Klotho (rs1207568 and rs564481) gene variants and colorectal cancer risk

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ABSTRACT

Background/Aims: Whereas colorectal cancer (CRC) is the third most common cancer worldwide, klotho gene has been reported as a tumor suppressor gene. Therefore, the aim of this study was to investigate the association between klotho (rs1207568 and rs564481) variants and CRC in Egyptian patients.

Materials and Methods: A case-control study comprising 100 patients with CRC and 100 age- and sex-matched healthy controls was conducted. Genotyping of klotho was performed by polymerase chain reaction with confronting two-pair primers.

Results: The frequencies of the A allele of rs1207568 and the AC haplotype were significantly higher in patients with CRC than in the controls (p=0.019 and p=0.005, respectively).

Conclusion: We propose that klotho (rs1207568 and rs564481) variants play a significant role in colorectal carcinogenesis and that the klotho protein could be a target for oncotherapy.

Keywords: Klotho, colorectal cancer, gene variation, klotho rs1207568, klotho rs564481

INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers, ranking as the third most common cancer worldwide (1). The incidence rate of CRC has been increasing steadily; thus, CRC is of great international concern for researchers (2).

Although CRC ranks second in terms of cancer-related deaths (1), it is considered a preventable cancer because genetic changes precede the onset of the disease by approximately 10 years (3). Thus, the development of CRC can be prevented, and numerous research groups are currently studying these genetic changes.

Screening for precancerous colorectal lesions results in improved outcomes and is important for disease prevention. Although considerable advances have been made in diagnostic procedures for CRC, many patients with CRC are still diagnosed in late stages (4). Thus, additional studies are needed to improve our understanding of the molecular alterations involved in CRC to identify diagnostic, prognostic, and predictive markers that can enhance prevention, early detection, and treatment (5).

In 1997, the klotho protein was identified as an anti-aging protein by studies showing that mice with a klotho protein deficiency were more susceptible to aging diseases (6,7). Previous scientific research indicates an anticancer role for klotho protein in addition to its anti-aging properties, and this anticancer role has been studied in many cancers, including lung cancer, breast cancer, colon cancer, and pancreatic cancer. The anticancer activity of klotho is thought to be attributed to the dysregulation of tumor cell proliferation and apoptosis (8–11). Further research on klotho is anticipated to provide new insight into targeted cancer therapy (12).

The aim of this study was to investigate the association between two single nucleotide variants (SNVs) in the *klotho* gene (*rs1207568*, in the promoter region and *rs564481*, located in exon 4) and CRC in Egyptian patients.

Klotho rs1207568 (c.-395G>A) is an SNV, located in the promoter region of the *klotho* gene, in which guanine nucleotide is replaced by adenine (13), whereas *klotho rs564481*(c.1767C>T, p.His589=) is a synonymous variant, located in exon 4, where cytosine nucleotide is sub-

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stituted by thymine at codon 1767 resulting in no amino acid (Histidine) change (14).

Despite the abundance of previous studies suggesting a significant role of klotho in tumorigenesis and cancer progression, data and published studies regarding the role of variations in the *klotho* gene (*rs1207568* and *rs564481*) and their association with CRC are scarce. Therefore, studies in numerous populations are needed to clarify this association.

Table 1. Descriptive data of the study subjects.

Demographics	Patients with CRC (n=100)	Healthy Controls (n=100)	p
Age (years)	46.6±13.9	43.4±9.9	0.061
Male Sex, %	46.0	37.0	0.169
Site, %			
Colon	53.0	—	—
Rectum	47.0	—	—
Differentiation Degree, %			
Low	17.0	—	—
Moderate and High	83.0	—	—
Histological type, %			
Mucinous	29.0	—	—
Adenocarcinoma	71.0	—	—
Tumor Stage, %			
I + II	30.0	—	—
III + IV	70.0	—	—
Polyp, %	22.0	—	—
CEA (ng/mL)	2.2 (1.2-19.4)	—	—
CA 19-9 (IU/mL)	14.2 (3.7-49.0)	—	—

Normally distributed variables are presented as mean ± standard deviation. Skewed variables are presented as median (interquartile range).
CRC: colorectal carcinoma; CEA: carcinoembryonic antigen; CA 19-9: cancer antigen 19-9.

MAIN POINTS

- *Colorectal cancer (CRC) is ranking as the third most common cancer worldwide and ranks second in terms of cancer-related deaths.*
- *klotho protein has both antiaging and anticancer properties.*
- *klotho downregulation was found to enhance cancer cell proliferation and decrease apoptosis.*
- *The A allele of klotho rs1207568 variant was associated with increased risk of CRC in Egyptian patients.*
- *klotho (rs1207568 and rs564481) variants play a significant role in colorectal carcinogenesis.*

MATERIALS AND METHODS

This study included 100 histopathologically confirmed CRC cases and 100 healthy controls. Clinical data, including age; sex; and tumor localization, staging, and grading, were collected from these patients. The characteristics of the patients with CRC and controls are summarized in Table 1.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Genotyping of SNVs in the klotho gene

Blood samples of 2 ml were collected from each study participant into ethylenediaminetetraacetic acid–containing tubes. Genomic DNA was extracted using a TINAamp genomic DNA extraction kit (Tiangen Biotech, Beijing, China). Genotyping of the *rs1207568* and *rs564481* variants in the *klotho* gene was performed using polymerase chain reaction (PCR) with confronting two-pair primers (15) according to the protocol proposed by Shimoyama et al. (16). In this assay, confronting pairs of primers (a total of four primers) were used (Operon Biotechnologies, Germany).

The following primers were used to amplify the *klotho rs1207568*—Forward primer 1, 5′-GTTTCGTGGACGCTCAGGTTCATTCTC-3′; Reverse primer 1, 5′-GATCCCGCCCCCAAGTCGGGA-3′; Forward primer 2, 5′-GAGAAAAGGCGCCGACCAACTTTC-3′; and Reverse primer 2, 5′-GTCCCTCTAGGATTTCGGCCAG-3′.

The PCR conditions were as follows: an initial denaturation step at 95 °C for 10 min; 35 cycles at 95 °C for 1 min, annealing at 65 °C for 1 min, and 72 °C for 1 min; and a final elongation step at 72 °C for 5 min. PCR products were evaluated using 2% agarose gels stained with ethidium bromide. The GG genotype appeared as two bands of 252 and 175 bp; the GA genotype appeared as three bands of 252, 175, and 121 bp; and the AA genotype appeared as two bands of 252 and 121 bp (Figure 1).

The following primers were used to amplify the *klotho rs564481*—Forward primer 1, 5′-CTCAGTTTACCGACCTGAATGTTTACCTG-3′; Reverse primer 1, 5′-GTCCAGGGAGAAGCGAAAATGTGTAACA-3′; Forward primer

2, 5′-CAGATCGCTTTACTCCAGGAAATGCAC-3′; and Reverse primer 2, 5′-GAGCTCTTGAAAGCACAGTCGGGC-3′.

The PCR conditions were as follows: an initial denaturation step at 95 °C for 10 min; 30 cycles at 95 °C for 1 min, annealing at 69 °C for 1 min, and 72 °C for 1 min; and a final elongation step at 72 °C for 5 min. PCR products were evaluated using 2% agarose gels stained with ethidium bromide. The CC genotype appeared as two bands of 416 and 291 bp; the CT genotype appeared as three bands of 416, 291, and 179 bp; and the TT genotype appeared as two bands of 416 and 179 bp (Figure 2).

Statistical Analysis

The Statistical Package for Social Science, version 16.0 (SPSS Inc.; Chicago, IL, USA), was used for statistical analysis in this study. In the controls, each polymorphism was tested for Hardy–Weinberg equilibrium (HWE). The haplotype frequencies were analyzed using the Web-based calculator SNPstats (17). The chi-square test and Fisher's exact test were used to analyze the association between *klotho* gene variants and CRC. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to determine the strength of the association.

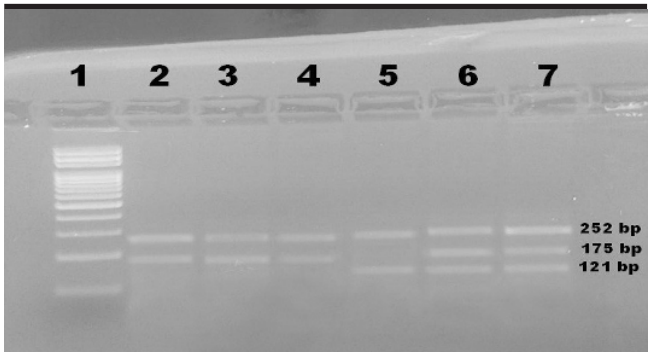

Figure 1. Agarose gel showing PCR-CTPP analysis of the *klotho* c.-395G>A variant. Lane 1 shows 100 bp DNA ladder, lanes 2, 3 & 4 show the homozygous GG genotype, lane 5 shows the homozygous AA genotype, lanes 6 and 7 show the heterozygous GA genotype.

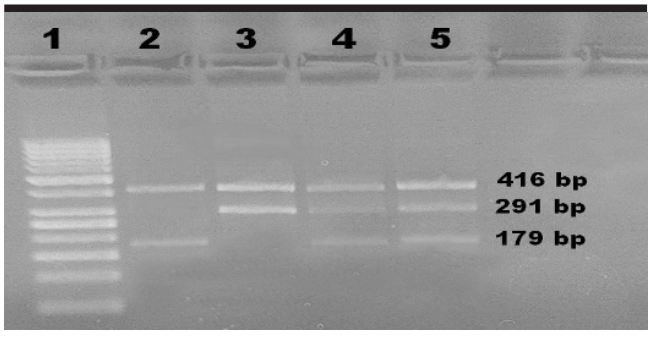

Figure 2. Agarose gel showing PCR-CTPP analysis of the *klotho* c.1767C>T variant. Lane 1 shows 50 bp DNA ladder, lane 2 shows the homozygous TT genotype, lane 3 shows the homozygous CC genotype, lanes 4 and 5 show the heterozygous CT genotype.

RESULTS

The descriptive data of the patients with CRC and healthy control subjects are summarized in Table 1. The patients with CRC and healthy controls were age- and sex-matched.

In the control group, both the *klotho rs1207568* and *rs564481* SNVs were in HWE. The *minor allele frequencies* of the *klotho rs1207568* and *rs564481* SNVs were 0.29 and 0.22, respectively, in patients with CRC.

Table 2 shows the *klotho* genotypes and allele frequencies in the patients with CRC and healthy control volunteers. The frequencies of both the A allele and the combined (G/A–A/A) genotype of the *klotho rs1207568* variant were significantly higher in the patients with CRC than in the controls (p=0.019 and p=0.007, respectively). However, no significant difference was found in the distribution of the *klotho rs564481* variant or the allele fre-

Table 2. The genotype and allele frequencies of *klotho* genetic variations in the CRC and healthy control groups.

Variant	Healthy Controls (n=100)	Patients with CRC (n=100)	OR (95% CI)	p
rs1207568, %				
G/G	63.0	44.0	1.00	—
G/A	36.0	54.0	2.15 (1.21-3.80)	0.008
A/A	1.0	2.0	2.86 (0.25-32.56)	0.570
G/G	63.0	44.0	1.00	
G/A–A/A	37.0	56.0	2.2 (1.2-3.8)	0.007
G allele	81.0	71.0	1.00	
A allele	19.0	29.0	1.7 (1.1-2.8)	0.019
rs564481, %				
C/C	62.0	57.0	1.00	—
C/T	34.0	41.0	1.31 (0.73-2.34)	0.843
T/T	4.0	2.0	0.54 (0.10-3.08)	0.683
C/C	62.0	57.0	1.00	
C/T–T/T	38.0	43.0	1.2 (0.7-2.2)	0.470
C allele	79.0	78.0	1.00	
T allele	21.0	22.0	1.1 (0.7-1.8)	0.716

CI: confidence interval; CRC: colorectal cancer; OR: odds ratio.

Table 3. Analysis of klotho haplotype frequencies with the risk of CRC.

Haplotype (rs1207568 and rs564481)	Total Frequency	Control Frequency	CRC Frequency	OR (95% CI)	p
GC	0.5797	0.6401	0.5071	1.00	—
AC	0.2028	0.1499	0.2679	2.66 (1.35-5.25)	0.005
GT	0.1803	0.1699	0.2029	1.49 (0.77-2.87)	0.24
AT	0.0372	0.0401	0.0221	0.88 (0.09-8.25)	0.91

CI: confidence interval; CRC: colorectal cancer; OR: odds ratio.

quencies between the patients with CRC and the healthy controls (Table 2).

Regarding the *klotho* haplotype distribution, the AC haplotype was significantly associated with an increased risk of CRC development (OR=2.66; 95% CI 1.35-5.25; *p*=0.005; Table 3).

We further analyzed the association between *klotho* genotypes and clinical manifestations of CRC. However, no significant associations were found between *klotho* variants and any of the clinical characteristics of CRC.

DISCUSSION

In this study, we analyzed the relationship between *klotho* gene variants (*rs1207568* and *rs564481*) and CRC in Egyptian patients to better understand the ways in which these genetic alterations influence CRC. To our knowledge, this study is the first to investigate the relationship between *klotho* variants (*rs1207568* and *rs564481*) and CRC in this population.

The A allele of *klotho rs1207568* was found to be significantly associated with an increased risk of CRC development. On the other hand, no significant association was found between the *klotho rs564481* variant and CRC. However, when both the *rs1207568* and *rs564481* variants were considered, the frequency of the AC haplotype was significantly higher in the patients with CRC than in the healthy controls, indicating that the *rs564481* variant influences CRC development, potentially through synergistic action with the *rs1207568* variant.

We further studied the association of the *klotho rs1207568* and *rs564481* genotypes with different demographic characteristics, histological tumor types, tumor stages, and CRC tumor markers, but no significant relationships were found.

In humans, the *klotho* gene is located at chromosome region 13q13.1 and consists of six exons (18). Humans express two types of the klotho protein: membrane-bound klotho, which is a coreceptor of fibroblast growth factor (FGF) 23 and regulates phosphate and vitamin D homeostasis (19, 20), and secreted klotho, which acts as a humoral factor that mediates anti-aging effects through the regulation of oxidative stress, receptors of multiple growth factors, and ion channels (21, 22).

Previous research has reported a link between a low klotho protein level in the blood and an increased risk of cancers, for example, pancreatic cancer and hepatocellular cancer. The suggested mechanism acts through epigenetic modulation, including promoter methylation and histone deacetylation. In addition, *klotho* downregulation was found to enhance cancer cell proliferation and decrease apoptosis through possible mechanisms involving the FGF signaling pathway, the insulin-like growth factor 1 receptor pathway, or the wnt/β-catenin signaling pathway (12).

Approximately 10 variations have been detected in the klotho gene (16). In this study, we focused on only two, *rs1207568* (c.-395G>A), located in the promoter region, and *rs564481* (p.His589=), located in exon 4, and their relation to CRC. We found that the *rs1207568* variant and the AC haplotype were significantly associated with increased CRC risk. Our results agree with those of Liu et al. (23) in Chinese patients.

Study of the functional mechanism of the *klotho rs1207568* variant revealed a correlation between the A allele and a low level of klotho protein expression in human vascular tissue (24). This finding is consistent with those of previous studies, which speculated that the A allele of this genetic variant reduces transcription factor binding SP1 and hence klotho expression levels (25). However, Kawano et al. (25) failed to find such an association between the G and A alleles and klotho protein expression levels in cultured human cells.

The potential effect of the *rs1207568* variant on *klotho* gene function remains speculative. The variation in the results of different studies could be due to the existence of regulatory sites other than those related to the *rs1207568* variant that also regulate the expression and function of *klotho*. In addition, *rs1207568* is in strong linkage disequilibrium with other functional genetic variations (such as c.110G>C, p.His589= and c.2298C>T) and could act as a surrogate for these variants (26).

Based on these studies and our results, we hypothesized that the A allele of the *klotho rs1207568* variant could reduce the level or activity of klotho in humans, thereby increasing the risk of CRC.

Although *rs564481*, located in exon 4, is a synonymous variant where a nucleotide transition from C to T resulted in no amino acid change, previous studies have demonstrated the possibility that this type of variations affects protein function, potentially through effects on mRNA stability and processing, translation kinetics, and protein folding (27).

Aberrant expression of *klotho* has been observed in several cancers. Klotho acts as a tumor suppressor in most cancers, as *klotho* overexpression results in the suppression of cancer cell proliferation. Because *klotho* expression is downregulated in most cancers, knowledge about the klotho protein will likely provide new insight into therapy for cancers, including CRC (28–31).

In conclusion, our study suggests that *klotho* (*rs1207568* and *rs564481*) variants play a significant role in colorectal carcinogenesis. However, further investigations are required to elucidate the detailed mechanisms of *klotho* genetic variations in cancers in general and in CRC specifically.

Ethics Committee Approval: Ethics committee approval was received from the Ethics Committee of Faculty of medicine, Cairo University.

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

Peer-review: Externally peer-reviewed.

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