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Association between apolipoprotein E genotype, serum lipids, and colorectal cancer in Brazilian individuals

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We evaluated genetic variants of apolipoprotein E (*APOE Hha*I) and their association with serum lipids in colorectal cancer (CRC), together with eating habits and personal history. Eight-seven adults with CRC and 73 controls were studied. *APOE*2* (rs7412) and *APOE*4* (rs429358) were identified by polymerase chain reaction-restriction fragment length polymorphism. *APOE* gene polymorphisms were similar in both groups, but the ε4/ε4 genotype (6%) was present only in controls. The patients had reduced levels (mean ± SD) of total cholesterol and low-density lipoprotein cholesterol fraction (180.4 ± 49.5 and 116.1 ± 43.1 mg/dL, respectively) compared to controls (204.2 ± 55.6, P = 0.135 and 134.7 ± 50.8 mg/dL; P = 0.330, respectively) indicating that they were not statistically significant after the Bonferroni correction. The *APOE*4* allele was associated with lower levels of total cholesterol, low- and high-density lipoprotein cholesterol fraction and increased levels of very low-density lipoprotein cholesterol fraction and triglycerides only among patients (P = 0.014). There was a positive correlation between the altered lipid profile and increased body mass indexes in both groups (P < 0.010). Moreover, a higher rate of hypertension and overweight was observed in controls (P < 0.002). In conclusion, the presence of the ε4/ε4 genotype only in controls may be due to a protective effect against CRC. Lower lipid profile values among patients, even those on lipid-rich diets associated with the *APOE*4* allele, suggest alterations in the lipid synthesis and metabolism pathways in CRC.

Key words: Apolipoprotein E; Colorectal cancer; Lipid profile; Polymorphisms

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Introduction

Colorectal cancer (CRC) is a common cause of death in Western countries where fat-rich diets, at quantities of 40 to 45% of the total calories ingested by the population, have a close relationship with its incidence (1). In addition to the diet, other factors predispose individuals to CRC. These include age, with 90% of cases occurring in individuals older than 50 years, a personal history of adenomas and CRC, a family history of polyposis syndrome and Lynch syndrome, or first-degree relatives with CRC and inflammatory intestinal disease (2). Recent years have been marked by great discoveries about the basic mechanisms involved in the genesis of CRC, which have been possible due to the recognition and description of oncogenes, tumor suppression genes and DNA repair genes (2,3).

There is evidence that the serum levels of total cholesterol (TC) and low-density lipoprotein cholesterol fraction (LDL-C) are positively correlated with the appearance of CRC (4), although this has not always been demonstrated (5). Thus, it is possible that genetic factors that affect the

metabolism of cholesterol also influence susceptibility to cancer. Hence, apolipoprotein E (apo E), which is a multifunctional protein with a role in the transport and metabolism of lipids (6), has also been considered in the etiology of CRC (7-9).

Apo E modulates the concentration of fecal bile acids, substances that may participate in the genesis of colorectal adenocarcinomas, particularly of the proximal colon, where they are in direct contact with the mucous membrane. In this case, the presence of the *APOE*4* allele is associated with lower concentrations of these acids in the gastrointestinal tract, suggesting its protective role against CRC (10).

The *APOE*4* allele seems to exert a protective effect in populations with high exposure to environmental factors predisposing to colon carcinoma (7,8), considering the hypothesis that ε4/ε4 homozygosity at the *APOE* locus was just found among controls, although this effect has not been confirmed in low-risk populations (7-9). Ethnic diversity, distinct eating habits and environmental factors characterize populations, whose profile should be investigated in association with determined genetic variants representing risk factors for this disease. This is the first study including Brazilians to consider the possible relationship between *APOE* gene polymorphisms and CRC. The 5-year survival rate after curative resection of CRC ranges from 40 to 60%. This stresses the necessity of a better understanding of tumoral markers that may indicate the prognosis and permit the selection of patients for a more aggressive therapy aiming at increasing survival. Thus, the objectives of the present study were to analyze the allelic and genotypic distributions of the *APOE Hha*I polymorphism and their association with the lipid profile in patients with colorectal cancer and to characterize the habits and personal histories of patients compared to a control group without this disease.

Patients and Methods

Patients

A total of 160 unrelated individuals attended at the Division of Proctology, Faculdade de Medicina de São José do Rio Preto, from August 2002 to July 2003, were studied. The subjects were divided into two groups: CRC group, 46 women (52.9%) and 41 men (47.1%) with a diagnosis of colorectal neoplasia confirmed by histopathology and ranging on age between 27 and 89 years (mean: 60.6 ± 13.4 years), and control group, 41 women (56.0%) and 32 men (44.0%), clinically healthy individuals with normal results of colonoscopy, confirming the absence of colon neoplasia, during clinical investigation for complex illness involving obscure weakness or refractory anemia, and ranging in age from 21 to 86 years (mean: 61.6 ± 14.7 years). Patients with intestinal inflammatory disease or polyposis were excluded. The study was approved by the Research Ethics Committee of Faculdade de Medicina de São José do Rio Preto, SP, Brazil. All individuals involved in the study were informed in writing about its nature and signed consent forms.

Genomic DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes by the method of Gustincich et al. (11). *APOE* [*APOE*2* (Cys176Arg, rs7412) and *APOE*4* (Cys130Arg, rs429358)] single nucleotide polymorphisms were analyzed by the polymerase chain reaction (PCR) and by the restriction fragment length polymorphism technique. Amplification was carried out in an Eppendorf-Mastercycler thermal cycler (Eppendorf HQ, Germany). The sequences of the primers and the PCR conditions for the *APOE* variants were described by Hixson and Vernier (12). PCR products were analyzed by 1.5% agarose gel electrophoresis followed by ethidium bromide staining and were digested with the endonuclease *Hha*I (Amersham Pharmacia Biotech of Brazil), according to manufacturer instructions. Fragments were identified by 6% polyacrylamide gel electrophoresis. Genotyping was determined using a homozygous sample for the restriction site as positive control. In addition, 10% of all analyses were repeated randomly to confirm the results obtained.

Analysis of serum lipids, personal histories and eating habits

The lipid profile was determined in peripheral blood samples drawn after a 12-h fast. Individuals under medicinal treatment with lipid-lowering drugs and hormones were excluded. The serum concentrations of triglycerides (TG) and TC were determined by enzymatic colorimetric methods (13,14). The serum levels of the high-density lipoprotein cholesterol fraction (HDL-C) were analyzed by precipitation with dextran-magnesium chloride. The levels of LDL-C and of the very low-density lipoprotein cholesterol fraction (VLDL-C) were calculated by the Friedewald formula (15) for TG levels below 400 mg/dL. The reference values used were those recommended by the IV Brazilian Guideline for Dyslipidemia and Atherosclerosis prevention from the Department of Atherosclerosis of Sociedade Brasileira de Cardiologia (16).

Gender, ethnic group, hypertension, diabetes mellitus, overweight including obesity, smoking, alcoholism, and sedentary life style were considered in the personal history. Hypertension was defined as systolic arterial pres-

sure of 140 mmHg or higher and diastolic pressure of 90 mmHg or higher (17). Patients using anti-hypertensive medications were considered to be hypertensive. Body mass index (BMI) was calculated using the formula [weight (kg)/height squared (m^2)], with values of 18.5-24.9 kg/m^2 being considered eutrophic, values of 25.0-29.9 kg/m^2 being considered to indicate overweight, and values of ≥30 kg/m^2 to indicate obesity (18). A fasting blood sugar level ≥126 mg/dL was used for the diagnosis of diabetes mellitus (16). Smoking was analyzed in terms of the duration of the habit and the number of cigarettes per day. Individuals who ingested ≥30 mL alcohol per day were considered to be alcoholics. Absence of sedentary life style was understood as physical exercise on at least three occasions weekly with a minimum duration of 45 min.

All individuals were interviewed using a questionnaire regarding the intake of meat (pork, beef, chicken, and fish), fatty and fried food, fibers (vegetables, and cereals), margarine or butter, and milk (skimmed and whole). For the ingestion of meat, a single alternative identifying the frequency of its consumption per week was marked, indicating zero, once weekly, two or three times weekly or four or more times weekly. For the ingestion of fatty foods and fibers the alternatives little (1 or 2 times weekly), average (3 times weekly) or frequent (more than 3 times weekly) were used. For margarine, butter and milk, the respective alternatives either confirmed their use in the diet or not.

Statistical analysis

The exploratory analysis of the lipid profile included calculations of the means and standard deviation, with differences between groups being assessed by the *t*-test. For the comparative study of the allelic and genotypic frequencies for *APOE*, personal histories and eating habits, the Fisher exact test was applied. An analysis of the main components for the TC, LDL-C, HDL-C, VLDL-C, and TG variables was performed in order to determine hierarchical factors according to their influence on total variation. Variables such as gender, age, BMI, smoking, alcoholism, hypertension, diabetes, practice of physical exercise, and eating habits were analyzed with respect to the hierarchical factors obtained for the lipid profile by applying the Pearson correlation coefficient. Significance was defined as $P < 0.05$, after applying the Bonferroni correction to all P values.

Results

Regarding tumor location, rectal adenocarcinoma predominated (62.1%), followed by right-sided or proximal colon (20.7%), and left-sided or distal colon adenocarcinoma (17.2%), with a predominance of moderately differentiated tumors (Table 1).

*APOE Hha*I polymorphisms

The distribution of alleles and genotypes is presented in Table 2. Note the prevalence of the *APOE*3* allele (control: 0.78; CRC: 0.82), followed by the *APOE*4* allele (0.12 and 0.14, respectively). The ε3/ε3 genotype was the most common in the two groups, varying from 66% (con-

Table 1. Location and histopathological classification of tumors of individuals with colorectal neoplasia.

Histopathological classification of tumor	Location of neoplasia		
	Rectal	Proximal colon	Distal colon
Moderately or well-differentiated adenocarcinoma	48 (55.2%)	15 (17.2%)	14 (16.1%)
Poorly differentiated adenocarcinoma	2 (2.3%)	1 (1.2%)	–
Adenoma	4 (4.6%)	2 (2.3%)	1 (1.5%)

Data are reported as number of individuals and percent within parentheses.

Table 2. Allelic frequencies and genotypic distribution of the apolipoprotein E polymorphisms in individuals with colorectal cancer (CRC) and controls.

*APOE Hha*I	CRC		Control	
Allele	N	Frequency	N	Frequency
*APOE*2*	11	0.06	12	0.08
*APOE*3*	143	0.82	116	0.78
*APOE*4*	20	0.12	18	0.14
N_{Total}	174	1.00	146	1.00
Genotype	N	Percent	N	Percent
ε2/ε2	3	3	1	1
ε2/ε3	3	3	9	12
ε2/ε4	2	2	1	1
ε3/ε3	61	71	48	66
ε3/ε4	18	21	10	14
ε4/ε4	0	0	4	6
N_{Total}	87	100	73	100

N = number of alleles and genotypes. There were no statistical differences between groups when they were compared by the Fisher exact test.

trol) to 71% (CRC), followed by the ε3/ε4 genotype (control: 14%; CRC: 21%). The ε2/ε3 genotype was not significantly more frequent in the control group (12%) than in the CRC group (3%). On the other hand, the ε4/ε4 genotype was not observed in the CRC group and in only 6% of control subjects. The analysis comparing *APOE*2* allele carriers to *APOE*4* allele carriers (homozygous or heterozygous), excluding ε2/ε4 carriers, showed that both groups were similar (data not shown). The *APOE* gene polymorphisms did not exhibit the pattern predicted by Hardy-Weinberg (HW) equilibrium for the CRC group ($\chi^2_{(3)}$ = 28.5; P < 0.001) and for the control group ($\chi^2_{(3)}$ = 7.95; 0.02 < P < 0.05) (data not shown).

Table 3. Demographic characteristics and biochemical data of Brazilian individuals with colorectal cancer (CRC) and controls.

Variable	CRC (N = 79)	Control (N = 58)
Gender (men)	49.4%	46.6%
Ethnics (European descent)	84.8%	84.5%
Hypertension	32.9%	69.0%*
Obesity or overweight	27.8%	65.5%*
Diabetes mellitus	6.3%	5.2%
Smokers	16.5%	13.8%
Ex-smokers	32.9%	34.5%
Alcoholics	16.5%	3.5%
Ex-alcoholics	3.8%	6.9%
Sedentary life style	58.2%	63.8%
TC (mg/dL)	180.4 ± 49.5	204.2 ± 55.6
HDL-C (mg/dL)	39.6 ± 15.3	45.0 ± 13.9
LDL-C (mg/dL)	116.1 ± 43.1	134.7 ± 50.8
VLDL-C (mg/dL)	24.5 ± 17.5	25.6 ± 11.9
TG (mg/dL)	116.2 ± 56.6	128.0 ± 59.5

Continuous variables are reported as means ± SD or percent. TC = total cholesterol; HDL-C = high-density lipoprotein cholesterol fraction; LDL-C = low-density lipoprotein cholesterol fraction; VLDL-C = very low-density lipoprotein cholesterol fraction; TG = triglycerides. *P < 0.05 (Fisher exact test).

Personal history, eating habits and serum lipids

Table 3 presents the demographic characteristics (gender, ethnic background, hypertension, obesity or overweight, diabetes mellitus, smoking, alcoholism, and sedentary life style) and biochemical data of CRC and control groups. There was a prevalence of European descent in both groups (about 80%). Moreover, a higher rate of hypertension and overweight or obesity was observed in the control group (69.0 and 65.5%, respectively; P < 0.0015 for both). Altered values (mean ± SD) for TC (204.2 ± 55.6 mg/dL) and LDL-C (134.7 ± 50.8 mg/dL) in the control group compared to the CRC group (180.4 ± 49.5 and 116.1 ± 43.1 mg/dL, respectively) were not statistically significant after the Bonferroni correction.

Comparative analysis of the two groups for the ingestion of meat, fiber and fats showed similar intake of fat.

Lipid profile and *APOE Hha*I

The lipid profile is presented in association with the genotypes for *APOE*, with means ± SD for each variable for both groups (Table 4). Note that for the CRC group the mean TC value was within the reference range for all genotypes, while in the control group with the ε3/ε3 genotype, the mean value was apparently higher (213.7 ± 59.4 mg/dL) compared to the CRC group (182.3 ± 56.0 mg/dL, P = 0.05, after Bonferroni correction). Mean HDL-C values were below the reference values in the CRC group with the ε2/ε3 (33.3 ± 8.3 mg/dL) and ε3/ε4 genotypes (34.4 ± 13.5 mg/dL), while they remained within the recommended range in the control group. The mean TG levels for both groups were within the reference range for all genotypes.

Table 4. Serum lipid concentrations in Brazilian individuals with colorectal cancer (CRC) and controls according to *APOE* gene polymorphisms.

APOE gene polymorphisms	Lipids (mg/dL)				
	TC	HDL-C	LDL-C	VLDL-C	TG
ε2/ε3					
CRC (N = 3)	179.6 ± 13.0	33.3 ± 8.3	122.6 ± 0.57*	27.0 ± 2.0	134.0 ± 10.5
Control (N = 8)	161.2 ± 25.8	52.1 ± 15.4	93.2 ± 23.6	21.0 ± 13.2	106.0 ± 67.4
ε3/ε3					
CRC (N = 53)	182.3 ± 56.0*	41.9 ± 16.1	118.9 ± 48.5	21.2 ± 10.9	107.6 ± 55.8
Control (N = 41)	213.7 ± 59.4	43.4 ± 13.7	143.9 ± 54.1	26.5 ± 12.4	132.6 ± 61.6
ε3/ε4					
CRC (N = 18)	176.5 ± 36.5	34.4 ± 13.5	109.1 ± 32.4	32.7 ± 30.2	131.9 ± 63.4
Control (N = 6)	185.6 ± 36.9	45.1 ± 14.9	121.0 ± 25.7	22.1 ± 5.0	109.8 ± 24.8

Data are reported as mean ± SD. TC = total cholesterol; HDL-C = high-density lipoprotein cholesterol fraction; LDL-C = low-density lipoprotein cholesterol fraction; VLDL-C = very low-density lipoprotein cholesterol fraction; TG = triglycerides. *P = 0.05 compared to control (*t*-test).

Combination of variables

A multivariate logistic regression analysis performed to test the main

components, identified as Factors 1, 2, and 3, showed the relationship between the lipid profile variables. In this case, Factor 1, which selected individuals with high or low levels of TC, LDL-C, VLDL-C, and TG all together, was responsible for 50.39% of the total variants of the lipid profile among the individuals. The mean Factor 1 value was significantly lower in the CRC group (-0.25 ± 1.52) compared to controls (0.35 ± 1.63, P = 0.016). This means that the patients, in general, had lower levels of TC, LDL-C, VLDL-C, and TG compared to controls. Factor 2 explained 28.5% of the total variation, identifying individuals with high levels of TC, HDL-C and LDL-C and reduced levels of VLDL-C and TG and vice versa. The mean Factor 2 value was significantly higher in the CRC group (0.17 ± 1.24) compared to controls (-0.24 ± 1.10, P = 0.021). This indicates that the patients, in general, had reduced levels of TC, LDL-C and HDL-C and high levels of the VLDL-C and TG subset compared to controls, with a tendency to the inverse result for the controls. Factor 3 explained 16.6% of the total variation of the lipid profile and identified higher levels of HDL-C and VLDL-C and reduced levels of LDL-C or vice versa, with similar means for the two groups (P = 0.370).

Regarding the analysis of factors in relation to alleles, there was a significant relationship only among patients, whose mean Factor 2 value was significantly higher in individuals with the *APOE*4* allele (0.60 ± 1.48) compared with individuals with the *APOE*3* allele (-0.28 ± 1.02; P = 0.014). This means that higher levels of TC, LDL-C and HDL-C and lower levels of VLDL-C and TG were, in general, seen in individuals with the *APOE*3* allele compared to individuals with the *APOE*4* allele.

Factors 1, 2, and 3, representing the lipid profile, were also analyzed with respect to BMI, age, smoking, hypertension, diabetes, and eating habits. A significant positive correlation was noted between Factor 1 and BMI in CRC (r = 0.306, P = 0.007) and control groups (r = 0.388, P = 0.003). This means that individuals with altered lipid profile levels, in general had increased BMI values. Moreover, in the controls, Factor 1 was also positively correlated with age (r = 0.367, P = 0.005). The other variables did not show statistically significant relationships with the main components identified as Factors 1, 2, and 3 in either group (data not shown).

Discussion

The present data indicate that the allelic and genotypic distributions for *APOE* gene polymorphisms were similar between patients with CRC and controls (8,9). Moreover, our study failed to provide outright confirmation of the conclusions of Kervinen et al. (10) in view of the significantly lower frequency of the *APOE*4* allele in patients with proximal carcinoma or adenoma compared to patients with distal CRC and controls. However, the ε4/ε4 genotype detected only in controls suggests a possible protective effect of the *APOE*4* allele, as reported by others (8,10). In contrast with the current results of a prevalence of female patients and rectal adenocarcinoma, the increased risk of colon cancer in individuals with the ε2/ε3 genotype was reported particularly in men (8).

In our series, the genotypic distribution observed for *APOE* in both groups was not in HW equilibrium. Similar situations in case-control type studies with analysis of single nucleotide polymorphisms have also been reported by others (19-21). The absence of the HW equilibrium suggests doubts including that evolutive factors and the criteria utilized in the selection of randomized patients may have changed the genotypic frequencies.

The analysis of the main components identified by the relationship between variables of the lipid profile confirmed the presence of reduced levels of TC, LDL-C, VLDL-C, and TG as a whole in CRC, with an association among the variables responsible for 50.3% of the total variation in the lipid profile. In fact, the association between CRC and reduced cholesterol levels was also reported by others (22,23), although its combination with obesity seems to indicate a four-times greater risk for colon cancer in men compared with a group showing average values of both variables (23), data not available from the current study with a predominance of rectal adenocarcinoma. In contrast, overweight and obesity were prevalent in controls, although both groups showed a positive correlation between BMI and lipid profile, in agreement with other reports (24). It is possible that the period of evolution of the disease during the selection of the patients (in the current study only 2.3% of the subjects had slightly differentiated adenocarcinoma), the gender (23) and age (25) may contribute to the differences in results between the studies. In addition, visceral obesity has also been reported to be a risk factor for the disease (26). Thus, Schoen et al. (27), in a study of patients with CRC, even without an association between BMI and CRC, observed a relationship between waist circumference and waist/hip index and the disease. Moreover, the discrepancy in the distribution of fat between men and women should be emphasized, as also should hormone replacement therapy for postmenopausal women, which is associated with a lower risk of colon cancer (28).

In the present study, the frequency of ingestion of fibers and red and white meat was similar for both groups. However, it may be suggested that cooking methods such

as frying meat excessively may influence the production of heterocyclic amines or polycyclic aromatic hydrocarbonates, reactive metabolites with a toxic action, increasing the risk of CRC (29), while dietary constituents such as folic acid from vegetables may reduce the risk for the disease (26). Studies of an association between eating habits and risk for CRC require careful evaluation with the identification not only of food classes, but also of food types and cooking practices, which were not evaluated in the current study. Also, sedentary life style prevailed among both CRC and control subjects, probably due to the older age range (mean age was about 60 years) in both groups. Participants, in general, did not practice physical exercises in a regular manner. In fact, physical exercise has been considered to be a protective factor against CRC (30).

The lipid profile was positively correlated with age only for the control group, with values similar to those of the general population (24), except for women, probably because postmenopausal women were included in this study, as were alcoholics. Alcoholism was prevalent in patients as also reported by other investigators, with a co-carcinogenic effect together with cigarettes (30), that in our series was similar between both groups. Fujimori et al. (31), in an evaluation of the influence of the consumption of alcohol on the association between serum cholesterol levels and colorectal adenoma or adenocarcinoma, observed significantly lower levels of TG and cholesterol in patients with adenoma who consumed alcoholic beverages on a daily basis, compared to those without the disease. These results suggest modifications in the lipid synthesis and metabolism secondary to CRC.

Studies have demonstrated a significantly increased risk of CRC, and particularly colon cancer, in subjects with a cluster of three conditions: hypertension, excessive weight and lower HDL-C levels (32). In the present study, hypertension was more prevalent in the control group than in the CRC group, in agreement with other studies (33), in which data showed the absence of any association between CRC and hypertension (34). However, in an Australian population, hypertension, coronary artery disease and chronic arthritis were identified as independent risk factors for CRC (33). Moreover, a meta-analysis showed a 30% increase in relative risk for the development of CRC in patients with diabetes mellitus compared to those without the disease (35). Thus, it is possible that the association between insulin resistance and CRC is mainly determined by adiposity (32), which was not analyzed in the present study due to the low frequency of diabetes mellitus in both groups.

The presence of the ε4/ε4 genotype only in control subjects suggests its protective effect against CRC. The association of the *APOE*4* allele with lower levels of TC and LDL-C in the CRC group, different from that which occurs in the general population, suggests alterations in the lipid synthesis and metabolism pathways in CRC. However, the mechanism of the involvement of *APOE* in carcinogenesis is not clear and further studies with larger samples are necessary to confirm this in the Brazilian population.

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