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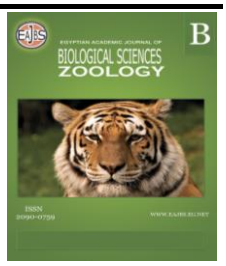

Study of UGT1A1 Gene Mutation in People Suspected of Hyperbilirubinemia and Its Relationship with Fatty Liver Syndrome by Tetra ARMS-PCR Technique

Ali Janbaz[1] and Abolhasan Rezaei[2*]
1- M.Sc in Genetics, Department of Biological Sciences, Islamic Azad University, Tonekabon Branch, IRAN
[*]2- Department of Biological Sciences, Faculty of Genetics, Islamic Azad University, Tonekabon Branch, IRAN
Email: a.rezaei@tonekaboniau.ac.ir

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ABSTRACT

Hyperbilirubinemia is one of the most common disorders in the world. The disease has spread out the Middle East to Africa. The importance of this disease is high due to its widespread prevalence in the world. In this study, to investigate hyperbilirubinemia and the difference between the mutations in the UGTA1 gene with hyperbilirubinemia and the fatty liver syndrome was selected from the SNPs involved in expressing the UGTA1 gene named rs372326047.DNA genomic was extracted and one pair of primer for amplifying of the rs372326047 was designed with the help of the Oligo Version 7.0. After the PCR reaction, samples were sequenced to determine of the fragment of UGTA1 gene. Figures 1 and 2 showed the sequences were analyzed using the Blast program. Results observed that no mutation between UGTA1 gene. In Table 1. Ten cases from forty samples were selected including, healthy, carrier, sick and different parameters that were questioned from them. Patients have been heart diseases, diabetic-hepatic, and some patients did not have a history of inheritance diseases in family, rate of Hb in some cases was high (more than ten). Most of the cases also had high anemia and regarding some cases, the history of hyperbilirubinemia and its relationship with fatty liver diseases in family was positive and some cases was negative. Table 2. One-way ANOVA followed by Tuckey's post hoc test and HWE frequency of allelic and genotyping of nucleotide of TT, TC and CC of rs372326047 of UGTA1 gene in cases and normal population respectively. Therefore, the results of this study show that there is no relationship between mutations in the rs372326047 of UGTA1 gene in hyperbilirubinemia and its relationship with fatty liver syndrome.

INTRODUCTION

The severity of hyperbilirubinimia is determined by the amount of hemoglobin synthesis that is associated with a defect at the molecular level. Studies have shown that increased HB in patients with hyperbilirubinemia is associated with improved disease phenotypes (Amandito et al., 2018; Judith Meza-Junco et al., 2009). Studies have shown this trait can be due to mutations in the UGTA1 gene or point mutations in the UGTA1 gene promoter, which is a pan cellular type in high red blood cells. Studies have shown that HB diversity in a single genetic population is not inherited, but depends on many gene sites

(Higgs et al. 2012; Kukreti et al. 2010). GWAS (Genome-Wide Association Study) studies have shown that the region of polymorphisms of UGTA1 gene is placed on chromosome 2 (Ambalavanon et al., 2011). However, the mechanism of the effect of these polymorphisms on the variation of HB level is still unknown but more researchers proposed that the basis of genetic variation in HB levels with GWAS studies (Higgs et al. 2012; Agrawal et al. 2009). Serum bilirubin, the end product of heam metabolism, has been found to possess potential antagonizing oxidative stress and inflammatory properties by acting as an antioxidant and cytoprotectant in vitro and in vivo. Besides, there has been accumulating evidence frequently documenting not only oxidative stress, but insulin resistance was considered to be major triggers to NAFLD pathogenesis and progression (Choi et al., 2013). NAFLD is frequently demonstrated to be strikingly associated with the risk of metabolic syndrome, type2 diabetes, and cardiovascular diseases independent of other classical risk factors (Dam-Larsen et al., 2004; Lalymp 2005). Therefore, a straightforward hypothesis has been proposed that bilirubin may contribute to protection against NAFLD risk, probably based on the antioxidant effects of bilirubin. So far, several previous studies have been performed to examine the association between bilirubin levels and the risk of NAFLD (Menzel et al. 2007; Rinella et al., 2006; Rodrigues et al., 2012). However, most of these studies were performed based on relatively small sample sizes. Moreover, certain limitations of evaluating causal relation and reliability of the results have been posed when interpreting the association by the cross-sectional or case-control study. In addition, the majority of these studies only accessed the effect of one type bilirubin on the risk of NAFLD but not all subtypes.

In this study, we aimed to respond to three questions including is their relation between a mutation in UGTA1 gene and hyperbilirubinimia in the Iranian population. Is there a relationship between the mutation in UGTA1 and fatty liver syndrome? Is there a relationship between a mutation in UGTA1 gene, hyperbilirubinimia, and fatty liver syndrome?

MATERIALS AND METHODS

The Samples:

The samples including forty numbers, twenty samples are healthy and twenty samples are cases collected from Mazandaran province. Interview face to face and questionnaires had done from healthy and cases group. The questionnaires were like :(age, sex, the behavior of feeding, pedigree family of both hyperbilirubinimia and fatty liver syndrome, marital status, and what treatment have you received).

Isolation of DNA Genomic for Tetra ARMS PCR and PCR Sequencing of UGTA1 Gene:

DNA genomic were isolated from 100CC of fresh blood from healthy and cases using standard kit GENET Bio(blood extraction kit-10000. The primers were designed according to the region of our SNPs in this study from 300bp from before and after the position of SNPs of rs372326047 of UGTA1 gene**.**

SNP(rs37232604**)**	**Forward Primer**	**Reverse Primer 5′→3′**
FNormal	GCTATGGUAATTGCTGATGCTTT	TCCACCATAGGGCGTTTATCC
FMutant	TCTCAAACACGCATGCCTTT	CAATGACAACAACCACAACAACA

According to above primers, PCR program for Tetra ARMS PCR and its reaction were applied for getting sharp band on the gel electrophoresis as follows:

Template DNA (100ng/µl)	3.0
Forward primer (10pmol/µl)	0.5
Reverse primer (10pmol/µl)	0.5
dNTP mix (2.5mM each).	1.0

10X buffer	2.5
$MgCl_2$	2.5
Taq enzyme (3U/ µl)	0.5
ddw	14.5
Total Reaction volume	25.0 µl

Moreover, for PCR-Sequencing of rs372326047 were designed primer with Primer3 online software according below:

SNP	Forward Primer	Reverse Primer	Product size
Rs372326047	TTCTGTAAGCAGGAACCCTTCT	ACTTCTCAGTGTGGTGGATCA	250bp

RESULTS

In this study, forty samples were collected from hospitals in Mazandaran province. All of the cases and the healthy group were used questionnaire reports for them. Table 1 showed the program of the questionnaire for both group, healthy and cases in Table 1. Ten cases from forty samples were selected including, healthy, carrier and sick, and different parameters that were questioned from them. Patients had heart diseases, diabetic-hepatic, and some patients did not have a history of inheritance diseases in family. Most of the cases also have high anemia and regarding some cases the history of both hyperbilirubinimia and fatty liver syndrome diseases in family was positive and some cases was negative. The primers are conferred (SNPs) of rs372326047 of UGTA1 gene both hyperbilirubinimia and fatty liver syndrome diseases by the identity of forty samples with the either wild type or the mutant (homozygosity and heterozygosity situation between normal and mutants) were studied (Table 2). According that the frequency of genotyping, allelic and HWE of nucleotides CC, TC more than TT in normal and TT, TC and CC of UGTA1 gene in cases was high respectively. One way of ANOVA followed by Tuckey's post hoc test also showed no significant variation between the frequency of allelic and genotyping of nucleotide of TT, TC and CC in rs372326047 of UGTA1 gene in cases and normal population respectively.

Table 1. questionnaire report of cases and healthy including blood group, the history of inheritance and both hyperbilirubinimia and fatty liver syndrome and the clinical symptoms showed. According that cases have been diseases and some patients negative about the history of inheritance of family and hyperbilirubinimia

Sex (male and female)	Blood group	The history of inheritance diseases in family	The history of both hyperbilirubinimia and fatty liver syndrome diseases in family	The rate of HB before injection of blood	Transfusion	The checking samples	The clinical symptoms
Male	B+	NO	Yes	<10	One time for per 30 days	RBC are highly microsetic and WBC and plackets are normal	High anemia
Male	AB+	Heart diseases	No	<10	One time for per 30 days	RBC are highly microsetic and WBC and plackets are normal	Hyper anemia
Male	B+	No	Both hyperbilirubinimia and fatty liver in sister	<10	One time for per 30 days	RBC are highly microsetic and WBC and plackets are normal	High anemia
Female	AB-	Diabetic-Hepatic	Both hyperbilirubinimia and fatty liver in her brother	<10	One time for per 30 days	RBC are highly microsetic and WBC and plackets are normal	High anemia
Female	A+	No	No	<10	One time for per 30 days	RBC are highly microsetic and WBC and plackets are normal	High anemia
Male	O+	No	No	<10	One time for per 30 days	RBC are highly microsetic and WBC and plackets are normal	High anemia
Female	A+	No	No	One time for per 30 days	RBC are highly micros etic and WBC and plackets are normal	High anemia	
Female	AB+	No	both hyperbilirubinimia and fatty liver syndrome	<10	One time for per 30 days	RBC are highly microsetic and WBC and plackets are normal	High anemia
Female	A+	No	both hyperbilirubinimia and fatty liver syndrome (her brother and sister)	<10	One time for per 30 days	RBC are highly microsetic and WBC and plackets are normal	High anemia

Table 2. Genotyping and allelic frequencies nucleotide of TT, TC and CC of rs372326047 of ugta1 gene with both hyperbilirubinimia and fatty liver syndrome in cases and normal population respectively.

Rs372326047	Genotype	N (n=40)	Frequency (%)	Expected genotype frequency under HWE	Allele	Allele frequency(%)	HW (*p*-value)
Normal	TT	5	9.10	9.56	C	65.21	0.31^{NS}
	TC	18	48.30	46.76	T	34.69	
	CC	21	42.60	51.78			
Cases	TT	24	18.0	44.34	T	32.67	0.23^{NS}
	TC	11	28.0	24.23	C	67.23	
	CC	17	54.0	29.32			

NS. NS mean non-significant variation.

Tetra ARMS-PCR of UGTA1 Gene:

In Figure 1. Tetra ARMS-PCR product position of rs372326047 of UGTA1 gene was run on the 1.5% gel electrophoresis showed H. Heterozygosity (120 and 250bp), N. Normal (200 and 250bp)**.** The result showed between forty samples, a heterozigosity variation between samples and other samples no mutation was observed. Figure 2. Showed the PCR product of the position of rs372326047 of ugta1 gene were run on the 1.5% gel electrophoresis showed a fragment of 300bp. Moreover, PCR products were sequenced and no variation in the position of the rs372326047 ugta1 gene. (T no converted to A) The electrogram curves were visualized by Chromas software. (Figure 3). Tetra ARMS PCR for normal primers was analysed with a blast online program for confirmation of primers. The result showed a hundred percent is matched with UGTA1 gene (Figure 4). Moreover, Tetra ARMS PCR for mutant primers was analysed with a blast online program to confirm of primers. The result showed a hundred percent is matched with UGTA1 gene (Figure 5). In Figure 6. PCR sequencing of primers was analysed with a blast online program for confirmation of primers. The result showed a hundred percent is matched with UGTA1 gene. Moreover, in Figure 7. The position of rs372326047 of UGTA1 gene was analysed with a blast online program for confirmation of primers. The result showed the position of T no mutation was observed (blue marked).

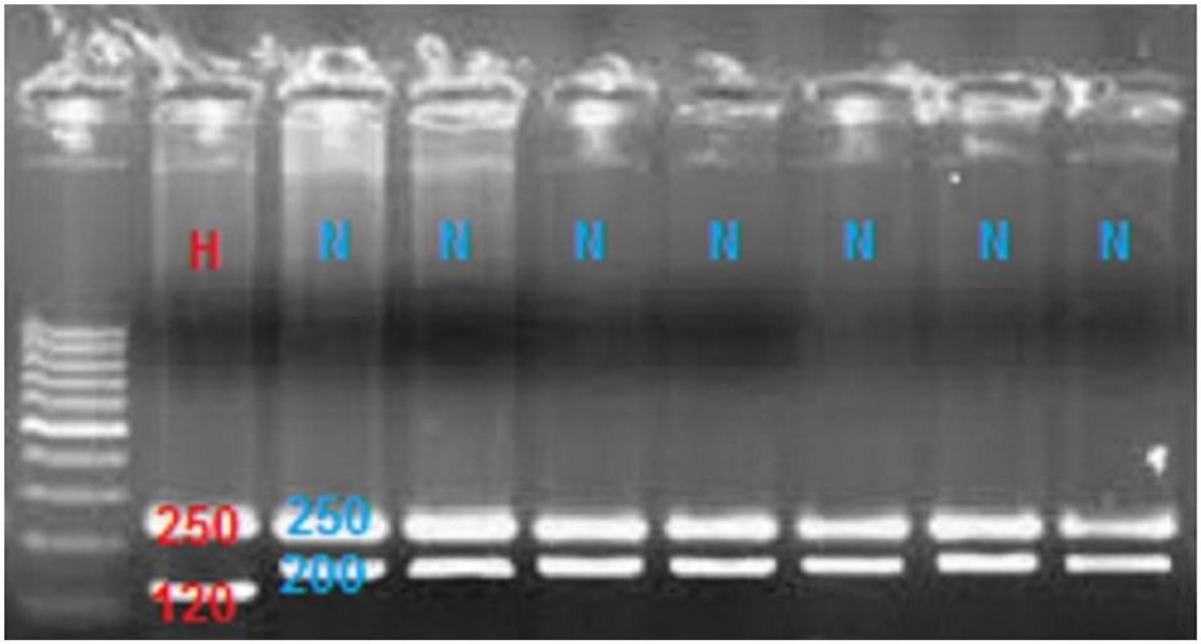


Fig. 1. Tetra ARMS-PCR product of position of rs372326047 UGTA1 gene with both hyperbilirubinimia and fatty liver syndrome (H. Heterozygosity (120 and 250bp), N. Normal (200 and 250bp)**.**

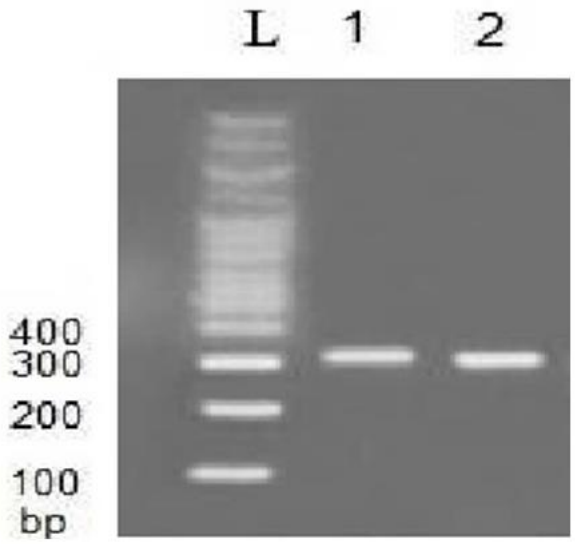


Fig. 2. PCR product of position of rs372326047 UGTA1 gene with both hyperbilirubinimia and fatty liver syndrome (300bp, L, 100bp).

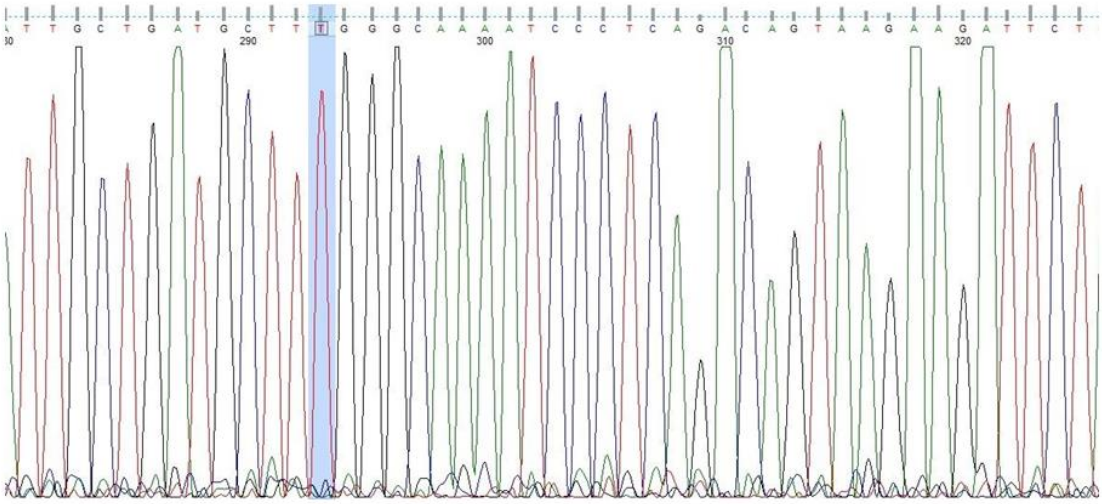


Fig. 3. The image of electrogram of the position of rs372326047 UGTA1 gene. The result showed no variation in the position of rs372326047 UGTA1 gene. (T no converted to A) The electrogram curves were visualized by Chromas software.

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity
Forward primer	GCTATGGCAATTGCTGATGCTTT	23	60.74	43.48	8.00
Reverse primer	ACTGTCTGAGGGATTTTGCCCA	22	61.90	50.00	3.00

Products on target templates

>NC_000002.12 Homo sapiens chromosome 2, GRCh38.p13 Primary Assembly

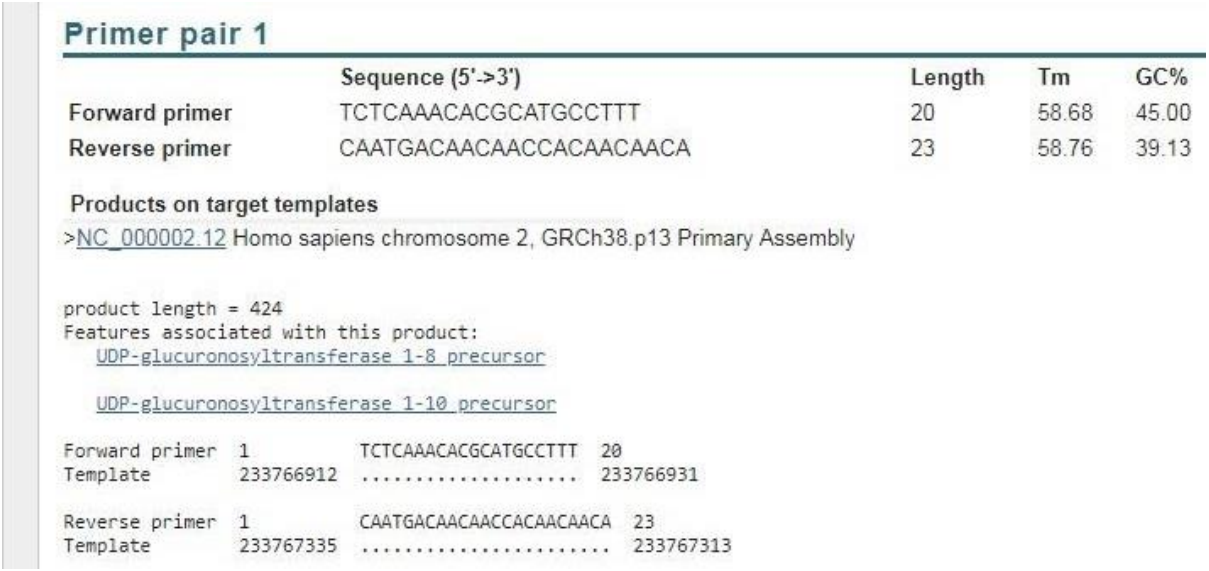
```
product length = 44
Features associated with this product:
   UDP-glucuronosyltransferase 1-8 precursor

   UDP-glucuronosyltransferase 1-10 precursor

Forward primer  1          GCTATGGCAATTGCTGATGCTTT  23
Template        233767124  .......................  233767146

Reverse primer  1          ACTGTCTGAGGGATTTTGCCCA  22
Template        233767167  ......................  233767146
```

Fig. 4. Tetra ARMS PCR for normal primers was analysed with a blast online program for confirmation of primers. The result showed a hundred percent is matched with UGTA1 gene.



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	TCTCAAACACGCATGCCTTT	20	58.68	45.00
Reverse primer	CAATGACAACAACCACAACAACA	23	58.76	39.13

Products on target templates

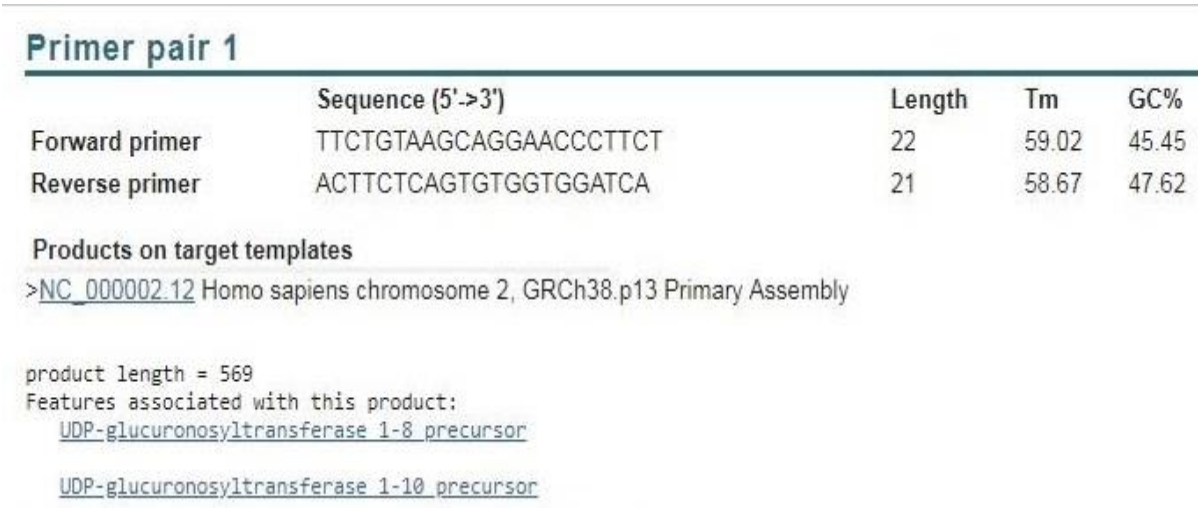
>NC_000002.12 Homo sapiens chromosome 2, GRCh38.p13 Primary Assembly

product length = 424
Features associated with this product:
UDP-glucuronosyltransferase 1-8 precursor

UDP-glucuronosyltransferase 1-10 precursor

Forward primer	1	TCTCAAACACGCATGCCTTT	20
Template	233766912	.....	233766931
Reverse primer	1	CAATGACAACAACCACAACAACA	23
Template	233767335	.....	233767313

Fig.5. Tetra ARMS PCR for mutant primers was analysed with a blast online program for confirmation of primers. The result showed a hundred percent is matched with UGTA1 gene.



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	TTCTGTAAGCAGGAACCCTTCT	22	59.02	45.45
Reverse primer	ACTTCTCAGTGTGGTGGATCA	21	58.67	47.62

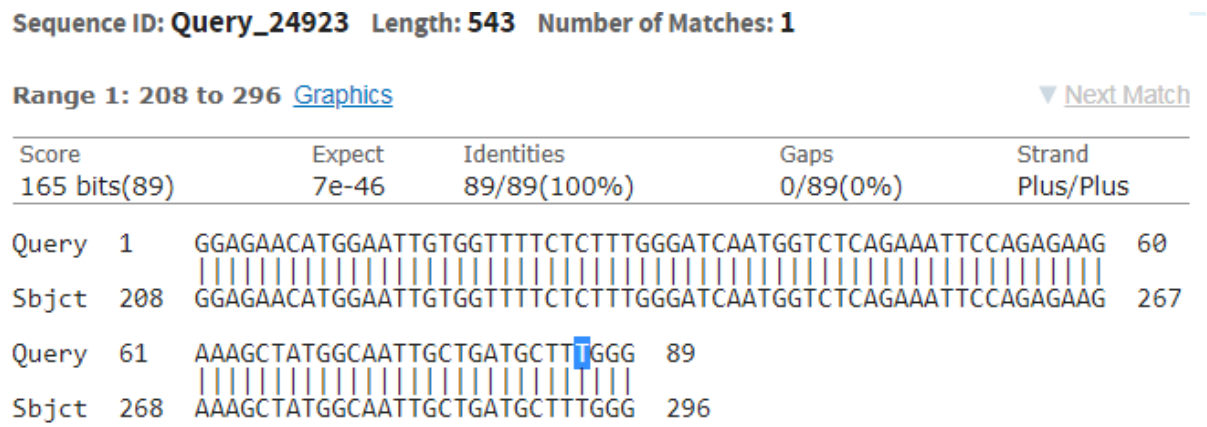
Products on target templates

>NC_000002.12 Homo sapiens chromosome 2, GRCh38.p13 Primary Assembly

product length = 569
Features associated with this product:
UDP-glucuronosyltransferase 1-8 precursor

UDP-glucuronosyltransferase 1-10 precursor

Fig.6. PCR sequencing of primers was analysed with a blast online program for confirmation of primers. The result showed a hundred percent is matched with UGTA1 gene.



Sequence ID: Query_24923 Length: 543 Number of Matches: 1

Range 1: 208 to 296 Graphics ▼ Next Match

Score	Expect	Identities	Gaps	Strand
165 bits(89)	7e-46	89/89(100%)	0/89(0%)	Plus/Plus

```
Query  1    GGAGAACATGGAATTGTGGTTTTCTCTTTGGGATCAATGGTCTCAGAAATTCCAGAGAAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208  GGAGAACATGGAATTGTGGTTTTCTCTTTGGGATCAATGGTCTCAGAAATTCCAGAGAAG  267

Query  61   AAAGCTATGGCAATTGCTGATGCTTTGGG  89
            |||||||||||||||||||||||||||||
Sbjct  268  AAAGCTATGGCAATTGCTGATGCTTTGGG  296
```

Fig. 7. The position of rs372326047 of UGTA1 gene was analysed with blast online program for confirmation of primers. The result showed the position of T no mutation was observed (blue marked).

DISCUSSION

A study by Xu et al., in the year 2008 found a relationship between mutations in UGTA1 gene and fatty liver syndrome. There are many genes related to fatty liver syndrome including ABCC2, STAT6, PPARa, PEMT, PNPLA3. In PNPLA6 gene the variation of isoleucine to methionine (I148M), disease including non-alcoholic fatty liver disease (NAFLD), although its role in the development of non-alcoholic fatty liver fibrosis is not fully understood (Yang *et al.,* 2013). In connection with mutations in the creation of the fatty liver, genetic factors play an important role. PNPLA6 is a protein with 721 amino acids which is highly expressed in hepatocytes as triglyceride hydrolysis) which represents it acts as a catabolic lipase (and an acetyl CoA) that represents anabolic lipogenetic activity (Singer *et al.,* 2007; Thein *et al.,* 2007; Thein *et al.,* 2009). The aim of this study was to investigate the relationship between hyperbilirubinemia and fatty liver disease is suspected and sick individuals are PNPLA6 gene is mentioned as a gene in which mutation causes fatty liver disease it is possible that one of its SNPs, which is pathogenic, will be used in this study. The present study was to investigate the UGT1A1 mutation in individuals suspected of having hyperbilirubinemia and its association with fatty liver by ARMS-PCR. Hyperbilirubinemia is a common manifestation in infancy and is benign in most infants. The yellow color is usually caused by the accumulation of non-conjugated bilirubin pigment (indirect type) which is fat-soluble and non-polar (Thein and Menzel 2009). Although bilirubin may play an antioxidant role in small amounts, high levels of bilirubin may not. Conjugated (indirect) is potentially neurotoxic. Although the conjugated form of bilirubin is not neurotoxic, In hyperbilirubinemia indicates potentially serious liver disorders or underlying systemic diseases. ARMS (Amplification Refractory Mutation System), a simple, fast and reliable method for detection of point mutations, deletions, or additions in molecular sequences primers are based on different DNA alleles (Singer *et al.,* 2007). The basis of this method is based on the differences between nucleotides. This method also is named PCR Amplification Specific Alleles (AS-PCR). In this method, two PCR reactions are performed using a template DNA molecule, in each reaction, a common primer is used along with one of the two allele-specific primers. The specificity of these primers for each of these alleles is due to differences in their nucleotides. It is related to the location of the difference between the two alleles. It should be noted that in this reaction, the use of Taq DNA Polymerase or a similar DNA polymerase that lacks exonuclease properties is essential. UGT1A1 gene activity is enhanced by alcohol or certain medications. Recent evidence has shown that low serum levels of bilirubinemia are a powerful physiological antioxidant that can protect against oxidative stress conditions such as atherosclerosis, coronary heart disease, and inflammation. But at high concentrations, bilirubin is a dangerous metabolite for human health, especially in infants, and toxicity with unconjugated bilirubin, which, if not treated immediately, can lead to corneal ectrosis. The results showed that TA duplication in the promoter of UGTA1 gene, hemoglobin concentration, fasting time, and BMI are the main factors determining bilirubin level (Xu *et al.,* 2010). Research has shown that there is a link between fatty liver disease and Gilbert's syndrome. Studies have shown that there is a link between liver dysfunction and blood bilirubin levels, both conjugated and non-conjugated. There is a conjugate connection. In a study of 27000 people with fatty liver, they had a basil level high bilirubinimia has been reported. Fatty liver disease is known as the most common liver disease. Cirrhosis and Cell Cancer (NASH) is a liver disorder ranging from simple osteotosis to non-allergic cancerous hepatitis. Liver Fatty has also been linked to insulin resistance, obesity, dyslipidemia, type 2 diabetes, and cardiovascular disease. Therefore, it is considered a hepatic manifestation of metabolic syndrome. In the present study, the aim was to investigate the mutation in a part of the UGT1A1 gene, rs372326047.The results of the present study showed that, among the

samples suspected of hyperbilirubinemia, in none of the samples in no mutation was observed at the study site. The age of the patients was often over 32 years old, and was collected from the region of Mazandaran province-Tonekabon city ARMS-PCR results were observed in healthy individuals. Finally, it is concluded that there is a link between mutations in the UGT1A1 gene locus and hyperbilirubinemia. Different reasons can be considered, these reasons can include the age of patients, geographical area, etc. Research on laboratory mice has shown that in newborn mice their blood bilirubin levels were very high, about 23% to 62% higher than in adult mice. In the present patients, they were often older. Meals and fiber foods are also effective in treating hyperbilirubinemia.

Suggestions: a. Given that different racial groups live in Iran, it is recommended that this screening for UGT1A1 mutations in different strains. B. For better and more review, other methods can be designed for the accuracy of primers used and reviewed the results. c. More samples can be considered, and these samples are better than regions be a different country and so-called jaundice.

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