

RESEARCH ARTICLE

Lack of *CHEK2* Gene Mutations in Differentiated Thyroid Carcinoma Patients using High Resolution Melting Analysis

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Abstract

Recently, mutations in the genes involved in cell cycle control, including *CHEK2*, are being considered as etiological factors in different kinds of cancers. The *CHEK2* protein plays an important role in protecting damaged DNA from entering mitosis. In this study the potential effects of two common mutations IVS2+1G→A and Ile157Thr of *CHEK2* gene in differentiated thyroid carcinoma (DTC) were evaluated. A total of 100 patients admitted to the Research Institute for Nuclear Medicine were diagnosed with DTC based on pathology reports of surgery samples. An additional 100 people were selected as a control group with no cancer history. PCR-HRM (high resolution melting) analysis was performed to deal with each of mutations in all case and control samples separately. During the analysis of IVS2+1G→A and Ile157Thr mutations of *CHEK2* gene in the case and control groups, all the samples were identified as wild homozygote type. The finding suggests that IVS2+1G→A and Ile157Thr mutations of *CHEK2* gene do not constitute a risk factor for DTC in the Iranian population. However, further studies with larger population are required to confirm the outcome.

Keywords: Differentiated thyroid carcinoma (DTC) - *CHEK2* gene - polymorphism

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Introduction

Thyroid cancer is the most common malignant tumor of endocrine glands in the world and it is the 7th most common cancer in females, 14th in the males and the 11th most frequent cancer in both sexes in Iranian population (Khayamzadeh et al., 2011). Differentiated thyroid cancer is the sum of papillary follicular types consisting of almost 90% of total malignancies of thyroid gland (Hundahl et al., 1998). The precise cause of the disease is not clear yet. The restricted risk factors are including food habit, race and exposure to external cancer factors namely ionizing radiation in adult hood. Based on the recent progress in molecular sciences, various relations between the genetic changes and various cancers have been proposed. The tissues with impaired control ability in cell cycle become cancerous. Recently, mutation in the genes involved in the cell cycle control including *CHEK2* is considered in different kinds of cancers (Cybulski et al., 2004).

As DNA molecule is broken, a series of proteins including RAD family proteins are activated and send a signal. The signal is transferred to Ataxia Telangiectasia Mutated (ATM) protein by various proteins. Then, ATM protein transfers the required signal to various proteins including BRCA, P53, *CHEK2* (Checkpoint kinase 2) and the proteins arrest the cell cycle altogether as receiving

the signal. *CHEK2* is one of the most important signal proteins being phosphorylated after receiving the message from ATM. *CHEK2* transfers signal phosphorylation to the other proteins including CDC25C. The final process of transduction of the signals is the cell cycle arrest in G1 phase (Chehab et al., 2000, Liang et al., 2000). Following the cell cycle arrest, DNA repair system is activated. If DNA molecule break is not repaired, apoptosis is activated to remove the damaged cell from cell cycle and DNA damaged molecule is not transferred to the future cell generations. Mutation in each of the proteins involving the processes can lead into various diseases including cancer. *CHEK2* protein plays an important role in protecting the damaged DNA from entering mitosis. It also stabilizes tumor suppressor P53. A few studies have been conducted regarding the relation between the existing genetic changes in *CHEK2* gene and thyroid cancer. Based on the significant association between mutations of IVS2+1G→A, Ile157Thr of *CHEK2* gene and various cancers, the present study aimed to evaluate the genetic changes in differentiated thyroid cancer.

Materials and Methods

A total of 100 patients admitting Research Institute for Nuclear Medicine were diagnosed with Differentiated

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Thyroid Cancer (DTC) based on pathology report of the surgery sample. Also, 100 people were selected as control group with no cancer history. Age and gender characteristics of the case and control groups are shown in Table 1.

3-4cc peripheral blood sample was taken of the cases and controls after obtaining the consent and filling out the questionnaire. The blood sample was collected in tube consisting EDTA 1ml (1g/dl) and it was kept at 2-8°C. DNA was extracted of peripheral blood samples by salting out method (Miller et al., 1988).

PCR-High resolution melting (HRM) analysis

A pair of primer was designed to evaluate each of mutations of IVS2+1G→A, Ile157Thr of *CHEK2* gene (Table 1). Real Time PCR (Corbett Life Sciences- Qiagen) was applied for each of two amplicons of *CHEK2* gene, in all cases, controls and negative controls. PCR profile was given for each of the mutation and it is shown in Table 1. Then, High Resolution Melting Analysis was conducted. To do this, HRM™ PCR Kit Type-it®, Consisting Eva-green florescent dye was applied. The volume of reactions was considered 10 µl in 0.1 ml strip tubes, 72-well rotor. The concentration of DNA was 50 mg in each reaction. HRM profile was introduced as following:

For generating heteroduplex, at first, the temperature was set at 95°C for 10s and following 60°C for 1min, then it was increased gradually (0.2°C increase in each stage for 2s) ranging 70°C to 80°C.

After plotting melt curves, the florescent of the curves was normalized in pre melt phase and post melt phase and then it was compared. To confirm the genotype of the samples, two samples with the same melt curve were sent for DNA sequencing.

In the process of evaluating of Ile157Thr mutation, for

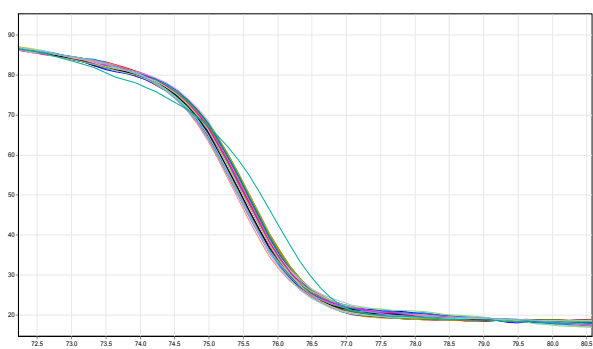


Figure 1. The Evaluation of Ile157Thr Mutation to Compare the Melting Curves to Determine the Samples Genotype, HRM Curve Consisting of CC Mutation being Generated by Site-directed Mutagenesis as Positive Control was Distinguished from Other Samples

comparing the melt curves to determine the genotype of the samples, a positive control sample was generated by Site-Directed Mutagenesis as a new forward primer which was designed to cover the site of the new mutation but to have its variant type nucleotide.

PCR-HRM was performed on one of the samples being shown as wild genotype on this polymorphism by sequencing results by a new forward primer (5'-TGT TCT CTA TTT TAG GAA GTG GGT CCT AAA AAC TCT TAC ACT GC-3') and previous reverse (beside other samples with the same forward primers and previous reverse). To be ensured of the completion of mutation in the sample, its PCR product was sent for sequencing. The results of sequencing confirmed the mutation in the sample being provided by site-directed mutagenesis. The melt curves being obtained of PCR-HRM showed the presence of mutant as positive control (Figure 1).

To evaluate IVS2+1G→A mutation, two samples were sent for sequencing and both of them were wild type. In each run of PCR-HRM assay of IVS2+1G→A mutation, these two samples were run as control beside other samples.

Results

The prevalence of IVS2+1G→A, Ile157Thr mutations of *CHEK2* gene was evaluated by High Resolution Melting (HRM) Analysis among patients and controls. The age and gender of cases and controls are shown in Table 2. Chi square test was applied for the age and gender of two groups using SPSS v13 software and there was no significant difference between two groups in age and gender (Table 2).

In the evaluation of Ile157Thr mutation, sequencing results confirmed a mutation in a sample generated by Site-Directed Mutagenesis as a positive control sample. The melt curves being obtained of PCR-HRM showed the presence of the mutant as positive control (Figure 1) and other samples with wild type genotype were different from this sample.

To evaluate IVS2+1G→A, the curve of all the samples of case and control groups were consistent with the wild type control samples had been sequenced (Figure 2).

Table 2. Age and Gender Parameters in Case and Control Groups

Variable	Case n (%)	Control n (%)	*p value	
Age	>50	18 (18%)	25 (25%)	0.228
	≤50	82 (82%)	75 (75%)	
Sex	Male	22 (22%)	23 (23%)	0.866
	Female	78 (78%)	77 (77%)	

*p value<0.05 represents statistical significance

Table 1. Primers, the Amplicon Length and PCR Profile of IVS2+1G→A and Ile157Thr Mutations of *CHEK2* Gene

Mutation	Primers*	Amplicon length	PCR Profile
IVS2+1G→A	F: 5'-GTGAATATTGCTTTGATGAACCACTGC-3' R: 5'-CAATGACCAAATTACCAGCTCTCCTAG-3'	133(bp)	95°C 5 min; 45X (95°C 10 s, 61°C 30 s, 72oC 10 s); 72°C 4 min
Ile157Thr	F: 5'-TGTCTCTATTTTAGGAAGTGGGTC-3' R: 5'-CAAGCTCTGTATTACAAAGGTTCC-3'	94 (bp)	95°C 5 min; 45X (95°C 10 s, 62°C 30 s, 72oC 10 s); 72°C 4 min

*F and R imply Forward and Reverse primers

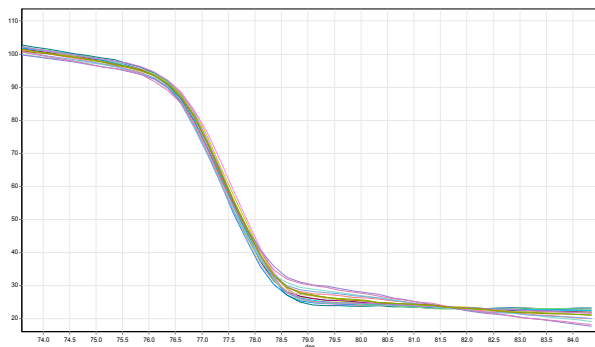


Figure 2. HRM Analysis of IVS2+1G→A Mutation, all the Samples are Consisting with the Sequenced Sample of Wild Type

Finally, all the case and control samples were identified as wild type in IVS2+1G→A, Ile157Thr mutation of *CHEK2* gene.

Discussion

For the first time in Iran, IVS2+1G→A, Ile157Thr mutation of *CHEK2* gene was evaluated by High Resolution Melting (HRM) Analysis to identify the predisposition to differentiated thyroid cancer among 200 samples 100 (cases) and 100 (controls) by High Resolution Melting (HRM) Analysis.

HRM analysis is a new technique in genotyping, based on double-strand DNA melting with high resolution. Therefore, the genotype changes can be shown in the displacement of the curves obtained of HRM and mutant genotypes are distinguished from wild genotype. This technique is economical in costs and time. Despite other screening methods of genetic changes including Single strand conformational polymorphism analysis (SSCP-analysis) and Denaturing gradient gel electrophoresis (DGGE), this method doesn't require gel electrophoresis in the next phases. As all the phases are occurred in a close system in this method, the cross contamination is minimal. Based on the recent progress in molecular sciences, various relations between the genetic changes and various cancers have been proposed. In Ile157Thr (470T>C) mutation of *CHEK2* gene, it was observed that 157Thr was located in the second fork head-associated domain (FHA) of the protein and the mutation resulted into the reduction of the activity of this protein in response to DNA damage (Ahn et al., 2002). IVS2+1G→A mutation revealed abnormal splicing and it led into frame shift and synthesis of truncated protein (Kleibl et al., 2008).

A few studies have been conducted regarding the relation between the two mutations and thyroid cancer. The only study regarding the relation between the two mutations and thyroid cancer was conducted in Poland during 2003-2004 on 4000 controls and 173 cancer cases. There was a strong association between IVS2+1G→A (P=0.0003) and Ile157Thr (P=0.04) of *CHEK2* gene with thyroid cancer and cancers of breast, colon, kidney and prostate and the mutations of the gene were the strong risk factors in various cancers (Cybulski et al., 2004). However, none of IVS2+1G→A and Ile157Thr mutations of *CHEK2* gene was identified among the participants

in the present study. Various studies showed different results regarding the relationship with other cancers: Some studies were demonstrated *CHEK2* Ile157Thr variant may be one of the predisposing gene in breast and colorectal cancer susceptibility (Liu C et al., 2012 a; Liu C et al., 2012 b). Another study was conducted to evaluate the effect of *CHEK2* variants and predisposition to ovarian cancer in 2006. 1108 Polish women were genotyped with various types of ovarian tumors and 4000 controls for the three *CHEK2* variants. Positive associations were seen with the ovarian cystadenomas (P=0.005), borderline ovarian cancers (P=0.002) and low-grade invasive cancers (P=0.04) with I157T missense but there was no significant association in high grade invasive cancer (Szymanska-Pasternak et al., 2006). In a study done regarding the effect of Ile157Thr mutation among 631 patients with colorectal cancer (CRC) in Czechoslovakia, the results were as following: Ile157Thr mutation of *CHEK2* gene had significant association with sporadic CRC but there was no significant association with familial CRC (Kleibl et al., 2009). A research done regarding the evaluation of the relation between the variants of *CHEK2* gene and malignant melanoma among 630 cases and 3700 controls in Poland, there was not significant association between the two factors (Debniak et al., 2008).

In a recent study which was carried out to investigate the effect of *CHEK2* gene mutations among 210 patients with Colorectal Cancer (CRC) in Turkey, mutation was not observed among the participants and they found that the mutations had not been a genetic susceptibility factor for CRC in the Turkish population (Bayram et al., 2012).

In conclusion, like the Turkish population, the frequency of IVS2+1G→A and Ile157Thr mutations of *CHEK2* gene in Iranian population was rare compared to the population of northern Europe countries (Bayram et al., 2012) and the introduction of the mutations as good risk factors is not recommended. The ethnical and geographical differences are some of the causes. As the present study was the first study regarding the evaluation of the mutations of *CHEK2* gene among the patients with thyroid cancer in Iran and the number of the participants was limited, rejecting the hypothesis of the relation of IVS2+1G→A and Ile157Thr mutations of *CHEK2* gene with differentiated thyroid cancer requires extensive geographical studies with more samples in Iran.

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