Serum levels of apolipoprotein E in colorectal cancer patients and its relationship with the diagnosis of the disease

Apolipoprotein E and colorectal cancer

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Abstract
Aim: The reprogramming of energy metabolism is one of the emerging hallmarks of cancer. Lipid metabolism plays a critical role in the pathogenesis of many diseases including cancer. Colorectal cancer (CRC) is one of the most aggressive tumors and death-related cancers worldwide. Apolipoprotein E (ApoE) is widely involved in the metabolism of many lipids including cholesterol and chylomicron. Therefore, this study aims to determine the serum ApoE levels in CRC patients and to assess its relationship with the diagnosis and prognosis of CRC. Materials and Methods: Blood samples were collected from 46 normal controls and 62 CRC patients to determine the lipid profile and serum ApoE levels. The demographic characteristics of all the participants were recorded. The expression of ApoE levels in the blood of CRC patients was determined by real-time polymerase chain reaction and compared with the adjusted lipid profile controls. Results: CRC patients had lower ApoE levels in their serum than the controls (mean of ApoE in the CRC patients was 7.346±0.71 vs. 14.86±1.03 in the controls, p < 0.0001). However, this reduced level was not correlated with the progression of CRC. Moreover, the expression of mRNA for ApoE showed a non-significant difference between the groups. Discussion: The current data revealed for the first time that ApoE levels were highly significantly reduced among the CRC patients, but it might not be appropriate to be used as a prognostic marker for CRC progression.
Keywords
Colorectal Cancer; ApoE Expression; Lipid Metabolism

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide. One to two million new cases are diagnosed every year [1]. Overall, CRC ranks third in terms of incidence and second in terms of mortality [2]. CRC is considered the third and the second most common type of cancer occurring in men and women, respectively, worldwide [2]. It is one of the main health problems in Saudi Arabia and ranks first in men and third in women as the most commonly occurring cancer [3]. The middle age for occurrence is 60 years for men and 55 years for women [4]. Although a remarkable improvement has been made in the cancer treatment field, the mortality rate of some cancer types, such as CRC, remains high especially in patients with high-grade tumors. One of the most studied reasons for the increased mortality rate for CRC is drug resistance. This resistance is not only caused by disorders in genetic and epigenetic factors but also by energy metabolism reprogramming in cancer [5]. Recently, advanced cancer research has shown that energy metabolism, especially lipid metabolism, is markedly increased during carcinogenesis [6,7]. Disorders of lipid metabolism have been found to promote cancer development, invasion, and metastasis, indicating that the targeting of lipid metabolism could be a new way to prevent and treat cancer [8].

Apolipoprotein E (ApoE) plays a major role in lipid metabolism, especially in cholesterol metabolism, as it controls the cellular uptake of lipoprotein particles by binding to low-density lipoprotein (LDL) and chylomicron receptors [9]. It has many other functions that may be important for neoplastic growth, such as cell reproduction, immune regulation, and angiogenesis [10,11]. ApoE overexpression has been reported in different carcinomas and adenocarcinomas, and it has been related to different aspects of tumorigenesis such as invasion, metastasis, and carcinogenesis of gastric tumor [12], progression of endometrium cancer [13], increase in lung cancer cells proliferation, migration, and resistance to chemotherapy [14], and moderation of the colon homeostasis, thus making it a risk factor for colon carcinogenesis [15]. Although many studies have shown that ApoE may influence CRC development through inflammation and other pathways such as lipid metabolism reprogramming and insulin regulation [16-18], studies on its functional expression and prognostic significance for CRC, especially in Saudi Arabia, have not been conducted yet. Studies performed on CRC patients in Saudi Arabia showed that most patients developed this type of cancer because of their poor lifestyle choices and limited physical activity. Therefore, this case-control study was performed to determine the expression of ApoE in the blood of CRC patients and to compare it with that in healthy subjects with a normal body mass index (BMI) and lipid profile to elucidate the role of ApoE in the lipid metabolism of CRC patients with different clinical stages and thus its contribution to CRC carcinogenesis. To the best of our knowledge, this study is the first to determine the novel relationship between ApoE levels and CRC tumorigenesis in Saudi Arabia.

Materials and Methods

The extraction of RNA from whole blood was conducted using the QIAamp RNA blood mini kit (catalog no. 52304, Qiagen, Germany). The cDNA samples were prepared using a high-capacity cDNA reverse transcription kit (catalog no. 4368814, Thermo Fisher Scientific, Lithuania). The 2X PowerUP™ SYBR® Green master mix (catalog no. A25741, Thermo Fisher Scientific, USA) was used in the quantitative polymerase chain reaction (qPCR) workflows. The human apolipoprotein E (Apo-E) ELISA kit (catalog no. SG-00319, SinoGeneClon Biotech Co., Ltd., China) was used for measuring serum levels of ApoE.

Subjects and Samples

This case-control study was approved by the research committee of the Unit of Biomedical Ethics at the Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia (reference number 379-17). The study was conducted on 108 male and female volunteers (n=62 CRC patients and n=46 controls) who were interested in participating after signing a research consent form and agreeing to give two blood samples. The blood samples were collected in a lavender top vacutainer containing an EDTA anticoagulant for the RNA extraction and in a red top vacutainer for the serum collection for the biochemical analysis. Samples were collected from September 2017 to August 2018 from the day care unit at King Abdulaziz University Hospital for the CRC patients and from blood banks in King Fahad General Hospital in Jeddah for the healthy controls. The practical work was performed at the Central Lab and Cancer and Mutagenesis Unit at the King Fahd Medical Research Center at King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

Demographic Characteristics

Body weight was measured to the nearest 0.1 kg with a balance scale, and height was measured to the nearest centimeter. BMI was estimated by dividing the body weight (in kilograms) by the square of height (in meters). Waist circumference was measured to the nearest centimeter at the level of the umbilicus with the subject standing and breathing normally. Hip circumference was measured to the nearest centimeter at the level of the iliac crest. The waist-to-hip ratio (WHR) was calculated manually by dividing the waist circumference by the hip circumference.

Determination of the Lipid Profile

The serum levels of triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low- density lipoprotein cholesterol (LDL-C), and cholesterol in mg/dl were calculated automatically by the colorimetric test on the ADVIA Centaur using a chemiluminescent reaction at the biochemistry lab in King Abdulaziz University Hospital.

Determination of the Serum ApoE Levels

The quantitative measurement of the human ApoE level (ng/ml) in serum was performed using a sandwich ELISA commercial kit. Each sample measurement was performed in duplicate for a more accurate result. The reagents of the kit were prepared just before running the experiment based on the manufacturer's instructions. The absorbance of the standard and samples was measured at 450 nm within 15 min after adding the stop solution, and the absorbance of the blank was subtracted from the readings for each standard and sample. A standard

curve was drawn on a linear-log graph paper with the optical densities on the y-axis and the ApoE concentration in (ng/ml) on the x-axis. The concentrations of the samples were calculated by interpolating the regression curve formula ($Y=0.0061X + 0.0637$) and then by multiplying the samples by the serum dilution factor. The measuring range was 1–60 ng/ml, and the minimum detectable concentration was 0.1 ng/ml.

Extraction of the RNA Samples and the qPCR Primer Design
Following the manufacturer's instructions for the QIAGEN-QIAmp RNA blood mini kit, the RNA samples were extracted from the whole blood EDTA samples. The final RNA concentration was determined by reading the 260 nm wavelength absorbance with the DeNovix DS-11 spectrophotometer. Any RNA samples that had an A260 measurement of greater than 0.15 and a reading percentage of A260/A280 ratio in the range of 1.9–2.1 were excluded from the experiments.
The qPCR primers for the human ApoE and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed using the University of California Santa Cruz Genomic Institute (USCS) genome browser. Using the primer 3 website, the primers were designed after confirming all the requirements needed for the qPCR primers. To validate the design, the sequences of the primers were confirmed again in *in silico* PCR using the USCS browser. For the human ApoE, the forward primer was (5'- CTCAGCTCCCAGGTCACC-3'), and the reverse primer was (5'-GGGTCAGTTGTTCCTCCAGT-3'). For the GAPDH, the forward primer was (5'-CACATCGCTCAGACACCATG-3'), and the reverse primer was (5'-ACCAGAGTTAAAAGCAGCCC-3')

Table 1. Mean±SEM of demographic measurements and lipid profile in CRC patients and controls

Variables	CRC patients n= 62	Controls n= 46	p- value
Age (years)	56.62±1.543	52.13±1.826	0.07
Height (cm)	165.1±1.183	164.5±1.168	0.82
Weight (kg)	73.76±2.006	83.78±2.582	0.005**
Body mass index (kg/m²)	27.08±0.728	31.04±0.978	0.004**
Hip (cm)	110.5±2.386	106.5±2.798	0.13
Waist (cm)	101.1±2.542	104.0±2.832	0.96
WHR	0.921±0.017	0.990±0.023	0.02*
Cholesterol (mg/dl)	194.3±8.251	189.9±6.821	0.82
Triglyceride (mg/dl)	187.7±13.24	128.7±12.79	0.0002***
HDL (mg/dl)	55.65±2.523	54.26±3.328	0.44
LDL (mg/dl)	102.3±7.096	109.2±4.835	0.17
Total lipid (mg/dl)	7.263±0.076	7.307±0.059	0.89

*p<0.05, **p<0.001, ***p<0.0001

Synthesis of the Complementary DNA (cDNA)
The cDNA samples were synthesized from highly purified RNA samples using the instructions for the random primer scheme mentioned in the high-capacity cDNA reverse transcription kit. The reagents of the kit were combined to form a 20 μL reverse transcription master mix as follows: 10 μL of the 300 ng RNA samples was added into an Eppendorf tube after a normalization step with nuclease-free water. Then, 2 μL of 10X

RT buffer, 0.8 μL of 100 mM 25X dNTP mix, 2.0 μL of 10X RT random primers, 1.0 μL of Multi Scribe™ reverse transcriptase, 1.0 μL of RNase inhibitor, and 3.2 μL of nuclease-free water were added into the RNA samples in the same tube. The cDNA synthesis was completed using the following four-step thermocycler reaction: step 1) incubation for 10 min at 25 °C, step 2) incubation for 120 min at 37 °C, step 3) incubation for 5 min at 85 °C, and step 4) maintaining of samples for ∞ at 4 °C. All of the synthesized cDNA samples were kept at –20 °C until use for the qPCR experiments.

Real-time qPCR
The SYBR® Green master mix was used to perform the qPCR experiments to measure the expression level of ApoE. To prepare a 20 μL/well reaction, the following mixture was prepared and added to each assigned well: 10 μL of PowerUP™ SYBR™ Green master mix (2X), 1 μL of each forward and reverse primers, 8 μL of nuclease- free water, and 1 μL of template cDNA. The experiment was performed in duplicate for each sample. The optical plate was sealed using an adhesive cover. Depending on the primer melting temperature (Tm) of less than 60 °C, the thermal cycler condition settings were applied for the two genes as shown in the protocol of the SYBR Green's kit as follows: step 1) Uracil-DNA glycosylase (UDG) activation for 2 min at 50 °C), step 2) Dual-Lock™ DNA polymerase for 2 min at 95 °C), step 3) Denaturation at 40 cycles for 15 s at 95 °C), step 4) Annealing at 40 cycles for 15 s at 55 °C–60 °C depending on the primer Tm), and step 5) Extension at 40 cycles for 1 min at 72 °C).

Table 2. The Spearman rank-order correlation between ApoE level and other variables in patients and controls group

Variables	CRC patients		Controls	
	r	p	r	p
Age	-0.1245	0.40	0.0845	0.58
Height	-0.0017	0.99	-0.1342	0.37
Weight	0.0957	0.52	0.1159	0.44
Body mass index (BMI)	0.0951	0.52	0.1878	0.21
Hip	0.0263	0.86	-0.0229	0.88
Waist	0.1132	0.45	0.2268	0.13
WHR	0.0013	0.99	0.2788	0.06
Cholesterol	0.0015	0.99	-0.2053	0.17
Triglyceride	0.1814	0.22	0.1506	0.32
HDL	0.1116	0.46	-0.2359	0.11
LDL	-0.0270	0.86	-0.3281	0.03*
Total lipids	-0.0540	0.72	-0.2269	0.13

*p<0.05

Statistical Analysis
The laboratory data were recorded on an investigative report form. Statistical analysis of the data was performed using GraphPad Prism version 5. The descriptive data were presented as the mean ± standard error of the mean (SEM). For the other statistics, the Mann-Whitney U was used to compare between two groups and the Kruskal-Wallis (H) test was employed to compare among more than two groups. The Spearman's rank-

order correlation was used to examine the relation between two variables in the same group. Furthermore, the expression levels of mRNA were determined with the REST2009 software after comparing them with the expression levels of GAPDH (housekeeping gene). A p- value less than 0.05 (level of significance) was considered statistically significant.

Results

Determination of the Demographic Characteristics and Lipid Profiles of the Study Subjects

This study included CRC patients (n=62) who were grouped by their gender into males (n=47, 75.8%) and females (n=15, 24.2%). The controls (n=46) were grouped into males (n=28, 60.9%) and females (n=18, 39.1%). The comparison between the CRC patients and the controls was conducted using the Mann-Whitney test (Table 1). To analyze the relationship of the ApoE level with the physical and biochemical parameters (lipid profile), the Spearman's rank-order correlation (r) was used. The results showed no significant correlation between the ApoE level and any of the physical and biochemical parameters in the patient group. In the control group, a significant correlation was observed between the ApoE level and the LDL level (p=0.03). This result was independent of the other variables obtained from the partial correlation (Table 2).

Determination of the Serum ApoE Level and its Correlation with CRC Progression

Analysis of the data from the ELISA assay showed a highly significant difference ApoE level in the serum (p < 0.0001). The mean of the ApoE level was 7.346±0.71 in the serum of the CRC patients compared with 14.86±1.03 of the controls (Figure 1). In this study, most of the CRC patients were clinically classified as high-grade CRC patients: stage I (n=7), stage II (n=7), stage III (n=17), and stage IV (n=31). The comparison of the mean values of the serum ApoE levels in each clinical stage by the Kruskal-Wallis analysis with the Dunn's multiple comparison post-test (Table 3) showed no significant association between the serum ApoE levels and the clinical progression of CRC (Figure 1).

Expression of ApoE mRNA in the CRC Patients

The analysis of the qPCR data in this study showed that the expression of ApoE mRNA in the CRC blood samples was not significantly different in comparison with the control samples (p=0.23). The mean of the mRNA expression level of ApoE was 34.45±0.32 in the CRC patients and 33.77±0.39 in the controls.

Table 3. Serum level of ApoE in different clinical stages of CRC

CRC clinical stages	Mean±SEM of serum ApoE level	95% CI		Kruskal-Wallis test p- value
		Lower bound	Upper bound	
Stage I	4.310±1.404	0.6998	7.920	
Stage II	5.950±0.8983	3.456	8.444	0.1828
Stage III	7.888±1.187	5.243	10.53	
Stage IV	7.667±1.221	5.120	10.21	

Discussion

CRC is considered the third and the second most commonly occurring cancer in men and women worldwide, respectively [2]. In Saudi Arabia, CRC has been considered the first and third most commonly occurring cancer in men and women since 2002. The median age for occurrence is 60 years in men and 55 years in women [4]. CRC begins in the large intestine (colon) or rectum. In classic adenoma-carcinoma sequence, it begins as benign polyp (adenoma) and over a period of time with the accumulation of additional mutations, some of these colonic adenomas give rise to colorectal adenocarcinomas. The signs and symptoms of CRC include change in bowel habits, for example, diarrhea or constipation, blood in stool, abdominal pain, incomplete bowel emptying, weakness and fatigue, and weight loss. Some of the factors increasing the risk of CRC include older age, family history of CRC or polyps, chronic colon inflammation, obesity, smoking, alcohol abuse, and physical inactivity. The treatment for CRC depends on the stage of cancer, but there are three main treatment options that are widely used in CRC based on the clinical diagnosis: surgery, chemotherapy, and radiation therapy [19].

Lipid metabolism is strongly associated with cancer tumorigenesis. Reprogramming the energy metabolism is known to be one of the emerging factors of cancer. Knowledge about the correlation between the ApoE expression and CRC tumorigenesis in patients in Saudi Arabia is limited. Studies performed on CRC patients in Saudi Arabia showed that most of the patients developed this type of cancer because of their poor lifestyle choices and limited physical activity. Therefore, this case-control study determined the profile of different lipids and the ApoE expression in the blood of CRC patients and compared them with those of healthy subjects with a normal lipid profile to elucidate the correlation between ApoE and lipid metabolism in CRC patients at different clinical stages and therefore its contribution to CRC progression. To the best of our knowledge, the current study is the first to determine the
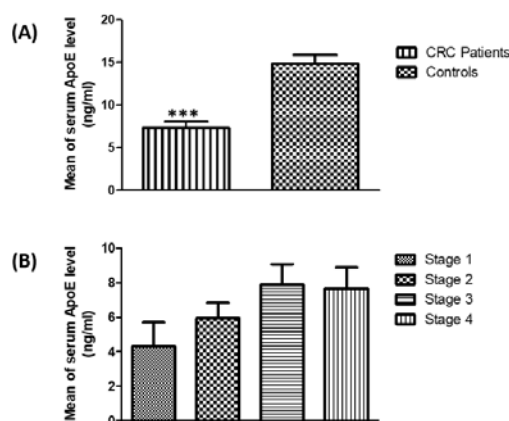

Figure1. Serum level of ApoE in CRC patients. The analysis in figure (A) revealed that the level of ApoE is significantly reduced in the serum of CRC patients compared to healthy controls (p<0.0001). However, this reduction in ApoE serum level is not correlated to CRC progression (p=0.2) as shown by the mean of ApoE level in each stage (B).

relationship between ApoE and CRC tumorigenesis in Saudi CRC patients.

ApoE is a 299-amino-acid glycoprotein [20]. The ApoE gene is located on chromosome 19 and expressed in three isoforms or alleles: Ɛ2, Ɛ3, and Ɛ4; the most common isoform is Ɛ3 [20]. ApoE is not only produced in the liver but also in the brain, kidney, adrenal glands, and macrophages [21]. ApoE has a molecular weight of 34 kDa and a variable plasma concentration based on the assessment and calibration methods that were used to identify ApoE blood level, ethnic group and gender [22]. ApoE is an essential component in the blood plasma because it is responsible for the transportation of cholesterol and its metabolites [21]. Therefore, it reduces the plasma cholesterol level and controls lipid metabolism [20,23]. It also plays a major role in altering the metabolism of cholesterol and bile acids, which are required by carcinogenic cells for their growth and metastasis [23]. In the current study, ApoE was found to be significantly reduced in the serum of CRC patients in comparison with the controls. This finding may help in the diagnosis of CRC. However, the correlation test showed that ApoE was not correlated with any of the physical, biochemical, and clinical stages of CRC patients. These data indicate that the serum level of ApoE may not be helpful in the clinical prognosis of CRC patients.

Less is known about the correlation between ApoE serum or plasma level and carcinogenesis as most published articles on different tumors examined only the effect of the mutations and the single nucleotide polymorphisms in the *ApoE* gene on the lipid profile levels. The few studies that examined the effect of serum or plasma ApoE level were performed either on healthy subjects or in other diseases and disorders rather than cancer. A study performed on 815 healthy Chinese subjects to determine the effect of ApoE polymorphisms and serum ApoE level on the lipid profile found that the ApoE level was influenced by *ApoE* polymorphism in a gene-dependent manner and had different effects on the serum lipid parameters with increasing age and BMI [24]. In a meta-analysis on 1,498 Alzheimer's disease (AD) patients, the level of blood ApoE was reduced in AD patients but not in the healthy controls, and this reduction is a potential risk factor for AD [25]. A case-control study performed on 47 breast cancer patients and 165 controls revealed that the participants with genotypes that include either ε2 or ε3 had lower triglyceride, cholesterol, and LDL-C levels and higher HDL-C levels than those with homozygous ε3 and heterozygous ε3 and ε4. Moreover, the serum levels of the lipids in the blood of breast cancer patients were found to be higher than those of the controls. Therefore, *ApoE* polymorphisms play a major role in the development of breast cancer especially when it is combined with high triglyceride levels [26]. Shinomiya et al. [27] found that altered lipid metabolism may be differentially associated with tumorigenesis in the proximal and distal colorectal cancers. Interestingly, a recent cohort research on 105,949 individuals to determine whether the plasma ApoE level and *ApoE* polymorphisms are associated with all-cause and cause-specific mortality found that the elevated plasma levels of ApoE were associated with the increased risk of cardiovascular and cancer mortality in the ApoE-adjusted group and Ɛ3 Ɛ3 carriers only, and that the reduced plasma levels of

ApoE were associated with the increased dementia-associated mortality in the same adjusted group and carriers [28].

In conclusion, to the best of our knowledge, this study is the first to examine the serum level of ApoE in CRC patients as a diagnostic marker. The serum level tended to be significantly lower in the CRC patients than in the healthy controls. However, this decrease was not correlated with the lipid profile or with the progression of cancer. Further studies are required to confirm this association in a larger population by performing cohort or meta-analysis studies and to reveal the mechanisms behind this association by examining the signaling pathways related to lipid metabolism.

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