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Increased Expression of *CYP2E1* Gene in Gastric Cancer May be a Molecular Marker for Mazandaran Province Population

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Abstract

Cytochrome P450 2E1 (CYP2E1) enzyme metabolically activates a large number of low molecular mass xenobiotics probably involved in gastric cancer incidence through activation of procarcinogens. North of Iran is amongst high incidence rate areas of gastric carcinoma where environmental carcinogenic compounds, including agricultural pesticides, are massively used. In this report, we quantitatively compared *CYP2E1* gene expression between tumoral and nontumoral-marginal tissues of gastric cancer patients as well as normal healthy gastric tissues by real-time PCR. Results showed that *CYP2E1* gene cDNA copy numbers were relatively increased in tumoral group vs. nontumoral-marginal and normal healthy groups. Comparison of means $\Delta\Delta C_T$ by Dunkan's test statistically verified significant differences of *cyp2E1* cDNA copy numbers between tumoral and healthy tissues (P=0.0018). It seems that the increased *CYP2E1* gene expression may be associated with increased risk for gastric cancer. So, we recommended that *CYP2E1* gene expression may be an appropriate molecular marker to determine individual sensitivity to gastric cancer and also for designing cancer prevention programs.

Key words: *CYP2E1*; Gastric cancer; Mazandaran province; Pesticide; Real-time PCR

Introduction

Most exogenous (xenobiotics) and endogenous chemical carcinogens undergo biotransformation by phases I and II enzyme systems to activate and subsequently detoxify in human body (Nebert & Dalton 2006). Cytochrome P450 (CYP), mainly localized in the liver, is a superfamily of hemoproteins and a major kind of phase I enzymes detoxification that play a central role in metabolism of many xenobiotics and endogenous compounds (Coon, 2005; Khalatbari *et al.,* 2017). Apart from the liver, CYPs are also expressed in other tissues such as lung, kidney, gastrointestinal tract and hematopoietic cells (Gonzalez, 2005). The aberrant function of the metabolizing enzyme system may influence the balance of the metabolizing capacity between bioactivation and detoxification of xenobiotics leading to activation of many carcinogens (Guengerich, 1988; Nair *et al.,* 2016). Stomach cancer is the second leading cause of cancer related death resulting in 10.4% annual deaths and the fourth most frequent cancer, accounting for 8% of the total cancer cases, worldwide (Lao *et al*., 2010; Siegel *et al*., 2016). Despite decrease of the incidence rate of gastric cancer in the western world, its incidence and mortality have increased or remained stable in developing countries. Accordingly, gastric cancer has been ranked as the leading and the third cause of cancer related death in Iranian men and women, respectively (Mousavi *et al*., 2009). Northern parts of Iran along with Northwestern regions are the highest risk areas for gastric cancer with an age-standardized incidence rate (ASR) of 49.1 for males and 25.4 for females (Malekzadeh *et al*., 2009). Gastric cancer mostly occurs as a result of complex interaction between genetic factors such as single nucleotide polymorphism (SNP) within key genes, and environmental risk factors such as *Helicobacter pylori* colonization and exposure to xenobiotics (Correa, 1992; Shikata *et al*., 2008). Pesticides as the most carcinogenic compounds massively using in northern Iran are assumed to be the most important risk factors for cancer incidence (Shokrzadeh *et al*., 2013).

Cytochrome P450 2E1 (CYP2E1), a member of the cytochrome P450 superfamily is an alcohol-inducible enzyme that plays a key role in the metabolic activation of many low molecular weight compounds such as benzene, vinyl chloride and N-nitrosamines and also has been implicated in a variety of pathological conditions such as diabetes, non-alcoholic steatohepatitis (NASH) and cancer (Kim *et al*., 2008; Rutkowski & Kaufman 2004; Vaclavikova *et al*., 2007). *CYP2E1* is located on chromosome 10q26.3 as an 11.7 kbp gene consisting of 9 exons and 8 introns that encodes for a 493 amino acid protein. Some functional *CYP2E1* gene SNPs have been identified that alter the transcriptional activity of the gene (Gao *et al.,* 2007). Indeed, variable *CYP2E1* gene expression has been shown in numerous inflammatory diseases including cancers (Helmig *et al*., 2010; Danko & Chaschin 2005). Differential expression of *CYP2E1* in different tumors and various stages of cancer, with its capacity to induce ROS production (Caro & Cederbaum 2004), provide opportunity to use *CYP2E1* cellular levels as an indicator of cancer progression and to find factors involved in its differential regulation of gene expression. High incidence rate of gastric cancer in the north of Iran, where pesticides are being massively used, and variable toxicity of chemical carcinogens in relation to metabolizing enzymes level have prompted us to analyze interindividual variation of *CYP2E1* gene expression as a key enzyme for xenobiotics metabolism. So, the aim of this research was to study the *CYP2E1* gene mRNA level in cancerous and normal stomach tissues and its association with the risk of gastric cancer in Mazandaran province.

Materials and methods

Samples collection

The present case-control study was comprised 20 gastric cancer patients who underwent surgery in Ayatollah Rouhani hospital of Babol city during 2014 to 2016. A piece of cancerous as well as its adjacent normal tissue was dissected from surgical specimens of stomach cancer. None of these patients received chemotherapy before surgical resection. Twenty normal tissue samples from healthy individuals whose stomach tissues were reported non-tumoral after pathology assays were also included in this analysis to compare *CYP2E1* gene expression between cancerous and normal samples. All specimens were analyzed by experienced pathologists and their clinopathological characteristics were extracted from their files. Tissue samples were snap frozen in liquid nitrogen and stored at -80^{o}C until further analysis. All patients had given informed consent form and the study was approved by the ethic committee of Ayatollah Rouhani hospital.

Total RNA isolation and cDNA synthesis

A small piece of each tissue samples, approximately 30 mg, were grounded in liquid nitrogen using prechilled mortar and pestle. Total RNA extraction was carried out by Total RNA Extraction Kit (Jena bioscience, Germany) based on the manufacturer's instruction. *DNase* treatment (Qiagen, Hilden, Germany) has been included in the procedure for elimination of any genomic DNA contamination. Extracted RNAs were eluted in 40µl *RNase*-free ddH_2O and stored at −80^{o}C. The integrity of RNA samples was assessed by agarose gel electrophoresis and its quantity (A260) and purity (A260/A280 and A260/A230) were measured by UV spectrophotometry (Sambrook *et al*., 1989). cDNA was synthesized from 100 ng total RNA using AccuPower RocketScript RT PreMix kit (Bioneer, South Korea). Reaction mixture contained Reverse Transcriptase (RT) enzyme (1.5 U/µL), 1X cDNA synthesis buffer, 0.5 pM oligo dT primer, 4 U/µL *RNase* out, and 2 mM dNTPs. The cDNA synthesis continued for 32 min at 42^{o}C, followed by an inactivation cycle at 95^{o}C for 3 min to stop the reaction. Two µl of this RT product was used for subsequent PCR amplification.

Real-time PCR

Quantitative real-time PCR was carried out using specific primer pairs for *CYP2E1* and β-actin housekeeping gene, chosen as a reference (Table 1) with the Quantive Nova SYBER Green PCR kit (Qiagen, Hilden, Germany) based on the manufacturer's instruction and run in the Rotor-Gene 6000 (Corbett Research, Sydney, Australia). The primer pairs were designed with Oligo software (National Biosciences Inc., ver 7.00) based on the human *CYP2E1* (NM_000499.3) and *β-actin* cDNA sequences (NM_001101.3) available from GenBank. Real-time PCR amplification was performed in a 15µL reaction containing 1X Quantive Nova

SYBER Green master mix, 0.3 pM each of forward and reverse primers, and 100 ng first-strand cDNA as template. Thermocycling conditions composed of an initial heating at 95°C for 5 min as an activation step, and 40 cycles of denaturation at 95°C for 15 sec. and annealing and extension at 60°C for 40 sec. Each RNA sample was divided into equal amounts and subsequently, *CYP2E1* and *β-actin* cDNAs were simultaneously amplified by real-time PCR in triplicate. In each PCR reaction, a six points 1/10-1/1,000,000 serial dilution of composite cDNAs was used to generate standard curve. *CYP2E1* gene mRNA expression was calculated by comparing the C_T values with the standard curves of the *CYP2E1* and *β-actin* genes in each amplification run using $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Also, a melting curve has been designed to confirm that a single amplicon has been generated by real-time PCR.

Statistical analysis

All statistical analyzes were carried out by SAS 9.1 software and $P<0.05$ was considered as statistical significance. Comparison of means of *CYP2E1* gene mRNA level between three tumoral, nontumoral-marginal and healthy control groups were calculated using Duncan's test (Yuan *et al*., 2006).

Table 1. Primer pairs used for Real-time PCR amplification of *CYP2E1* and β-actin genes

Gene	Sequence	Amplicon size (bp)	Tm (ºC)
CYP2E1	5′- TGAAGCAACCCGAGACACC -3′ 5′- TTCTCCAGCACACACTCGTT -3′	211	59.93 59.54
β-actin	5′-GGCACCCAGCACAATGAAG-3′ 5′-GCCGATCCACACGGAGTACT-3′	67	60 60

Results

CYP2E1 gene expression

The expression of *CYP2E1* gene was quantitatively examined in 20 tumoral as well as 20 normal stomach tissues. An intense band of expected size was appeared in agarose gel following electrophoresis of real-time PCR amplified *CYP2E1* and *β-actin* cDNAs (Fig. 1). No major primer-dimer was generated during the 40 real-time PCR amplification cycles. The standard curve plotted based on the logarithm of the cDNAs concentration against threshold cycle (C_T) was linear helping to determine cDNA concentration needed for real-time PCR (Fig. 2A). The real-time PCR amplification curves of *CYP2E1* and *β-Actin* genes using real-time PCR showed logarithmic amplification of cDNAs (Fig. 2B). Analysis of melting curve for the designed primers showed that each primer pairs specifically bind to the template and no non-specific bands or secondary structures were created in the reaction mixture (Fig. 2C).

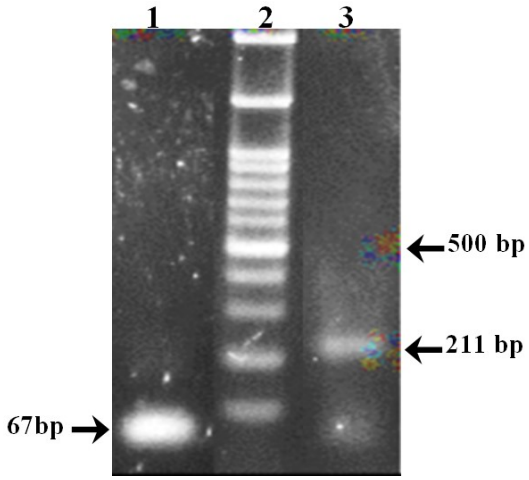

Fig. 1. Agarose gel electrophoresis of real-time PCR amplified *CYP2E1* and β-Actin genes on 1.5% agarose gel: line1= β-actin gene amplified fragment; Line2= 100 bp ladder (Fermentas); line3= *CYP2E1* gene amplified fragment.

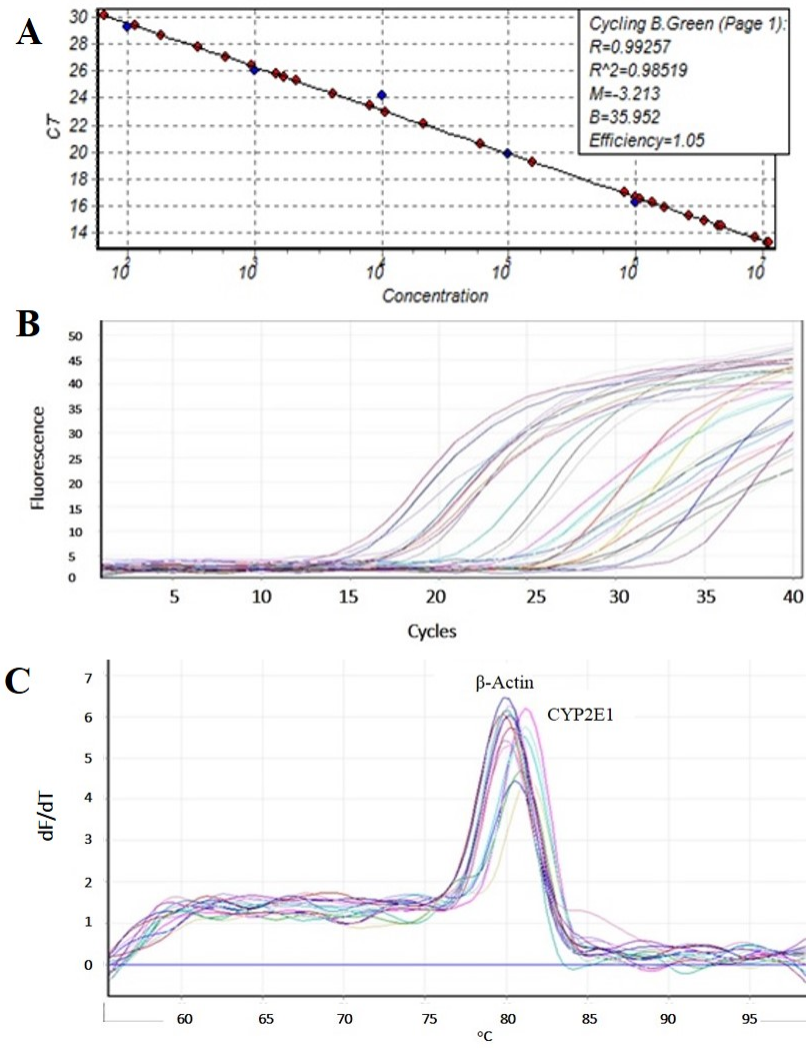

Fig. 2. Real-time PCR amplification graphs. A) Six points standard curve plotted based on copy numbers of the composite cDNAs vs. threshold cycle (C_T); B) Real-time PCR amplification curves of *CYP2E1* and β-actin genes cDNA from tumoral and normal gastric tissues plotted based on the C_T value against fluorescent intensities. The C_T value of each curve confirms different cDNA copy numbers of *CYP2E1* genes in tumoral and normal gastric tissues; C) Melting curve plotted based on -ΔF/ΔT vs. temperature. The represented peaks show specific amplification of *CYP2E1* and β-actin cDNAs using real-time PCR without primer dimers and non-specific products.

Association between *CYP2E1* gene expression and gastric cancer risk

Results of real-time PCR amplification showed that *CYP2E1* gene was expressed in each of three gastric samples including tumoral, nontumoral-marginal and normal-healthy tissues. Comparison of the expression level of *CYP2E1* gene showed that the cDNA copy numbers of the *CYP2E1* gene was relatively increased in tumoral tissues (mean $\Delta\Delta C_T$=2.352±0.47) relative to nontumoral-marginal (mean $\Delta\Delta C_T$=1.698±0.29) and control (mean $\Delta\Delta C_T$=0.656±0.23) groups (Table 2). Comparison of means by Dunkan's test statistically verified significant differences (P=0.0018) of *CYP2E1* cDNA copy numbers between tumoral and healthy tissues (Table 2). However, no significant differences found between nontumoral-marginal and tumoral groups.

Table 2. *CYP2E1* gene expression level between tumoral, nontumoral-marginal and normal groups

Group of sample	Mean $\Delta\Delta C_T \pm$ SE	Duncan's Grouping	P value
Tumoral	2.352 ±0.47	A	0.0018
Nontumoral-marginal	1.698 ±0.29	A	-
Normal healthy	0.656 ±0.23	B	-

Discussion

In spite of decrease in the incidence rate of gastric cancer in western world, north and northwestern parts of Iran are still amongst high incidence rate areas of gastric carcinoma (Jemal *et al*., 2011; Mohebbi *et al*., 2008; Colagar & Amjadi Souraki, 2012). Cytochrome P450 2E1 is a member of cytochrome P450 monooxygenase superfamily and phase I detoxification enzymes that involved in the metabolic activation of some environmental carcinogens (Glatt, 2000; Cai *et al*., 2005; Tang *et al*., 2010). Many xenobiotics and chemical carcinogens such as pesticides turn to carcinogenesis form via biotransformation system of the body. Over the past several years, there has been intense focus on identifying molecular markers to better predict the aggressiveness of certain cancers. In this reports we quantitatively measured *CYP2E1* gene expression rate in tumoral, nontumoral-marginal and normal gastric tissues. Results of real-time PCR amplification indicated that the *CYP2E1* cDNA copy numbers was gradually and relatively increased in tumoral vs. nontumoral-marginal vs. normal groups. Interestingly, *CYP2E1* gene expression difference between normal and cancerous individuals was more than the difference between nontumoral-marginal and tumoral tissues of each patients. Over-expression of *CYP2E1* gene in nontumoral-marginal tissues may be related to the changed signaling processes occurs in the course of tumorigenesis. Comparison of means $\Delta\Delta C_T$ by Duncan's test statistically verified significant differences of *CYP2E1* cDNA copy numbers between tumoral and healthy tissues (P=0.0018). It seems that the increased *CYP2E1* gene expression may be associated with the risk for gastric cancer. Differential expression of *CYP2E1* gene was documented in various cancers such as breast cell metastasis (Leung *et al.,* 2013) and non-small cell lung cancer (Oyama *et al*., 2007). In addition to inter-ethnic differences of *CYP2E1* gene and their discrete effects on cancer susceptibility, the interaction of genetic and environmental risk factors may be involved in cancer development (Shahriary *et al*., 2012). In this regard, we recently reported lack of the association between *Pst*I/*Rsa*I polymorphism and gastric cancer (Kamalipour *et al*., 2016). However, in an effort to find environmental risk factors, we successfully detected Diazinone pesticide in the blood serum of some gastric cancer patients (0.155 ppm) using GC-mass analysis (unpublished data). Pesticides, as a major group of environmental risk factors mostly exert their effects through epigenetic modifications on the regulatory regions of key genes including cytochrome P450 superfamily members having major role in xenobiotic metabolism. The increased expression of *CYP2E1* gene detected in this study may be linked to the potential of pesticides to change epigenetic moieties of *CYP2E1* gene controlling elements. Taken together, searching for epigenetic changes in *CYP2E1* gene control elements would be the key point in differential expression of *CYP2E1* gene in various cancers.

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