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Correlation between Certain *Klotho* Gene Polymorphisms and IGF-1 Levels of Colorectal Cancer Patients in Northern Iran

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Abstract

Background: Colorectal cancer susceptibility may correlate with the *Klotho* gene G-395A and C1818T polymorphisms. This study aims to evaluate the relationship between a *Klotho* single nucleotide polymorphism and IGF-1 with risk of colorectal cancer.

Methods: This study enrolled 60 colorectal cancer patients and 60 age-matched healthy persons who referred to Razi Hospital, Rasht, and Northern Iran in September 2013. Patients enrolled under supervision of a gastro-intestinal specialist and according to the ethics right. G-395A and C1818T polymorphisms were genotyped with polymerase chain confronting two pair primer technology. IGF-1 and certain biochemistry analytes were assayed. Statistical analysis was used to compare appropriate relationships.

Results: There were different base pair partitions for G395A and C1818T. Odds ratio and 95% confidence interval were used to analyze the correlation of genotypes and haplotypes with colorectal cancer susceptibility. The AA (odds ratio: 1.437, 95% confidence interval: 0.596) and GA (odds ratio: 1.958, 95% confidence interval: 1.133-3.385) genotypes of the G-395A polymorphisms showed a slight relationship to the risk of colorectal cancer. The A allele had a much higher frequency in the case group (31.2%) compared with the control group (17.6%). There was no significant relationship with the C1818T polymorphism between the case and control groups.

Conclusion: The *Klotho* gene polymorphism did not significantly increase the risk of colorectal cancer. Therefore, these genotypes might not have a correlation with IGF-1.

Keywords: *Klotho* polymorphisms, IGF-1, Colorectal cancer

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Introduction

Klotho was originally characterized as an aging suppressor gene, and identified as a tumor suppressor gene in a variety of cancers, including colon cancer.[1] Over-expression of *Klotho in vitro* has been shown to inhibit cell proliferation activities and invasive potential in colon cancer cells, accompanied by decreased expression of the p-IGF1 receptor.[2] *Klotho* is a tumor suppressor in cancer by inhibiting insulin and insulin-like growth factor-1 (IGF-1).[2] It has been suggested that *Klotho* is a potential tumor suppressor and an inhibitor of the IGF-1 pathway. Recent findings indicate that its expression down-regulates in human colon cancer, and correlates with tumor invasion and Dukes staging. Over-expression of *Klotho* suppresses growth and invasion through inhibition of the IGF1R-mediated PI3K/AKT pathway in colon cancer cells, which suggests that *Klotho* may serve as a potential therapeutic target for the treatment of colon cancer.[3] Colorectal cancer (CRC) is a major burden to health care systems worldwide and accounts for approximately one million new cancer cases.[4] The mortality associated with CRC remains high despite advances made in adjuvant and neo-adjuvant therapy.[5] A search for new and effective anti-cancer treatments is necessary. An attractive avenue of cancer research is gene intervention therapy. Recent studies show that *Klotho* is involved in the progression of several types of human cancers, and plays an important role in tumor genesis, proliferation, survival, autophagy, and resistance to antitumor therapies.[6] Colorectal cancer mortality is reportedly increasing and has become one of the leading causes of cancer-associated deaths.[7]

More than 10 mutations or single nucleotide polymorphisms (SNPs) have been reported in the human *Klotho* gene. G-395A polymorphisms in the promoter region and C1818T in exon 4 are reportedly associated with many physiological processes. Shimoyama et al. have found that *Klotho* gene SNPs G-395A and C1818T were associated with lipid metabolism in Japanese men, and glucose metabolism, bone mineral density and systolic blood pressure in Japanese women.[8] However, studies on the correlation of the *Klotho* gene with CRC are few. We sought to explore the relationship between CRC risk and

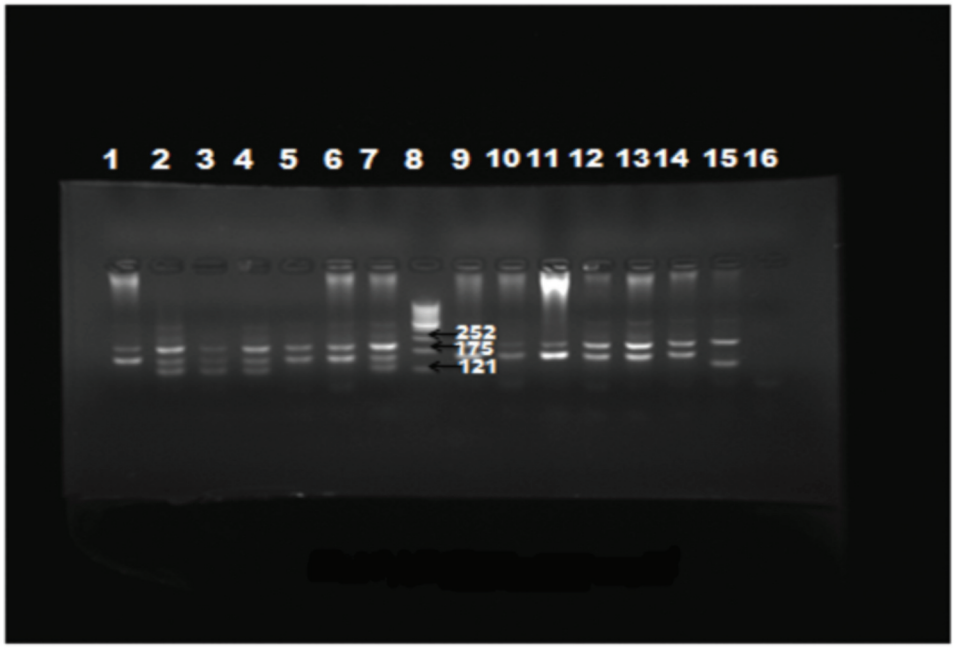

Figure 1. G395A *Klotho* polymorphism. Different lanes (1-16) show various genotypes as follows: negative control (16), size markers (8), GG(1,5,6,9,10,11,12,13,14), GA (2,3,4,7), and AA (15). Agarose gel electrophoresis showed a few bands. The 252bp was a common band for all lanes (control), GG (175bpand 252bp), AA (252bp and 121bp), and GA (252bp, 175bp, and 121bp).

Table 1. Demographic characteristics for age-matched healthy individuals

Subject	All	Male	Female
N	60	27	33
Age(years)	55.0±11.13	55.83±11.33	55.22±11.12
FBS(mg/dl)	104.73±14.73	104.37±14.63	104.11±14.74
Urea(mg/dl)	17.6±5.535	17.8±5.49	17.6± 5.93
Creat (mg/dl)	1.2±2.11	1.26±2.51	1.35±2.36
Calcium(mg/dl)	9.46±0.56	9.47±0.53	9.46 ±1.31
Phosphorus(mg/dl)	4.025± 0.49	4.025± 0.05	4.025± 0.49
Vitamin D3 (ng/ml)	25.30± 14.09	25.36± 13.71	25.36± 14.35
PTH (pg/ml)	47.60± 12.24	47.68± 14.90	47.80± 15.24

FBS: Fasting blood glucose, Creat: Creatinine, PTH: Para- thyroid hormone, Vitamin D3: cholecalciferol.

Klotho gene polymorphism (G-395A and C1818T). In addition to genetic variations of the *Klotho* sequence, possibly epigenetic modifications such as methylation, acetylation, and chromatin remodeling contribute to CRC.[9]

Materials and Methods

Subjects

This case-control study enrolled 60 patients from Razi Hospital who had a median age of 47 (22-65) years and 60 controls who had a median age of 51(35-63) years. The control participants consisted of non-cancerous individuals who referred for routine experiment (Tables 1, 2). Patients with CRC were diagnosed by a histopathology test. All patients were not related by other diseases except CRC (patients only had CRC) and had not undergone radiotherapy or chemotherapy. Samples were collected in accordance with national ethics standards for human genome study and participants provided written informed consent. (Satisfaction letter that allowed sampling according standard guideline).

All CRC patients and healthy controls were admitted under the supervision of a GI specialist and completed questionnaire about demographics,

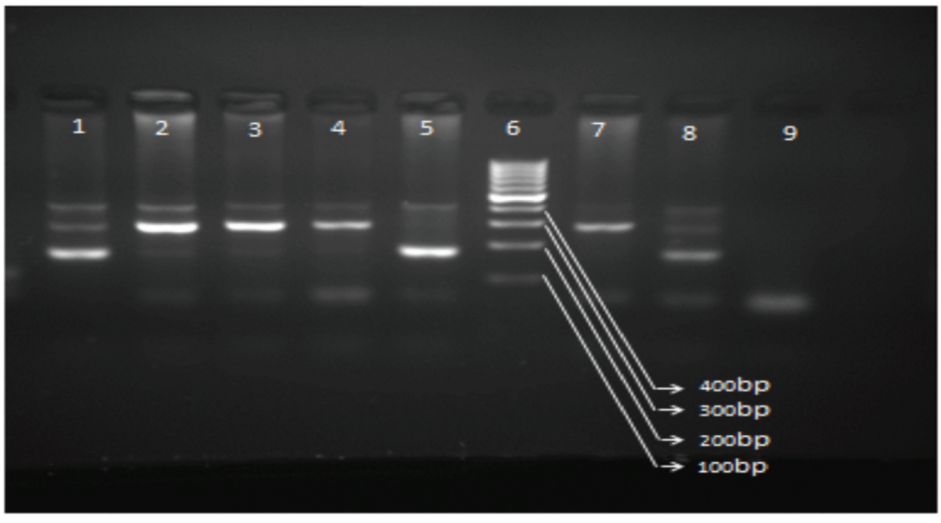

Figure 2. C1818T *Klotho* polymorphism. Different lanes (1-9) show various genotypes as follows: negative control (9), size marker (6), CC (2, 3, 4, 7), CT (1, 8), and TT (5). Agarose gel electrophoresis showed a few bands. The 416 bp was common for all lanes (control). CC (416bpand 291bp), TT (416bpand 179bp), and CT (416bp, 291bp, and 179bp).

Table 2. Demographic characteristics for colorectal cancer (CRC) patients.

Subject	All	Male	Female
N	60	27	33
Age(years)	58.63±11.51	56.88±15.75	52.41± 9.8
FBS(mg/dl)	112.16±28.64	114.07±24.10	113.97±31.90
Urea(mg/dl)	50.2±10.52	52.44±9.54	48.55±10.65
Creat (mg/dl)	5.27±1.21	5.48±1.29	5.09±1.18
Calcium(mg/dl)	9.46±0.74	9.47±083	9.43±0.63
Phosphorus(mg/dl)	4.61±0.71	4.062±0.59	4.66±0.79
Vitamin D3 (ng/ml)	21.97±7.52	24.42±7.86	19.43±6.62
PTH (pg/ml)	80.23±23	89.78±32	72.50±33.60

FBS: Fasting blood glucose, Creat: Creatinine, PTH: Para- thyroid hormone, Vitamin D3: cholecalciferol.

public health, and the possibility of confounding data, while present at the hospital.

DNA extraction

Participants provided 5 ml of venous blood from elbow and performed anticoagulant through ethylenediamine tetraacetic acid (EDTA), not allowed to coagulate (preparing plasma). DNA was extracted by the NaCl-ethanol method by a blood genome DNA Extraction Kit (Fermentaze Co., Ltd., Iran). Genomic DNA was stored at -80°C until use.

Polymerase chain reaction (PCR)

Klotho gene polymorphisms were analyzed using polymerase chain reaction-restriction two primer (PCR-CTPP) technology. The primers were synthesized by Blast Technology as Gene Runner software. PCR primers for amplification of the *Klotho* SNPs (G-359A) were as follows: G-395A forward 1(5'-GTTTCGTGGACGCTCAG GTTCAT TCTC-3') and reverse 1(5'-GATCC-CGCCCCCAAG TCG GGA-3'), and forward 2(5'- GAGAAAAGGCGCCGACCAACTTTC-3') and reverse 2 (5'- GTCCCTCTAGGA TTTCGGCCAG-3').

C1818T forward1(5'-CTCAGTTTACCGAC-CTGAATGTTTACCTG-3') and reverse 1(5'-GTCCAGGGAGAAGCGAAAATGTG-TAACA-3'), and forward 2 (5'- CAGATCGCTTT ACTCCAGGAAATGCAC-3') and reverse 2 (5'-GAGCTCTTGAAAGCACAGTCGGGC-3').The PCR reaction mixture (24 µL) consisted of 0.7 µl of each primer (20 µmol/l), 0.8 µl ,50mmol/l of MgCl2, 0.5mmol/l of dNTP, 0.08 µl Taq DNA polymerase, 1 µl of genome DNA (80 µg/µl), and 17.32 µl of ddH2O. A Bio Rad Thermal cycler was used for the following PCR procedure: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 11 s, annealing at 61°C for 45 s, extension at 72°C for 45 s, continuation at 72°C for 5 min after all cycles, and finally at 10°C for not limited time. The digestion products were separated through a 1.5% agarose gel elec-

Table 3. Genotype, allele frequencies in A and B groups.

Genotype	N (%)	N(%)	N(%)	*P*-value
G395A	G/G	G/A	A/A	
A	42 (70)	15 (25)	3 (5)	0.28
B	49 (81.7)	10 (16.7)	1 (1.6)	
G395A	GG	GA+AA	-	
A	42 (70)	18 (30)	-	0.063
B	49 (81.7)	11 (18.3)	-	
C1818T	C/C	C/T	T/T	
A	14 (23.3)	43 (71.6)	3 (5)	0.9
C1818T	CC	CT+TT	-	
A	14 (23.3)	46 (76.6)	-	1.04
B	14 (23.3)	46 (76.6)	-	

Table 4. Odds ratio (OR) with 95% confidence interval (CI) for biochemical parameters using binary logistic regression.

Subject	*P*-value	%95 CI	OR
Vitamin D	0.122	0.993-1.057	1.025
PTH	0.000	0.940 -0.978	0.959
Calcium	0.990	0.619-1.606	0.997
Phosphorus	0.000	0.1810.583	0.322
Creatinine	0.000	0.171-0.391	0.259
Urea	0.000	0.605-0.824	0.706
FBS	0.392	0.996-1.011	1.003

trophoresis with ethidium bromide staining.

Statistical analysis

We used the chi-square and t-tests for assessment of the genotype distribution of the control group with SPSS-16 software (Table 3). The association between risk of CRC and *Klotho* polymorphisms was tested with odds ratio (OR) and 95% confidence interval (CI), calculated by the chi-square test. Analyses of haplotypes were conducted with Haplo view software. $P<0.05$was considered statistically significant.

Results

Essential features of participants

Figures 1 and 2 show different bands for G395A and C1818T polymorphisms in agarose for: GG (175,252), GA (121,175,252), and CC (291,416), CT (179,416), and TT (179,291,416). There were no significant differences between the two groups in age or gender. The genotype distributions of G-395A and C1818T polymorphisms of the controls were in accordance with Hardy–Weinberg equilibrium (HWE) ($P>0.05$).

Correlation analysis between Klotho polymorphisms and colorectal cancer (CRC) risk

Analysis of the results showed that the frequencies of AA ($P=0.016$) and GA ($P=0.005$) genotypes in G-395A were not significantly higher in CRC patients compared to controls. AA or GA was characterized as a different genotype in the G-395 polymorphism. Odds ratio and 95% CI indicated that persons at increased risk for CRC had the AA (OR: 4.161, 95% CI: 1.437-12.053) and GA (OR: 1.958, 95% CI: 1.133-3.385) genotype. We found that the A allele was a low risk factor for CRC (OR: 2.123, 95% CI: 1.393-3.236). However, the genotype and allele distributions of the C1818T polymorphism showed no significant difference between CRC patients and controls ($P>0.05$)

Association between base biochemical elements with colorectal cancer (CRC) risk

We analyzed base biochemical elements -PTH, vitamin D, calcium, phosphorus, urea, creatinine, and FBS as possible risk markers for CRC patients (Table 4).

Correlation between Klotho polymorphisms (G-395A, C1818T) and IGF-1

Table 5 shows that the *Klotho* polymorphisms (G-395A, C1818A) correlated with IGF-1 in CRC patients.

Table 5. Correlation between *Klotho* polymorphisms (G-395A & C1818T) and IGF-1.

Genotype	Frequency	IGF-1Mean (mg/dl)	*P*-value
GG	95	1.465±67	0.13
GA+AA	25	1.243±56	
CC+TT	29	1.511±66	0.4
CT	91	1.387±68	

Discussion

CRC, developed in colon or rectum, is one of the most common malignant tumors, the morbidity of which is rising year by year. As its pathogenesis is extremely complex, 9 deeper studies are required to improve the identification and treatment of patients with CRC. In recent years, many researchers focused on exploring new biomarkers that were used in early diagnosis and treatment of tumors. Lots of biomarkers have been found and *Klotho* gene is one of them.

The present study was the first Iranian study that assessed the possibility of a correlation among *Klotho* gene variations, IGF-1, and signal transduction factors in CRC patients.

The human *Klotho* gene located at chromosome 13q12 is composed of five exons and ranges over 50 kb. Found in 1997, *Klotho* is regarded as one anti-aging gene by earliest studies.[10-12] Recently, researches on the functions of *Klotho* gene have found its close correlation with malignant tumors and possible functions varied in different tumors. Lee et al. reported that epigenetic silencing of *Klotho* may occur during the late phase of cervical tumor genesis, and consequent functional loss of *Klotho* may lead to aberrant activation of the canonical Wnt pathway in cervical carcinoma.[13] Besides, *Klotho* could inhibit multiple growth factor signaling pathways and serve as an endogenous anti-EMT factor in mice.[14] Meanwhile *Klotho* plays an anti-oncogene in human lung cancer cell line A549 by inhibiting growth and promoting apoptosis.[15]

However, there were few reports about the *Klotho* gene and CRC. C1818T polymorphism showed no effects on the pathogenesis of CRC, which was similar with our study and consistent with the researches coronary heart disease, but not with vaso-spastic angina.[16,17] A case-control study involved 125 patients and 125 healthy persons were performed to analyze the correlation of *Klotho* polymorphisms (G-395A and C1818T) with CRC susceptibility.[18] In addition, the haplotype analysis indicated that A-C and A-T haplotypes were both significantly associated with CRC risk. HWE test on controls showed that study population was representative. From the analysis, we found that AA and GA genotypes of G-395A served as risk factors for CRC.[18] Studies show that *Klotho* expression universally stopped in a wide range of malignancies, like breast, pancreatic, ovarian, lung, colorectal, and melanoma and that *Klotho*'s expression can serve as prognostic marker. Epigenetic model, i.e., promoter hyper methylation and histone deacetylation, are mainly associated with *Klotho*'s down-regulation, thus some results suggest that *Klotho* is inactivated through promoter hyper methylation and potentially acts as a tumor suppressor gene in CRC.[19]

Klotho as novel tumor suppressor gene is epigenetically inactivated in colorectal cancer that suggested *Klotho* promoter hyper methylation as a predictor of the prognosis in colorectal cancer patients.[20,21]

In conclusion, G-395A polymorphism in human *Klotho* gene was related with CRC low risk but C1818T was not effective. Since the precise function mechanism is still not clear, so further studies are required to clarify the issue.

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Conflict of Interest

No conflict of interest is declared.

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