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RESEARCH ARTICLE

Molecular detection of GSTM1 and GSTT1 for bladder cancer patients in Iraq

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Abstract

The current study was done during the period from December 2013 to January 2015 to detect the mutations of GSTM1 and GSTT1 in bladder cancer patients. A total sixty fresh tumor biopsies from patients of Transitional cell carcinoma (TCC) and thirty fresh biopsies of cystitis as control used in this study. GSTM1 mutation of patient group was significantly higher (P<0.001) than that of control group while GSTT1 mutation was not significant in bladder cancer patients. There are a significant correlation between GSTM1 mutation with smoking (P=0.047) and advanced tumor stage (P=0.006) and also between GSTT1 mutation with smoking (P=0.002) and higher mean age (P = 0.025).

INTRODUCTION

Urinary bladder carcinoma considered as the second most frequent malignancy of the genitourinary tract worldwide (Kirkali *et al*., 2005; Kaufman *et al*., 2009). Statistical frequency of bladder cancer in Iraq was fourth prevalent in males and eighth prevalent in females (Al-Foudi and Parkin, 2006). Bladder cancer in males more than in females by four times, Also the vast majority of patients' ages are more than 60 years (Begum *et al*., 2004). This cancer regarded as the foremost costly cancer in health care because of its frequent recurrence and relatively long life span of patients (Parkin, 2008). Although most of bladder cancer causes are exogenous carcinogens such as smoking, occupational exposure, radiation and infections, the genetic predisposing factor can be added to these causes (Botteman *et al*., 2013). There are several types of bladder cancer, more than "90%" of bladder cancer types was Transitional cell carcinoma (TCC) (Al-Sukhun and Hussain, 2003).

Because the metabolism of tobacco-related carcinogens and environmental risk factors may be influenced by the activity of xenobiotic metabolizing enzymes (Glutathione S Transferase enzymes, GSTs), genetic polymorphisms in mu "GSTM1" and theta "GSTT1" genes may increase individual susceptibility to various environmental pollutants which regarded as one of the most causes of bladder cancer (Stern *et al*., 2002).GSTMI and GSTTI null genotypes arise from genes deletion of both alleles that lead to absence of GSTMI and GSTTI enzymes activities which causes decreased its ability to detoxify carcinogens and increased risks of bladder cancer occurrence (Pradubkaew *et al*., 2009).

It looks that the prevalence of most types of tumors were increased in Iraq at multi-fold as expected from the exposure to hazardous pollution of last wars against our country, this may reflects some specific-mutational patterns in genes that play a role in urinary bladder carcinoma development of Iraq patients and these genes may regarded as genetic markers added to clinical features (Tawfiq *et al*., 2002).

Material and Methods

The current study was conducts through the period from January 2013 to May 2014 in molecular laboratory of Faculty of veterinary in Al-Qadsia University. Sixty fresh tumor biopsy from patients of Transitional cell carcinoma

(TCC) diagnosed by transurethral resection (TUR-biopsy) used in this study. Fresh tumor specimens were obtained from each patients put in container contain normal saline then transferred to laboratory for DNA extraction in order to prepared for molecular study by detection of mutations in xenobiotic-metabolizing genes (GSTM1 and GSTT1).At first, the biopsies fixed in formalin 10% to prepare tissues blocks embedded in paraffin wax for prepared of Hematoxylin and Eosin (H&E) stained slides which examined for histopathological assessment of grade , stage and morphology of tumors.

Thirty specimens of fresh benign bladder lesions (cystitis) biopsies as control collected randomly during collection of malignant biopsies with matched age, sex and smoking.

Extraction of genomic DNA:

DNA extracted from fresh tissues of TCC samples as well as from fresh benign bladder lesions (cystitis) samples as control by using AccuPrep®Genomic DNA extraction kit (Bioneer, Korea).

Multiplex PCR Test:

This assay was used to determine present or absent the GSTM1 and GSTT1genes mutations (deletion) as well as the Albumin gene was used as internal control gene in fresh tissues of TCC patients' samples and fresh benign bladder lesions (cystitis) samples as control. This assay was conducted depending on the method of (Markoulatos *et al*., 2002) as following steps:

Multiplex PCR master mix preparation:

Preparation of master mix for multiplex PCR occurs by used (PCR PreMix Kit AccuPower) that carried out according to instructions of company in this table:

Multiplex PCR Master Mix contents

Multiplex PCR Master mix	Volume
DNA template	5 µl
Forward primer (10pmol) for GSTM1, GSTT1, & Albumin primer	2.5µl 2.5µl 2.5µl
Forward primer (10pmol) for GSTM1, GSTT1, & Albumin primer	2.5µl 2.5µl 2.5µl
PCR water	30 µl
Total volume	50 µl

After that, these multiplex PCR master mix components that was mentioned above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Tris-HCl pH: 9.0, $MgCl_2$, dNTPs, KCl, Taq DNA polymerase and stabilizer and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. After that, it placed in PCR Thermocycler.

Conditions of PCR Thermocycler:

Conditions of multiplex PCR thermocycler were carried out by used convential PCR thermocycler that reported in the following table:

Conditions of multiplex PCR thermocycler

PCR steps	Temp.	Time	Repeat cycle
Initial Denaturation	95°C	3min	1
Denaturation	94°C	1min	30 cycle
Annealing	59°C	1min	
Extension	72°C	1min	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

Then, The PCR products of genes for multiplex PCR assay were analyzed by agarose gel electrophoresis.

Statistical analysis:

Data have been analyzed by using SPSS version 16 and Microsoft Office Excel 2007. Numeric variables were expressed as mean±SE, while nominal variables were expressed as number and percent. Student t-test was used to compare mean between two groups, when variables were normally distributed while Mann Whitney U test was used to compare mean between two groups, when variables were not normally distributed. Chi-square test was used to compare frequencies, and when it was not valid corrected chi-square was used instead. Risk estimation was done using odd ratio with 95 % confidence interval and etiologic fraction. P-value was regarded significant when it was less than or equal to 0.05.

Results

Clinicopathologic characteristics of patient group and control group

Mean age of patients enrolled in the present study was 71.67±7.39 years. Mean age of control subjects was 69.27±10.22 years. Male to female ratio was 2:1 and 3.28:1 in control and patient group respectively. The ratio of smokers was exclusively significant in patients with bladder carcinoma (BC) than in control group, 63.33% Vs. 0.00%. Majority of cases were of the papillary subtype, accounting for 38 (63%). Non-papillary subtype was seen in 22 (37%) of patients. Most of the patients enrolled in the present study exhibited a low grade histologic pattern, accounting for 42 (70%). High grade tumors were seen in 18 (30%) of patients. While, majority of patients had Ta stage tumors, 30 (50%), stage T1 was found in 14 (23%) but stage T2 was seen in 16 (27%).

Detection of mutation in GSTM1 and GSTT1 in bladder cancer patients

Rate of GSTM1 mutation (deletion) in patient group was more significant than that of control group, 42 (70%) Vs. 8 (26.67%),(P<0.001). Odd ratio was 6.417; 95% CI was (2.409-17.091); Etiologic fraction was 0.709.

Rate of GSTT1 mutation (deletion) of patient group was not significantly different from that of control group, 20 (33%) Vs. 6 (20%), (P=0.224).

Null genotype (when both genes are deleted) was more frequent in patient group than control group, 12 (20%) Vs. 2 (6.67%). Despite that, P-value was not significant (P=0.129), as shown in table (1):

Table (1): Comparison of GSTM1 and GSTT1 mutations rate between control group and patient group

Gene	Mutation	Control (n=30)		Patients (n=60)		P-value	Odd ratio	95 % CI*		ET†
		No.	%	No.	%			Lower	Upper	
GSTM1	Positive	8	26.67	42	70.00	<0.001	6.417	2.409	17.091	0.709
	Negative	22	73.33	18	30.00					
GSTT1	Positive	6	20.00	20	33.33	0.224	2	0.705	5.677	0.385
	Negative	24	80.00	40	66.67					
Null↑ genotype	Positive	2	6.67	12	20.00	0.129	3.500	0.730	16.787	0.612
	Negative	28	93.33	48	80.00					

*Confidence interval; †Etiologic fraction; ↑ both genes are deleted

The mutational analysis carried out by using multiplex PCR for finding deletion mutations in GSTM1 and GSTT1 with used albumin as internal control, these mutations observed in the following figure (1):

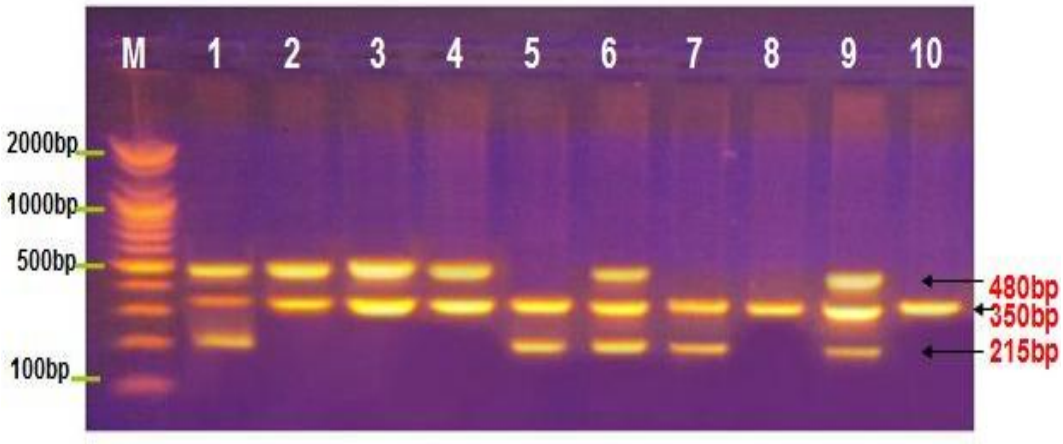

Figure (1): Agarose gel (2%) electrophoresis image that shows the Multiplex PCR products analysis of GSTs genes deletion from TCC patient samples and control sample. Where **M**: Marker (2000-100bp), **Lane (1)**: control sample that show no deletion as normal genotype (480bp: GSTT1, 350bp: albumin as internal control gene and 215bp: GSTM1), **Lanes (6 and 9)**: patient samples that also show no deletion as normal genotype, **Lanes (2,3&4):** patient samples that show positive GSTM1 deletion, **Lanes (5 and 7)**: patient samples that show positive GSTT1 deletion. Finally, Lanes **(8 and 10)**: patient samples that show two deletion in GSTM1and GSTT1 genes as null genotype.

Association between clinicopathologic parameters and GSTM1 and GSTT1 mutations:

Mean age of patients with positive GSTT1 mutation was more significant than of patients with negative mutation, 74.65±8.01 Vs. 70.18±6.68 years; (P = 0.025), as presented in table (2):

Table (2): Association between age and GSTM1 and GSTT1 mutation:

Genes	Negative mutation		Positive mutation		P.
	Mean age	SE	Mean age	SE	
GSTM1	69.11	9.41	72.76	6.15	0.080
GSTT1	70.18	6.68	74.65	8.01	**0.025**

SE: Stander error, P: Probability.

No significant association was found between gender of patients with GSTM1 and GSTT1.Proportion of smokers with positive GSTM1 mutation was significantly higher than that of non-smokers, 78.95 % Vs. 54.55%; (P=0.047).The ratio of smokers with positive GSTT1 mutation was significantly higher than that of non-smokers, 47.37 % Vs. 9.09%; (P=0.002).No significant association was found between grade of tumor and GSTM1 and GSTT1 mutations. There was no association between morphology of tumor and other genes mutations as shown in table (3):

Table (3): Association between mutation in GSTM1 and GSTT1 and clinicopathologic characteristics:

		GSTM1					GSTT1				
		Negative		Positive			Negative		Positive		
Parameter		**No.**	**%**	**No.**	**%**	**P.**	**No.**	**%**	**No.**	**%**	**P.**
gender	Male	12	26.09	34	73.91	0.319	28	60.87	18	39.13	0.119
	Female	6	42.86	8	57.14		12	85.71	2	14.29	
Smoking	No	10	45.45	12	54.55	**0.047**	20	90.91	20	52.63	**0.002**
	Yes	8	21.05	30	78.95		4	10.53	18	47.37	
Grade	Low	14	33.33	28	66.67	0.389	30	71.43	12	28.57	0.232
	High	4	22.22	14	77.78		10	55.56	8	44.44	
Stage	Ta	14	46.67	16	53.33	0.523	22	73.33	8	26.67	**0.006**
	T1	0	0.00	14	100.00		8	57.14	6	42.86	
	T2	4	25.00	12	75.00		10	62.50	6	37.50	
Morphology	Papillary	14	36.84	24	63.16	0.129	26	68.42	12	31.58	0.705
	Non-papillary	4	18.18	18	81.82		14	63.64	8	36.36	

P: Probability.

Disscussion

In this study, the rate of GSTM1 mutation (deletion) in TCC patient group was significantly higher than that of control group (70% Vs 26.67%), while no significant mutation of GSTT1 (deletion) was detected in patients group when compared with control (33.33% Vs 20%). Similar results have been reported by Salagovic *et al*. (2008), Steinhoff *et al*.(2010) and Safarinejad *et al*. (2013) ,they reported a significant association between GSTM1 mutation and TCC cases. While other researchers revealed no significant association between GSTT1 mutation and TCC cases (Pradubkaew *et al*., 2009; Toruner *et al*., 2011).

In controversy to this finding, other studies had exhibited increased risk of TCC with null (deleted) genotype of GSTT1 (Martone *et al*., 2000; Cao *et al*., 2005).

The present study found a significant association between smoking and mutation (deletions) of GSTM1 and GSTT1 in TCC cases. This finding is similar many studies worldwide as smoking has a core effect in the pathogenesis of BC (Kempkes *et al*., 2013; Okkels *et al*., 2014).

It can be explained that GSTM1 gene codes for GSTM1 cytosolic enzyme which has as an important role in TCC incidence. This gene is related to smoking because of its role in the detoxification of some toxic substances which found in tobacco smoke like polycyclic aromatic hydrocarbons (PHA) and benzo [a] pyrene by conjugating them with glutathione (an antioxidant) (Engel *et al*., 2002). The GSTT1 metabolizes some toxic chemical substances present in tobacco smoke like monohalomethanes and others which have a potential carcinogens effect (Abdel-Rahman *et al*., 2012).

These genes have a vital role in individual susceptibility to environmental substances induced TCC because of their detoxification role (Fishbain *et al*., 2004). Decreased activity of GSTM1 and GSTT1 due to deletion was be considered as an early step in the bladder pathogenesis (Srivastava *et al*.,2005). The null genotype in GSTM1 and GSTT1 in TCC patients may be linked to an increased DNA damage from experimental carcinogens such as smoking (Wiencke *et al.*, 2006).

In the bladder, GSTMl enzyme has a protective role against environmental chemicals. Impaired detoxification of reactive oxygen species (ROS) may be caused by increased susceptibility of the GSTMl null genotype in individuals (Strange,2000). According to this assumption , it has been suggested that individuals who have null genotype of GSTMl would be more susceptible to oxidative stress - induced damage, this leads to increase free radicals formation which may has a role in progression of malignancy (Weitzman and Gordon,2003). This finding indicates that smoking causes deletion in GSTM1 due to polycyclic aromatic hydrocarbons (PAHs) in tobacco smoke which result in impairment of reparative mechanism of DNA repairing genes with subsequent genetic alteration that increases the individual susceptibility to TCC through the oxidative damage by effect of the

accumulated reactive oxygen species. This finding supports the proposed hypothesis of these enzymes role in detoxification of the tobacco carcinogens (Salinas-Sanchez *et al*.,2011).

Furthermore, mutations of these genes (GSTM1 26.67% & GSTT1 33.33%) were also reported in normal looking healthy persons (control group) in spite of the significant difference with BC group. This means that normal looking persons may carry baseline genetic alterations in their epithelial cells and could be considered as prone persons for development of TCC in spite of normal histological appearance of bladder biopsy. This necessitates considering these people as potentially prone and at risk of development of BC, and it may be essential to investigate these genes in normal looking healthy and to introduce a screening molecular study for patients with cystitis.

This document has also been reported Caucasian and Asian people, the mutation frequency in GSTM1 or GSTT1 has been reported in these ethnic groups. It's about 50% of the Caucasian people in Europe (Nelson *et al*.,2013) and about 15-30% in Asian population (Steinhoff *et al*., 2000) and 23%-48% in African population (Cotton *et al*.,2000). In a local study, Al-Awadi *et al*. (2009) showed that the deletions proportion in GSTT1 and GSTM1 of Iraq people were 30%, this percentage lower than European population and similar to those in Asia. Deletion of these genes can cause lack of GSTs (GSTM1 and GSTT1) enzymes expression which has a diverse catalytic activity than that in wild-type. GSTs enzymes protect the cells against environmental carcinogens and deletions of these genes may predispose them to a different diseases that caused by xenobiotics especially cancers.

This study recorded a significant relationship between GSTT1 mutation in TCC patients with advanced patient's age and advanced stages of tumor. These results are in accordance with that presented by (Altayli *et al*., 2009; Bell *et al*.,2012).

The relationship between advanced ages of TCC patients and GSTs polymorphism may be represented by accumulation of ROS which result from environmental hazards particularly tobacco smoke that causes DNA damage in epithelial cells which increased with advanced ages and causes deletions of GSTM1 and GSTT1.

Also, the current study was showed that both genes GSTM1 and GSTT1 are deleted (null genotype) at same time in TCC patients , this state doesn't relate significantly with TCC occurrence , this finding consistence with (Brockmoller *et al*.,2006; Lee *et al*.,2012).

Also, the gender of TCC patients doesn't significantly effect on the rate of mutations of GSTM1 and GSTT1. This study agrees with the result of (Toruner *et al*., 2011; Lee *et al*., 2012). As it is known that smoking is significantly frequent among males than females for GSTM1 (73.91% Vs. 57.14%) and GSTT1 (39.13% Vs. 14.29%), it looks that there is evident increased frequency of mutation of these genes among males but without significant difference with females for both genes. This result reflects that both sexes are equally exposed and affected by environmental hazards and pollutions including smoking.

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