downstream (Fig. 1).

To get this sequence, the UCSC bioinformatics website [17], their web based software and database were explored.

gtctgtccctgccccctttgctattttgcccgtgcctcacagtgctctctgcctgtgacgacagctccgcagaagttcggaggatataatgga
attcattgtgtactgaagaatggatagagaactcaagaaggaaattggaaactggaagcaaatgtaggggtaattagacacctggg
gcttgtgtgggggtctgcttggcggtgaggggctctacacaagcttcctttccgtcatgccggcccccaccctggctctgaccattctgtt
ctctctggcagGTCATGATGATGGGCAGCGCCCGAGTGGCGGAGCTGCTGCTGCTCCACGGC
GCGGAGCCCAACTGCGCCGACCCCGCCACTCTCACCCGACCCGTGCACGACGCTGCCC
GGGAGGGCTTCCTGGACACGCTGGTGGTGCTGCACCGGGCCGGGGCGCGGCTGGACGT
GCGCGATGCCTGGGGCCGTCTGCCCGTGGACCTGGCTGAGGAGCTGGGCCATCGCGAT
GTCGCACGGTACCTGCGCGCGGCTGCGGGGGGCACCAGAGGCAGTAACCATGCCCGCA
TAGATGCCGCGGAAGGTCCCTCAGgtgaggactgatgatctgagaatttgtaccctgagagcttccaaagctcaga
gcattcattttccagcacagaaagttcagcccgggagaccagtctccggtcttgcctcagctcacgcgccaatcggtgggacggcctg
agtctccctatcgccctgccccgccagggcggcaaatgggaaataatcccgaaatggacttgcgcacgtgaa

Fig. 1: Selected region from CDKN2A exon 2 with some introns upstreams and downstreams.

Primer Design

Web-based bioinformatics software "Primer 3" [18] was used which provided information on primers, showed the exact annealing positions, size of the product and as well as the temperature of melting point.

Performing UCSC In-slilico PCR

The design of the primers was verified by software based program in UCSC bioinformatics software.

Performing Polymerase Chain Reaction (PCR)

To amplify the selected target sequence, several polymerase chain reactions were carried out in this project. The steps involved to perform this reaction are discussed as follows:

1. Preparation primer solution (10 µmol/µl): For PCR, both primers were diluted to 10 µmol/µl. Both primer solutions were then kept in the labeled box and stored in a freezer (at -20 degree Celsius) before commencing further work.
2. Preparation of Master Mix: Master mix solution was prepared with the primers, enzyme mix (Taq polymerase, reaction buffer, dNTPs and MgCl2) and DNAase/RNAase free water.

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3. Preparing DNA replicates and Control:Once, the master-mix was ready, 19 µl of the mixture was poured three separate tubes. Then in each of the two replicate tubes, 1 µl of placental DNA (~250ng) was added, whereas in control tube, only 1 µl of water was added. All three tubes were centrifuged and kept in the cool box.

PCR Programme

The PCR tubes (2 DNA replicates and 1 control) were then inserted in the PCR machine (Thermocycler). The Thermocycler was started as per the conditions mentioned below:

Initial denaturation: 94°C for 5 minutes, denaturation: 94°C for 1 minutes, annealing: 58-62°C for 30 cycles, extension: 72°C for 0.5 minute, and final extension: 72°C for 1 minute.

Annealing Temperatures and DNA concentrations in PCR:

A number of PCR were carried out at various temperatures from 58°C -62°C to determine the best probable annealing temperature and to obtain the best product. Of the mentioned temperature range, 60°C showed the best annealing.

Gel Electrophoresis

After the PCR, the samples were investigated through gel electrophoresis which was performed as previously described method [7].

Sequence Data Analysis

When the sequences of both primers arrived, they were analyzed using bioinformatics software "Chromas". For both primer sequences, the unidentified nucleotides were removed from both ends. After the removal, the reverse primer was prepared as reverse complement using the web-based software "Bioweb" [19]. Then both sequences were taken in a single notepad for joining. The overlapping region of these two sequences was detected. Then the end part from forward primer and the starting part from reverse complement was removed. Next, they were joined together and this gave the final sequence of amplified DNA.

This sequence was then submitted to NCBI nucleotide blast and compared with the database to see whether there was any mutation or SNP or deletion or any other abnormalities. The sequence was also translated to amino acids using Expasy web-based software [20].

III. RESULTS

Primer Design Obtained By "Primer3"

Using "Primer3"[18] web based software the primers were designed along with their probable annealing positions and tms (melting point temperature) as well. The output of this software is shown below:

No mis-priming library specified.

Table I: Using 1-Based Sequence Positions

OLIGO	start	Length	tm	gc%	Any	3' seq
LEFT PRIMER	250	20	60.26	55.00	5.00	0.00 cctggctctgaccattctgt
RIGHT PRIMER	791	20	59.90	45.00	3.00	2.00 gcaagtccatttcgggatta

SEQUENCE SIZE: 800

INCLUDED REGION SIZE: 800

PRODUCT SIZE: 542, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

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UCSC In-silico PCR

To verify the primer design UCSC in-silico PCR was run [17] (Fig. 2).

>chr9:21970698-21971239 542bp CCTGGCTCTGACCATTCTGTGCAAGTCCATTT
CGGGATTACCTGGCTCTGACCATTCTGTtctctctggcaggtcatgatgatgggcagcgcccgagtggcggagct
gctgctgctccacggcgcggagcccaactgcgccgaccccgccactctcacccgacccgtgcacgacgctgcccgggagggcttc
ctggacacgctggtggtgctgcaccgggccggggcgcggctggacgtgcgcgatgcctggggccgtctgcccgtggacctggctga
ggagctgggccatcgcgatgtcgcacggtacctgcgcgcggctgcgggggggcaccagaggcagtaaccatgcccgcatagatgc
cgcggaaggtccctcaggtgaggactgatgatctgagaatttgtaccctgagagcttccaaagctcagagcattcattttccagcacag
aaagttcagcccgggagaccagtctccggtcttgcctcagctcacgcgccaatcggtgggacggcctgagtctccctatcgccctgcc
ccgccagggcggcaaatgggaaaTAATCCCGAAATGGACTTGC

Fig. 2: Result of UCSC in-silico PCR, demonstrating the verification of primers designed by the product outcome.

From this result, It was seen that the primers worked perfectly with In-silico PCR and gave exactly the same product shown by primer3 software. Moreover, the Tms for both primers were also found to be same here which was 60.3°C for forward and 59.9°C for reverse primer.

Outcomes from Gel Electrophoresis after PCR

All of the PCR products were analyzed with gel electrophoresis to see whether the product of desired size was been produced. PCRs were run at 60°C with 250ng of human placental DNA (Fig. 3). The reason for running the same PCRs several times was to ensure reproducibility as well as to ensure enough samples for DNA quantification and sequencing. Finally, the PCR products were mixed together and centrifuged. This PCR mix was then ready to be used for DNA quantification as well as sequencing.

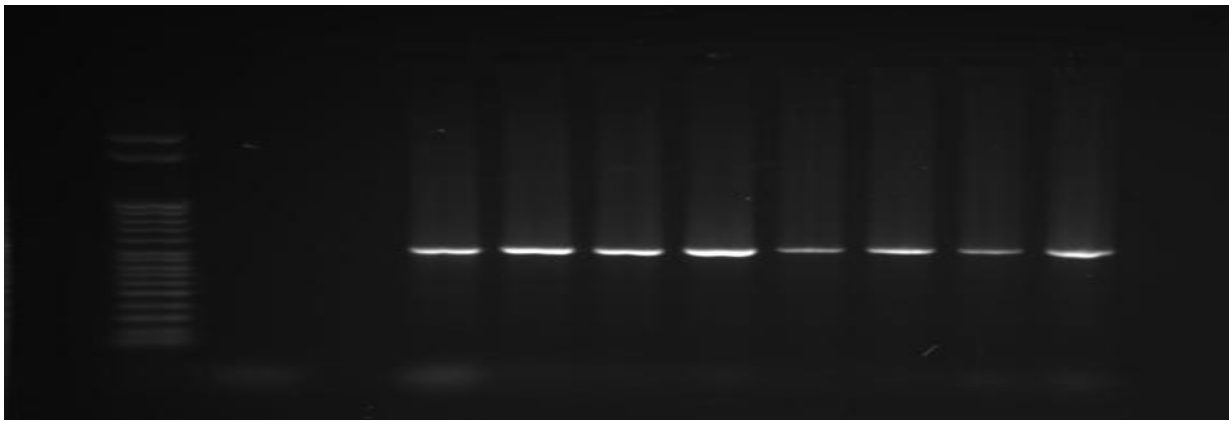

Fig. 3: Agarose gel Electrophoresis of PCR at 60°C (all bands appeared around 542 bp)

DNA Sequencing

The following DNA sequence was generated (Fig. 4).

CTGCTGCTGCTCCACGGCGCGGAGCCCAACTGCGCCGACCCCGCCACTCTCACCCGACC
CGTGCACGACGCTGCCCGGGAGGGCTTCCTGGACACGCTGGTGGTGCTGCACCGGGCC
GGGGCGCGGCTGGACGTGCGCGATGCCTGGGGCCGTCTGCCCGTGGACCTGGCTGAGG
AGCTGGGCCATCGCGATGTCGCACGGTACCTGCGCGCGGCTGCGGGGGGCACCAGAGG
CAGTAACCATGCCCGCATAGATGCCGCGGAAGGTCCCTCAGGTGAGGACTGATGATCTGA
GAATTTGTACCCTGAGAGCTTCCAAAGCTCAGAGCATTCATTTTCCAGCACAGAAAGTTCA
GCCCGGGAGACCAGTCTACCGGTCTTGCCTCAGCTCACGCGCCAAT

Fig. 4: Final DNA sequnce generated with PCR at 60°C.

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Blasting the Final Sequence

The final sequence was then submitted to NCBI nucleotide BLAST in order to compare the generated sequence with those in the database. The outcomes of the blast are shown as Follows [21]:

Table II: Sequences Producing Significant Alignments

Description	Max score	Total score	Query coverage	E value	Max ident
Transcripts					
Homo sapiens cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) (CDKN2A), transcript variant 1, mRNA	359	359	60%	6e-97	100%
Homo sapiens cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) (CDKN2A), transcript variant 4, Mrna	359	359	60%	6e-97	100%
Homo sapiens cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) (CDKN2A), transcript variant 3, mRNA	359	359	60%	6e-97	100%
Homo sapiens cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) (CDKN2B), transcript variant 2, mRNA	198	198	44%	1e-48	91%
Homo sapiens cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) (CDKN2B), transcript variant 1, mRNA	198	198	44%	1e-48	91%
Genomic Sequences					
Homo sapiens chromosome 9 genomic contig, GRCh37 reference primary assembly	584	783	100%	8e-165	99%
Homo sapiens chromosome 9 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence	584	783	100%	8e-165	99%

From the above table, it is noted that three of the transcripts matched 100% with the human CDKN2A gene. Moreover, two CDKN2B transcripts were also produced and matched to 91% of the database. Besides, the two genomic sequences matched 99% with the data base.

Translation of the sequence to Protein

The sequence was then submitted to a web based protein translation tool [20] and the following reading frame (Fig. 5) was found in which they can be expressed-

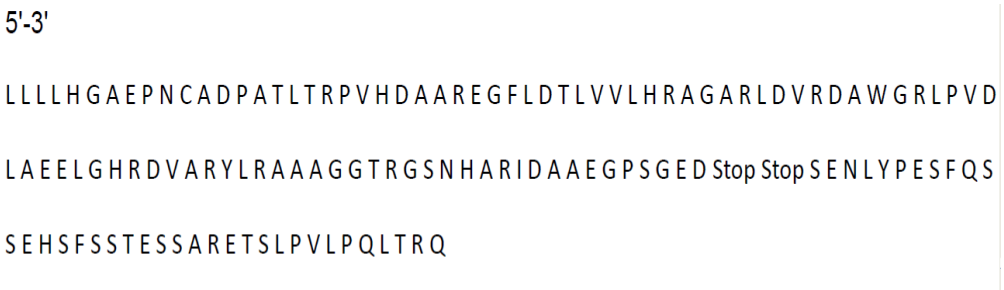

Fig. 5: Protein translation with 5'-3' open reading frame

Here, for 5'3' strand, the frame-1 seems to be the most widely open frame since as it has very few "Stop" codons. So, this was supposed to give the best protein translation. This amino acid sequence was then blasted in SIB (Swiss Institute of Bioinformatics) blast network service and the sequence matched 51 different proteins, principally, with the protein products of CDKN2A gene [22].

IV. DISCUSSION

Many studies previously reviewed showed the activities of CDKN2A gene and its association with many diseases particularly malignant melanoma. After PCR amplification of exon-2 of this gene, no mutation or SNP was detected in this project. A little insertion was found with the genomic sequence comparing the database may not be very harmful as it was detected in the intron. One of the reasons for not finding any SNP may be the placental DNA used. The donor of that DNA might not be a melanoma prone. However, in protein translation, "3" frame-1 gave the desired protein which matched 100% with the database.

V. CONCLUSION

Although in this analysis no mutation in the transcripts was identified, it cannot be concluded that this part of the CDKN2A gene does not have mutation. For future directions, these experiments can be repeated with the same region of interest and can be continued by changing some parameters such as choosing the length of introns upstream and downstream of the exon-2.

Moreover, the same region of CDKN2A (including exon-2 and same intron length) can be amplified by using the DNA from the melanoma patients or a group of melanoma prone patients.

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