SNPs Analysis in the CDKN2A /CDKN2B Genes in human assayed by DNA sequencing and ARMS™ testing

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ABSTRACT

This research facilitates the detection process of single-nucleotide polymorphism (SNP) by DNA sequencing and Amplification Refractory Mutation System™ (ARMS™). Alterations of four SNPs have been investigated at the specific location in CDKN2A and CDKN2B genes. In fact, these are tumor suppressor genes. The DNA from the cultured cell were extracted and then by using specific Oligonucleotide for SNPs, the CDKN2A and CDKN2B genes were amplified by PCR later separated by gel electrophoresis. Finally, the ARMS test and sequencing test were applied for all samples to find out allele frequency and genotype frequency in this population. The result presented that the efficient selection of oligonucleotides able to discriminate a single nucleotide mismatch also shows that different people may have different SNP on the same region in the chromosome. These data will facilitate the design of custom made primer viable to gene expression profiling and sequencing analyses even it will be very interesting to compare the Kurdish People genomes to find the correlation between the SNP with several genetic backgrounds diseases. Both results ARMS and sequencing test have compared together. It was illustrated that there are different in genotype results by ARMS test and sequencing test for the same sample.

After the sequencing stage, it was concluded that ARMs test can be used for sequencing the genomes of a large number of people, additionally it is very sensitive, inexpensive and rapid to find out different SNPs of each individual and generate a single map of the human genome containing all possible SNPs (SNP maps). Depending on this map we can group the people based on the SNP profile to determine if SNP associated with a disease, also explain why some people respond to treatment and not others?

1. INTRODUCTION

The single nucleotide polymorphism (SNP) and refers to the predominant form of segregating variation at the molecular level. SNPs are extremely important in a wide range of biological investigation, ranging from biomedical research to ecology and evolutionary biology as well in the characterization of population structure. The human DNA sequences are 99.9% identical with small genetic differences (0.1%) between humans. it has been found that variants of the letter sequence frequently occur in the genome nucleobases can be missing, exchanged or inserted at a certain point in the genome. Many geneticists regard this genetic variation at the level of DNA structure ("DNA sequence variation") as the key to innate susceptibilities
to folk diseases, congenital responses to drugs and other differences in the phenotype of humans. By far the largest part of the variation is made by single, exchanged nucleotides, so-called (SNPs) (Hart et al., 2004).

Such SNPs happen once in every 300 nucleotides on average, which implies there are roughly around 10 million SNPs in large genomes like ours, there are many polymorphic positions (Ali et al., 2017). Some SNP is only present in large groups of people at all time, so that groups of people with the same haplotype can be formed. For this purpose, the members of a family clan or resident of an inaccessible region are to be counted. Numerous diseases may be linked to specific haplotypes and could be easily identified.

Generally, lots of SNP are found in the DNA between genes. They can act as biological markers, helping researchers to locate genes that are related to the disease (Helyar et al., 2011). When SNPs arise within a gene, they may play a more direct role in disease by affecting the gene’s activity. When this project is completed, the analysis of certain positions for broad statements is sufficient. In principle, the individuality of a person can be clearly defined by less than 100 polymorphisms and linked with all possible predispositions.

In the HapMap project, haplotypes are being studied to identify genetic similarities and differences in individuals. Using this data researcher will be able to find genes involved in disease and responses to therapeutic drugs at International HapMap Project (Jing et al., 2014). Researcher work to identify all SNPs in the genome and to develop technologies (microarrays or "DNA chips") that allow a person's genome to be tested in a short time to see if certain SNPs exist (Govindarajan et al., 2012).

In this study, I have been investigated alterations of two SNPs at the specific location in cyclin dependent kinase inhibitor 2A (CDKN2A) and CDKN2B genes which exist on P arm of chromosome 9 in human genome. In fact, these are tumor suppressor genes by cell cycle inhibiting. The CDKN2A gene in Homo sapiens locus in chromosome band 9 p21.3, complement (21,967,751.21,994,490) also stated that the CDKN2 gene consists of 3 coding exons (1β, 1α, 2 and 3) (Rahman et al., 2014). CDKN2A gene makes several proteins, p16 (INK4a) and the p14 (ARF) proteins are most common. Both work as tumor suppressors, by keeping the cells from dividing too quickly. The p14(ARF) protein transcript from the first exon which protects a protein called p53 from being degraded by E3 ubiquitin-protein ligase MDM2. The p53 protein is an important tumor suppressor that is essential for regulating cell division and apoptosis as well induces G2 arrest and prevent tumor formation. Also, the p16 and p15 share a controlling role in cell cycle G1 checkpoint (McWilliams et al., 2011).

The cyclin-dependent kinase inhibitor 2B (CDKN2B) gene in Homo sapiens locus in chromosome band 9 p21.3, also stated that the CDKN2B gene has 2 coding exons (E1, and E2). It is linked to p16INK4A and p14ARF (Fares et al., 2012). CDKN2B gene encodes two separate transcript variants: p15 and p10. The most common function of p15INK4B is protein kinase inhibitors specificity towards the (CDK4) or (CDK6) also play a critical role during differentiation of the cell and cellular senescence (Takahashi et al., 2007). CDKN2B is commonly mutated or deleted in wide variety diseases include Myelodysplastic syndromes, a group of clonal stem cell disorders, Acute myeloid leukemia (Brakensiek et al., 2007).
2. MATERIALS AND METHODS

2.1. Experimental background

In the present study, the SNPs rs199907548 and rs1024022739 have been investigated for different DNA samples. The ability to genotype large numbers of SNPs in large numbers of individuals rapidly and cost-effectively is essential for many applications, from linkage and association mapping to mutation detection and diagnosis and there are some methods for detection of SNP genotyping as following:
1) Low-Technology Methods, (ASO)
2) Hybridization-Based Methods
3) Mini-sequencing Methods
4) Homogeneous Fluorogenic Dye-Based Methods
5) Haplotype phasing Methods
6) Amplification refractory mutation system (ARMS)
7) DNA sequencing assay

In this study, the ARMS test and DNA sequencing assay was used.

2.2. DNA samples

A total of 11 human fibroblast cultures, established from Lung and skin samples derived from fetal (14–20 wk gestational age) and two samples were 43 and 47 years old, all the samples were ordered from the National Institute on Aging cell repository at the Coriell Institute for Medical Research (CIMR) Camden, NJ. All the samples were medically examined and diagnosed as “healthy” as well they have a normal karyotype, i.e., 46, XX or 46, XY. Cells were grown without antibiotics and supplied with 2 mM l-glutamine and 10% (vol/vol) fetal bovine serum. 100 ng of genomic DNA was taken from each sample to identify CDKN2A/B genes and amplify it by available PCR kits.

The starting material for amplification The DNA extraction was done from 11 cultured cell samples that has been mention above (Table 1) as described by the manufacturer from Coriell institute and Lundby laboratory from Gothenburg University.

Table 1- show the name of samples, age, type of cell, source and karyotype.

| Sample   | Taken from | Source | cell     | Origin    | Karyotype |
|----------|------------|--------|----------|-----------|-----------|
| 1 AG01522p3 | aborted fetus 20 w | Skin   | Fibroblast | Caucasian | 46, XY    |
| 2 AG06556   | aborted fetus 20 w | Lung   | Fibroblast | Caucasian | 46, XY    |
| 3 AG04393p3 | aborted fetus 16 w | Lung   | Fibroblast | Caucasian | 46, XX    |
| 4 AG04452   | aborted fetus 16 w | Lung   | Fibroblast | Black     | 46, XX    |
| 5 AG09033   | aborted fetus 17 w | Skin   | Fibroblast | Caucasian | 46, XY    |
| 6 AG09117   | 49 years    | Lung   | Fibroblast | Caucasian | 46, XY    |
| 7 AG04525   | aborted fetus 17 w | Skin   | Fibroblast | Caucasian | 46, XY    |
| 8 AG06173   | aborted fetus 5 m  | Lung   | Fibroblast | Asian     | 46, XX    |
| 9 AG04931   | 49 years    | Lung   | Fibroblast | Caucasian | 46, XY    |
2.2 PCR Amplification and Sequencing of CDKN2A and CDKN2B genes.

Oligonucleotide primers were synthesized by cyanoethyl phosphonamidite chemistry on an Applied Biosystems model 392 DNA synthesizer (Applied Biosystems Inc., Foster City, CA). Multiplex PCR was performed in a final volume of 25 μL containing 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 2.5 mM MgCl2, 5% (vol/vol) DMSO, 200 μM deoxyribonucleotide triphosphates, 1 U of Taq polymerase and 25 pmol of each of the primers used.

Primers used to amplify p14, p15 and p16 exons of CDKN2A/B are listed in Table 2. were reported elsewhere (Fujiwara-Igarashi et al., 2013).

Table 2. Primer sequences for p14, p15 and p16 analyses.

| Primer  | Amplify       | Fragment |
|---------|---------------|----------|
| P14-1F  | 5’-GGGAGCAGCAGCATGGAGGAGGGCG-3’ | p14. exon 1 | 204 bp |
| P14-1R  | 5’-AGTCGCCGCCCCCATCCCCCT-3’     | p14, exon 1 |        |
| P14-2F  | 5’-GGAAATTGGAAGACTGGAGGAGCG-3’  | p14, exon 2 | 498 bp |
| P14-2R  | 5’-TCTGAGGCTTTCAGAGCTTCT-3’     | p14, exon 2 |        |
| P15-1F  | 5’-CCAGAAAGCAATCCAGCGGCGGCG-3’  | p15, exon 1 | 400 bp |
| P15-1R  | 5’-AAATGCGACACACCTGCAGGGCAGCG-3’ | p15, exon 1 |        |
| P15-2F  | 5’-CTTTAATGGCTCCACCTGC-3’       | p15, exon 2 |        |
| P15-2R  | 5’-CGTTGCAGCCTTTTCATCG-3’       | p15, exon 2 |        |
| P16-1F  | 5’-GAAAGGAGAGGAGGGGCTTCATCG-3’ | p16, exon 1 | 340 bp |
| P16-1R  | 5’-GCGCTACCTGTGATTCCATCCATCG-3’ | p16, exon 1 |        |
| P16-2F  | 5’-GGAAATTGGAAGACTGGAGGAGCG-3’  | p16, exon 2 |        |
| P16-2R  | 5’-TCTGAGGCTTTCAGAGCTTCT-3’     | p16, exon 2 |        |
| P16-3F  | 5’-TTTTTCTTCTGCCCCTGAC-3’       | plo, exon 3 |        |
Primers used to amplify p16 exons are listed in Table 2 and named A and G according to the consequence of the Allele, also primers used to amplify p15 exons are listed in Table 2 and named T and G. All PCR reactions were carried out and mixed with 100 ng genomic DNA. The PCR solutions are prepared as have been described by (Lorenz, 2012). The PCR program used for all samples by using different specific primer for each sample as showing in table 3, 4 and 5 in Appendix. The PCR products were separated by gel electrophoresis on 1.5% agarose or 10% nondenaturing polyacrylamide gels Figure 5.

The Pure Link® Quick Gel Extraction Kit from thermos fisher scientific is used to purify CDKN2A and CDKN2B fragments from agarose gels in less than 30 minutes. For sequencing the CDKN2A and CDKN2B fragments the ABI PRISM 377 DNA sequencer are used and the sample prepared according to the procedure from the AB Applied Biosystems. The ABI PRISM 377 DNA Sequencer analyzes fluorescently labeled DNA fragments by gel electrophoresis. The DNA sections labeled with four diverse fluorescent colors and stacked into a gel made of polymerized acrylamide. Once the samples are loaded, voltage is applied, causing the fragments to move through the gel and separate according to size. Later the gel exposed to the laser and the fragments which attached by fluorescent dyes will produce the light at a specific wavelength. Then the results from the sequencer collected and blasted in database program.

The amplification refractory mutation system (ARMS) are used for detecting any mutation relating to SNP. ARMS method depends on the use of allele-specific PCR primers (ASP) that permit enhancement of the DNA fragment just when the objective allele is contained inside the sample. Subsequent presence of a PCR product is diagnostic for the presence of the target allele and vice versa.

A total of 11 samples of CDKN2A and CDKN2B fragments was extracted, and PCR reactions were prepared in two separate tubes for each sample. One test tube was used for the amplification of the normal ARMS primer and the second for the amplification of the mutant ARMS primer. The primers used for ARMS were kindly provided in Table 2. The procedure used according to the instructions provided by the manufacturer and Institute of Clinical Chemistry, Rudolfstiftung Hospital (Najmabadi et al., 2001).

3. Results

One µl of extracted DNA from different samples were run on 1% agarose gel to be sure if the DNA is extracted. The size is approximately between 11000-12000 bp.
Figure 6. The extracted DNA from 11 samples

**ARMS test**

Five µl of each DNA sample was run on 1% agarose gel.

Figure 7- ARMS test for some samples with four allele specific primer.

Figure 8. Represent bands for purification of P15 DNA and P16 DNA fragment.
Table 6 - Genotypes of SNPs rs199907548 Alleles: A/G in CDKN2A (P16) and SNPs rs1024022739, Alleles: T/G in CDKN2B (P15) in 11 human samples assayed by ARMS test

| Samples   | Amplified fragment by specific primer | Size bp | Genotype |
|-----------|--------------------------------------|---------|----------|
| AGO1522p3 | T15- G15                             | 400-420 | T/G      |
| AGO1522p3 | A16- G16                             | 280-300 | A/G      |
| AGO6556   | T15- G15                             | 340-380 | T/G      |
| AGO6556   | A16- G16                             | 280-300 | A/G      |
| AGO4393p3 | T15- G15                             | 340-380 | T/G      |
| AGO4393p3 | A16- G16                             | 280-300 | A/G      |
| AGO4452   | T15- G15                             | 340-380 | T/G      |
| AGO4452   | A16- G16                             | 280-300 | A/G      |
| AGO9033   | T15- G15                             | 400-420 | T/G      |
| AGO9033   | A16- G16                             | 280-300 | A/G      |
| AGO9117   | T15- G15                             | -       | - / -    |
| AGO9117   | A16- G16                             | -       | G/G      |
| AGO4525   | T15- G15                             | 400-420 | T/G      |
| AGO4525   | A16- G16                             | 280-300 | A/G      |
| AGO6173   | T15- G15                             | 400-420 | T/G      |
| AGO6173   | A16- G16                             | -       | G/G      |
| AGO4931   | T15- G15                             | 400-420 | T/G      |
| AGO4931   | A16- G16                             | -       | G/G      |
| AGO6814   | T15- G15                             | 400-420 | T/G      |
| AGO6814   | A16- G16                             | -       | - / -    |
| AGO5965   | T15- G15                             | 400-420 | T/G      |
| AGO5965   | A16- G16                             | -       | - / -    |
The results mentioned above shows that different people may have different SNP on the same region. The remaining of the same samples that had been amplified for both primers (R and L), were run on agarose gel for purification and sequencing test. Both results ARMS and sequencing test have compared together in (Table 7). It was illustrated that there are different in genotype results by ARMS test and sequencing test for the same sample especially in P15 but the result was same in P16.

Table 7- comparison between genotypes of SNPs rs199907548 Alleles: A/G in CDKN2A (P16) and SNPs rs1024022739, Alleles: G/T in CDKN2B (P15) in 11 human samples assayed by ARMS test and sequencing test.

| Row | samples       | ARMS test p16 | Sequencing test p16 | ARMS test p15 | Sequencing test p15 |
|-----|---------------|---------------|---------------------|---------------|---------------------|
| 1   | AGO1522P3     | A/G           | A/G                 | T/G           | T/T                 |
| 2   | AGO6556       | A/G           | A/G                 | T/G           | T/T                 |
| 3   | AGO4393P3     | A/G           | A/G                 | T/G           | -/-                 |
| 4   | AGO4452       | A/G           | A/G                 | T/G           | -/-                 |
| 5   | AGO9033       | A/G           | A/G                 | T/G           | T/G                 |
| 6   | AGO9117       | G/G           | G/G                 | -/-           | T/T                 |
| 7   | AGO4525       | A/G           | A/G                 | T/G           | T/T                 |
| 8   | AGO6173       | G/G           | G/G                 | T/G           | T/T                 |
| 9   | AGO4931       | G/G           | G/G                 | T/G           | T/T                 |
| 10  | AGO6814       | -/-           | G/G                 | T/G           | T/T                 |
| 11  | AGO5965       | -/-           | A/G                 | T/G           | T/G                 |

4. Discussion

DNA amplification by methods such as primer extension pre-amplification, produce relatively low molecular weight DNA that is not representative of the entire genome. The product is often error prone and unlikely to completely preserve heterozygosity information or correct copying of short repeated elements, but the allele-specific PCR has been rationalized by using primary allele-specific PCR primers that contain a universal 5’-tail sequence that becomes part of the PCR product of amplification, this technique is the best principle of choice genotyping of SNPs, and has been improved in numerous assay formats.

Allele-specific primer extension, two primers that anneal to their target sequence adjacent to the SNP and have the nucleotide complementary to the allelic variant at their 3’-
end are used in primer extension reactions catalyzed by a DNA polymerase. Only primers with perfectly matched 3’-ends will be extended. The Allele-specific PCR depend on exact base pairing at the 3’end of the primer that can distinction between alleles that differ at just a single nucleotide. In the popular ARMS method, primers are designed with their 3’ end nucleotides designed to base-pair with the variable nucleotide which distinguishes the two alleles, and with the remaining primer sequence designed to be complementary to the sequence immediately adjacent to the variable nucleotide. Under suitable experimental conditions, amplification will not take place where the 3’ end nucleotide is not perfectly base-paired thereby distinguishing the two alleles.

APS1 will bind perfectly to the complementary strand of the allele 1 sequence, permitting amplification with the conserved primer. However, the 3’ terminal C of the ASP2 primer mismatches with the T of the allele 1 sequence, making amplification impossible. Similarly, ASP2 can bind perfectly to allele 2 and initiate amplification, unlike ASP1.

There are a lot of SNPs in CDKN2A and CDKN2B genes and two of them are SNPs rs199907548, Alleles: A/G in CDKN2A (P16) that exist on Exon 3 and SNPs rs1024022739, Alleles: G/T in CDKN2B (P15) that exist on Exon 2 respectively. In this experiment, the mentioned SNPs detected by using the Allele-specific primer in 11 human DNA samples then by running DNA sequencing and ARMS™ test analysis the result collected and compared.

In contrast to results from the Table 6 that show different people may have different SNP on the same region. likewise, there was a different result in DNA sequencing and ARMS™ test for the same SNP at the same region as showing in Table 7.

This is the first study to report on resequencing of the locus 9p21.3 containing CDKN2A and CDKN2B. This region is one of the most imperative loci in modern genetic studies, other studies have reported sequencing 9p21.3 with sign that supports risk associated alleles having effects on expression of CDKN2A and CDKN2B (Pilbrow et al., 2012).

After the sequencing stage, it was concluded that ARMs test can be used for sequencing the genomes of a large number of people, additionally it is very sensitive, inexpensive and rapid to find out different SNPs of each individual and generate a single map of the human genome containing all possible SNPs (SNP maps). Depending on this map we can group the people based on the SNP profile to determine if SNP associated with a disease, also explain why some people respond to treatment and not others?

According to our results it can be proposed that the genetic variance is mainly accounted by many variants and loci while there are significant differences in tissues, investigations, and isoforms targeted across these studies that may limit direct comparisons. The overall findings of the present study are consistent with several prior findings.

Additionally, detailed studies in multiple tissues and in large clinical accomplices are necessary to determine how these variations influence disease and which ones are most important for potential clinical applications.

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