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Screening for variants in the *MUTYH* gene in Saudis

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ABSTRACT

***MUTYH* is a base excision repair glycosylase responsible for correcting the G:A mismatches that arise from replication of a damaged DNA, such impairment results from attacks by reactive oxygen species that are produced from different sources including cigarette smoking. The produced reactive oxygen species trigger the oxidation of guanine in to 8-oxo-G and the latter mispairs with adenine. Alterations in the *MUTYH* gene can affect its glycosylase function and hence the DNA repair capacity that is tightly linked to cancer development. Defects in the *MUTYH* were found to be associated with predisposition to colorectal cancer; the third most common cancer in the world. Although some studies suggested the existence of an ethnic differentiation among *MUTYH* mutations, there are no documented reports regarding this gene in Saudi cases or controls so far. 153 healthy Saudi individuals including smokers were screened for the IVS1 + 5 G/C, V22M, Y165C, R231C, H324Q and G382D *MUTYH* variants using either ARMS or RFLP or direct sequencing. Allelic frequencies were calculated and were found to be as follows: IVS1 + 5 G/C = 1/0, V22M = 0.99/0.01, Y165C = 1/0, R231C = 1/0, H324Q = 0.29/0.71 and G382D = 0.997/0.003. Comparison of the allele frequencies between Saudis and other populations revealed a significant difference between the Saudis and Europeans for the V22M (p-value: 0.0003, OR: 5.899, 95% CI: 1.999 - 17.408); and between the Saudis and Asians for the H324Q (p-value: <0.0001, OR: 0.473, 95% CI: 0.341-0.6559). No significant differences were found between smokers and non-smokers groups. These data document the allele frequencies of different *MUTYH* variants in Saudi populations and support the existence of an ethnic difference between *MUTYH* variants which is beneficial as some *MUTYH* common polymorphisms in certain populations may correlate with disease predisposition in other rare populations.**

Keywords: *MUTYH*; Allele Frequencies; Single Nucleotide Polymorphism; Mutations; Colorectal Cancer

1. INTRODUCTION

Genomic DNA damage occurs as a result of chemical events that are triggered by endogenous processes or the exposure to exogenous substances [1]. The most frequent DNA damage is caused by Reactive Oxygen Species (ROS) [2-3]. These species are generated by normal cellular metabolism, exogenous chemicals, ionizing radiation [4] and chronic infection that triggers inflammatory response [3].

Although ROS are capable of oxidizing the DNA, guanine is the most susceptible base to oxidation [5]. The oxidized 8-hydroxyguanosine (8-oxo-G) has the ability to mispair with Adenine and results in G:C to T:A and A:T to C:G transversions after replication. *MUTYH* enzyme is a member of the mammalian Base Excision Repair (BER) glycosylases that excise adenines misincorporated opposite to 8-oxo-G [6-9] thereby correcting the G: A mismatches arising from replication of a damaged DNA and protecting the cell from the mutagenic effect of guanine oxidation [10].

Defects in the BER pathway contribute to tumorigenesis as these defects may increase the frequency of mutations in tumour suppressor genes and oncogenes [11]. Earlier, a recessive familial form of colorectal cancer (CRC) known as the *MUTYH* associated polyposis (MAP) was linked to the base excision repair gene *MUTYH* [12], this was pursued by the identification of several polymorphic forms of *MUTYH* that were most likely associated with cancer susceptibility and were present in different human populations [4]. For instance, different studies reported on CRC patients that were compound heterozygous for the two missence variants Y165C and G382D of *MUTYH* and it is now an accepted fact that biallelic germline mutations in the *MUTYH* gene significantly increase the risk for colorectal cancer [13-18].

Yamaguchi *et al*., 2002 described a lung cancer cell line with an alternative splicing event in *MUTYH* intron 1 triggered by the IVS1 + 5 G/C variant. This variant was also found in an oesophageal cancer, a skin squamouse cell cancer, and two colon adenocarcinoma patients [19-20]. Additionally, Miyaki *et al*., 2005 reported a patient with multiple colorectal adenomas to be homozygous for the *MUTYH* R231C (891 C > T) mutation [21]. Moreover, the 972C allele of the *MUTYH* common polymorphism H324Q (972 C > G) was suggested to be inversely associated with the risk of proximal CRC in the Japanese population due to its interaction with other alleles [21,22]. On the other hand, V22M (66G > A) polymorphism was not associated directly with any disease state but rather it may be associated with localization of the *MUTYH* to the site of DNA replication [23].

Although carriers of biallelic *MUTYH* mutations have a 60% risk to develop CRC [24] it is now clear that some genes which cause autosomal recessive cancers predisposition are related to fairly increased risk of adult cancer in monoallelic carriers [25]. Such monoallelic mutations may be strongly related to cancer when combined with other genes mutations [26] or carcinogen exposure [27] and they also can influence genetic pathways in CRC [19].

The wide range of variation in the *MUTYH* reported frequencies for control populations [28] and the considerable inter-individuals variation in DNA repair capacity in the general population, emphasizes on the importance of screening for *MUTYH* genetic variants in the normal Saudi population.

This study aimed to determine allele frequencies for different *MUTYH* gene variants in Saudis to investigate the possible role of these variants in increasing the susceptibility to cancer and to compare the results with reported frequencies of this gene in other populations. Additionally, the study compared allele frequencies between smokers and non-smokers since cigarette smoke may induces production of 8-oxo-G that is considered the natural cause of genomic diversity and SNPs in organisms because of its mutagenic capability [5].

2. METHODS

2.1. Subjects, Samples and Polymerase Chain Reaction

Subjects enrolled in this study were healthy Blood donors attending local hospitals in Riyadh, Saudi Arabia. The study group included 153 healthy unrelated Saudi subjects (113 non-smokers and 40 smokers); peripheral blood was drawn by venepuncture in EDTA tubes after obtaining written informed consent. Blood samples were stored at –20˚C until required for analysis. DNA was extracted from the whole blood using the QIAmp blood mini kit (Qiagen) according to the manufacture's instruction. Amplifications were performed in a DNA thermal cycler, using 2.5 units of pureTaq DNA polymeras, 5 pmole primer, 10 - 60 ng genomic DNA, 200 µM dNTP, 50 mM KCl and 1.5 mM $MgCl_2$.

Cycling parameters were 95˚C for 10 minutes, 34 - 40 cycles of 94˚C for 30 seconds, annealing temperature from 52˚C - 60˚C for 30 seconds and 72˚C for 30 seconds followed by a final elongation step at 72˚C for 10 minutes.

2.2. Restriction Endonuclease Digestion

Exon 2 enclosing the V22M (66 G > A) polymorphism was amplified at 58˚C for 35 cycles using primers reported before [12]. The 285 bp PCR product was digested with *Nco*I restriction digest, 10 µl of the PCR product of each sample was mixed with 10U (1µl) of the *Nco1* restriction enzyme (10U/µl), followed by incubation at 37˚C for 3 hours and finally analyzed by agarose gel electrophoresis.

Exon 13 harbouring the G382D (1145 G > A) mutation was amplified at 56˚C for 35 cycles using primers described before [12]. The 242 bp PCR product was digested with *Bgl*II restriction enzyme. 10 µl of exon 13 PCR product of each sample was mixed with 10U (0.1667 µl) of the *Bgl*II restriction enzyme (60 U/µl) and incubated at 37˚C for 3 hours and finally analyzed by agarose gel electrophoresis.

2.3. Sequencing

Sequencing was carried out using DYEnamic™ ET Terminator Cycle Sequencing Kit from Amersham Biosciences UK Limited (Littele Chalfont Buckinghamshire, UK). The mixture was combined with 5 µM of *MUTYH* exon 9 PCR product and 5 µM forward primer then thermo cycled (95˚C for 30 seconds, followed by 24 cycles of 95˚C for 20 seconds, 50°C for 15 seconds and 60˚C for 1 minute) then the products were passed through a gel-filtration resin and finally resuspended in a formamide loading buffer, the sequencing were preformed in the MegaBACE 1000 genetic analyzer from Amersham and analyzed using the lasergene6 package (DNAStar, WI, USA).

2.4. Amplification Refractory Mutation System (ARMS)

IVS1+5 G < C variant in intron 1 was assayed using a common (IVS1 + 5 C) primer and a normal (IVS1 + 5 N) or mutant (IVS1 + 5 M) ARMS primers as previously reported [12]. In addition, an internal control primer *SETX* (exon 20) was co-amplified in a di-plex reaction to ensure that the ARMS PCRs were efficient in each reaction tube and to avoid any false negative results. Annealing temperature of 58˚C and 33 cycles were the op-

timum conditions selected for equal amplification.

Y165C (494 A > G) in exon 7 was assayed using a common (IVS1 + 5 C) primer and a normal (165N) or mutant (165M) ARMS primers [12]. The internal control primer *SETX* (exon 20) was amplified together with the ARMS primers. Annealing temperature of 58˚C and 35 cycles were the optimum conditions selected for equal amplification.

H324Q (972 C > G) in exon 12 was assayed using a common (324C) and a normal (324N) or mutant (324M) ARMS primers [12]. The internal control primer DBTE (exon 2) was amplified together with the ARMS primer. Annealing temperature of 62˚C and 39 cycles were the optimum conditions selected for equal amplification. All products were visualized by regular agarose gel electrophoresis .

2.5. Statistical Analysis

Allele frequencies were obtained by allele counting and errors were assigned. Differences in allele frequencies between populations and between smokers and non-smokers were tested using Fisher's exact test, a P-value of ≤0.05 was considered statistically significant. For sample size larger than 1000, χ^2 test was performed and in both cases 95% CI was calculated, all computations were undertaken using the statistical software GraphPad InStat version 2.04 (Ralf Stahlman, Purdue Univ. 931897S).

3. RESULTS

3.1. *MUTYH* Genotypes Assays

Agarose gel images for exon 2 digests showed that the wild type allele remained undigested and that the V22M allele was digested generating 207 and 78 bp products. In exon 13, the wild type allele remained undigested, whilst the G382D allele was digested generating 160bp and 82bp fragments (**Figure 1(a)**).

Exon 9 was amplified then sequenced and the electropherograms showed all the samples to be homozygous for the wild type allele (R231/R231) harbouring a C at position 891 and lacking the T at this position, no sample was found to carry the mutant allele T (**Figure 1(b)**).

Intron 1 ARMS primers generated a 178 bp product only in the normal reaction and a 300 bp product was generated for the internal control. Similarly, exon 7 ARMS primers generated a 148 bp product only in the normal reaction and a 300 bp product was generated for the internal control.

Finally, exon12 ARMS primers generated a 150 bp product in some normal and some mutant reactions and a 300bp product was generated for the internal control (**Figure 1(c)**).

3.2. *MUTYH* Genotype Frequencies

The genotype frequencies for all the genetic variants of the *MUTYH* are summarized in **Table 1**. Allele frequencies were as follows: IVS1 + 5 G > C 1/0, V22M (66 G > A) 0.99/0.01, Y165C (494 A > G) 1/0, R231C (891 C<T) 1/0, H324Q (972 C > G) 0.29/0.71 and G382D (1145 G > A) 0.997/0.003.

4. DISCUSSION

Several defence mechanisms that avoid accumulation of oxidative DNA damages exist in mammalian cells. The two BER glycosylases; *MUTYH* and OGG1 play important roles in the repair of modified bases including 8-oxo-G [29-32] *MUTYH* removes the mismatched adenine and helps in base excision repair of 8-oxo-G:A and G:A, while OGG1 removes 8-oxo-G from duplex DNA.

Different variants of the *MUTYH* gene can affect the protein function and hence the DNA repair capacity that is tightly linked to cancer development. Biallelic germline mutations in the *MUTYH* lead to *MUTYH* associated polyposis (MAP) and provide a significant risk for colorectal cancer [24]. Different studies supported the role of *MUTYH* in colorectal adenoma and carcinoma pre- disposition [33,34].

Table 1. The genotype frequencies for the *MUTYH* variants in Saudi samples.

N= 153 samples			
MUTYH variants	Genotypes Frequencies (%)	*MUTYH* variants	Genotypes Frequencies (%)
IVS1+5 G > C		R231C (891 C < T)	
G/G	100%	C/C	100%
G/C	0%	C/T	0%
C/C	0%	T/T	0%
V22M (66G > A)		H324Q (972 C > G)	
G/G	97.37%	C/C	8.72%
G/A	2.63%	C/G	39.60%
A/A	0%	G/G	51.68%
Y165C (494 A > G)		G382D (1145 G > A)	
A/A	100%	G/G	99.32%
A/G	0%	G/A	0.68%
G/G	0%	A/A	0%

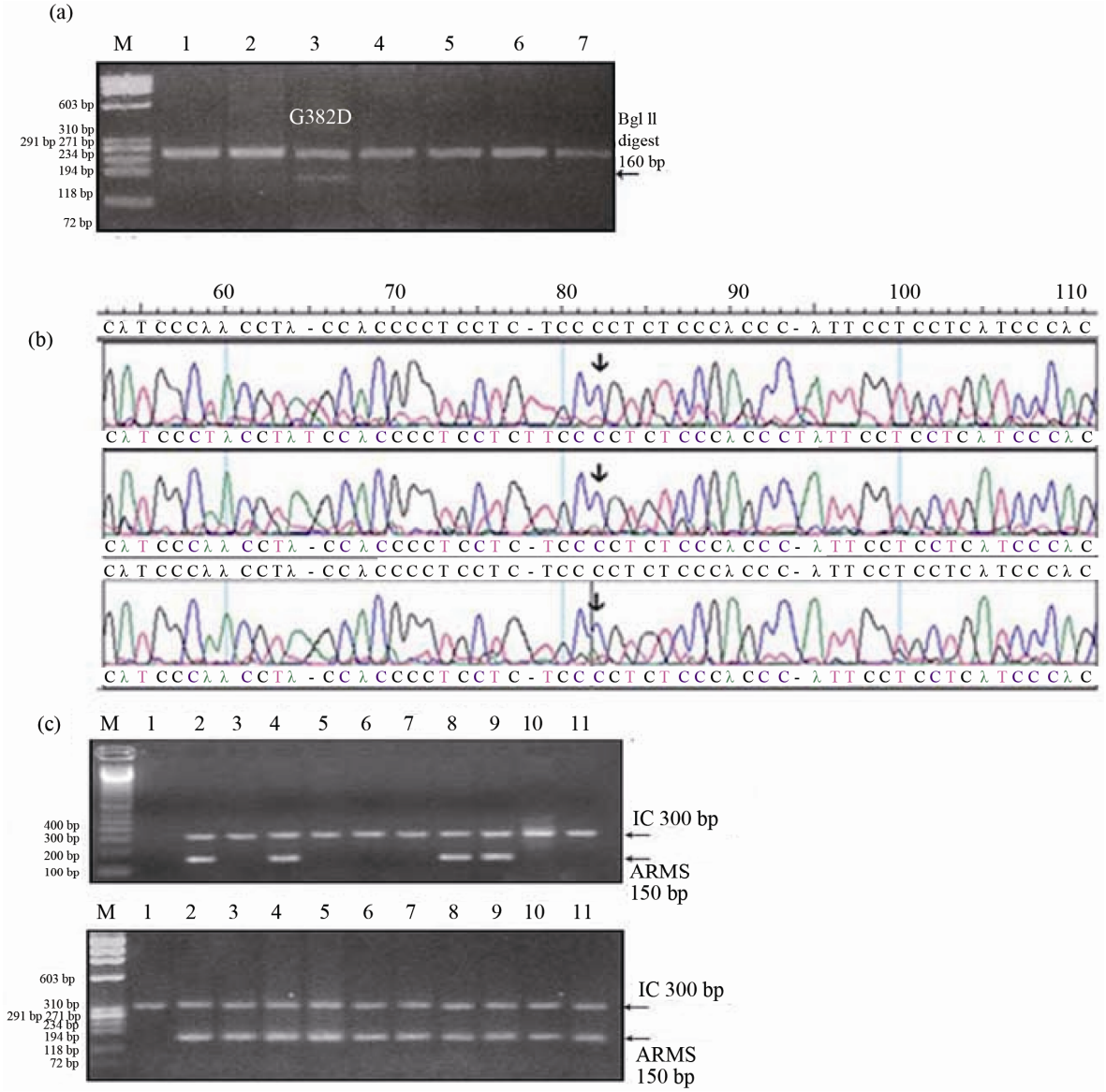

Figure 1. (a) RFLP assay for G382D (1145 G > A) polymorphism in Saudi controls. *Bgl*II restriction digests of the PCR product for different samples, lane 3 shows the G382D allele in a heterozygous sample, the wild type allele remained undigested (242 bp) and the mutant allele was digested generating a 160bp and 82 bp, all the other lanes had only the wild type allele. Lane 1: *ΦX* 174/*Hae*III DNA size marker (M). (b) Electropherograms obtained by sequencing of the *MUTYH* exon 9 using the reverse primer and complimentary conting. The samples were screened for the R231C (891 C < T) mutation. Arrows point to the wild type allele (C) found in all the samples, mutant allele (T) was absent.(c) Agarose gel pictures resulting from the ARMS assay of the H324Q polymorphism in Saudi controls. Top: The gel pattern using the normal primer: No normal allele bands were detected in the different samples in lanes 3, 5, 6, 7, 10 and 11. Different individual samples in lanes 2, 4, 8, and 9 had a normal allele band, lanes 1 contained 100bp DNA size markers. Bottom: The gel pattern using the mutant primer: All except the first lane had mutant bands, lane 1 contained *Φ X* 174/*Hae*III DNA size markers. Internal Control: *SETX* exon 20, 300 bp (M).

The IVS1+5 G/C SNP is located in intron 1 at the splice donor site where a G nucleotide is replaced by a "C". Studies from lung cancer cell lines showed that such replacement causes the retention of the first 237 bp of intron 1 sequence between exon 1 and exon 2 of the transcript. The SNP was found to cause the activation of a cryptic splice donor site and the alternative splicing in intron 1 [20]. Comparison of the allele frequencies for IVS1+5 G/C mutation between the Saudi samples and an Australian study [19] revealed no significant difference (Fisher's p-value = 0.0765, OR = 0.07559, 95% CI = 0.003596 - 1.589).

The V22M (66G > A) is located in exon 2 at the RPA binding domain of the *MUTYH* protein. This SNP may be associated with localization of *MUTYH* to the site of DNA replication [23] and is associated with substitution of hydrophobic valine for hydrophobic methionine resulting in a conservative change.

The study sample presented a frequency of 0.99 for the normal allele and of 0.01 for the mutant allele with no homozygous samples for the mutant allele. Interestingly, when the Saudi V22M allele frequencies were compared with four different populations (Europeans, Asians, Sub-Saharan Africans and African Americans), there was a significant difference between the Saudis and Europeans but a statistical similarity between the Saudis and the three other populations. Moreover, when compared with the same four populations, the Saudi H324Q allele frequencies were similar to Europeans, SubSaharan Africans and African Americans but were signifycantly different from the Asian population. These results are summarized in **Table 3**.

The Y165C (494 A > G) located in exon 7 of the *MUTYH*, causes the substitution of the hydrophobic tyrosine residue for a polar uncharged cysteine and results in a non-conservative mutation. This variation resides in the HhH motif of the *MUTYH* protein which is part of the catalytic core, and hence the mutation completely abolishes the adenine glycosylase activity towards the A.8-oxo-G mispairs. The G382D (1145 G > A) mutation located in exon 13 involves a substitution of a nonpolar uncharged glycine for a negatively charged aspartic acid, resulting in a non conservative pathogenic mutation. This mutation is located in the MutT like domain and it gives rise to decreased adenine removal [35]. In the present study on normal Saudis the allele frequencies for Y165C and G382D are similar to controls of Caucasians, Australians, Canadians, British populations [28]. Other studies showed that Y165C and G382D mutations were identified commonly in British, Italian, American, Portuguese and Dutch populations and never detected in East Asians including Japanese populations [21,22,36-38]. The low and similar Y165C and G382D allele frequencies between the Saudis and the other control populations further confirms that they are disease causing mutations.

The R231C (891 C > T) mutation located in exon 9 of the *MUTYH* protein causes the substitution of a charged arginine for an uncharged polar cysteine resulting in a non conservative mutation; the R231C appears to affect the interaction between the *MUTYH* and MSH6 and is assumed to be pathogenic [21]. No significant difference was observed in the allele frequencies for the R231C mutation between the study sample and that conducted by Miyaki *et al*. 2005 [21]. These results are summarized in **Table 2**.

Table 2. Comparison of Saudi IVS1 + 5 G/C and R231C *MUTYH* variations with other populations.

Ethnicity	Reference	N	*MUTYH* alteration	Major Allele frequency	Minor Allele frequency	Comparison with the corresponding Saudi alleles
Australians	(Kambara *et al*., 2004)	53	IVS1+5 G/C	G = 0.98	C = 0.02	p-value: 0.0765, OR: 0.07559, 95% CI: 0.003596 - 1.589
Japanese	(Miyaki *et al*., 2004)	80	R231C	C = 1	T = 0	-

Table 3. Comparison of Saudi V22M and H324Q *MUTYH* variations with other populations.

Ethnicity	Reference	N	*MUTYH* alteration	Major Allele frequency	Minor Allele frequency	Comparison with the corresponding Saudi alleles
Europeans	SNP database	144	V22M	G = 0.93	A = 0.07	p-value: 0.0003, OR: 5.899, 95% CI: 1.999 - 17.408
Europeans	SNP database	118	H324Q	G = 0.72	C = 0.28	p-value: 1, OR: 1, 95% CI: 0.6894 - 1.4698
Asians	SNP database	202	V22M	G = 0.998	A = 0.002	p-value:0.1706, OR: 0.1861, 95% CI: 0.02068 - 1.674
Asians	SNP database	177	H324Q	G = 0.54	C = 0.46	p-value: <0.0001, OR: 0.473, 95% CI: 0.341 - 0.6559
Sub-Saharan Africans	SNP database	120	V22M	G = 0.996	A = 0.004	p-value: 0.39, OR: 0.3138, 95% CI: 0.03483 - 2.828
Sub-Saharan Africans	SNP database	119	H324Q	G = 0.70	C = 0.30	p-value: 0.7026 , OR: 0.9201, 95% CI: 0.6331 - 1.3371
African Americans	SNP database	55	V22M	G = 0.982	A = 0.018	p-value: 0.6585, OR: 0.3138, 95% CI: 0.2507 - 7.694
African Americans	SNP database	60	H324Q	G = 0.66	C = 0.34	p-value: 0.2891, OR: 0.7689, 95% CI: 0.4886 - 1.21

The H324Q (972C > G) variation in exon 12 is associated with amino acid substitution of histidine to glutamine, where the replacement leads to a non-conservative polymorphism as a charged residue is substituted for an uncharged polar residue. There is no difference in the repair activities for the two types of polymorphic *MUTYH* proteins. This polymorphism is located near the APE1 binding site which is not a key functional site. The Saudi population H324Q allele frequency was similar to the Europeans, Sub-Saharan-Africans and African Americans. However, the Saudi H324Q allele frequency was significantly different compared to the Asians. These results are summarized in **Table 3**.

Genetic drift possibly contributed to the difference in allele frequency between the Asians and Saudis. The alleles subjected to random genetic drift would eventually reach fitness and hence the H324Q event in the Asians may be more recent than the Saudis and thus later in approaching 100% fitness.

Cigarette smoke is a rich source of ROS that induce a variety of DNA damage including oxidative DNA damages that can result in gene mutations, since guanine is the most susceptible base to oxidation resulting in 8-oxo- G production; the 8-oxo-G has been recently considered the natural cause of genomic diversity and SNPs in organisms because of its mutagenic capability [5].

The allele frequencies for V22M, H324Q and G382D were compared between smoker and non-smoker subdivisions of the study group (113 smokers and 40 non-smokers). No significant difference was observed in any of these variants allele frequencies between smokers and non-smokers, the comparison resulted in the following statistical parameters (Fisher's p-value = 0.5762, OR = 0.3043, 95% CI = 0.01619 - 5.720), (Fisher's p-value = 0.5563, OR = 0.8209, 95% CI = 0.4659 - 1.4463), (Fisher's p-value = 1, OR = 0.954, 95% CI = 0.03852 - 23.746) for V22M, H324Q and G382D, respectively.

In a recent study it was found that smokers carrying the wild type of OGG1 S326C and variant genotype of XRCC1 R194W and variant genotype of *MUTYH* H324Q had a 31.86 fold increased risk for bladder cancer [39]. Another study suggested that the *MUTYH* H324Q polymorphism appears to play an important role in lung cancer risk in the Japanese population [40]. Moreover, the Saudis were found in this study to be statistically different from Asians (including Japanese) in the H324Q polymorphism as the mutant form was more common in the Asians. Also the Saudis were found to be statistically different from Europeans in the V22M polymorphism and the mutant form was more common in Saudis.

Although different studies showed a significant effect between smoking and genetic polymorphisms in the *MUTYH* yet there were no significant difference in any of the *MUTYH* allele frequency between smokers and non-smokers in this study.

In terms of future work, it is important to determine the allele frequencies for these *MUTYH* variations in the Saudi Colorectal cancer patients, endometrial cancer and liver cancer patients as some *MUTYH* mutations may be associated with increased susceptibility to these cancers.

5. CONCLUSIONS

This detailed report on *MUTYH* variants in Saudis and comparison with other populations highlights the existence of ethnic differences between *MUTYH* variants and further confirms the pathogenic role of some *MUTYH* mutations previously linked to disease.

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