

RESEARCH ARTICLE

Cytochrome P450 1A1, 2E1 and GSTM1 Gene Polymorphisms and Susceptibility to Colorectal Cancer in the Saudi Population

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Abstract

Background: The Saudi population has experienced a sharp increase in colorectal and gastric cancer incidences within the last few years. The relationship between gene polymorphisms of xenobiotic metabolizing enzymes and colorectal cancer (CRC) incidence has not previously investigated among the Saudi population. The aim of the present study was to investigate contributions of CYP1A1, CYP2E1, and GSTM1 gene polymorphisms. **Materials and Methods:** Blood samples were collected from CRC patients and healthy controls and genotypes were determined by polymerase chain reaction restriction fragment length polymorphism and sequencing. **Results and Conclusions:** CYP2E1*6 was not significantly associated with CRC development (odd ratio=1.29; confidence interval 0.68-2.45). A remarkable and statistically significant association was observed among patients with CYP1A1*2A (odd ratio=3.65; 95% confidence interval 1.39-9.57). The GSTM1*0/*0 genotype was found in 2% of CRC patients under investigation. The levels of CYP1A1, CYP2E1 and GSTM1 mRNA gene expression were found to be 4, 4.2 and 4.8 fold, respectively, by quantitative real time PCR. The results of the present case-control study show that the studied Saudi population resembles Caucasians with respect to the considered polymorphisms. Investigation of genetic risk factors and susceptibility gene polymorphisms in our Saudi population should be helpful for better understanding of CRC etiology.

Keywords: Cytochrome P450 - xenobiotic - colorectal cancer - single nucleotide polymorphism

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Introduction

Colorectal cancer (CRC) is one of the most frequent causes of cancer death in industrialized countries with a yearly incidence of 50 new cases for every 100,000 people in the population (Boyle and Ferlay, 2005). CRC is the third most common cancer in the world and its prevalence has been steadily increasing over the last century, while mortality rates have declined as a result of improved treatment and efficient screening and surveillance (Heavey et al., 2004; Parkin et al., 2005). CRC is traditionally classified into sporadic and familial or hereditary forms and represents a complex disease which development is mediated by genetic and environmental factors (Potter, 1999; Hemminki and Czene, 2002). Several studies have reported the association between polymorphisms for gene encoding enzymes involved in biotransformation of xenobiotics and susceptibility to cancers (Induski and Lutz, 2000; Chang et al., 2003; Terry et al., 2003). The human body is exposed daily to a number of xenobiotics

including drugs, dietary compounds and environmental carcinogens which are metabolized by a variety of enzymes through phase I and phase II reactions (Bozina et al., 2009). These enzymes participate in the conversion of xenobiotics to more water soluble metabolites which are readily excreted from the body. During metabolism of some of these xenobiotics a variety of unstable and reactive intermediates can be formed which could attack DNA causing cell toxicity and transformation (Bozina et al., 2009). The key enzyme system involved in such activation process are phase I xenobiotic metabolizing enzymes (XMEs) such as cytochrome P450s (CYPs) and detoxification by phase II XMEs such as glutathione S-transferases (GSTs) (Raunio et al., 1995). They are responsible for detoxification of a wide range of xenobiotics, including environmental carcinogens, chemotherapeutic agents and reactive oxygen species (Taspinar et al., 2008). Mutations in the genes that code for these enzymes can affect the metabolism of chemical carcinogens and which alters susceptibility to different

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types of cancers. The CYP1A1, an inducible CYP, is important for the conversion of carcinogenic polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene to mutagenic benzo[a]pyrene diol epoxide (BPDE) (Gelboin, 1980). The CYP1A1 gene is located on the long arm of chromosome 15q22-qter (Corchero et al., 2001). A phenotypic polymorphism in CYP1A1 inducibility was first reported in 10% of Caucasians who showed much higher CYP1A1 activity in lymphocytes after exposure to inducer than the non exposed group (Kellerman et al., 1973). In genotyping studies, two closely linked polymorphisms of the CYP1A1 gene have been well studied in Caucasian and Oriental populations, the 3'-flanking region MspI site that associated with increased sensitivity to inducer in some studies and the exon 7 Ile-Val substitution that appears to result in higher enzyme activity in vitro (Hayashi et al., 1991). Individuals possessing the CYP1A1*2A allele might thus exhibit higher rates of carcinogen activation than individuals with the wild-type allele. The CYP2E1 enzyme, a member of the cytochrome P450 superfamily, is a natural ethanol-inducible enzyme that is involved in the metabolic oxidation of low molecular weight carcinogens such as N-nitrosoamines, benzene and vinyl chloride (Liu et al., 2009). CYP2E1 gene is located on the 10q24.3-qter. It is 18,754 bp long consisting of nine exons and eight introns, which encodes a 493 amino acid protein. CYP2E1 gene contains six restriction fragment length polymorphisms, of which the RsaI/PstI polymorphism in its 50-flanking region has been shown to affect its transcriptional level. The variant type of this polymorphic site can enhance the transcription and increase the level of CYP2E1 activity in vitro (Hayashi et al., 1991). A nucleotide substitution (7632T>A) in intron 6 of the CYP2E1 causes the absence of a DraI restriction enzyme site (CYP2E1*6 allele, rs. 6413432). The effect of this mutation on enzyme activity is still not yet fully elucidated. However, one study showed a trend to lower 6-OH-chlorzoxazone/chlorzoxazone plasma ratios in healthy subjects possessing at least one CYP2E1*6 allele compared with wild type subjects (Haufrond et al., 2002). Moreover, induction of CYP2E1 by ethanol seems to be less efficient in patients with the mutated genotype in comparison with the wild-type genotype (Lucas et al., 1995). Glutathione S-transferases (GSTs) are family of phase II inducible enzymes that are essential in carcinogen detoxification. They catalyze the conjugation of a variety of different compounds with the endogenous tripeptide glutathione (GSH).

In humans GSTM1 gene is polymorphic and mapped on chromosome 1p13.3 (Gao et al., 2010). Both the expression and the protein level of GST isozymes vary between individuals, making them liable to the toxic effects of environmental carcinogens. Elevated levels of GSTs (especially GSTP) have been found in human cancer tumours compared with normal tissues (Ketterer et al., 1992). One of these GSTs is the GSTM, a protective enzyme that detoxifies a number of chemical carcinogens such as benzo[a]pyrene diol epoxide (BPDE) (Chang and Yang, 2000). An inherited homozygous deletion of the gene (GSTM1*0/*0 genotype) causes deficiency in enzyme activity. Individuals possessing this genotype

have reduced carcinogen-detoxification ability and are theoretically at a high risk of cancer development. Case control studies have shown that some of the mentioned gene polymorphisms are associated with a significant increase in the risk of cancer including CRC in some populations (Chang and Yang, 2000).

In the Kingdom of Saudi Arabia, the relationship between gene polymorphisms of CYPs and GSTs and cancer incidence has not been investigated previously. The aim of the present study was to test for potential association between the CYP1A1*2A, CYP2E1*6 and GSTM1*0/*0 polymorphisms and the risk of CRC in Saudi population.

Materials and Methods

Samples collection

This study was conducted after review and approval of the Institutional Review Board of the Ethics Committee at King Khalid University Hospital in Riyadh, KSA. Blood samples were collected from 100 confirmed colon cancer patients (65 males and 27 females, age range, 26-80 years; mean age, 58.4 years) and 79 healthy controls matched for age and sex. The control samples were collected from subjects referred to the hospital for general medical checkups. Colon cancer tissue samples were also collected from 12 unrelated colorectal cancer patients 8 males and 4 females and histologically normal tissues in the distant margin to the tumour were collected at the time of surgery from the patient who undergoing resection of colorectal tumours. The diagnosis of cancer was based on standard clinical, endoscopic, radiological, and histological criteria. Clinical and demographic characteristics were recorded, including age at diagnosis, gender, family history, smoking habits, disease behaviour, disease location, and need for surgery. Tissue samples to be used for RNA analysis were immediately submerged in RNAlater solution (Ambion, Courtabeuf, France) to avoid RNA degradation, stored at 4°C for 24 h, and then stored at -20°C until needed. Genomic DNA was isolated from blood samples using QIAampR DNA Blood Min Kit Cat. No. 51106 (Qiagen Co., Germany). Samples of 30-60 mg of the preserved colorectal tissues were homogenized in RLT lyses buffer (Qiagen Co., Germany) supplemented with 1% 2-mercaptoethanol, using a rotor-stator homogenizer. Total RNA was extracted using the RNeasy Mini kit (Qiagen Co., Germany), with a DNA digestion step, according to the manufacturer's instructions. Elution was performed with 50 µl nuclease-free water. Concentration, purity, and quality of the isolated RNA were determined using the Agilent 2100 Bioanalyzer System and Agilent Small RNA analysis kit according to instruction provided by the manufacturer (Agilent Technologies, Waldbronn, Germany). The RNA Integrity Numbers, RIN were ranged from 6.4-8.6 in CRC and normal control samples. Total RNA in aliquots of 1 µg was retro-transcribed into single-stranded c-DNA using the ImProm-II Reverse Transcription System (A3800, Promega USA). Complementary DNA was synthesized by reverse transcription and used as a template for the quantification of CYP1A1, 2E1 and GSTM1 gene

expression levels.

Cell lines and culture conditions

LoVo (human colon supraclavicular lymph node metastasis), HCT-116 (human epithelial colorectal carcinoma) and SW480 (human colorectal adenocarcinoma) cell lines were obtained from Dr Abdelillah Aboussekhra Research Laboratories, King Faisal hospital Riyadh, Arabia Saudi. Human lung adenocarcinoma epithelial cell line (A549) was obtained from ATCC (ATCC No.CCL-185TM). These cell lines were cultured in Dulbecco's medium (DMEM) supplemented with 100 IU/mL of penicillin G and 10% fetal bovine serum. The medium was changed three times a week and when the culture reached 90% confluence, the cells were detached from the flasks using a 0.05% trypsin–0.1% ethylenediaminetetraacetic acid (EDTA) solution, washed twice and finally resuspended in DMEH-supplemented medium at a final concentration of 106 cells/mL to use for RNA isolation and cDNA synthesis.

Genotyping

Genotyping for the CYP1A1*2A allele (6235T>C; rs4646903) was achieved by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). Briefly, a 340bp DNA fragment containing the polymorphic MspI restriction site, corresponding to the 3' end of CYP1A1, was amplified using the following primers: 5'-CAGTGAAGAGGTGTAGCCGCT-3' and 5'-TAGGAGTCTTGTCTCATGCCT-3' (Sivarman et al., 1994). The PCR was carried out in a final volume of 50 µl containing 25 µl 2x high-fidelity PCR master mix (GE Healthcare, USA), 30 pmoles of each primer and 100 ng of the extracted genomic DNA. Cycling conditions were as follows: preincubation step at 95°C for 5 min; 30 cycles consisting of denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and extension at 68°C for 1 min; followed by a final extension step at 68°C for 10 min. PCR products were analyzed using 2.5 % agarose gel electrophoresis and the size of the products were determined by including 100 bp DNA ladder on the gel and visualization using AlphaImager (Alpha Innotech Version 2.0.0). Finally, 20 µl of each PCR product was digested overnight at 37°C with 20 units of MspI restriction enzyme. The digestion products were subjected to electrophoresis on 2.5% agarose and on GeneGel Excel pre-made polyacrylamide gel (T=12.5%, C= 2%, Kit Code No.17-6000-14, Pharmacia Biotech) using GenePhor (Pharmacia Biotech.) electrophoresis system at 350 V and stained with DNA Silver staining Kit, Cat.No. 10007697 (GE Healthcare, USA).

The genotyping for the CYP2E1*6 allele (7632T>A; rs6413432) was also achieved by PCR-RFLP (Darazy et al., 2011). First, a 327-bp DNA fragment containing the polymorphic DraI restriction site in intron 6 of the CYP2E1 was amplified using the following primers: 5'-CGACATGTGATGGATGGATCCAGGG-3' and 5'-TCGTGATCGCCTGCCTCA-3'. The PCR mixture and conditions were the same as applied for CYP1A1*2A except that annealing temperature was 63°C. 20 µl of each PCR product was digested overnight at 37°C with 20 units

of DraI restriction enzyme and analyzed on 2.5% agarose gel.

Genotyping for the GSTM1 gene deletion (GSTM1*0/*0 genotype) was performed by PCR as previously described (Darazy et al., 2011). A 219 bp amplicon of the GSTM1 gene was amplified using forward and reverse primers: 5'-GAACTCCCTGAAAAGCTAAAGC-3' and 5'-GTTGGGGTCAAATATACGGTGG-3'. In addition, a 268 bp amplicon of the β-globin gene (5'-CAACTTCATCCACGTTTACC-3' and 5'-GAAGAGCCAAGGACAGGTAC-3') was used as an internal positive control. The PCR mixture contained 30 pmole of each primer and 100 ng of genomic DNA. The same PCR program was applied as for the other two genes except that the annealing temperature was 55°C.

Sequencing of PCR products

Sequencing of the PCR products was carried out according to Sanger et al. (1977), using the MegaBACE 1000 DNA Sequencing System (Pharmacia/Amersham Co.). The chain termination sequencing reaction was conducted utilizing the DYEnamic ET terminator kit as an integral part of the MegaBACE 1000 DNA sequencing system. The sequencing reaction products were purified using DyeEx 2.0 Spin Kit (63206 Qiagen Co., Germany) and applied to MegaBace 1000 Sequencing machine.

Quantitative real-time RT-PCR

Quantitative PCR (qPCR) was carried out as previously described (Lionel et al., 2010). mRNA transcripts for CYP1A1, CYP2E1 and GSTM1 were measured using the Applied Biosystem 7500 Fast real-time PCR detection system. Reactions were performed using a PCR SYBR Green supermix from Applied Biosystem. Primers used were as follows; CYP1A1 forward and reverse primers: 5'- GAATAGGGATGAAGTCAGCTG-3', 5'- ACACCTTCACCCTCATCAGTA-3'; CYP2E1: 5'- TAATGGACCTACCTGGAAGGA-3'; 5'- CAAGTAGTGTAGAAAGCTGGG-3'; GSTM1: 5'- GTGGGGAGACAGAAGAGGAG-3'; 5'- AATTCTGGATTGTAGCAGATCAT-GCC-3'; GAPDH: 5'- GGTATCGTCGAAGGACTCATGAC-3'; 5'- ATGCCAGTGAGCT-TCCCGTTCAGC-3'. Primers were added to the reaction mix at a final concentration of 250 nM. Five microlitres of each cDNA sample was added to a 20 µl PCR mixture containing 12.5 µl of SYBR Green supermix (Applied Biosystem), 0.5 µl of specific primers (1A1, 2E1, GSTM1 or GAPDH) (eurofins MWG/Operon) and 7 µl of RNase/DNase-free water. Each reaction was performed in a 7500 fast real time PCR Thermal Cycler. The thermocycling conditions for CYP1A1 and CYP2E1 were established as 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C, with each reaction done in triplicate. The specificity of each primer pair was verified by the presence of a single melting temperature peak. GAPDH produced uniform expression levels varying by less than 0.5 CTs between sample conditions and was therefore used as a reference gene for this study. The amplified products were run on an agarose gel to confirm that there were no spurious products amplified during the cycles. Results were analysed using

the $2^{-\Delta\Delta Ct}$ (Livak) relative expression method.

Detection of cytochrome P450 1A1 by immunohistochemistry

Slides were deparaffinized in three changes of xylene for 5 min each. They were hydrated in decreasing concentrations of ethanol and rinsed in 1X PBS. A hydrophobic barrier created around the section using an Immerge pent pen (Dako, Cambridgeshire, UK). Antigen retrieval was performed by immersing the slides in 0.01 M citrate buffer pH 6.0 and heating for 2-3 minutes microwaving at 100% power followed by 10-30 minutes at 20-30% power using an 800-900 Watt maximum capacity microwave oven. Endogenous peroxidase was quenched with 3% H₂O₂ for 6 min at room temperature. Slides were incubated overnight at 4°C with a 1:50 dilution of CYP1A1 (H-7) rabbit polyclonal antibody raised against amino acids 246-315 mapping to an internal region of CYP1A1 of human origin (Santa Cruz Biotechnology, INC). A biotin-streptavidin detection system was employed with diaminobenzidine (DAB) as the chromogen. Slides were washed twice with PBS and incubated with the linking reagent (biotinylated anti-rabbit IgM antibody) for 1 hour at room temperature. After rinsing in 1X PBS, the slides were incubated with the peroxidase-conjugated streptavidin label for 20 min. The sections were again rinsed with 1X PBS and incubated with DAB for 10 min in the dark. After chromogen development, slides were washed in two changes of water for 8 min each and counterstained with 0.2% methyl green (MD Supplies, UK) in sodium acetate buffer, pH 4.0. The sections were then dehydrated, cleared in xylene, and mounted with DPX mounting medium (Raymond A. Lamb Laboratory supplies, UK).

Microscopical analysis

Two investigators independently evaluated CYP1A1 staining under a light microscope at a magnification of 10X and 40X. Five images of representative areas were acquired for each specimen.

Statistical analysis

Genotype and allelic frequencies were computed and were checked for deviation from Hardy-Weinberg equilibrium (<http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>). Case-control and other genetic comparisons were performed using the chi-square test and allelic odds

ratios (OR), and 95% confidence intervals (CI) were calculated by Fisher's exact test (two-tailed). Statistical analysis was done using SPSS 16.0 for Windows. We considered p-value of <0.05 as significant.

Results

It should be noted that the molecular tools used for the genotyping do not determine whether other polymorphisms in the same gene are also present in the tested subject. The wild type (wt) denotation thus refers to the wild type allele at the investigated polymorphic site only, regardless of other unstudied polymorphisms for that particular gene. Furthermore, in this study, there were no significant differences in the distribution of genotypes for the three investigated genes when male groups were compared with females groups (data not shown).

CYP1A1 genotyping

The CYP1A1 genotypes are illustrated in Figure 1. The distribution of CYP1A1 genotypes among the tested subjects and statistical analysis of the obtained data are detailed in Table 1. The frequency of the CYP1A1 wt/*2A allele was 22.3% and 6.05% in controls. The distribution of CYP1A1 wt/*2A genotype reflected a statistically significant increase of cancer risk associated with this genotype (OR=3.65; CI=1.39-9.57; $\chi^2=7.59$ and $p=0.0058$). The distribution of CYP1A1 wt/*2A and *2A/*2A genotype in patients was also statistically different from the control healthy individuals (OR=4.17; CI=1.6-10.81; $\chi^2=9.63$ and $p=0.0019$).

CYP2E1 genotyping

The CYP2E1 genotypes are illustrated in Figure 2A and B. The distribution of CYP2E1 genotypes among the tested subjects and the statistical analysis of the obtained data are detailed in Table 1. The frequency of the CYP2E1*6 allele was 24.46% in CRC patients and 35.44% in the healthy controls. The homozygous variant allele, CYP2E1*6/*6 was detected in 5.31% of the CRC patients but not in the control group. The distribution of CYP2E1 wt/*6 genotype in CRC patients was not statistically different from that of healthy controls (Fisher's exact $p=0.17$), the OR being 1.57 (95% CI: 0.81-3.05). Moreover, the results remained statistically non-significant when both CYP2E1*6/*6 and wt/*6 were considered together as one group (OR=1.29 at CI 0.68-

Table 1. Statistical Analysis and Distribution of CYP1A1*2A (MspI, 6235T>C, rs4646903 and CYP2E1*6 (DraI, 7632 T>A rs6413432 Genotypes for Colorectal Cancer Patients and Controls

Genotype	Cases No. (Freq)	Control No. (Freq)	OR	95%CI	c ²	p value
CYP2E1 (A>T) rs6413432:						
AA (wt)	66 (0.70)	51 (0.65)	Ref			
AT (wt/2E*6)	23 (0.25)	28 (0.35)	1.570	0.81-3.05	1.82	0.17
TT (variant) (2E*6/2E*6)	5 (0.05)	0	0.117	0.006-2.172	3.2	0.95
AT+TT (wt/2E*6 and 2E*6/2E*6)	28 (0.3)	28 (0.35)	1.290	0.68-2.45	0.63	0.42
CYP1A1 (C>T) rs4646903:						
CC (wt)	70 (0.745)	73 (0.924)	Ref			
CT (wt/*2A)	21 (0.223)	6 (0.076)	3.650	1.39-9.57	7.59	0.0058
TT (variant) (*2A/*2A)	3 (0.032)	0	4.245	2.452-16.91	7.93	0.00002
CT+TT (wt/*2A and *2A/*2A)	24 (0.255)	6 (0.076)	4.170	1.6-10.81	9.63	0.0019

*N, number of cases; OR, odd ratio; p, Fisher's exact p; CI, confidence interval; wt, wild-type allele for investigated polymorphism; CRC, colorectal cancer; Ref, referent

2.45) as shown in Table 1.

GSTM1 genotyping

The GSTM1 genotype is illustrated in Figure 2C. Among the CRC patients, 2% were homozygous for the deletion polymorphism (GSTM1*0/*0). On the other hand, the control group included 100% GSTM1 positives and possessed at least one copy of the GSTM1 gene (either GSTM1 wt/wt or GSTM1 wt/*0).

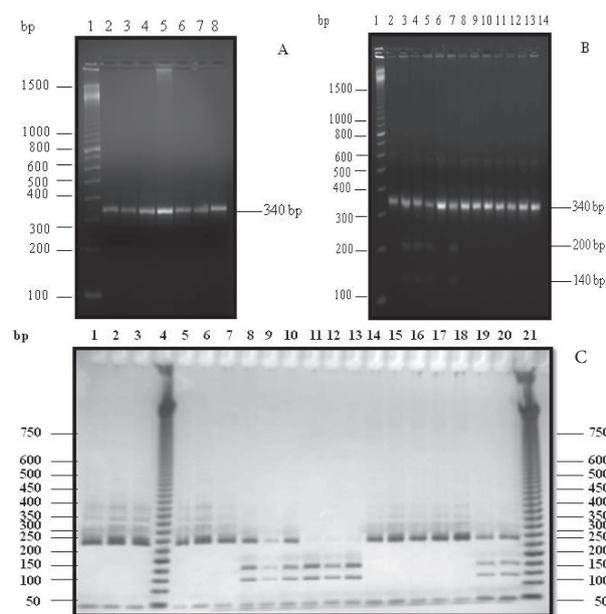


Figure 1. (A) Agarose gel (2.5%) Electrophoresis for PCR Products of CYP1A1 in Colon Cancer Patients Samples (Lanes 2-5) and Control Samples (Lanes 6-8). Lane 1 Represents 100 bp DNA Molecular Weight Markers. (B) Agarose Gel (2.5%) Electrophoresis for PCR Products of CYP1A1 in Colon Cancer Patients Samples (2-14) Digested with MspI Prior to Electrophoresis. (C) Polyacrylamide Gel (12.5%) Electrophoresis for Genotyping Results of CYP1A1 of Colon Cancer Patients (Lanes 1-21). The amplicon is subjected to digestion with MspI prior to electrophoresis. The 340 bp uncut amplicon (upper band) reveals CYP1A1 wt/wt homozygous wild type, the mutant homozygous CYP1A1*2A/*2A shows two fragments (200 and 140 bp), and the heterozygous genotype CYP1A1wt/*2A presents three fragments (the uncut 340-bp fragment and two restriction fragments of 200 and 140 bp). Lanes 1-3 represent control uncut PCR products. Lanes 4 and 21 represent 50 bp DNA ladder molecular weight markers

Analysis of genotype combinations for the three polymorphic sites in the three investigated genes was conducted (data not shown). None of the tested subjects carried the variant allele for the three gene polymorphisms simultaneously. All other combinations between the genotypes of the three genes showed no significant variation from the results of the analysis of each gene separately.

Gene expression analysis of CYP1A1, 2E1 and GSTM1 by quantitative real time PCR

The levels of gene expression of CYP1A1, 2E1 and GSTM1 were determined in twelve colon cancer samples and twelve normal tissues in the distant margin to the tumour using qPCR. Results are shown in Figure 3A. It was observed that CYP1A1, CYP2E1 and GSTM1 were highly expressed in colon cancer tissues as compared with normal control adjacent tissues and their expression levels were found to be 4, 4.2 and 4.8 times as compared to the control respectively. Gene expression levels of CYP1A1, CYP2E1 and GSTM1 were also examined in colon cancer cell lines LoVo, HCT-116 and SW480 and in human lung adenocarcinoma epithelial cell line (A549). It was noticed that CYP1A1 was highly expressed in LoVo and HCT-116 cell lines as compared with SW480 and A549 cell

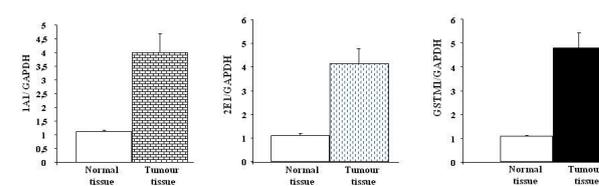


Figure 3A

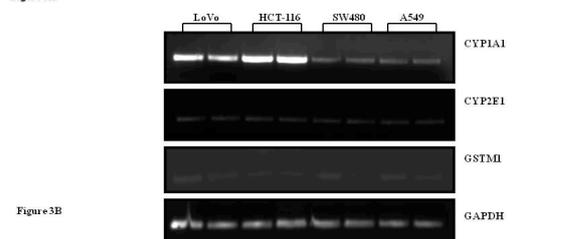


Figure 3B

Figure 3. (A) Real Time PCR Analysis of CYP1A1, CYP2E1 and GSTM1 mRNA in Normal (control) and Colon Cancer Tumor Tissues. (B) Expression of CYP1A1, 2E1 and GSTM1 in Different Cancer Cell Lines as Compared to GAPDH

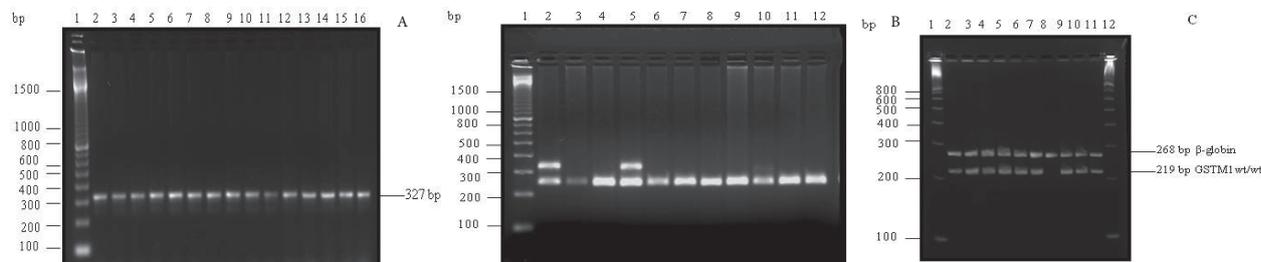


Figure 2. (A) Agarose gel (2.5%) Electrophoresis for PCR Products of CYP2E1 in Colon Cancer Patients Samples (Lanes 2-9) and Control Samples (Lanes 10-16). Lane 1 Represents 100 bp DNA Molecular Weight Markers. (B) Agarose gel (2.5%) Electrophoresis for PCR Products of CYP2E1 in Colon Cancer Patients Samples (2-12) Digested with DraI Prior to Electrophoresis. (C) Agarose gel (2.5%) Electrophoresis for PCR Products of Genotyping Results of 10 Samples for GSTM1 (Lanes 2-11). The presence of a 219 bp amplicon reveals the GSTM1wt/wt and wt/*0; absence of amplicon reveals the variant genotype GSTM1*0/*0. The 268 bp amplicon of β -globin is the internal positive control. Lanes 1 and 12 represent 100 bp DNA molecular weight markers

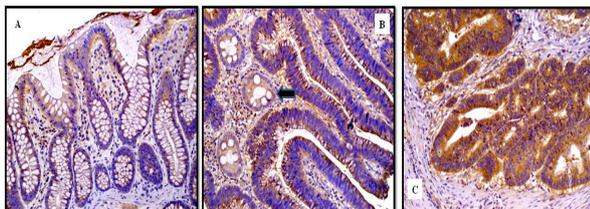


Figure 4. Microphotograph of CYP1A1 Immunohistochemistry in Colorectal Adenocarcinoma (C), Normal Non-Neoplastic Colon (A) and Poly (pre-malignant) Normal Colon (B). Colorectal Adenocarcinoma Overexpressed CYP1A1 Protein and Showed a Strong Staining in a Granular Cytoplasmic Pattern. Non-Neoplastic Normal Colon Epithelial Cells were CYP1A1 Weak Staining (A, B) in Comparison with Adenocarcinoma (C)

lines (Figure 3B). On the other hand, detectable levels of CYP2E1 and GSTM1 were found in all cancer cell lines under investigation (Figure 3B).

Detection of CYP1A1 expression in colon cancer

The reliability of the expression qPCR data was confirmed for CYP1A1 by performing immunohistochemistry on colon cancer tumour samples and normal controls. Cytochrome P450 1A1 was found to be highly expressed in colon cancer tissues as indicated by strong staining (Figure 4C) when compared with control tissues (weak staining) (Figure 4A) and premalignant tissues (polyp) (weak staining) (Figure 4B). Over-expression of CYP1A1 in tumour tissues correlated with tumour progression indicating a potential role in tumour development.

Discussion

Acknowledging the fact that cancer is a multi-factorial disease involving inherited and environmental factors, the present study focused on the genetic basis of cancer susceptibility especially the SNPs. Single nucleotide polymorphisms are the most abundant forms among variations in human genomes, and it has been demonstrated that some SNPs are strongly associated with pathophysiological properties of individuals such as responses to medications and mortalities of hereditary diseases (Brookes, 1999; Roses, 2000; Sachidanandam et al., 2001). Hence, genotyping these biallelic markers may convey great potential for the identification of disease-causing agents, definition of drug targets and establishment of markers for individual medications (Carlson et al., 2001). Moreover, susceptibility to cancer is determined by the activation of enzymes involved in carcinogens activation or deactivation. Genetic variations in these genes encoding the enzymes, possibly by altering their expression levels and functions, may increase or decrease carcinogen activation/detoxification and modulate DNA repair. Recently, a commonly studied SNP in the CYP1A1 has been implied to associate with cancer risk (Guengerich and Shimada, 1998; Wenlei et al., 2012). The SNP located at nucleotide 3801 in the 3' non-coding region containing a single T to C substitution that results in a polymorphic

restriction site for the MspI enzyme (MspI or CYP1A1*2A polymorphism, rs4646903). The MspI restriction site polymorphism results in three genotypes: a predominant homozygous m1 allele without the MspI site (type A, TT), the heterozygote (type B, TC) and a homozygous rare m2 allele with the MspI site (type C, CC) (Zhou et al., 2009). The CYP1A1*2A polymorphism has been associated experimentally with increased catalytic activity and individuals possessing CYP1A1*2A are expected to exhibit high rates of carcinogen activation (Landi et al., 1994). The present study showed that the variant CYP1A1*2A allele is significantly associated with CRC in Saudi population (OR=3.65; CI=1.39-9.57). This is in agreement with studies in other populations, for example statistically significant eight fold increased in risk for CRC was reported in Japanese living in Hawaii and homozygous for this allele (Nisa et al., 2010). No statistically significant differences in CYP1A1 MspI genetic variations have been shown in Italy (Boccia et al., 2007), the United Kingdom (Ye and Parry, 2002) and in Lebanon (Slattery et al., 2004). It has been reported that the detection of an association between CYP1A1*2A and cancer could not be confirmed in Caucasian populations because of the low frequency of this allele among these population (Garte et al., 2001). The frequency of the CYP1A1*2A allele is about 9.4% in Caucasians, 35.8% in Asians, and 23.8% in Africans (Wu et al., 2002). The CYP1A1*2A polymorphism has been associated experimentally with increased catalytic activity and individuals possessing CYP1A1*2A are expected to exhibit high rates of carcinogen activation (Lindi et al., 1994). The present study showed that the CYP1A1 gene expression level was four times higher in CRC compared to control normal tissues as measured by qPCR (Figure 3A) indicated that the high mRNA level for CYP1A1 was detected in tumour tissues as compared with normal tissues. This finding was confirmed by immunohistochemistry study using anti-cytochrome P450 1A1 antibody (Figure 4). It was clearly observed that colon cancer tumour tissues expressed more CYP1A1 protein than normal adjacent control tissues.

Many studies have investigated the relationship between CYP2E1 gene variation and the risk of CRC. The most extensively studied SNPs of CYP2E1 are RsaI/PstI site in the 5' flanking region and the DraI site in intron 6 (Hayashi et al., 1991; Liu et al., 2009). The present study indicated that the polymorphism caused by the presence or absence of a DraI site in CYP2E1 intron 6 has no significant association with CRC in Saudi populations. This is in agreement with a previous study about CRC in a Taiwanese population (Dil Lio et al., 1987) and Lebanese population (Darazy et al., 2011). The effect of this mutation on CYP2E1 enzyme activity is still not well established; however, an increased risk for CRC was detected among Italian population ever drinkers carrying CYP2E1*6 (Boccia et al., 2007). It seems that the low frequency of this allele and the low number of samples would make it difficult to detect an association with CRC in Saudi population. Study of CYP2E1 expression by qPCR showed a 4.2 fold induction in mRNA levels in CRC samples as compared with control tissues (Figure 3A).

The genotyping for GSTM1 in the present study

was based on PCR assay that identified the GSTM1 and the GSTM1 (null/-) genotypes but did not distinguish homozygous wild type ++ and heterozygous +/- individuals. The present study demonstrated that the GSTM1*0/*0 genotype is found in 2% of CRC patients under investigation indicating low frequency of GSTM1 (null -/-) genotype in Saudi population. Meanwhile, the expression level of GSTM1 mRNA was found to be 4.8 times higher in CRC patients (Figure 3C) compared with normal control healthy adjacent tissues. GSTM1 is generally considered as a protective enzyme because it detoxifies a number of toxic and carcinogenic substances such as nitrosamines and PAHs including BPDE (Ketterer et al., 1992). Moreover, GSTM1 protect cells against endogenous agents, preventing DNA damage and carcinogenesis. The increase of GSTM1 gene expression in larger tumour could comply with an adaptive cellular response to disease progression and this response was less evident in the deletion variants. GSTM1 gene expression varies widely inter-individually and this may be due to tumour-specific expression or to metabolic specialization. Some of these variations are genetically linked and may affect an individual's susceptibility for cancer (Kato et al., 1996; Rawal et al., 1999; Martinez et al., 2006). GSTM1*0/*0 genotype was linked to an elevated risk of CRC in several populations such as the Spanish (Ates et al., 2005), Turkish (Kiss et al., 2004), and Italian (Sgambato et al., 2002). It was linked also to an elevated risk of gastric cancer in Turkish and Iranian populations (Saadat and Saadat, 2001; Tamer et al., 2005). Other studies investigating the association between the GSTM1*0/*0 gene polymorphism and gastric cancer risk have reported conflicting results. Case-control studies in Korean and England populations reported no significant increase in the risk of gastric cancer or CRC (Welfare et al., 1999; Piao et al., 2009). Meta analysis of 49 published case-control and cohort studies showed that there was a significant association between the GSTM1*0/*0 genotype and gastric cancer among Asians, but not among Caucasians (Wang et al., 2010). Another meta analysis of 36 case-control studies observed significant associations with CRC in Caucasians but not in other ethnic groups (Gao et al., 2010). The population-dependent difference in term of association might be due to a difference in the frequency of this polymorphism among these populations as well as to other factors related to the diet and the environment. In addition, the presence of other genetic polymorphisms affecting XMEs similar to GSTM1 might also affect the strength of the association. For instance, GSTT1, which is involved in the detoxification of chemical carcinogens, also exhibits a gene deletion mutation. A case-control study in a Spanish population observed that the GSTM1*0/*0 genotype alone was associated with a 1.91-fold increase in the risk of CRC, but the combination of both GSTM1*0/*0 and GSTT1*0/*0 genotypes was associated with an even higher OR of 4.98 (Ates et al., 2005).

In conclusion, the work undertaken in this study raises a number of interesting observations. First, the CYP1A*2A genotype is a significant risk factor for CRC in Saudi population. Second, the CYP2E1*6 and GSTM1

*0/*0 independently do not seem to be significantly risk factors in the analyzed population. Third, all genes under investigation were found to be highly expressed in CRC patients which is likely to be an important determinant in predicting the outcome of cancer chemotherapy. Finally, large-scale studies with more CRC patients are required to draw a clearer picture about the genetic factors of CRC etiology in Saudi population.

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References

- Ates NA, Tamer L, Ates C, et al (2005). Glutathione S-transferase M1, T1, P1 genotypes and risk for development of colorectal cancer. *Biochem Genet*, **43**, 149-63.
- Bell DA, Taylor JA, Paulson DF, et al (1993). Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen metabolism gene glutathione S-transferase M1(GSTM1) that increases susceptibility to bladder cancer. *J Natl Cancer Inst*, **85**, 1159-64.
- Boccia S, Sayed-Tabatabaei FA, Persiani R, et al (2007). Polymorphisms in metabolic genes, their combination and interaction with tobacco smoke and alcohol consumption and risk of gastric cancer: a case-control study in an Italian population. *BMC Cancer*, **7**, 206.
- Boyle P, Ferlay J (2005). Cancer incidence and mortality in Europe. *Ann Oncol*, **16**, 481-8.
- Bozina N, Bradamante V, Lovric M (2009). Genetic polymorphism of metabolic enzymes P450 (CYP) as a susceptibility factor for drug response, toxicity and cancer risk. *Arch Hig Rada Toksikol*, **60**, 217-42.
- Brookes AJ (1999). The essence of SNPs. *Gene*, **234**, 177-86.
- Carlson CS, Newman TL, Nickerson DA (2001). SNPing in the human genome. *Curr Opin Chem Biol*, **5**, 78-85.
- Chang BL, Zheng SL, Isaacs SD, et al (2003). Polymorphisms in the CYP1A1 gene are associated with prostate cancer risk. *Int J Cancer*, **106**, 375-8.
- Chang HY, Yang X (2000). Proteases for cell suicide: functions and regulation of caspases. *Microbiol Mol Biol Rev*, **64**, 821-46.
- Corchero J, Primprale S, Kimura S, et al (2001) Organization of the CYP1A cluster on human chromosome 15: implications for gene regulation. *Pharmacogenetics*, **11**, 1-6.
- Darazy M, Balbaa M, Mugharbil A, et al (2011). CYP1A1, CYP2E1, and GSTM1 Gene Polymorphisms and Susceptibility to Colorectal and Gastric Cancer Among Lebanese. *Genet Test Mol Biomarkers*, **15**, 423-9.
- Dil Llio C, Del Boccio G, Casaccia R, et al (1987). Selenium level and glutathione dependent enzyme activities in normal and neoplastic human lung tissues. *Carcinogenesis*, **8**, 281-4.
- Gao Y, Cao Y, Tan A, et al (2010). Glutathione S-transferase M1 polymorphisms and sporadic colorectal cancer risk: an updating meta analysis and HuGE review of 36 case-control studies. *Ann Epidemiol*, **20**, 108-21.
- Garte S, Gaspari L, Alexandrie AK, et al (2001). Metabolic gene polymorphism frequencies in control populations. *Cancer Epidemiol Biomarkers Prev*, **10**, 1239-48.
- Gelboin H (1980). Benzo[a]pyrene metabolism, activation and carcinogenesis: role of regulation of mixed-function oxidases

- and related enzymes. *Physio Rev*, **60**, 1107-66.
- Guengerich FP, Shimada T (1998). Activation of procarcinogens by human cytochrome P450 enzymes. *Mutat Res*, **400**, 201-13.
- Haufroid V, Buchet JP, Gardinal S, et al (2002). Cytochrome P450 2E1 phenotyping by the measurement of the chloroxazone metabolic ratio: assessment of its usefulness in workers exposed to styrene. *Int Arch Occup Environ Health*, **75**, 453-8.
- Hayashi S, Watanabe J, Nakachi K, et al (1991). Genetic linkage of lung cancer-associated MspI polymorphisms with amino acid replacement in the heme binding region of the human cytochrome P450 1A1 gene. *J Biochem (Tokyo)*, **110**, 407-11.
- Hayashi S, Watanabe J, Kawajiri K (1991). Genetic polymorphisms in the 5'-flanking region change transcriptional regulation of the human cytochrome P450IIE1 gene. *J Biochem*, **110**, 559-65.
- Heavey PM, McKenna D, Rowland IR (2004). Colorectal cancer and relationship between genes and the environment. *Nutr Cancer*, **48**, 124-41.
- Hemminki K, Czene K (2002). Attributable risks of familial cancer from the family-cancer database. *Cancer Epidemiol Biomarkers Prev*, **12**, 1638-44.
- Induski JA, Lutz W (2000). Metabolic genotype in relation to individual susceptibility to environmental carcinogens. *Int Arch Occup Environ Health*, **73**, 71-85.
- Katoh T, Nagata N, Kuroda Y, et al (1996). Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) genetic polymorphism and susceptibility to gastric and colorectal adenocarcinoma. *Carcinogenesis*, **17**, 1855-9.
- Kellerman G, Shaw CR, Luyten-Kellerman M (1973). Arylhydrocarbon hydroxylase and bronchogenic carcinoma. *N Engl J Med*, **289**, 934-7.
- Ketterer B, Harris JM, Talaska G, et al (1992). The human glutathione S-transferase supergene family, its polymorphism, and its effects on susceptibility to lung cancer. *Environ Health Perspect*, **98**, 87-94.
- Kiss I, Ne'meth A, Bogner B, et al (2004) Polymorphisms of glutathione-S-transferase and arylamine N- acetyltransferase enzymes and susceptibility to colorectal cancer. *Anticancer Res*, **24**, 3965-70.
- Landi MT, Bertazzi PA, Shields PG, et al (1994). Association between CYP1A1 genotype, mRNA expression and enzymatic activity in humans. *Pharmacogenetics*, **4**, 242-6.
- Lionel L, Abdelhabib S, Marc B, et al (2010). Crosstalk between T cells and bronchial fibroblasts obtained from asthmatic subjects involves CD40L/ $\alpha 5\beta 1$ interaction. *Mol Immunol*, **47**, 2112-8.
- Liu Y, Meng XW, Zhou LY, et al (2009). Genetic polymorphism and mRNA levels of cytochrome P450IIE1 and glutathione S-transferase P1 in patients with alcoholic liver disease in different nationalities. *Hepatobiliary Pancreat Dis Int*, **8**, 162-7.
- Lucas D, Menez C, Girre C, et al (1995). Cytochrome P450 2E1 genotype and chlorzoxazone metabolism in healthy and alcoholic Caucasian subjects. *Pharmacogenetics*, **5**, 298-304.
- Martinez C, Martin F, Fernandez JM, et al (2006). Glutathione S-transferases mu 1, theta 1, pi 1, alpha 1 and mu 3 genetic polymorphisms and the risk of colorectal and gastric cancers in humans. *Pharmacogenomics*, **7**, 711-8.
- Nisa H, Kono S, Yin G, et al (2010). Cigarette smoking, genetic polymorphisms and colorectal cancer risk: the Fukuoka colorectal cancer study. *BMC Cancer*, **10**, 274.
- Parkin DM, Bray F, Ferlay J, et al (2005). Global cancer statistics 2002. *CA Cancer J Clin*, **55**, 74-108.
- Piao JM, Shin MH, Kweon SS, et al (2009). Glutathione-S-transferase (GSTM1, GSTT1) and the risk of gastrointestinal cancer in a Korean population. *World J Gastroenterol*, **15**, 5716-21.
- Potter JD (1999). Colorectal cancer: molecules and populations. *J Natl Cancer Inst*, **91**, 916-32.
- Rawal RM, Patel DD, Patel BP, et al (1999). Assessment of glutathione S-transferase and glutathione reductase in patients with squamous cell carcinoma of buccal musosa. *Int J Cancer*, **83**, 727-31.
- Raunio H, Husgafvel-Pursiainen K, Anttila S, et al (1995). Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility. *Gene*, **159**, 113-21.
- Roses AD (2000). Pharmacogenetics and the practice of medicine. *Nature*, **405**, 857-65.
- Sachidanandam R, Weissman D, Schmidt SC, et al (2001). A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature*, **409**, 928-33.
- Saadat I, Saadat M (2001). Glutathione S-transferase M1 and T1 null genotypes and the risk of gastric and colorectal cancers. *Cancer Lett*, **169**, 21-6.
- Sanger F, Nicklen S, Coulson A (1977). DNA sequencing with chain terminating inhibitor. *Proc Natl Acad Sci (USA)*, **74**, 5463-7.
- Sgambato A, Campisi B, Zupa A, et al (2002). Glutathione S-transferase (GST) polymorphisms as risk factors for cancer in a highly homogeneous population from southern Italy. *Anticancer Res*, **22**, 3647-52.
- Slattery ML, Samowitz W, Ma K, et al (2004). CYP1A1, cigarette smoking, and colon and rectal cancer. *Am J Epidemiol*, **160**, 842-52.
- Sivarman L, Leatham MP, Yee J (1994). CYP1A1 genetic polymorphisms and in situ colorectal cancer. *Cancer Res*, **54**, 3692-5.
- Tamer L, Ates NA, Ates C, et al (2005). Glutathione S-transferase M1, T1 and P1 genetic polymorphisms, cigarette smoking and gastric cancer risk. *Cell Biochem Funct*, **23**, 267-72.
- Taspinar M, Ayodos SE, Comez O, et al (2008). CYP1A1, GST gene polymorphisms and risk of chronic myeloid leukaemia. *Swiss Med Wkly*, **138**, 12-7.
- Terry KL, Garner EO, Vitonis AF, et al (2003). Interaction between CYP1A1 polymorphic variants and dietary exposures influencing ovarian cancer risk. *Cancer Epidemiol Biomarkers Prev*, **12**, 187-90.
- Wang H, Zhou Y, Zhuang W, et al (2010). Glutathione S-transferase M1 null genotype associated with gastric cancer among Asians. *Dig Dis Sci*, **55**, 1824-30.
- Welfare M, Monesola Adeokun A, Bassendine MF, et al (1999). Polymorphism in GSTP1, GSTM1, and susceptibility to colorectal cancer. *Cancer Epidemiol Biomarkers Prev*, **8**, 289-92.
- Wenlei Z, Liang Z, Zhinqun Q, et al (2012). Does cytochrome P450 1A1 MspI polymorphism increase acute lymphoblastic leukemia risk? Evidence from 2013 cases and 2903 controls. *Gene*, **510**, 14-21.
- Wu MS, Chen CJ, Lin MT, et al (2002). Genetic polymorphisms of cytochrome P450 2E1, glutathione S-transferase M1 and T1, and susceptibility to gastric carcinoma in Taiwan. *Int J Colorectal Dis*, **17**, 338-43.
- Ye Z, Parry JM (2002). Genetic polymorphisms in the cytochrome P450 1A1, glutathione S-transferase M1 and T1, and susceptibility to colon cancer. *Teratog Carcinog Mutagen*, **22**, 385-92.
- Zhou SF, Liu JP, Chowbay B (2009) Polymorphism of human cytochrome P450 enzymes and its clinical impact. *Drug Metab Rev*, **41**, 89-295.