Association of a New Germline Variant in the MUTYH DNA Glycosylase Gene with Colorectal Adenoma Transformation into Malignancy

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ABSTRACT

Background: MUTYH DNA glycosylase germline mutations are linked to the recessive inheritance of multiple adenoma. Studies have revealed that germline mutations in this gene are ethnicity related. This study aimed to identify the germline mutations in MUTYH gene and determine their prevalence among Jordanian patients with colorectal adenoma. Methods: In this study, 150 colorectal adenoma patients and 150 cancer-free individuals with no previous history of polyps were recruited. Sanger DNA sequencing of the MUTYH gene (accession number NG_008189.1) was carried out using 3130xL Genetic Analyzer. Sequencing results were analyzed by ChromasPro, and mutational effects were predicted by online bioinformatics tools. Results: Two novel variants, g.87C>T and c.1264G>C, were identified. g.87C>T was also found in 60 (40%) patients and 10 (6.7%) controls. However, c.1264G>C was detected in 90 (60%) patients and 7 (4.7%) controls. Thus, a significant association was observed between these two variants and colorectal adenoma (p value for both variants was <0.0001). Moreover, the newly identified germline variant, c.1264G>C, was found to be significantly associated with colorectal adenoma transformation into malignancy (p < 0.0001). Conclusion: The data showed high prevalence of two germline mutations in MUTYH gene among Jordanians with colorectal adenoma, which may make them as potential early biomarkers for diagnosis of colorectal adenoma. DOI: 10.29252/ibj.23.6.412

Keywords: Colorectal adenoma, Germline mutations, MUTYH gene

INTRODUCTION

Colorectal adenoma pathogenesis is a multistep process and can induce neoplastic transformation of normal cryptic epithelial cells into neoplasia¹. This stepwise transformation is due to multiple genetic and environmental factors, which disrupt the epithelial cells homeostasis by diminishing cellular apoptosis, persisting DNA replication, and losing the ability for differentiation and maturation²⁴. Colorectal adenoma can be classified into tubular, villous, and tubuvillous adenoma⁵. The age, gender, smoking, diet, and obesity are environmental factors that contribute to colorectal neoplasia⁶⁸. Genetic mechanisms such as chromosomal instability, CpG island hypermethylation, and microsatellite instability assist in colorectal neoplastic transformation. Microsatellite instability results from defect in DNA mismatch repair system that includes PCNA, MLH1, MSH6, XRCC1, and MUTYH genes⁹¹¹³. MUTYH comprises 16 exons encoding 535 amino acids protein and is mapped to the chromosome 1(1p32.1-p34.3)¹². The MUTYH glycosylase, a base excision repair enzyme¹³, detects and repairs DNA
damage including those generated by normal metabolic 
reactions such as alkylation, deamination, or 
oxidation\textsuperscript{[14]}. The exposure of reactive oxygen species 
produced during aerobic metabolism to some 
chemicals/radiation influence the DNA integrity\textsuperscript{[15]}. As 
a consequence, 7,8-dihydroxy-8-oxoguanine (8-oxo-G) 
often pairs with adenine (A), resulting in transversion of 
guanine:cytosine into thymidine:adenine (G:C\textrightarrow 
T:A) after two replication rounds of DNA\textsuperscript{[16]}. The 8-oxo-G:A base-pair detection is initiated by MUTYH 
glycosylase\textsuperscript{[14]}.

*MUTYH* germline mutations have been linked to 
multiple colorectal adenomas inheritance in Caucasian 
populations\textsuperscript{[17]}. Therefore, their identification greatly 
enhances our understanding of the colorectal cancer 
(CRC) potential causes and attributes in 
implementation of screening and management 
measures. Importantly, the early detection of CRC can 
be effective by establishing panels of genetic 
biomarkers. There are many genes and proteins 
currently used in the clinical settings in USA\textsuperscript{[18]}, but 
they differ significantly in their predictive and 
prognostic values among populations\textsuperscript{[19]}. Supposing 
that Jordanian patients with colorectal adenoma have a 
novel germline mutation(s) in *MUTYH* gene, this study 
aimed to identify these mutations among those patients 
and to determine their prevalence in relation to their 
ethnic background.

**MATERIALS AND METHODS**

**Study subjects**

Colorectal adenoma patients (n = 150) were recruited 
from King Abdullah University Hospital (Ramtha, 
Jordan) during the period between January 2016 and 
February 2017. To select these patients, the biopsy 
samples from 400 subjects who visited the Endoscopy 
Unit at King Abdullah University Hospital were 
examined by a pathologist. Subjects (n = 150) who 
were confirmed pathologically to have tubular 
adenoa, villous adenoma, or tubulovillous adenoma 
were investigated in the study. Inclusion criteria 
include the number of polyps and the patient's previous 
treatment status. Patients with more than 10 polyps and 
without any surgical removal of polyps were enrolled 
in this study. However, those with a previous history of 
irritable bowel syndrome or Crohn's disease were 
excluded. Clinical data were collected from patient's 
history files. The study was approved in advance by 
the Institution Review Board at King Abdullah 
University Hospital and Jordan University of Science 
and Technology (Ramtha, Jordan). Informed written 
consents were obtained from all participants. Besides, 
150 cancer-free individuals with no previous family 
history of polyps were recruited as controls. A 
structured questionnaire interview was established to 
collect data on the characteristics of the study 
participants.

**Sample collection and handling**

One blood sample was collected from each 
participant. Five milliliters of whole blood was 
collected in EDTA tubes. Samples were transported on 
ice to the DNA extraction laboratory at Princess Haya 
Biotechnology Center (Ramtha, Jordan) and either 
processed immediately or stored at 4 °C and extracted 
the next day.

**Genomic DNA extraction**

DNA was extracted from whole blood samples using 
Qiagen\textsuperscript{®} genomic DNA purification kit (Qiagen, 
USA) according to the manufacturer’s instructions. 
The concentration of DNA was determined using Nano 
Drop 2000 (Thermo Scientific, USA). To check the 
quality of extracted DNAs, two microliters from each 
extracted DNA sample was subjected to agarose gel 
electrophoresis. Extracted DNA samples were stored at 
-80 °C.

**Polymerase chain reaction (PCR) procedure**

All exons of *MUTYH* gene were amplified using 
PCR. The primers were designed using Primer3 Plus 
(http://www.bioinformatics.nl/cgi-bin/ primer3plus/ 
primer3plus.cgi/). Eight amplicons were designed 
comprising the whole coding regions of *MUTYH* gene 
as well as the 5’ untranslated region and exon-intron 
boundaries (Fig. 1). Amplification of the target 
sequences was performed using conventional PCR 
(Verit\textsuperscript{TM} Thermal Cycler from Applied Bioscience). 
PCR was carried out in a reaction volume of 25 µL 
containing 12.5 µL commercial 2× Master Mix (2.5 U 
*Taq* of DNA polymerase, 3 mM of MgCl\textsubscript{2}, and 0.5 mM 
of dNTPs), and buffer (100 mM of KCl, 20 mM of Tris 
HCl [pH8.3]), as well as 2µL of each forward and 
reverse primer (10µM), 6.5 µl of nuclease-free water, 
and 2-3 µl of sample DNA. The list of primers and 
sequences for each amplicon are shown in Table 1.

**Gel electrophoresis**

Five microliters from each PCR product was loaded 
on 2% agarose gel. The electrophoresis was run at 140 
volts for 40 minutes. The running Tris/Borate/EDTA 
buffer was diluted to 1×, and the bands were then 
detected under a UV light in Gel Doc\textsuperscript{TM} XR system 
(BioRad, USA) using Red Safe stain (iNiRON). Images 
were processed and analyzed by Quantity One 
4.6.9 software.
Cycler sequencing, cleaning and Sanger sequencing

Purified PCR products were cycle-sequenced using Big Dye Terminator Ready Reaction (R-R) mix. The reaction components were 4 µL of sequencing buffer, 4 µL of nuclease-free water, 1 µL of R-R, 1 µL of forward or reverse primer, and 4 µL of purified DNA. The excess of Dye Deoxy™ terminator, primer dimmers, and the other impurities were removed from DNA sequencing reaction using Qiagen cleaning kit. Sequencing of cleaned cycle-sequenced products was carried out on an ABI prism 3130xL Genetic Analyzer (Applied Biosystems, USA) in the Genomics Sequencing Laboratory at Princess Haya Biotechnology Center (PHBC).

Sequencing data analysis

Sequence analysis was carried out using ChromasPro software (http://technelysium.com.au/wp/chromas/).

Bioinformatics and computational analysis of variants

Several bioinformatics tools were used in this study. Primer3Plus online software (http://www.primer3plus.com/cgi-bin/primer3plus/primer3plus.cgi/) was used for primer design. MutationTaster software (http://www.mutationtaster.org/).PROVEAN (http://provean.jcvi.org/index.php) was applied for mutational effect prediction and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) for protein prediction analyses.

Statistical analysis

GraphPad Prism 6.0 was used for statistical analyses. In particular, Chi-square test was used. Statistical analyses with p value less than 0.05 were considered statistically significant.

RESULTS

Characteristics of the study participants

The recruited Jordanian subjects with colorectal adenoma were 81 (54%) males and 69 (46%) females, and the controls were 78 (52%) males and 72 (48%) females. There was no age difference between the

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Reverse primer</th>
<th>Forward primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cttggatcacaacgcctaa</td>
<td>agaggacgacccgcaagt</td>
<td>410</td>
</tr>
<tr>
<td>2</td>
<td>gccagagtaaacacgttag</td>
<td>cttgggccacaacctagtgc</td>
<td>300</td>
</tr>
<tr>
<td>3</td>
<td>cactgtggacactg</td>
<td>ggtctgacccgaccttc</td>
<td>590</td>
</tr>
<tr>
<td>4</td>
<td>tggcgtatagagtggtctaga</td>
<td>accccaacatctaccagag</td>
<td>593</td>
</tr>
<tr>
<td>5</td>
<td>gcaaggttctctccctagt</td>
<td>cagcgcggagtcaccgta</td>
<td>690</td>
</tr>
<tr>
<td>6</td>
<td>agggatgctggcttga</td>
<td>aaggaatgcaacagccagaa</td>
<td>459</td>
</tr>
<tr>
<td>7</td>
<td>acaaaagtaaacgcctgaagtt</td>
<td>cctggagcgattttctga</td>
<td>249</td>
</tr>
<tr>
<td>8</td>
<td>gctctagagccccccggtt</td>
<td>aatcactggagccagatca</td>
<td>410</td>
</tr>
</tbody>
</table>
patients and controls ($p = 0.37$, Table 2). Tubular adenoma was the most prevalent histopathological group among 103 (68.6%) patients. Also, 28 subjects (18.6%) had villous adenoma, and 19 (12.7%) had tubulovillous adenoma. There was no significant difference in gender among the study subjects ($p = 0.29$; Table 3). Regarding the progression of colorectal adenoma into CRC, 107 (71.4%) subjects had progressed to CRC, while 43 (28.6%) had recovered after polypectomy. In addition, there was no significant association between CRC progression and gender ($p = 0.31$). On the other hand, 86 (80.3%) patients, who progressed into CRC, continued to metastasis, compared to 21 (19.7%) who recovered after surgery and chemotherapy ($p = 0.28$).

**Mutational analyses**

Interestingly, we identified five germline variants (g.87C>T, c.381A>C, c.724G>A, c.1264G>C, and c.1760C>T) in Jordanian subjects with colorectal adenoma (Table 4); two of which (g.87C>T and c.1760C>T) were novel. The c.1264G>C was the most common variant found in 90 patients (60%) with colorectal adenoma. Three variants (c.381A>C, c.724G>A, and c.1264G>C) were found to be disease predisposing, and two variants (g.87C>T and c.1760C>T) were considered as benign (Table 4). Based on Polyphen-2 protein prediction pipeline, c.724G>A and c.1264G>C were probably damaging to DNA glycosylase. However, c.381A>C and c.1760C>T were benign. These findings are consistent with those obtained from PROVEAN v1.1.3 software (http://provean.jcvi.org/seq_submit.php), which indicates that c.724G>A and c.1264G>C are predicted to be deleterious to DNA glycosylase.

The heterozygous variant g.87C>T was identified in amplicon 1 in 60 subjects with colorectal adenoma and in 11 controls. Searching the ExAc and 1000 genome databases showed that g.87C>T was a novel variant found in this study. The variant was mapped to the

### Table 2. General characteristics of the participants (n = 300)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control n (%)</th>
<th>Patient n (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>78 (52)</td>
<td>81 (54.0)</td>
<td>0.73*</td>
</tr>
<tr>
<td>Female</td>
<td>72 (48)</td>
<td>69 (46.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Age groups (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-50</td>
<td>84 (56)</td>
<td>80 (53.3)</td>
<td></td>
</tr>
<tr>
<td>51-70</td>
<td>45 (30)</td>
<td>40 (26.7)</td>
<td>0.37</td>
</tr>
<tr>
<td>&lt;60</td>
<td>21 (14)</td>
<td>30 (20.0)</td>
<td></td>
</tr>
</tbody>
</table>

*Odds ratio: 0.922 and relative risk: 0.960

### Table 3. Clinical characteristics of the patients (n=150)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male n (%)</th>
<th>Female n (%)</th>
<th>Total n (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histopathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular adenoma</td>
<td>60 (58.25)</td>
<td>43 (41.17)</td>
<td>103 (68.6)</td>
<td>0.29</td>
</tr>
<tr>
<td>Villous adenoma</td>
<td>13 (46.4)</td>
<td>15 (53.6)</td>
<td>28 (18.6)</td>
<td></td>
</tr>
<tr>
<td>Tubulovillous adenoma</td>
<td>8 (42.10)</td>
<td>11 (57.9)</td>
<td>19 (12.7)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>69</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>CRC progression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>55 (51.5)</td>
<td>52 (48.5)</td>
<td>107 (71.4)</td>
<td>0.31*</td>
</tr>
<tr>
<td>No</td>
<td>26 (60.4)</td>
<td>17 (39.6)</td>
<td>43 (28.6)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>69</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>42 (48.9)</td>
<td>44 (51.1)</td>
<td>86 (80.3)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>13 (61.9)</td>
<td>8 (38.1)</td>
<td>21 (19.7)</td>
<td>0.28**</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>52</td>
<td>107</td>
<td></td>
</tr>
</tbody>
</table>

*Odds ratio: 0.692 and relative risk: 0.850; **odds ratio: 0.58 and relative risk: 0.788
Table 5. Genotype and allele frequencies of germline mutation c.1264G>C in MUTYH gene in study subjects (n = 300)

<table>
<thead>
<tr>
<th>Genotypes and Alleles</th>
<th>Control n (%)</th>
<th>Patient n (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>143 (95.3)</td>
<td>60 (40)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G/C</td>
<td>7 (4.7)</td>
<td>5 (3.3)</td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>0 (0)</td>
<td>85 (56.7)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Allele G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele C</td>
<td>293 (97.7)</td>
<td>125 (41.7)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Total</td>
<td>300</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Familial</td>
<td>6 (85)</td>
<td>63 (70)</td>
<td>0.376**</td>
</tr>
<tr>
<td>Sporadic</td>
<td>1 (15)</td>
<td>27 (30)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

*Odds ratio: 58.6 and relative risk: 18.22; **Odds ratio: 2.57 and relative risk: 2.43.

5' untranslated region (5'UTR) of MUTYH gene (Fig. 2) and was named according to Human Genome Variation Society guidelines. Table 5 shows that g.87C>T is significantly associated with colorectal adenoma in comparison to the controls (p < 0.0001). Statistical analysis of allele frequencies of both groups showed that the wild-type allele (C) was more common in the control group (96.7%) than the patients group (80%). However, the mutant allele (T) was found in 20% among patients compared to 3.3% among the controls. These data are statistically significant with p < 0.0001.

Amplicon 2 contains exon number 2 of MUTYH gene. It has been amplified to yield 300 bp PCR products. The PCR products were purified, and DNA cycle sequencing was carried out using the forward primer. However, no variant was found in this exon in the study subjects. Likewise, we carried out a PCR to amplify amplicon number 3, which contains three exons, 3, 4, and 5. This amplicon spans 590 bp as visualized by 2% gel electrophoresis. Analysis of amplicon 3 showed a reported single-nucleotide polymorphism called c.381A>C in 11 Jordanians with colorectal adenoma. c.381A>C was found to be disease predisposing with Baye’s probability score of 50 (Table 7). Genotype frequency of both mutant (C) and wild type (A) among the study patients were 12 (8%) and 138 (92%), and their allele frequencies were 288 (96%) and 12 (4%), respectively among study patients (Table 6).

Table 6. Genotype and allele frequency of germline variants found in Jordanian patients with colorectal adenoma (n =150)

<table>
<thead>
<tr>
<th>Amplicon number</th>
<th>Variant identified</th>
<th>Nucleotide (wt:vt)</th>
<th>Genotype frequency (wt:vt) n (%)</th>
<th>Allele frequency (wt:vt) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>g.87C&gt;T</td>
<td>C:T</td>
<td>90 (60)</td>
<td>C:240 (80) T:60 (20)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>60 (40)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>c.381A&gt;C</td>
<td>A:C</td>
<td>138 (92)</td>
<td>A:288 (96) C:12 (4)</td>
</tr>
<tr>
<td>4</td>
<td>c.724G&gt;A</td>
<td>G:A</td>
<td>143 (95)</td>
<td>G:293 (97.6) A:7 (2.4)</td>
</tr>
<tr>
<td>5</td>
<td>c.1264G&gt;C</td>
<td>G:C</td>
<td>60 (40)</td>
<td>G:125 (41.7) C:175 (58.3)</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>5 (3.3)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>85 (56.7)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>c.1760C&gt;T</td>
<td>C:T</td>
<td>145 (96.7)</td>
<td>C:295 (98.3) T:5 (1.7)</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>5 (3.3)</td>
<td></td>
</tr>
</tbody>
</table>

Wt, wild type; vt, variant type
Fig. 2. Mutational analysis of amplicon 1 of MUTYH gene. (a) Schematic representation of MUTYH gene showing that amplicon 1 contains 5' untranslated region, exon 1, and the intronic boundaries. (b) The reference sequence of amplicon 1 obtained from Gene Bank (NG_008189.1) and the highlighted sequences are orange for TATA box, green for initiator, blue for downstream promoter elements (DPE), and yellow for exon 1. The red box contains the sequence in which g.87C>T variant was identified. The PCR was run using the forward and reverse primers (sequences above and below the block arrows). The expected PCR product was 410 bp. (c) 2% agarose gel electrophoresis of amplicon 1. The amplicon was successfully amplified and 410 bp bands were visualized using ethidium bromide staining. The size was compared to 100 bp ladder (lane 1), and the negative control (lane 2) was included. Lanes from 3-12 and from 13-18 are representative of patients and controls, respectively. (d) Partial chromatogram for forward primer sequencing of amplicon 1 from normal control, showing wild-type sequence (wt/wt). (e) Partial chromatogram for forward primer sequencing from patients illustrating heterozygous, g.87C>T variant (wt/vt). (f) Partial chromatogram for reverse primer sequencing from normal control indicates wild type (wt/wt). (g) Partial Chromatogram for reverse sequencing from patients, showing heterozygous g.87C>T variant (wt/vt).
Fig. 3. Mutational analysis of the amplicon 5 of MUTYH gene. (a) Schematic representation of MUTYH, showing that amplicon 5 contains three exons (10, 11, and 12) and the intronic boundaries. (b) The reference sequence of amplicon 5 obtained from Gene Bank (NG_008189.1) and the yellow highlighted sequences are for exons 10, 11, and 12. The PCR was run using the forward and reverse primers (sequences above and below the block arrows). The expected PCR product was 690 bp. The red box shows the position of c.1264G>C mutation. (c) 2% gel agarose gel electrophoresis for amplicon 5. The amplicon was successfully amplified, and 690 bp bands were visualized using ethidium bromide staining. The size was compared to 100 bp ladder (lane 1), and negative control (lane 2) was included. Lanes from 3-12 and 13-20 are representative patients and controls samples, respectively. (d) Partial chromatogram for forward primer sequencing of amplicon 5 from normal control showing wild-type sequence (wt/wt). (e) Partial chromatogram for forward primer sequencing from patients illustrating heterozygous, c.1264G>C, variant (wt/vt). (f) Partial chromatogram for forward primer sequencing from patient indicates homozygous c.1264 G>C mutation (vt/vt). (g) Partial chromatogram for reverse primer sequencing from normal control showing wild-type sequence (wt/wt). (h) Partial chromatogram for reverse primer sequencing from patient with heterozygous, c.1264 C>G, mutation (wt/vt). (i) Partial chromatogram for reverse primer sequence of amplicon 5 from patient with homozygous mutation, c.1264 C>G (vt/vt).
the probability of colorectal adenoma. However, no rs140118273, was successfully identified in Jordanians using 3 products. The products were purified and sequenced (amplicon 8) were amplified using the primers listed as 7 in the Table 8. PCR was successful and resulted in amplification of 230 bp products. The products were purified and sequenced using 3130XL Genetic Analyzer. A reported variant, rs140118273, was successfully identified in Jordanians with colorectal adenoma. However, the variant was found to be neutral.

deleterious to DNA glycosylase based on data obtained from PolyPhen2 and PROVEAN, which scored 0.58 and -2.98, respectively (Table 7). The high prevalence of c.1264G>C in Jordanian patients with colorectal adenoma stressed the importance of investigating the association between this mutation and the progression of adenoma into carcinoma (Fig. 4). Interestingly, a significant association was observed between c.1264G>C mutation and malignant transformation of colorectal adenoma among patients (p < 0.001). This observation brings the necessity to evaluate the relationship between c.1264G>C and colorectal cancer metastasis. Hence, the disease pattern was followed, and the c.1264G>C was found to be significantly associated with CRC metastasis (Fig. 5).

Exon 15 and its intronic boundaries (amplicon 7) were also amplified using the primers listed as 7 in the Table 1. PCR was successfully performed and resulted in amplification of 230-bp products. The products were purified and sequenced using 3130XL Genetic Analyzer (Applied Biosystem, USA). However, no mutation was found in this exon among the study subjects.

Finally, exon 16 and its intronic boundaries (amplicon 8) were amplified using the listed primer 8 (Table 1). PCR successfully amplified 410-bp products. The products were purified and sequenced using 3130XL Genetic Analyzer. A reported variant, rs140118273, was successfully identified in Jordanians with colorectal adenoma. However, the variant was found to be neutral.

### DISCUSSION

In this study, five variants were found in Jordanian patients with colorectal adenoma, of which three (c.381A>C, c.724G>C, and c.1760 C>T) has previously been reported, and two (g.87C>T and c.1264G>C [Q350H]) were novel. These findings are in contrast to Caucasian populations in which Y165C and G382D are the most common mutations associated with multiple polyposis syndrome[18]. The Y165C and G382D mutations have been shown to impair the MUTYH repair activity and significantly contribute to CRC development[19]. The missense R245C and the splice site IVS10-2A>G variants have been identified in Japanese and Korean patients[15,20]. In another studies conducted in Korean and Japanese patients (n = 97), 7.2% were bi-allelic carriers for the germline mutations c.1-18G>T, A359V, and R170G in the MUTYH gene[21,22]. After screening the entire gene, Vandrovcová et al.[23] found no mutation in MUTYH

<table>
<thead>
<tr>
<th>Amplicon Number</th>
<th>Variant</th>
<th>dbSNP ID (rs)</th>
<th>Mutation/Taster Prediction(score)</th>
<th>PolyPhen-2 prediction(score)</th>
<th>PROVEAN prediction(score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>g.87C&gt;T</td>
<td>Novel</td>
<td>Polymorphism (12)*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>c. 381A&gt;C</td>
<td>rs199929178</td>
<td>Disease predisposing (50)*</td>
<td>Benign (0.002)**</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>c.724G&gt;A</td>
<td>rs786203212</td>
<td>Disease predisposing (61)*</td>
<td>Probably damaging (1.0)**</td>
<td>Deleterious (-2.86)**</td>
</tr>
<tr>
<td>5</td>
<td>c.1264G&gt;C</td>
<td>Novel</td>
<td>Disease predisposing (81)*</td>
<td>Probably damaging (0.58)**</td>
<td>Deleterious (-2.98)**</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
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<tr>
<td>7</td>
<td></td>
<td></td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>c.1760C&gt;T</td>
<td>rs140118273</td>
<td>Polymorphism (15)*</td>
<td>Benign (0.003)**</td>
<td>Neutral (-1.0)</td>
</tr>
</tbody>
</table>

Bayes classifier score, which calculates the actual probability that certain alteration causes a disease. the range of result values is between 10 and 100. Therefore, the higher score, the more harmful alteration for human health. **This score indicates the probability that certain mutation is damaging. Hence, variants scores between 0.0 and 0.15 are benign, and variants with scores between 0.15 and 0.40 are probably damaging. However, variants that score between 0.51 and 1.0 are probably damaging.***Delta alignment score of PROVEAN: if the score is below or equal to the threshold (-2.5), the alteration is predicted to have deleterious effect. However, if the variant has a score above -2.5, the variant is predicted to be neutral.

![Fig. 4. Association between c.1264G>C and colorectal adenoma malignant transformation. A strong association was found among the study subjects (p < 0.001). Of 90 subjects, who harbored the c.1264G>C germline mutation, 82 (91.2%) had progressed to colorectal cancer. In contrast, only 25 wild-type patients had progressed into CRC, comparable to 35 subjects who recovered after polypectomy.](https://example.com/final-figure.png)
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Fig. 5. The association between c.1264G>C and CRC metastasis. A significant (p < 0.001) association was observed between c.1264G>C and CRC metastasis. Sixty four subjects included in this study with c.1264G>C germline mutation had CRC metastasis, comparable to 12 who had recovered after colectomy and chemotherapy.

in Singaporean population, though this may be a biased result due to the small sample size (n = 63). Furthermore, five unrelated Indians patients with colorectal adenoma were homozygous for the missense mutation E480X[23]. Other MUTYH variants have been found in Italian (A473D), Finnish (p.P391L), Swedish (G175E), and Portuguese (E383fsX45) [24-27]. In a case-control study and meta-analysis, Win et al. [26] have indicated an association between MUTYH gene mutations and the increased CRC risk. Another meta-analysis demonstrated the risk of MUTYH in monallelic and biallelic carriers [28]. MUTYH gene loss has been linked to colorectal carcinogenesis due to immunosuppression and altered immune response [27]. Grasso et al. [28] have shown that MUTYH mediates the toxicity of DNA 6-thioguanine and UV radiation. MUTYH-OGG1 XRCC1-PARP1-MMP1 is a linear interacting susceptibility locus for CRC [29].

In Arabian descent populations, four studies have been performed; one in Tunisia that showed high prevalence of c.1227-1228dup [30], the second in Morocco that introduced three variants (c.494A>G, c.1145G>A, and c.1185_1186dup) [31], the third in Saudi Arabia that revealed the presence of V22M, Y165C, H324Q, and G382D variants [32], and the last in Egypt that found a significant association between G396D and Y179C mutations and colorectal carcinogenicity [33]. These results contradict with those obtained from a study involved 360 Arabie patients [34]. The best explanation for these discrepancies between data is that these studies only targeted either specific reported mutations, such as G396D and Y179C, or familial cases. However, in the present study, MUTYH gene was sequenced thoroughly for the patients with colorectal adenoma and the controls from the same ethnic group. It has recently been shown that MUTYH interacts with Rad9-Rad1-Hus1 complex (9-1-1 complex) to coordinate the cell cycle checkpoint. The interdomain connector (IDC) of MUTYH (residues between 65 and 350) is critical for 9-1-1 complex and MUTYH interaction [11]. The significance of the interaction between SpMyh1 I complex and 9-1-1 has been elucidated by Lunceford et al. [11] who tested in vivo the biological effects of V315 and I261 mutations in the impairment of 9-1-1 complex-MUTYH interaction. Interestingly, they showed that MUTYH IDC is important for MUTYH DNA repair function and interaction with 9-1-1 complex. MUTYH IDC aspartate at the position number 350 interacts with histidine at the position number 18 of 9-1-1 complex [35]. Therefore, c.1264G>C missense variant found in Jordanian patients would probably affect MUTYH-9-1-1 complex interaction. This finding explains the strong association between c.1264 G>C and colorectal malignant transformation among mutant colorectal adenoma patients. However, this hypothesis requires further functional testing using cell-based cloning and cell culture manipulation.

In conclusion, germline mutations in MUTYH gene are highly prevalent among patients with colorectal adenoma from Jordan. In particular, c.1264 G>C is the most common variant associated with malignant transformation of colorectal adenoma into carcinoma. This variant has a potential to be an early biomarker for diagnosis of CRC in Jordan.

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CONFLICT OF INTEREST. None declared.

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