ABSTRACT

Objective: The objectives of this study were to evaluate the impact of two X-ray repair cross complementing 1 (XRCC1) gene polymorphisms (Arg194Trp and Arg399Gln) on the risk of development of colorectal cancer (CRC) and to assess the expression levels of microRNA-21 (miR-21) in CRC patients.

Materials and Methods: A case-control cross sectional study was conducted on 50 CRC patients and 50 cancer-free subjects. DNA and miR-21 were extracted from whole blood samples. The expression levels of the XRCC1 polymorphisms and miR-21 were assessed by real-time PCR in all subjects of the study.

Results: Genotype analysis revealed a significant association between CRC risk and both the Arg194Trp genotype (OR=11.407, 95% CI=4.039-32.221, p<0.001) and the Arg399Gln genotype (OR=3.778, 95% CI=1.6-8.919, p=0.002). The expression levels of circulating miR-21 were able to detect CRC cases significantly (p=0.022) with a sensitivity of 82% and a specificity of 56% (Area under the curve (AUC)=0.633) but were unable to distinguish between early and late cases (AJCC classification) (p=0.194).

Conclusion: The XRCC1 Arg194Trp and Arg399Gln polymorphisms both confer high susceptibility for the development of CRC. Circulating miR-21 expression levels are a potentially diagnostic non-invasive genetic marker of CRC.

Keywords: Colorectal cancer, miRNA-21, XRCC1, DNA repair, single nucleotide polymorphisms

Introduction

It has been reported in many previous studies that most nucleotide polymorphisms (SNPs) have no deleterious effects or negative health impacts because they are usually located in the non-coding regions of genes. SNPs are often used as genetic markers for the prediction of patient response to therapy and to predict susceptibility to the development or diagnosis of a particular disease [1, 2].

Expression of the X-ray cross-complementing group 1 (XRCC1) gene yields a scaffolding protein that binds to many repair enzymes, such as poly-ADP ribose polymerase, DNA ligase III, DNA polymerase β, polynucleotide kinase, AP endonuclease, proliferating cell nuclear antigen,
and 8-oxoguanine DNA glycosylase [3]. These DNA-repair enzymes have critical protective functions against all kinds of environmental carcinogens that damage human genome. The XRCC1 gene codes for a DNA-repair enzyme that is involved in base excision repair of oxidative DNA damage as well as single-strand break repair. Moreover, several studies have reported an association between XRCC1 Arg/Gln or Gln/Gln genotypes and the clinical response to chemotherapy in colorectal cancer (CRC) [4]. Huang et al. [3] found a strong relation between environmental risk factors such as smoking or drinking alcohol and the presence of XRCC1 gene polymorphisms and the risk of CRC. They reported that individuals who have the XRCC1 194Arg allele and are heavy smokers or subjects reported that individuals who have the XRCC1 gene polymorphisms and the risk of CRC. They drinking alcohol and the presence of XRCC1 environmental risk factors such as smoking or Group 1

Fifty CRC patients (26 males and 24 females) with an average age of 49.5±15.37 [mean±standard deviation (SD)] years. Inclusion criteria were pathologically confirmed CRC cases by pathological examination of the colonoscopy findings from both sexes; ascending, transverse, or left-sided rectosigmoid cancer; and not having previously been treated. Exclusion criteria were non-eligible patients; recurrent tumor cases or a history of secondary tumors; and having received any intervention, including surgery, chemotherapy, or radiotherapy.

DNA extraction
Whole blood DNA was extracted with the Quick-gDNA™ MiniPrep kit (Zymo Research, CA, USA, Catalogue no. D3024) according to the manufacturer's directions.

Real-time PCR
This step involved DNA amplification and detection of Arg194Trp (rs1799782) and Arg-399Gln (rs 25487) SNPs using Real MOD™ Real-time PCR Master Mixture (Catalog no. 25341, Intrion Biotechnology, Korea).

DNA template concentration used for TaqMan SNP genotyping was 20 ng/µl in the quantitative (qRT-PCR) reaction mix. DNA concentration was determined by measuring the absorption at 260 nm using a Nanodrop ND-1000* spectrophotometer.

The qRT-PCR reaction mix per well consisted of 10 µl of 2x RealMOD™ Real-time PCR Master Mix solution, 7 µl nuclease-free water, 2 µl PCR primers, TaqMan® probe, and 1 µl template DNA. The Real-time PCR instrument (Applied Biosystems, SDS v 2.1 software, Step One System, RQ Manager 1.2) was programmed as follows: denaturation (95°C, 10 min) followed by 40 cycles of denaturation (95°C for 15 sec) and annealing/extension (60°C for 60 sec).

Statistical analysis
Data were analyzed using the Statistical Package for Social Sciences version 22 (IBM Corp.; Armonk, NY, USA). Data were calculated as frequencies and relative frequencies for categorical variables and mean±SD for quantitative variables. Comparisons between groups were calculated with an unpaired t-test. For categorical data, the comparison was done using chi-square (c2) test. When the expected frequency was less than 5, exact test was used. The receiver operating characteristic (ROC) curve was constructed, and the area under the curve (AUC) was calculated to determine the best cutoff value for miR-21 detection. Genotype frequencies were compared between groups using c2 test. p<0.05 was considered statistically significant.

Materials and Methods

Study population
Overall, 50 CRC patients and 50 cancer-free control subjects were included in the study. All subjects signed a written informed consent to participate in the study. This study got approval of Ethical committee of Faculty of Medicine Cairo University. The current study was conducted in compliance with Declaration of Helsinki ethical principles. Data for all subjects were obtained from medical records and personal interviews. CRC patients were classified according to the American Joint Committee on Cancer staging (AJCC).

Subjects of the study were divided into the following groups

Group 1
Fifty CRC patients (26 males and 24 females) with an average age of 49.5±15.37 [mean±standard deviation (SD)] years. Inclusion criteria were pathologically confirmed CRC cases by pathological examination of the colonoscopy findings from both sexes; ascending, transverse, or left-sided rectosigmoid cancer; and not having previously been treated. Exclusion criteria were non-eligible patients; recurrent tumor cases or a history of secondary tumors; and having received any intervention, including surgery, chemotherapy, or radiotherapy.

Group 2
Fifty cancer-free control subjects of both sexes (50 males and 50 females) with an average age of 49.18±16.17 years were included. They were chosen from symptomatic patients who were undergoing colonoscopy with confirmed non-cancerous lesions (with or without inflammatory bowel disease).

miRNA extraction
mRNAs were extracted from 300 µl whole blood with the miRNeasy extraction kit according to the manufacturer’s directions (Catalogue no. 217004, Qiagen, Valencia, CA, USA).

Quantification of miR-21 by reverse-transcriptase real-time PCR
The miRNAs were converted into cDNA with the TaqMan micro-RNA kit (Catalogue no. 4427975, ID 000397, Applied Biosystems) according to the manufacturer’s directions.

b. Real-time PCR (qPCR):
The cDNA generated from the RT reaction was amplified using the TaqMan PCR Master Mix kit (Applied Biosystems, Cat No. 4440040), and a Step One System (RQ Manager 1.2, software v 2.1, Applied Biosystem) was used for the reactions. To determine the threshold cycle (CT) value from the amplification plot, RNU6B (Applied Biosystems) was used as the endogenous reference control gene for normalization control. Negative controls were used to exclude any contamination.

The cDNA was amplified using FAM/MGB probe (5’ FAM-TCCGGCCCTGTTAGT MGB 3’), forward primer (5’ CGTCTGTATCTCG- GAAGCT AAGC 3’), and reverse primer (5’ GGGCGGTCTCCTCCATCAA 3’) (Gene Bank accession number NG043905.1)

The qRT-PCR reaction mix per well consisted of 2 µl nuclease-free water, 0.5 µl TaqMan microRNA Assay (20×), 6.5 µl TaqMan Universal Master Mix, and 3 µl cDNA. The RT-PCR instrument was programmed as follows: denaturation (95°C, 10 min) followed by 40 cycles of denaturation (95°C for 15 sec) and annealing/extension (60°C for 60 sec).

The cDNA was amplified using FAM/MGB probe (5’ FAM-TCCGGCCCTGTTAGT MGB 3’), forward primer (5’ CGTCTGTATCTCG-GAAGCT AAGC 3’), and reverse primer (5’ GGGCGGTCTCCTCCATCAA 3’) (Gene Bank accession number NG043905.1)
Results

Demographic results
Colorectal cancer patients were stratified as described by the AJCC staging system. Findings of the present study revealed that most of the CRC patients (78%) had an early-stage disease, in which there were no lymph node involvement, no metastases, and no local invasion beyond the colon. Most of the patients (82%) presented with a left-sided tumour, with or without extensions to the transverse or even the ascending colon. More than one-third of the CRC patients presented with low-grade adenocarcinoma.

Diagnostic results

XRCC1 gene polymorphisms
The homozygous wild types Arg/Arg for exon 399 and Arg/Arg for exon 194 were significantly more frequent in the control group compared to the CRC group. The homozygous 194Trp/Trp genotype was not found in control subjects but was detected in 8% of CRC patients, while the heterozygous 194Trp genotype was found in 12% of the controls and 56% of the cases. The frequency of the 194Trp allele was significantly higher in CRC cases (36%) in comparison to control subjects (6%). CRC cases showed significantly higher expression of the 399Gln allele (36%) compared to control subjects. The homozygous 399Gln/Gln polymorphism was detected in 8% of CRC cases and 4% of the control subjects. The heterozygous 399Arg/399Gln genotype was found in 56% of CRC cases and 28% of the control subjects (Table 1).

Table 1. XRCC1 genotype in CRC cases and matched controls

<table>
<thead>
<tr>
<th>Exon 194</th>
<th>Exon 399</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>Arg/Arg</td>
</tr>
<tr>
<td>Group</td>
<td>No. (%)</td>
</tr>
<tr>
<td>Patients (n=50)</td>
<td>18 (36%)</td>
</tr>
</tbody>
</table>

CRC: colorectal cancer

Diagnosis of the ROC curve of miR-21 for the detection of CRC cases

ROC curve analysis (Figure 1) showed that the miR-21 levels had a significant impact on discriminating CRC cases from control subjects. The AUC was 0.633 (95% CI=0.519-0.746), and the specificity and sensitivity were 56% and 82%, respectively (Table 3) using a cutoff value of 30.795 (Youden index=0.38). We found no statistically significant diagnostic value of miR-21 expression to discriminate between early and late stages of CRC (p=0.194).

Discussion
A case-control study was conducted in 50 CRC cases and 50 control subjects to assess the impact of two XRCC1 gene polymorphisms (194Arg/Trp and 399Arg/Gln) and to assess miR-21 expression as a risk factor for the development of CRC. We found significantly higher expression of the heterozygous Arg194Trp genotype in CRC cases, and the 194Trp allele was significantly more frequent in CRC cases than in control subjects. Moreover, expression of the heterozygous 399Arg/Gln genotype was significantly elevated in CRC cases compared to control subjects. The 399Gln allele frequency was highly significant in CRC cases. Our findings are comparable to those reported by Karam et al. [11] who found a significant association between both XRCC1 A399G polymorphisms and histological grading of CRC.
cur in conserved sequences, which influence the gene-coding regions. The XRCC1 gene is a DNA-repair gene that encodes a protein that plays a major role in single-strand break repair and base excision repair systems. A recent large-scale meta-analysis confirmed the presence of a significant link between XRCC1 399Arg/Gln polymorphism and the development of CRC [13].

In agreement with our results, Halim et al. [14] reported that the 194Trp allele is significantly associated with high risk for the development of CRC. The frequency of the 194Trp allele in our study was similar to that previously reported in studies from Egypt and Mexico [15, 16].

Moreover, Dai et al. [17] showed that the XRCC1 194Arg/Trp gene polymorphism is associated with increased risk for CRC, reduced chemotherapy response, and reduced survival. Another study conducted by Nissar et al. [18] among a Kashmiri population found a significant impact of the Trp/Trp and Arg/Trp genotypes on the development of CRC.

Some studies have reported contradictory results regarding the association between the risk of CRC development and the presence of XRCC1 polymorphisms [19, 20]. These findings might be explained by the fact that the development of CRC is influenced by interactions between environmental factors and genetic factors. Zhang et al. [21] found that smoking significantly affects the association between XRCC1 399Arg/Gln and CRC risk in certain Chinese populations. Moreover, Brevik et al. [22] showed that free radical damage generated by a diet rich in red meat plays a significant contribution to the development of CRC.

We found significantly higher expression levels of circulating miR-21 in CRC patients compared to controls, and the ROC curve showed an 82% sensitivity and 56% specificity. However, the ROC curve was unable to discriminate between early and late CRC cases. Bastaminejad et al. [23] reported that miR-21 expression levels in serum were significantly elevated in CRC cases with an 86.05% sensitivity and 72.97% specificity. A meta-analysis that included articles published from 2009 to 2014 where the participants include Caucasians, Asians, and a few Africans showed that circulating miR-21 is useful as an early diagnostic biomarker for several malignant tumors, including CRC [24, 25]. Moreover, Sheng et al. [26] found that the protein kinase/phosphatase/phosphatidyl-inositol kinase (AKT/PTEN/PI3K) signaling pathway is intimately linked with many malignant tumors and their progression, drug resistance, immunity, angiogenesis, and metastasis [27, 28]. Furthermore, there have been several studies showing that miR-21 has a significant role in regulating the AKT/PTEN/PI3K signaling pathway and contributes to the initiation of carcinogenesis, tumor progression, and metastasis [29, 30]. Sheng et al. [26] reported that the PTEN gene is a direct target gene of miR-21. Thus, gene expression levels of phosphatase and its downstream AKT and PI3K genes might be controlled by regulating miR-21 expression, which in turn regulates the development of CRC.
Limitations of the present study include the small number of CRC cases as well as the presence of small number in each CRC patient subgroup that was categorized according to the tumor grade or stage.

To the best of our knowledge there are no relations between miRNA21 and XRCC1 gene polymorphisms.

The presence of either the 194Trp allele or 399Gln allele is associated with greater risk for CRC. Circulating miR-21 expression levels can be used as an early diagnostic marker for CRC cases but cannot discriminate early from late cases of CRC.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethical Committee of Cairo University Faculty of Medicine (2015).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.


Conflict of Interest: No conflict of interest was declared by the authors.

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References