

RESEARCH COMMUNICATION

Glutathione S-transferase mu Gene Variants and Colorectal Cancer Development - Use of Sequence-specific Probes for an Iranian Population

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Abstract

Background: Enzymes of the glutathione S-transferase (GST) family are encoded by a set of polymorphic genes as an important part of cellular chemical defense. The aim of this study was to investigate the possible effect of GSTM1 deletion on susceptibility to developing clinical outcome of colorectal cancer in a group of CRC patients from Isfahan province, Iran, in comparison to age and gender matched control group. **Methods:** DNA was extracted from blood of 140 CRC patients and 90 healthy individuals and a set of sequence specific hybridization probes was used for GSTM1 genotyping by real-time PCR in Light-Cycler instrument. Chi-squared test was used to assess the statistical significance of observed differences between the patient and control subjects of different genders and ages. To estimate the risk for overall and stratified analyses, odds ratios (OR) with 95% confidence intervals (CI) computed with logistic regression. **Results:** No difference in GSTM1 null genotype frequency was found in CRC patients and controls stratified by gender (p value=0.14). The data were suggested a trend of increasing risk for GSTM1 null genotype in patients over 60 years old compared with controls (p value=0.05). GSTM1 null genotype carried an increase of the odds of developing CRC in patients over 60 years old (OR=2.7; 95% CI: 1.03-7.05). No significant association was found (P> 0.05) between the GSTM1 null genotype with tumor site (right, left, rectum) or tumor differentiation (well, moderately). **Conclusion:** Our findings suggest that the GSTM1 null genotype may contribute to colorectal cancer development in people over 60 years old.

Keywords: Colorectal cancer - glutathione S-transferase mu - sequence specific probe - RT-PCR - Iranian population

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Introduction

Worldwide, colorectal cancer is the second commonest form of cancer after lung cancer (Ferlay et al. 2004), with 1.02 million new cases and more than 500,000 deaths annually (Parkin et al., 2005). The gastrointestinal (GI) cancers are the most frequent cancer in Iranian males, the second to breast cancer among females (Mosavi-Jarrahi and Mohagheghi, 2006) and Colorectal Cancer (CRC) is the third most common cancer among males and the fourth amongst females (Kolahdoozan et al., 2010). Iran like some of the more developed Asian countries with traditionally low incidence of CRC has already experienced a rapidly rising trend in CRC incidence which is comparable to the rate of Western populations. The CRC incidence was reported to increase sharply by 82% during the last 30 years in Tehran, the capital of Iran (Sung et al., 2005; Yazdizadeh et al., 2005, Malekzadeh et al., 2009)

Morbidity and mortality rates have been shown wide differences by ethnic/racial group. Since the colorectal cancer is a multi factorial disease including intrinsic due to gene-environment interaction, therefore, its etiology is very complicated (Ahmed, 2006). The increasing incidence rates may be resulted from the increasing in exposure to risk factors as some environmental pollutants, toxic materials and the lifestyle risk factors (Slattery, 2000) such as diet high in red meat especially substances like heterocyclic amines and polycyclic aromatic hydrocarbons which are produced from well-done meats, low fibers and vegetables (Gonzalez, 2006), smoking (Giovannucci, 2001), obesity (Bianchini, 2002) and physical activity (Boutron-Ruault, 2001). The risk of sporadic CRC may modulated by genetic factors (Naccarati, 2007).

Genes encoding glutathione S-transferases (GSTs) as detoxifying enzymes are among various candidates genes implicated in colorectal cancer studies. GST is a multi

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gene family which encoded phase II detoxifying drugs and carcinogen metabolizing enzymes that inactivate the xenobiotics by conjugation to an endogenous water-soluble substrate such as glutathione so it can facilitate eventual elimination from cells. GSTs have a key role in the protection of human cells against a wide range of environmental carcinogens, pesticides, products of oxidative stress and chemotherapeutic agents (Hayes 1995, Abdollahi, 2004). To date, eight classes of cytosolic GSTs including alpha, kappa, mu, omega, pi, sigma, theta and zeta have been described with a high degree of expression in mammalian tissues.

Genes encoding the mu class of GST enzymes are organized in a gene cluster on chromosome 1p13.3. The polymorphic nature of GST encoding genes leads to variations in the susceptibility of individuals to carcinogens and toxins as well the toxicity and efficacy of certain drugs (Joseph, 2010). Homologous recombination in the left and right region of GSTM1 gene produces null genotype, so-called GSTM1*0. In addition, a missense single nucleotide polymorphism also reported in GSTM1 gene introduced a substitution G to C (172 Lys to Asn), corresponding to GSTM1*A and GSTM1*B genotype, which does not affect the enzyme activity. However, deletion of GSTM1, which associated with no enzyme activity and impaired ability to metabolically elimination of carcinogens, is more important and contributes in greater risk to cancers (Fritz, 2005).

More recently, contradictory results have been found about the association between GSTM1 null genotype and colorectal cancer risk. Although association was found in a line of studies (Sachse, 2002, Kiss 2004, Ates 2005 and Martinez, 2006), other authors have documented no contribution of this null genotype and CRC (Kato, 1999; Saadat 2001; Yeh 2007; Skjelbred, 2007; Kury, 2008; Zupa, 2009; Matakova 2009).

On the other hand, the new insight to drug treatment response and pharmacogenetics has opened a new field to investigate the role of the polymorphisms in drug metaboliser genes. Drug metabolising enzymes are responsible for the activation, inactivation and detoxification of many chemotherapeutic agents thereby deficiency in these enzymes may influence the efficacy of treatment. Many cancer chemotherapeutics are electrophile (or their metabolic precursors) and GSTs play an important role in inactivation and elimination of such drugs. It is accepted that GSTM1 null genotype have a clear association with longer survival in different malignancies when the substrates of the GSTM1 (mostly alkylating agents and platinum compounds) are used as chemotherapeutic drugs (Joseph, 2010).

The present study was designed to assess the GSTM1 genotypes compared to control group using blood samples and pathological information of colorectal cancer patients of Isfahan province, Iran. The aim of the study was to evaluate the relation of GSTM1 genotypes with the development of colorectal cancer. To the best of our knowledge, traditional PCR methods have been often used in the most (more than 85%) studies on GSTM1 genotyping and only a few studies used other methods such as real-time PCR. In comparison with the traditional

Table 1. GSTM1 Gene Primers and Hybridization Probe Sequences

Forward Primer	gAACTCCCTgAAAAgCTAAAAGCT
Reverse Primer	gTTgggCTCAAATATACggTgg
FLU probe	ACTCCTCCTTTACCTTgTTTCCTg CAAACC- -F
LCR640 probe	TggCCgCTTCCCCAgAAACTCTg- -PH

methods, real time PCR is considered as a safe, fast, and accurate procedure for genotyping studies, therefore, it was used in this study for GSTM1 genotyping.

Materials and Methods

Subjects

This case-control study consisted of one hundred and forty patients with operative and histological confirmation of adenocarcinoma of the colon and rectum. Only patients with sporadic colorectal cancer undergoing potentially curative surgery were included in the study. Exclusion criteria were any history of metastatic diseases and hereditary CRC. The patients group was comprised of 65 males and 75 females with the mean age of 51.7 years. For the control group, any history of cancer was considered as inclusion criteria. The control group included ninety individuals of both genders (41 males and 49 females). The mean age (Min-Max) of the control group was 51.8 years (22-76). The participants in this study were from the Isfahan province, Iran. The study was approved and conducted by Institutional Review Board of Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Iran.

After signing the informed consent, blood samples and defined data were collected. For the control group, the structured checklists consist of the demographic information and history of cancer was completed in an interview for each individual to document his or her personal and health history.

Patients' clinical data

Clinical details, including histological data, were obtained retrospectively from histology reports. Clinical data were collected for established outcome markers: tumor site (right, left and rectum), tumor grading in base of differentiation (well, moderate and poor). All data were registered in the databank and were used in the study as the source of demographic and clinical data.

Sampling and Laboratory Measurements

Genomic DNA was extracted from the blood of each subject using a Puregene DNA purification kit (Gentra Puregen, Germany). For genotyping, the thermocycler LightCycler (Roche, Mannheim, Germany) was used and hybridization probes were applied in combination with a QuantiFast Probe PCR kit (QIAGEN, Germany). The PCR primers and probes were synthesized by TIB MOLBIOL (Berlin, Germany) according to Ko et al (17) with some modifications.

The final PCR conditions were optimized as follows: 0.1 μ M of each hybridization probe, 0.3 μ M of each primer, 2 μ L of the QuantiFast Probe PCR which Contains

Table 2. GSTM1 Genotype Profile and Colorectal Cancer by Gender and Age

Group	Genotypes	Case	Control	OR	95%CI
All	GSTM1*1	55.7	44.3		
	GSTM1*0	66.1	33.9	1.54	0.90-2.64
Men	GSTM1*1	53.1	46.9		
	GSTM1*0	68.4	31.6	1.81	0.82-4.05
Women	GSTM1*1	57.6	42.4		
	GSTM1*0	63.8	36.2	0.96	0.38-2.43
<60	GSTM1*1	58.8	41.3		
	GSTM1*0	63.2	36.8	1.02	0.63-2.3
≥60	GSTM1*1	48.6	51.4		
	GSTM1*0	71.8	28.2	2.7	1.03-7.05

HotStarTaq® Plus DNA Polymerase, reaction buffer, MgCl₂, and dNTP mix, and 10-50 ng of genomic DNA in a final volume of 10 μL. The primers and probes sequence are shown in Table 1. The temperature profiling of PCR was started with 3 min Pre-incubation time at 95 °C. Then, 45 cycles of amplification were performed with initial denaturation for 10 sec at 95 °C which followed by 15 sec annealing at 55 °C for 15 sec and extension at 72 °C. Differentiation of GSTM1 genotype was performed by determination of melting curves after PCR. Melting curves were obtained 95 °C for 20 sec followed by an annealing period of 40 °C and a final temperature of 85 °C with a temperature gradient 0.2 / Sec followed by cooling cycle 40 °C for 30 Sec. Intra-assay (the amount of the error seen with the same assay in each run) and inter-assay (the error between separate assays) were performed.

Statistical Analysis

Applying chi-squared test, genotype frequencies among cases and controls were compared. Pearson chi-squared test or Fisher exact tests were used to assess differences between the case and control subjects in the distributions of gender, age and assessing the association between different genotypes in case and controls. To estimate the risk for overall and stratified analyses, odds ratios (OR) with 95% confidence intervals (CI) were computed with logistic regression. Statistical significance was considered as $p < 0.05$ and P-values were two-tailed. Statistical analyses were performed using statistical package SPSS for windows, version 12.0 (SPSS SAS Institute, NC, USA).

Results

The age of 40% of males in CRC patients was more than 60 years versus 25.4% in females. The frequency of individuals with age of more than 60 years was 32.1% and 32.2% in patients and control group, respectively. No differences in the age distribution were found in case compare with control group. The patients group consisted of 53.6% males and 46.4% females. No significant differences were seen in colorectal incidence rate by gender (see Table 2).

The total incidence of colon to rectum cancer ratio was exactly unity and the male to female ratio was 1 for colon as well as rectal cancers. Frequency of GSTM1*0 was 37.9% in the control group for individuals elder than

60 years versus 62.1% in subjects above 60 years in case group. Although significant differences were not observed between the GSTM1 polymorphisms and the age of more than 60 years, there was a trend of increasing risk for null genotype. Therefore, it is concluded that GSTM1 null genotype carried an increase risk of developing CRC in patients older than 60 years (OR=2.7; 95%CI: 1.03-7.05, Table 2).

The frequency of males and females were 46.4% and 53.6% in patients group and 45.6% and 54.45% in controls. No differences in GSTM1 null genotype frequency were perceived in the cases and control groups stratified by gender. In addition, no association between GSTM1 genotype and development of colorectal cancer was seen when stratified by gender.

In this study, it has been observed that of 49.1% of tumors located in rectum 37.1% and 12.8% were corresponded to right and left sides, respectively. No significant association between the GSTM1 null genotype was shown when tumor sites are considered as a colorectal outcome (Table 3). The grading of patients tumors were 70.5%, 25.9% and 3% corresponding to well, moderate, poor differentiation, respectively. No significant trend was observed between GSTM1 null genotype frequency and tumor differentiation (p value=0.15).

Discussion

The present study was conducted to assess the possible effects of GSTM1 genotypes in colorectal cancer patients in comparison to control group. The age of 40% of males in CRC patients was more than 60 years versus 25.4% in females. This finding is in agreement with the idea that the age incidence rate is larger for men than women (Parkin b, 1997).

No significant differences observed between colorectal cancer incidence in males (46.4%) and females (53.4%). This is inconsistent with the results of (Sainz et al., 2011) which reported that the incidence rates and relative risks for colorectal cancer (CRC) are higher in men than in women because sex steroids may play a role in this gender-associated difference in CRC risk.

In our study, the ratio of colon to rectum tumors was 1 while in most populations the colon cancer was reported to be the most common (Parkin b, 1997). The male to female ratio was 1 for colon cancer as well as for rectal cancer; whereas it was reported the rectal cancer incidence was 1.5 or greater in males (Parkin b, 1997).

In previous studies, (Katoh et al. 1999) found no association between GSTM1 genotype and CRC (OR=0.1, 95% (CI):0.01-1.89). Such results were also reported by Saadat et al. (2001), Yeh et al. (2007), Skjelbred et al. (2007), Zupa et al. (2009) and Matakova et al. (2009). In all of the mentioned studies, conventional PCR method has been used for GSTM1 genotyping. Kury et al. (2008) also confirmed no association between GSTM1 genotype and CRC by use of Taq-Man probes OR=1.15, 95% Confidence Interval (CI):0.98-1.37. In our study, the association between GSTM1 null genotype and developing CRC did not reach significant level OR=1.53, 95% (CI): 0.911-2.647. We used probe-based real-time

PCR assay for genotyping GSTM1.

A significant association between GSTM1 genotype and CRC was reported by Ates et al. (2005), Martinez et al. (2006), Kiss et al. (2004) and Sache et al. (2002). Ates used real-time PCR, OR=1.56, 95% (CI) 1.04-2.33, Martinez and Kiss used conventional PCR approach and Sache used Taq-Man probe method. Our findings are in disagreement with these studies which found association between GSTM1 genotype and development of CRC.

Colorectal cancer is a multifactorial condition and the consequence of gene-environmental interaction expected to be vary in different population. Genetic susceptibility to CRC might depend on genetic variations in carcinogens detoxifying enzymes. Epidemiologic evidence suggests that much of the geographic variation reflects differences in lifestyle and environment, perhaps acting with variations in genetic factors. It could be due to some critical reasons: the most important one may be ethnical differences in expression pattern of this polymorphic gene. It is documented that, the frequency of the GSTM1 null genotype ranges from 23 to 48 percent in Africans, from 33 to 63 percent in Asian populations and 39 to 62 percent in European (Cotton, 2000). Life style and food habits also have critical role in developing CRC. For example, consumption of broiled or grilled meats by browning of the meat surface have important effects especially in GSTM1 null genotype individuals because they have impaired catalytic activity of this enzyme to carcinogens detoxification such as heterocyclic amines and polycyclic aromatic hydrocarbons. Exposure to carcinogens and toxic materials as environmental pollutants is a consequence of industrialization in developing countries so it is unavoidable to expose for some population in different area.

The most interesting finding in this study was identification of an association between GSTM1 null genotype and developing CRC in patients over 60 years. It has been proved that the CRC incidence increases with age (Cotton, 2000) but it is not reported an association between GSTM1 null genotype and CRC development just in older group. A possible explanation for this might be that the impaired enzymatic capacity of GSTM1*0 in detoxification carcinogens. GSTM1 null genotype individuals fail to remove some highly potent carcinogens which induced formation of DNA adducts. The consequence of DNA-adducts formation may predispose DNA to damage. DNA damage in critical loci such an oncogene or tumor suppressor genes can lead to somatic mutation and disruption of cell cycle. Aggregation of these changes in DNA level may be in contribution with some other ageing process and induce susceptibility to colorectal cancer in older group.

However, given the lack of association between GSTM1 null genotype and developing CRC in our study for all patients (less and more than 60 years old), our finding on older group would require further confirmatory data. We didn't observe the distribution of GSTM1 genotype significantly different by tumor site. This finding is consistent with those of Holley et al. (2006, Deakin et al. (1996), Gertig et al. (1998), and Chenevix-Trench et al. (1995), who identified no significant associations between

GSTM1 genotypes and tumor site (right, left and rectum), but is in disagreement with Ates et al (2005) study that reported association between GSTM1 null genotype with risk of developing transverse or rectal tumors.

No associations were found between GSTM1 null genotypes and tumors differentiation. This result is in disagreement with (Holley et al., 2006) study that found an association between GSTM1 null and decreased frequency of poorly-differentiated tumors.

In this study, we used LightCycler real-time PCR assay and sequence specific hybridization probe for genotyping the GSTM1 gene. Unlike conventional PCR in which cycling takes several hours, the PCR and online melting curve analysis conducted with LightCycler took around 1 hour. The majority of authors have been used conventional PCR method which depends on the analysis of Ethidium bromide-stained gels. In addition to avoiding from the toxicity of ethidium bromide, no additional times is required for the preparation and documentation of agarose gel in the mean of analyzing the PCR product when the LightCycler used.

This study was undertaken to design a case-control study and evaluate the GSTM1 genotype and developing colorectal cancer by use of real-time PCR assay. There was no association between GSTM1*0 genotype and colorectal cancer risk. The most obvious finding of the present study is that the GSTM1*0 genotype increased the odds of colorectal cancer development in the age group of more than 60 years. Further molecular epidemiological studies are needed to investigate the association between GSTs in colorectal and other cancers in different geographical areas on various ethnic groups.

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