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Discrimination of Codon 72 p53 Polymorphisms using High Resolution Melting Curve Method

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ABSTRACT

One of the most important polymorphism in p53 gene occurs in codon 73 of exon 4. Individuals who have Arg/Arg allele may have an increase risk for progress of cancer. The present study was aimed to develop a rapid, inexpensive and simple high resolution melting curve analysis method to distinguish different genotype of codon 72 p53. This study was performed on 167 patients with four common cancers in Kurdistan. High resolution melt analysis of PCR products revealed different allele of codon 72 p53 gene. Genotyping by high resolution melt analysis was verified by DNA sequencing. The samples were categorized as Arg/Arg (31%), Arg/Pro (44%) and Pro/Pro (25%) in four cancer types. Patients with colorectal cancer had the highest frequency of Arg/Arg polymorphism (65%). High resolution melting analysis was completely compatible with sequencing results. The results indicate individuals with Arg/Arg allele of codon 72 p53 gene have an increased risk for development of cancer in Kurd ethnicity. In addition high resolution melting analysis is useful for rapid differentiation of p53 alleles and can be used for diagnostic purposes directly from clinical samples, as well as epidemiological studies.

Key words: High resolution melting analysis, p53, codon 72 polymorphism, cancer

INTRODUCTION

The p53 is a tumor suppressor protein produced by p53 gene that has an important role in prevention of cancer formation caused by environmental and genotoxic stress, through cell cycle inhibition and promotion of programmed cell death (Deppert *et al*., 1990; Diller *et al*., 1990). The p53 is located on the short arm of chromosome 17 (17p13, 1) and has 11 exon (Khan *et al*., 2005) and encodes a nuclear phosphoprotein with 393 amino acids (Salazar *et al*., 2004). The lack of p53 activity due to mutation, denaturation of protein and allelic loss occurs. Various stresses such as radiation, carcinogens, toxic drugs, hypoxia, nucleotide deficiency, the activity of oncogenes, the loss of binding microtubules and disorganization of normal cells activities the p53

(Kuerbitz *et al*., 1992; Lowe *et al*., 1993; Goi *et al*., 1997). The p53 gene is found to be mutated in almost all cancer in different incidence (Adhikari and Iwakuma, 2009). It has been recorded that the p53 gene is mutated in 50% of human cancers (Vogelstein *et al*., 2000). The high frequency of p53 mutation have been reported in ovarian cancer (48.3%), followed by colorectal cancer (43.6%), oesophageal cancer (42.6%), head and neck cancer (41.5%) and lung cancer (38.4%) (Petitjean *et al*., 2007). Together Single Nucleotide Polymorphisms (SNPs), rare single nucleotide substitutions and small insertion/deletion mutations constitute the most common forms of sequence variation in the human genome (Garritano *et al*., 2009). Codon 72 of exon 4 is one of the most important polymorphism (rs1042522) that encodes either an arginine amino acid (CGC) or a proline amino acid (CCC) in proline-rich region of the P53 protein that is required for growth suppression and apoptosis mediated by p53. These two p53 variants are functionally different based on the ability to bind components of the transcriptional machinery, activate transcription, induce apoptosis and suppress tumor growth (Thomas *et al*., 1999). Arginine/Arginine has been associated with a higher risk of cancer development by binding to p53-homolog p73 and neutralization of p73-induced apoptosis (Marin *et al*., 2000).

Different genotyping methods has been developed for evaluation of SNPs based on the analysis of one or few SNPs (Zhu *et al*., 2003; Yan *et al*., 2005) or to scan the whole genome (Meaburn *et al*., 2006). Direct sequencing has high sensitivity and specificity for detection of SNPs, However, these technique is expensive and time consuming and arguably to not provide useful solutions for larg samples. Interest in fast and reliable methods of mutation screening is increasing as well. For determination of genomic variation many methods have been developed, including HPLC (High Performance Liquid Chromatography) based methods, electrophoretic conformational changes and enzymatic or chemical cleavage reactions (Garritano *et al*., 2009). The goal of these screening techniques is to reduce the use of DNA sequencing and control costs while maintaining sensitivity and specificity. High Resolution Melting (HRM) analysis is a novel, homogeneous, close-tube, post-PCR method that has been developed and widely used for genotyping and mutation scanning of human genetic and medical research (Wittwer, 2009). This technique is appropriate for fast screening of large number of DNA samples. Samples can be discriminated according to their sequence, length, GC content or strand complementarily. Even single base changes such as SNPs (single nucleotide polymorphisms) can be readily identified. The most important high resolution melting application is gene scanning prior to or as an alternative to sequencing. This technique is an accurate, user friendly, cost-effective, fast and simple method and does not need post-PCR processes (Krypuy *et al*., 2007; Bastien *et al*., 2008; Millat *et al*., 2009). The technique has already been employed to scan for somatic mutations in the KIT, BRAF, EGFR, ERBB2, KRAS and recently for p53 exon 5-8 genes (Willmore-Payne *et al*., 2005, 2006; Krypuy *et al*., 2006). Genetically identification of at risk population by a rapid and inexpensive method would be highly desirable, therefore, this study was aimed to develop a HRM method for investigate of p53 codon 72 polymorphism so it can be used as an alternative to DNA sequencing for detecting nucleotide variations and genotyping large number of the p53 codon 72 polymorphism.

MATERIALS AND METHODS

Study population: All 167 patients (90 male and 77 female) with breast, esophageal, gastric and colorectal cancers who admitted to the Sanandaj Tohid Hospital (Kurdistan, Iran) during the period of November 2012 until November 2013 were enrolled in this study. Patients, aged 59.35±11.24 years (range between 36-85 years), identified by histopathology of the involved tissue.

All patients were from Kurdistan, a province in Western Iran with a population that is Kurds. Written informed consent for participation was obtained and the project was approved by the Research Ethics Committee of Kurdistan University of Medical Sciences (Iran) and conformed to the Declaration of Helsinki. Demographic, clinical and laboratory data and information on the specific therapy were recorded.

Blood sample collection and analysis: Aliquots of the EDTA-whole blood samples were used for DNA extraction. Genomic DNA was extracted from whole blood using DNA extraction Kit (DNP™) (CinnaGen Inc, Tehran, Iran) according to the manufacturer's instructions. Briefly, blood samples were incubated with lysis buffer then DNA selectively precipitated. The insoluble DNA has washed and desalted by wash buffer and it was stored at -20°C pending simultaneous analysis. The similar buffer solution was used for all DNA samples because different buffers can affect melting temperature.

Pre-amplification high resolution melting curve analysis: Before HRM analysis, a target sequence of exon 4 from p53 gene has been amplified. The PCR reaction was performed in a final volume of 20 μL using HOT Taq EvaGreen qPCR Mix kit (CinnaGen Inc, Tehran, Iran), 10 pmol of each primer with final concentration of 400 nM and 25 ng DNA. A 119 bp fragment of exon 4 of p53 gene were amplified by using two primers; 5'-CCAGATGAAGCTCCCAGAATG-3' as forward and 5'-CCCTGTCATCTTCTGTCCCTTC-3' as reverse primer. The PCR conditions was: 15 min at 95°C (initial denaturation), followed by 45 cycles of 95°C for 15 sec (denaturation) and 55°C for 30 sec (annealing) and 72°C for 10 sec using 36-well Rotor-Gene™ 6000 instrument (Corbett Life Science, Qiagen Inc.). Melting curve was obtained by increasing the temperature from 75 to 90°C at the default of Rotor Gene melting rate (0.1°C each step) with continuous fluorescence monitoring. Optical measurements (fluorescence signals) in the green channel (using a digital filter, with excitation at 479 nm and detection at 510 nm) were measured (Rostami *et al*., 2013). In each PCR run, samples with no DNA template were used as negative controls. Three previously sequenced DNA samples for p53 codon 72 and identified as Wild (GG), Mutant (CC) and Hetrozygote (GC) were included in each PCR set as standards. To identify alterations in the shape of the curve melting profiles, they were analyzed with the software Rotor Gene (Rostami *et al*., 2013). Briefly, the melting peak was illustrated by plotting derivative of the fluorescence signal against the temperature. Then, the original melting curve data were normalized by defines pre and post-melting transition. Finally, the analysis of difference graph by setting the curves from a base curve and generating a difference plot curve were done (Fig. 1).

DNA sequencing: PCR products from randomly selected individual of each genotype category were sequenced in an ABI-3730XL capillary machine by BioNeer Inc. (South Korea) to confirm and validate HRM results. Sequence data were adjusted manually and complete alignment was carried out using the CLC software (CLCbio, Denmark). The results were compared with published reference sequences and HRM analysis results.

Statistical analysis: Genotype frequencies were determined by direct counting and to estimate precision, within-run and between-run coefficient of variance (CV %) were analyzed using control samples. Within-run CV% was calculated on 10 replicates of each control. Between-run CV% was estimated by analysis of 5 replicates per day on 5 consecutive days. Statistical analysis was carried out using SPSS 16 (SPSS Inc., Chicago).

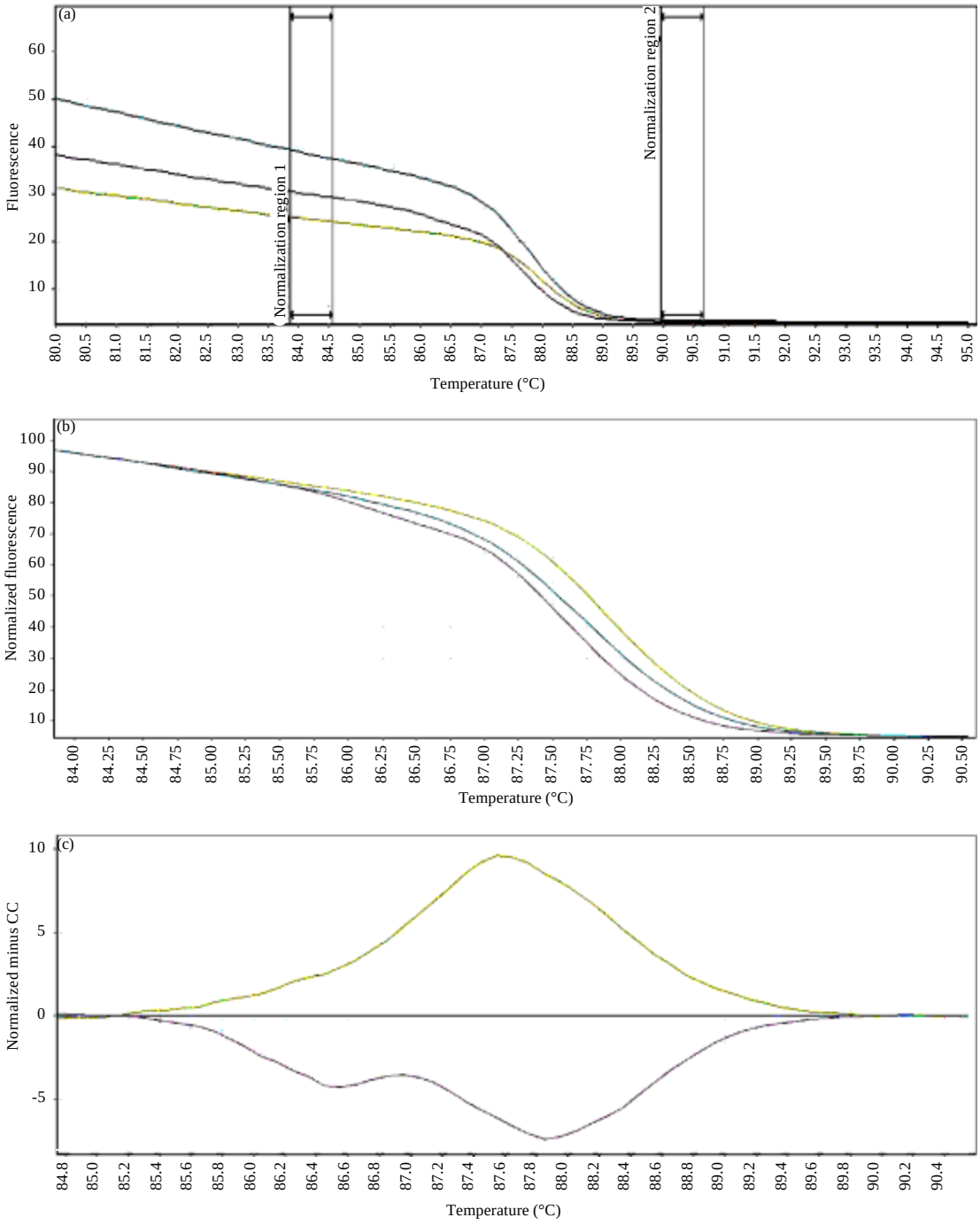

Fig. 1(a-c): Discrimination of human codon 72 p53 SNP genotypes (C to G substitution) using HRM curve analysis; homozygous wild type (blue), mutation (yellow) and heterozygote (violet) samples are shown on a standard normalized melt plot (a) Before, (b) After manual normalization and (c) A difference plot normalized to wild type (C/C or Pro/Pro) samples. HRM analysis was done using a Rotor-Gene™ 6000 instrument (Corbett Life Science) and genotypes were automatically assigned by the Rotor-Gene software. The fragment was pre-amplified using a 45 cycle

RESULTS

For HRM analysis, firstly, all graphs were manually normalized and then converted to a difference platform at with Arg/Pro standard as a baseline. Figure 1 shows normalized plots and difference plots of each sample compared to Pro/Pro control for amplicons. The difference between sample and Arg/Pro patterns was clearly shown. Sequencing results confirmed that all the samples were correctly differentiated by HRM. HRM-PCR amplifications were successfully performed on all 167 isolates (Fig. 2). According to HRM analysis performed on 167 subjects 52, 74 and 41 cases were identified as Arg/Arg, Arg/Pro and Pro/Pro genotypes. According to obtained results, the highest frequency of Arg/Arg polymorphism was seen in colorectal cancer (65% of patients) followed by breast (25%), esophagus (23%) and gastric (18%) cancer. The frequency of Arg/Pro polymorphism was the highest in gastric cancer (56%) versus other four cancers. Besides, our results shown that the frequency of Pro/Pro genotype was the highest in gastric cancer (26%). Evaluation of within and between run variability demonstrated low and acceptable CVs. Within and between run CVs were 0.0123 and 0.0168, respectively.

Table 1 shows the demographic and epidemiological characteristics of subjects. As noted, among the 167 studied individuals 52 (31%) persons were diagnosed to have Arg/Arg genotype, 66 (44%) subjects with Arg/Pro genotype and 41 (25%) were diagnosed to have Pro/Pro genotype. Overall, the number of female patients (60%) was higher than male (40%) subjects. However, there was not a significant correlation between polymorphism in codon 72 p53 and gender of subjects. Of the 56 Arg/Arg cases, 30 (54%) were male and 26 (46%) female. Of the 45 Pro/Pro cases, 21 (47%) were male and 25 (53%) female. In heterozygote group 28 (42%) were male and 38 (58%) were female. Furthermore, according to age of disease diagnosis of patients, samples were divided into four age

Table 1: Demographic properties of subjects

		Gender			Place of resistancy		Polymorphism		
				Age (year)					
Groups	N	Male	Female	Mean±SD	City	Rural	Arg/Arg	Arg/Pro	Pro/Pro
Breast cancer	57	0	57	49.8±7.3	42	15	14	29	14
Esophagus cancer	30	16	14	65.6±8.5	23	7	7	15	8
Colorectal cancer	36	22	14	59.2±11.2	25	11	23	5	8
Gastric cancer	44	29	15	63.1±10.9	32	12	8	25	11
Total	167	67	100	59.3±11.2	122	45	52	74	41

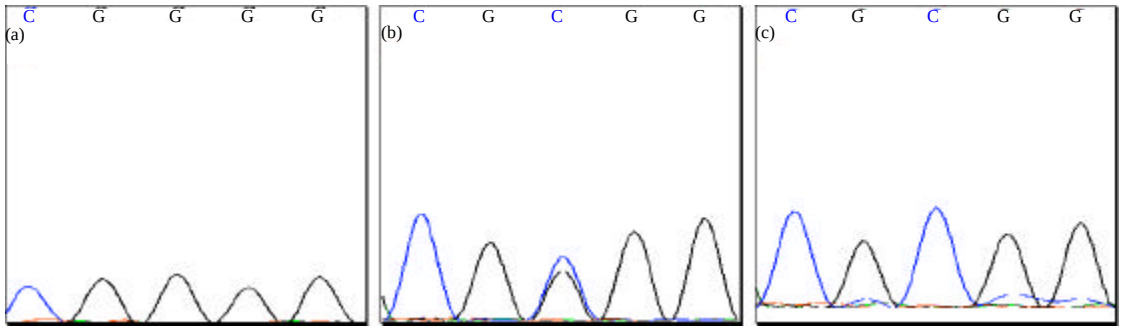

Fig. 2(a-c): Sequencing results shows the three different genotypes, (a) Pro/Pro, (b) Arg/Pro and (c) Arg/Arg

range (R1<50 years, R2: 50-60 years, R3: 60-70 years and R4>70 years). In addition, our results shown that the highest frequency of Arg/Arg polymorphism in each age range is seen in R4 (41%), followed by R1 (37%), R3 (35%) and R2 (20%). Based on this clustering, there were 10 (26%), 16 (39%), 12 (25%) and 9 (23%) cases of Pro/Pro polymorphism in R1, R2, R3 and R4 age group, respectively. The frequencies for Arg/Pro genotype were 37, 41, 40 and 36% for R1, R2, R3 and R4, respectively. Moreover, data analysis confirmed that there is a significant correlation between cancer and the place of resistance. Among 167 patients, 122 people lived in the city and there were only 45 patients living in rural areas (Table 1).

DISCUSSION

In this study we optimized a HRM based analysis method for detection of codon 72 p53 polymorphism. There have been developed numerous methods used to identify mutations such as SSCP and RFLP. The most important advantages of scanning methodologies such as SSCP, gradient gel electrophoresis and Denaturing High Performance Liquid Chromatography (DHPLC) are reduction the amount of sequencing that ultimately needs to be performed and improvement of the mutation detection process (Hensel *et al*., 1991). Because of performing directly after PCR process, HRM analysis is increasingly used as a reliable, cost-effective, simple and rapid method for identification and diagnosis of gene mutation. Recently, the method has been adopted for characterization and genotyping of photogenic mutations such as, human p53 mutation in exon 5-8 and characterization and genotyping of parasitic organisms (Krypuy *et al*., 2007; Bastien *et al*., 2008; Andriantsoanirina *et al*., 2009; Costa *et al*., 2011). In recent study done by Rostami *et al*. (2013) a high resolution melting technique were used for molecular epidemiological studies of cystic echinococcosis to differentiating G1, G3 and G6 genotypes of *E. granulosus*. The results of this study showed that HRM is completely compatible with those obtained from sequencing and rostellar hook measurement. They mentioned that HRM could be a valuable screening tool for large scale molecular epidemiological studies. In the other study, the diagnostic efficacy of HRM for mutation analysis of exon 5-8 p53 were evaluated by Krypuy *et al*. (2007). They designed PCR amplicons for HRM mutation scanning of TP53 exons 5 to 8 and tested them with DNA from cell lines heterozygous or homozygous for known mutations. They performed HRM analysis on ovarian and breast tumor for detection the mutation of p53 gene. They showed that HRM correctly could distinguish and differentiate the mutant exon. They concluded that HRM may be used as a rapid technique for detection of p53 exon 5-8 mutations and this method significantly reduced the amount of sequencing required in mutational studies of TP53. In the present study, we report the first application of HRM analysis for mutation analysis of codon 72 p53 gene. In line with previous data, our results confirm that use of HRM before sequencing, considerably cut down the number of samples that should be analyzed.

Previous studies proved that polymorphism in codon 72 of p53 gene have an important role in progression of cancer. It has been suggested that individuals with the Arg/Arg codon72 TP53 genotype have a higher risk of development of cancer (Marin *et al*., 2000). The most studied cancer in this topic is cervical cancer. However, the results of different studies show controversial results about relation between Arg/Arg polymorphism and development of cancer (Malisic *et al*., 2013). In the study was carried out on 49 Serbian women with cervical cancer, the influence of Arg/Arg polymorphism on enhancement the risk of cancer was not statically significant, however, this study could show that carriers of Arg/Arg allele of codon 72 p53 gene have an increased risk for development of cervical carcinoma in Serbian women. They have expressed that, the main

reason of non-significant difference is the small number of samples (Malisic *et al.*, 2013). In a meta-analysis published by Hou *et al.* (2013) clearly showed that there is not any significant correlation between allele's polymorphism and development of breast cancer. They suggested that to confirm the relationship between the codon 72 polymorphism of p53 gene and development of breast cancer, further studies with larger numbers of contributors in different region should be investigated. On the other hand, Biramijamal *et al.* (2001) studies the mutation of p53 in patients with esophageal cancer. The study subjects were lived in Northern Iran and have Turcoman and Persian ethnicity. They showed that there is a significant correlation between p53 mutations and risk of esophageal cancer, however, they could not prove the p53 mutation frequency or patterns in relation to genetic susceptibility or background. In line with this data, we showed that the overall frequency of Arg/Arg polymorphism in Kurdish patients with cancers is high. It appears that in addition the sample size, the ethnic factors, environmental factors and life style has important effects on results of different studies.

In summary, we showed high frequency of Arg/Arg genotype in Kurdish patients with cancers. Patients with colorectal cancer had the highest prevalence of Arg/Arg polymorphism, followed by breast cancer. It can be concluded that screening codon 72 of p53 gene by HRM technique is a cost effective and rapid method for large number of specimens that could be reduced the requirement of sequencing.

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