Combination of Cytochrome P450 Gene Polymorphisms Enhancing the Risk for Sporadic Colorectal Cancer Related to Red Meat Consumption

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Abstract

Susceptibility to sporadic colorectal cancers (CRC) is generally thought to be the sum of complex interactions between environmental and genetic factors, all of which contribute independently, producing only a modest effect on the whole phenomenon. However, to date, most research has concealed the notion of interaction and merely focused on dissociate analyses of risk factors to highlight associations with CRC. By contrast, we have chosen a combinative approach here to explore the joint effects of several factors at a time. Through an association study based on 1,023 cases and 1,121 controls, we examined the influence on CRC risk of environmental factors coanalyzed with combinations of six single nucleotide polymorphisms located in cytochrome P450 genes (c.–163>A>C and c.1548T>C in CYP1A2, g.–1293G>C and g.–1053C>T in CYP2E1, c.1294C>G in CYP1B1, and c.430C>T in CYP2C9). Whereas separate analyses of the SNPs showed no effect on CRC risk, three allelic variant combinations were found to be associated with a significant increase in CRC risk in interaction with an excessive red meat consumption, thereby exacerbating the intrinsic procarcinogenic effect of this dietary factor. One of these three predisposing combinations was also shown to interact positively with obesity. Provided that they are validated, our results suggest the need to develop robust combinative methods to improve genetic investigations into the susceptibility to CRC. (Cancer Epidemiol Biomarkers Prev 2007;16(7):1460-7)

Introduction

Given its high prevalence and poor prognosis, colorectal cancer (CRC) is very much a public health issue in industrialized Western countries. In France, it is the third most frequent cancer (>36,000 new cases were diagnosed in 2000) and the second cause of cancer-related death (16,000 deaths were caused by CRC in 2000 for both sexes; ref. 1). Moreover, the current aging of the French population tends to drastically increase the number of incident cases, which warrants research studies on strategy development for CRC prevention.

The vast majority of CRCs are sporadic disorders, the genesis of which includes both environmental and heritable factors (2). Epidemiologic studies on Western populations have emphasized the large contribution of food and lifestyle to sparcancer CRC risk (3-9). High-fat and low-fiber diets, as well as alcohol, tobacco, and red or processed meat consumption, have been shown to produce high levels of polycyclic aromatic hydrocarbons and heterocyclic aromatic amines. These procarcinogenic agents are potentially very harmful and may play a key role in the malignant transformation of cells by interacting with DNA (10, 11). However, their biological deleterious effects cannot be exerted without an oxidative activation by phase I cytochrome P450 (CYP) isoenzymes (12). CYPs are encoded by highly polymorphic genes, which results in a remarkable interindividual variability of enzymatic activity. Therefore, the level of procarcinogens activated by an individual depends on genetic background, which determines the degree of susceptibility to procarcinogen exposure and, consequently, to cancer.

Genetic association studies have reported several examples of single nucleotide polymorphisms (SNP) in CYP genes correlated to modulations of CRC risk, whether alone (13-22) or following interaction with environmental factors such as red meat or cigarette consumption (12, 23-26). Despite laying the foundations for further genetic investigations, these studies still provide too partial a view of the cancer susceptibility phenomenon. In fact, they do not take into account the functional redundancy of CYP enzymes and they usually only analyze the modifications of cancer risk associated with an environmental factor and a unique SNP at a time. In fact, the same alimentary compound can be the substrate of numerous CYP enzymes, the polymorphisms of which may all interfere with CRC risk. Rather than unique genetic polymorphisms, these are combinations of polymorphisms that determine the individual genetic background that will finally potentiate the intrinsic promoting or protective effect of xenobiotics on colorectal carcinogenesis (27). Thus, a clue to the understanding of susceptibility to CRC probably involves an examination of these combinations of polymorphisms conducted in the environmental setting.
This is precisely the target we set ourselves through the population-based case-reference study that we report here. In the present work, we coanalyzed six SNPs of CYP genes—c.−163A>C and c.1548T>C in CYP1A2, g.–1293G>C and g.–1053C>T in CYP2E1, c.1294C>G in CYP1B1, and c.430C>T in CYP2C9—and various environmental factors selected from a bibliographical meta-analysis among the factors presumably the most relevant to colorectal carcinogenesis. We investigated combinations of CYP allelic variants that could serve as biomarkers for CRC prevention and/or prognosis, regarding their possible influence on the relative risk for developing CRC when interacting with environmental factors.

Materials and Methods

Experimental Design and Assembly of the Cohorts. To investigate potential associations between sporadic CRCs and both environmental and genetic factors, we conducted a case-controlled association study. From December 2002 to March 2006, we assembled two cohorts of 1,023 patients and 1,121 controls. The recruitment of these individuals was carried out in the Pays de la Loire region, which is located in the west of France and incorporates the administrative districts of Loire Atlantique, Maine et Loire, Mayenne, Sarthe, and Vendée. Six public hospitals and five clinics situated within these districts gave their agreement to collaborate in this project, thereby constituting a network of hepatogastroenterologists and oncologists specialized in CRCs. All patients with a personal history of CRC diagnosed at an age ≥40 years were eligible for the case cohort provided that there was no suspicion of major genetic predisposition (familial form of CRC).

Most of the control individuals were selected during a routine check-up by physicians working in one of two Health Examination Centers of region Pays de la Loire. Recruitment of individuals ≥70 years was completed in the departments of internal medicine and hepatogastroenterology of the University Hospital of Nantes. In this way, we made up a control cohort that matched the case cohort according to sex, 5-year age groups, and geographic origins. Control individuals were eligible to be included in the study if they were ≥40 years of age, lived in the study region, spoke French, and were physically and mentally able to participate in a personal interview of ~15 to 20 min. Unaffected individuals with a familial history of CRC or polyps were noneligibile.

To meet the requirements of the local (Comité de Protection des Personnes dans la Recherche Biomédicale) and national (Commission nationale de l’Informatique et des Libertés) ethics committees that approved the study, a written informed consent was obtained from each individual included, after having been clearly explained the research protocol by a physician. During the examination, two venous blood samples of 4 mL each were collected in EDTA tubes from the participant and were used later on for extraction of genomic DNA. All information regarding participants was made anonymous after the collection of blood samples.

In the presence of the physician, each participant answered the same one-page standardized questionnaire that was especially drawn up to provide valuable information on demographic characteristics, regular diet, weight and height, personal history of diabetes mellitus and dyslipidemia, physical activity, and smoking habits (see Fig. 1). In this multiple-choice questionnaire, we did not take into account every potential risk factor reported in the literature but we focused more on some of the environmental factors among the most relevant to colorectal carcinogenesis. It is worth noting that we used the grandparents’ place of residence—or by default, the parents’ place of residence—to define the geographic origin of each participant. Questions about eating habits had been developed with the help of a nutritionist to cover the main categories of food components found in the French diet. Participants were questioned only about the average consumption frequency of foods, whereas quantitative questions were deliberately avoided to keep the questionnaire clear and simple.

For cases, we additionally requested endoscopy and histology reports from the study oncologist or hepatogastroenterologist. To obtain homogeneous data, a standardized report model designed as a multiple-choice questionnaire was provided to each physician, which notably included questions about tumor aspect and location, presence of metastasis, and tumor infiltration.

All data provided by both the participants and the physicians through the different questionnaires were stored in a common electronic SQL database set up for the sole purpose of the present study. This database also enables the collection of genetic data generated as explained below.

Genotype Analysis. Genomic DNA was extracted from collected blood samples using the Nucleon BACC2 kit (GE Healthcare). DNA concentrations were calculated using PicoGreen technology (Molecular Probes) and diluted to 10 ng/μL in 96-well format after a customized automated procedure developed by Microlab Star (Hamilton Robotics).

All study participants were genotyped for six SNPs related to drug-metabolizing enzymes CYP involved in heterocyclic aromatic amines and polycyclic aromatic hydrocarbons carcinogen metabolism: SNPs rs762551 (c.–163A>C; CYP1A2*F) and rs2470890 (c.1548T>C; p.N516N) in CYP1A2, rs3813867 (g.–1293G>C; CYP2E1*5A and CYP2E1*5B) and rs3031920 (g.–1053C>T; CYP2E1*5A and CYP2E1*5B) in CYP2E1, rs1058363 (c.1294C>G; p.V432L; CYP1B1*3) in CYP1B1, and rs1799853 (c.430C>T; p.R144C; CYP2C9*2A, CYP2C9*2B, and CYP2C9*3) in CYP2C9. These six SNPs were selected for their relevance to sporadic CRC according to a bibliographical meta-analysis done through PubMed.

Genotypes were determined using high-throughput TaqMan allelic discrimination tests. Primers and dye-labeled MGB-NFQ probes were designed and synthesized by Applied Biosystems (Supplementary Table S1). Reactions were set up in a 384-well plate in a 6 μL final volume, including 2.25 μL of 2× Universal Master Mix, 0.11 μL of 40× Assay Mix, 1.64 μL of water, and 20 ng of genomic DNA (10 ng/μL). Reaction plates were thermocycled in an i-cycler (Bio-Rad Laboratories): An initial 10 min denaturation at 95°C was followed by 45 cycles including a denaturation step at 92°C for 15 s and an annealing/extension step at 60°C for 1 min. After amplification, end-point fluorescence readings were conducted on an Applied Biosystems ABI 7900HT sequence detection system. Genotypes were assigned using the allelic discrimination software SDS v2.1 (Applied Biosystems).

Statistical Analysis

Determination of CRC Risk Associated with Environmental Factors Analyzed Independently. The statistical analysis was done with SAS 9.1 statistical software (SAS Institute, Inc.). Continuous variables were reported as median and range and compared between the case and control groups by Mann-Whitney U test. Categorical variables were reported as number of patients (percentages) and compared by χ² or Fisher’s exact tests when appropriate. P values <0.05 were considered to be statistically significant.

Univariate analyses were done using conditional logistic regression to account for the matching of cases and controls. An appraisal was made on the association between environmental variables adjusting for age followed by an assessment of the odds ratio (OR) of cancer cases together with 95% confidence intervals (95% CI). Multivariate analyses were then done using an iterative stepwise selection procedure to select the variables that were significantly associated with CRC, as...
assessed by the likelihood ratio test (variable candidates for the model were those associated with CRC in univariate analyses with the $P < 0.15$ criterion; variables were selected in the model using the $P < 0.05$ criterion).

**Determination of CRC Risk Associated with CYP Allelic Variants Analyzed Independently.** To assess the risk of CRC associated with each of the six CYP SNPs listed above, we used SNPStats, a free web-based tool designed for genetic epidemiology purposes (28). SNPStats has a user-friendly interface thanks to the PHP server programming language and uses the free software environment R for statistical computing implemented by the algorithm of the package genetics. As regards our study, the association with cancer was estimated for each SNP by OR of cancer cases along with 95% CI; calculations were adjusted for age and sex, and followed an unconditional logistic regression model. For each SNP, homozygous carriers of the

**QUESTIONNAIRE – LIFE HABITS to be completed by the patient**

**Name:**

**Grands-parents’ birthplace** (city or region or district): ... ... ... ... ... ... ... ...

**By default, parents’ birthplace:** ... ... ... ... ... ... ...

**Caution : a unique answer is required for each question**

**Are you:**

- Non-smoker? ❑
- Current smoker? ❑
- Former smoker? ❑ (stop date: ... ... ... ... ...)

**What amount of time do you spend on physical activity (e.g. walking, biking, or other sports):**

- Less than 1 hour / week? ❑
- 1 to 3 hours / week? ❑
- More than 3 hours / week? ❑

**How often do you drink alcohol:**

- Never? ❑
- Sometimes? ❑
- 2 to 3 times / week? ❑
- Everyday? ❑

**How often do you eat red meat (beef, lamb...):**

- Once / week or less? ❑
- 2 to 4 times / week? ❑
- More than 5 times / week? ❑

**How often do you eat poultry and/or white meat (chicken, guinea fowl, turkey, rabbit, calf...):**

- Once / week or less? ❑
- 2 to 4 times / week? ❑
- More than 5 times / week? ❑

**How often do you eat cold cuts:**

- Once / week or less? ❑
- 2 to 4 times / week? ❑
- More than 5 times / week? ❑

**How often do you eat fish:**

- Once / week or less? ❑
- 2 to 4 times / week? ❑
- More than 5 times / week? ❑

**What is your favorite cooking method (for meat, poultry and fish):**

- Roasting or frying with fats? ❑
- Roasting or frying without fats? ❑
- Grilling? ❑
- Steaming? ❑

**How often do you eat fruits:**

- Less than once / day? ❑
- Once / day? ❑
- Twice / day or more? ❑

**How often do you eat vegetables:**

- Less than once / day? ❑
- Once / day? ❑
- Twice / day or more? ❑

**How often do you eat pastries, viennoiseries, ice:**

- Less than once / week? ❑
- Once / week? ❑
- Twice / week or more? ❑

**How often do you eat dairy products (milk, cheese, yoghurt, custard dessert...):**

- Once / day or less? ❑
- Twice / day? ❑
- 3 times / day or more? ❑

**Do you rather eat:**

- White bread? ❑
- Wholemeal bread? ❑

Please, be sure that you answered to all the questions and thank you for your participation.

**Figure 1.** Standardized questionnaire on life habits fulfilled by the study participants.
Table 1. General characteristics of study patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Case (n = 1,023)</th>
<th>Control (n = 1,121)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>67 (40-99)*</td>
<td>62 (41-101)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;18.5</td>
<td>21 (1)*</td>
<td>11 (1)</td>
</tr>
<tr>
<td>18.5-24.9</td>
<td>411 (42)</td>
<td>544 (49)</td>
</tr>
<tr>
<td>25-29.9</td>
<td>393 (40)</td>
<td>405 (36)</td>
</tr>
<tr>
<td>&gt;30.0</td>
<td>152 (16)</td>
<td>157 (14)</td>
</tr>
<tr>
<td>Sex ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>632 (62)*</td>
<td>609 (54)</td>
</tr>
<tr>
<td>Female</td>
<td>391 (38)</td>
<td>512 (46)</td>
</tr>
</tbody>
</table>

NOTE: Data are median (range) for continuous variables or numbers of patients (percentages) for categorical variables.

*P < 0.01.

most frequent allele in the cohorts were used as the reference for the analyses. Calculations of ORs were double checked by statistical analysis done with SAS 9.1 statistical software (SAS Institute).

Analyses of gene-environment interactions were then done with SNPStats, which proposes statistical models for calculation of risk associated with one pair of interacting variables. For each of the six CYP SNPs, we investigated all the possible interactions with the environmental covariables of the questionnaire completed by the study participants, and we calculated 95% CI ORs of cancer cases to determine if any “SNP-environmental factor” pair could modify the risk for CRC in our cohorts. A likelihood ratio test was used to investigate interaction among variables of interest.

Analysis of CRC Risk Associated with Multiple-SNPs Combinations. A special option of the software SNPStats is the haplotype analysis done through R package haplo.stats.

Using genotypes obtained at different points of polymorphisms (SNPs) located on the same chromosomal region, the software makes it possible to predict and rebuild the haplotypes characterizing a group of individuals through a log-additive model. Although the six CYP SNPs chosen for our study are not all located on the same chromosome and, by definition, cannot belong to a common haplotype, we nevertheless used this SNPStats option to predict preferential combinations of these six SNPs. Based on the genotyping results determined for the six single SNPs in the patient and control cohorts, an evaluation was made of the relative frequencies of multiple-SNP combinations present in the cohorts and the corresponding samples sizes were calculated. Logistic regression was then applied to these data to analyze the associations of the SNP combinations to CRC risk translated into 95% CI ORs.

By use of SNPStats, we investigated the possible SNP combinations interacting with environmental factors and we estimated their associations to CRC risk with 95% CI ORs.

Results

The present work is based on 1,023 patients with sporadic CRC and 1,121 unaffected controls whose main general characteristics are indicated in Table 1. The mean age is slightly higher for cases than for controls (67 versus 62 years old; P 0.01), mainly because of the overrepresentation of individuals ≥70 years old in the case cohort [404 individuals (39%) versus 199 (18%) in the control cohort] despite our efforts to match the two cohorts by age. Mean body mass indexes are approximately equal between the two cohorts (26 kg/m² in patients versus 25 kg/m² in controls; P 0.01). As regards gender distribution, males are more numerous in both cohorts but they are slightly underrepresented in the control cohort (62% of

Table 2. Univariate and multiple variable models of promoting and protective factors independently associated with CRC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analyses</th>
<th>Multivariate analyses*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Female gender</td>
<td>0.71 (0.59-0.85)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Physical activity, &lt;1 h/wk</td>
<td>1.82 (1.44-2.29)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tobacco, former and current smokers</td>
<td>1.04 (0.86-1.24)</td>
<td>0.705</td>
</tr>
<tr>
<td>Alcohol consumption, never</td>
<td>1.66 (1.25-2.11)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cold cuts, ≤twice a week*</td>
<td>1.43 (1.19-1.71)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Red meat, ≥5 times a week**</td>
<td>3.41 (1.93-6.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>White meat, ≥2 times a week</td>
<td>1.04 (0.86-1.26)</td>
<td>0.701</td>
</tr>
<tr>
<td>Fish, ≥2 times a week</td>
<td>0.91 (0.76-1.08)</td>
<td>0.279</td>
</tr>
<tr>
<td>Vegetables, ≤once a day*</td>
<td>0.92 (0.67-1.26)</td>
<td>0.597</td>
</tr>
<tr>
<td>Fruits, ≤twice a day**</td>
<td>1.33 (1.11-1.60)</td>
<td>0.002</td>
</tr>
<tr>
<td>Dairy products, ≤once a day</td>
<td>1.50 (1.24-1.81)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cooking, grilling, roasting or frying without fats**</td>
<td>0.87 (0.58-1.31)</td>
<td>0.507</td>
</tr>
<tr>
<td>Cooking, roasting or frying with fats**</td>
<td>1.08 (0.71-1.64)</td>
<td>0.716</td>
</tr>
<tr>
<td>Bread, wholewheat bread***</td>
<td>0.56 (0.44-0.70)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes, yes</td>
<td>2.31 (1.62-3.28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dyslipidemia, yes</td>
<td>0.74 (0.60-0.92)</td>
<td>0.007</td>
</tr>
<tr>
<td>Pastry, once a week</td>
<td>1.22 (0.93-1.60)</td>
<td>0.146</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not analyzed.

*Multivariate analyses were done only on variables that were selected for their significant association with CRC according to univariate analyses (using the P < 0.05 criterion).

†References used for the variables were male gender.

‡One to 3 h or >3 h a week.

§Nonsmokers.

‖Sometimes or twice to thrice a week or everyday.

††Once a week or less.

‡‡Once a week at most.

†††Less than once a day.

‡‡‡Twice a day or more.

††††Twice a day or three times a day or more.

‡‡‡‡Steaming.

***White bread.

††††No.

†††††Less than once a week.
Table 3. Analysis of single-SNP association with CRC (n = 2,131; OR calculations were adjusted by sex and age)

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Genotype</th>
<th>Controls</th>
<th>Patients</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>c.–163A&gt;C (rs762551)</td>
<td>A/A</td>
<td>553 (49.5%)</td>
<td>514 (50.7%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/C</td>
<td>480 (42.9%)</td>
<td>420 (41.5%)</td>
<td>0.98 (0.81-1.17)</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>85 (7.6%)</td>
<td>79 (7.8%)</td>
<td>1.08 (0.77-1.51)</td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>c.1548T&gt;C (rs2470890)</td>
<td>T/T</td>
<td>454 (40.6%)</td>
<td>428 (42.2%)</td>
<td>0.98 (0.81-1.18)</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/C</td>
<td>523 (46.8%)</td>
<td>464 (45.8%)</td>
<td>0.98 (0.81-1.18)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>141 (12.6%)</td>
<td>121 (11.9%)</td>
<td>0.96 (0.72-1.27)</td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>g.–1293G&gt;C (rs3813867)</td>
<td>G/G</td>
<td>1,029 (92%)</td>
<td>944 (93.2%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G/C</td>
<td>88 (7.9%)</td>
<td>67 (6.6%)</td>
<td>0.81 (0.58-1.14)</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>1 (0.1%)</td>
<td>2 (0.2%)</td>
<td>2.17 (0.18-25.47)</td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>g.–1053C&gt;T (rs2031920)</td>
<td>C/C</td>
<td>1,027 (91.9%)</td>
<td>940 (92.8%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/T</td>
<td>90 (8.1%)</td>
<td>67 (6.6%)</td>
<td>0.80 (0.57-1.12)</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>1 (0.1%)</td>
<td>6 (0.6%)</td>
<td>5.77 (0.67-49.51)</td>
<td></td>
</tr>
<tr>
<td>CYP1B1</td>
<td>c.1294C&gt;G (rs1056836)</td>
<td>C/C</td>
<td>368 (32.9%)</td>
<td>317 (31.3%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/T</td>
<td>576 (51.5%)</td>
<td>507 (50%)</td>
<td>0.99 (0.81-1.20)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>1 (0.1%)</td>
<td>6 (0.6%)</td>
<td>5.77 (0.67-49.51)</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>c.430C&gt;T (rs1799853)</td>
<td>C/C</td>
<td>174 (15.6%)</td>
<td>189 (18.7%)</td>
<td>1.22 (0.94-1.58)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/T</td>
<td>821 (73.4%)</td>
<td>745 (73.5%)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>280 (25%)</td>
<td>247 (24.4%)</td>
<td>0.96 (0.78-1.17)</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17 (1.5%)</td>
<td>21 (2.1%)</td>
<td>1.34 (0.69-2.60)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Analysis of association to CRC risk of multiple CYP SNPs combinations interacting with the environmental variable “red meat consumption”

<table>
<thead>
<tr>
<th>Combination</th>
<th>No. individuals exhibiting the combination</th>
<th>Red meat consumers ≤4 times/wk</th>
<th>OR (95% CI)</th>
<th>Red meat consumers ≥5 times/wk</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Multiple-SNPs combination and red meat cross-classification interaction table (n = 2,087, adjusted by age and sex)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGCC</td>
<td>659 (31.6%)</td>
<td>1.00</td>
<td>13.72 (8.53-22.06)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCCGC</td>
<td>461 (22.1%)</td>
<td>1.09 (0.88-1.36)</td>
<td>4.81 (2.08-11.14)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCGGCC</td>
<td>280 (13.4%)</td>
<td>0.95 (0.73-1.24)</td>
<td>3.88 (1.20-12.55)</td>
<td>0.024</td>
<td></td>
<td></td>
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<tr>
<td>CCGGCC</td>
<td>204 (9.8%)</td>
<td>0.87 (0.66-1.15)</td>
<td>14.20 (7.64-26.42)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGCGT</td>
<td>92 (4.4%)</td>
<td>0.84 (0.54-1.32)</td>
<td>36.87 (18.93-71.81)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Multiple-SNPs combinations within red meat (n = 2,087, adjusted by age and sex)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGCC</td>
<td>659 (31.6%)</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCCGC</td>
<td>461 (22.1%)</td>
<td>1.09 (0.88-1.36)</td>
<td>0.35 (0.17-0.72)</td>
<td>0.004</td>
<td></td>
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</tr>
<tr>
<td>CCGGCC</td>
<td>280 (13.4%)</td>
<td>0.95 (0.73-1.24)</td>
<td>0.28 (0.12-0.72)</td>
<td>0.035</td>
<td></td>
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</tr>
<tr>
<td>CCGGCC</td>
<td>204 (9.8%)</td>
<td>0.87 (0.66-1.15)</td>
<td>1.04 (0.76-1.41)</td>
<td>0.804</td>
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<tr>
<td>ATGCGT</td>
<td>92 (4.4%)</td>
<td>0.84 (0.54-1.32)</td>
<td>2.69 (1.72-4.19)</td>
<td>&lt;0.001</td>
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<td></td>
</tr>
<tr>
<td>C. Red meat within multiple-SNPs combinations (n = 2,087, adjusted by age and sex)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ATGCC</td>
<td>659 (31.6%)</td>
<td>1.00</td>
<td>13.72 (8.53-22.06)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCCGC</td>
<td>461 (22.1%)</td>
<td>1.00</td>
<td>4.42 (1.92-10.15)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCGGCC</td>
<td>280 (13.4%)</td>
<td>1.00</td>
<td>4.08 (1.26-13.21)</td>
<td>0.019</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCGGCC</td>
<td>204 (9.8%)</td>
<td>1.00</td>
<td>16.34 (9.47-28.21)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGCGT</td>
<td>92 (4.4%)</td>
<td>1.00</td>
<td>43.78 (27.05-70.88)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Allelic variants composing the combinations of six SNPs are ordered as follows: first CYP1A2 c.–163A>C, second CYP1A2 c.1548T>C, third CYP2E1 g.–1293G>C, fourth CYP2E1 g.–1053C>T, fifth CYP1B1 c.1294C>G, and sixth CYP2C9 c.430C>T. Only the five more frequent combinations (>4%) are represented.

(A) Cross-classification using a common reference category for both interacting variables. The group of moderate red meat consumers (≤4 times/wk) exhibiting the most frequent combination of SNPs is selected as the reference category. ORs are estimated, together with 95% CI, for all other combinations distributed between the groups of moderate and great (≥5 times/wk) red meat consumers, according to a log-additive model. Signcant associations to modification of CRC risk are typed in bold characters. (B) The influence of SNP combination on modification of CRC risk is estimated by ORs calculation within each group of moderate and great red meat consumers. (C) Red meat consumption nested within SNP combinations: The influence of the frequency of red meat consumption is estimated by OR calculation within each combination of SNPs.

in patients versus 54% in controls; P = 0.01). Overall, study participants were very compliant as the global response rate to the questionnaire on life habits reached 97.5%; the only relatively weak response rate was in relation to the “method of cooking” item, giving a response of 88.2%.

Table 2 describes the more significant results obtained with the univariate and multivariate analyses of environmental factors. In the study cohorts, the consequence of red meat consumption on CRC risk was very noticeable and quite distinguishable from those of other environmental factors: Compared with the reference group of moderate eaters, high consumers (≥5 times a week) were at increased risk with an OR of 3.414 (95% CI, 1.93-6.04). To a lesser extent, diabetes status, lack of physical activity, as well as frequent cold cuts and pastry consumption, or, on the contrary, poor-quality fruit intake and low dairy product and fruit consumption were also associated with an increased risk of CRC. Paradoxically, the lack of alcohol consumption was also associated with a decreased CRC risk. By contrast, female gender, dyslipidemia status, and frequent consumption of wholemeal bread decreased the risk of CRC. In addition, we found no effect at all for fish, white meat and cigarette consumption, or cooking methods.

From these observations, we also examined combinations of environmental factors found to have a more obvious bearing on CRC risk. As expected, we observed that individuals with the lowest risk of CRC were those who combined the highest consumption of fruit (more than twice a day), vegetables (more than twice a day), dairy products (more than thrice a day), and wholemeal bread. They were associated with an OR of 0.78 (95% CI, 0.62-0.98, P = 0.036) compared with reference individuals who had the same nutritional habits except that they ate white bread instead of wholemeal bread. People with the highest risk of CRC were...
those eating fruit and vegetables once a day at the most, dairy products twice a day at the most, and white bread (OR, 1.36, 95% CI, 1.15-1.60, P = 0.0004).

Table 3 shows the results of the univariate analyses done with SNPStats for the six single SNP's selected: rs762531 (c.163A>C) and rs2470890 (c.1548T>C) in CYP1A2, rs318367 (g.−1293C>G) and rs2033192 (g.−1053C>T) in CYP2E1, rs1056836 (c.1294C>G) in CYP1B1, and rs1799853 (c.430C>T) in CYP2C9. We did not find any association with CRC for any of the allelic variants tested, as ORs never reached statistical significance either for proximal and distal colon, or rectal cancer. Moreover, analyses of interactions between single SNPs and environmental factors of the questionnaire did not show any interacting pair of variables that would modify the risk of CRC (Supplementary Table S2). The results obtained with SNPStats were confirmed with SAS 9.1, by performing univariate analyses adjusted for the covariate SNP of the environmental factors found to be potential risk factors (Table 3). As an example, ORs associated with a high consumption of red meat (more than five times a week) remained steady and statistically significant at about 3.4 (P < 0.001) when adjusted for any of the six SNPs.

We then analyzed the association between CRC and combinations of multiple SNPs. We tested combinations of two to six SNPs: None was found to be associated with a modification of CRC risk. Therefore, we investigated interactions between these combinations and environmental factors of the questionnaire. The integration of multiple-SNP combinations to the statistical analyses barely changed the ORs calculated above for most environmental factors, especially for the potential risk factors listed in Table 2. However, as summarized in Table 4, striking results were once again obtained with red meat consumption. Part A of Table 4 indicates that three multiple-SNP combinations—ATGCCGT, CCGCGC, and ATGCC (allelic variants in the combinations are ordered as follows: CYP1A2 c.−163A>C, then CYP1A2 c.1548T>C, CYP2E1 g.−1293C>G, CYP2E1 g.−1053C>T, CYP1B1 c.1294C>G, and CYP2C9 c.430C>T)—were associated with a strong elevation of CRC risk in significant red meat consumers when compared with a common reference group of moderate red meat consumers presenting the most frequent combination ATGCCGT. Part B shows that the risk of CRC did not vary significantly between moderate red meat consumers (<4 times a week) whatever SNP combination they exhibited; on the other hand, in significant red meat consumers, the influence of SNP combination was more perceptible as individuals with combination ATGCCGT were associated with a significantly increased risk (OR, 2.69; 95% CI, 1.72-4.19) compared with combination ATGCCGT; by contrast, combinations of ATGCCGT and CCGCGC were at lower risk with ORs dropping to 0.35 (0.17-0.72) and 0.28 (0.12-0.65). Part C of Table 4 takes into account the influence of red meat consumption in the different SNPs combinations; it shows that the intrinsic CRC risk of red meat consumption expressed in Table 2 (OR, 3.414 in great red meat consumers) was slightly increased for combinations (OR, 4.42 and 4.08) ATGCCGT and CCGCGC, but considerably enhanced for combinations CCGCGC (OR, 16.34) and ATGCCGT (OR, 13.72), and extremely high for combination ATGCCGT (OR, 43.78).

Further analyses of SNP’s combinations revealed an interaction between combination ATGCCGT and body weight (Supplementary Table S3). Compared with individuals with low to normal weight (body mass index <18.5-24.9; see Table 1), obese individuals (body mass index ≥30) were at a statistically significantly increased risk of CRC (OR, 12.55; 95% CI, 6.28-25.09), and overweight individuals (body mass index 25-29.9) were associated with a barely significantly increased risk (OR, 2.24; 95% CI, 0.94-5.31). None of the other most frequent SNP combinations (>4%) showed an interaction with body weight.

Discussion
Colorectal carcinogenesis has been the subject matter of many studies, all of which confirm an environmental and genetic bicomponent for carcinogenesis of sporadic CRC (2). Numerous population-based studies have underlined associations between risk for CRC and SNPs—notably including the SNPs of the CYP genes we have chosen in our study—interacting with at least one environmental factor. Nevertheless, most of these studies do not produce a sufficient statistical power, and are therefore not reproducible, because they rely upon a too small number of nonhomogeneous or even badly designed cohorts (usually 40-300 individuals; ref. 29). To circumvent this problem, we based our association study on two large cohorts including more than 1,000 individuals each. To date, only a few population-based association studies as large as ours have been reported on genetic susceptibility to CRC and, to our knowledge, none of them have comprised uniquely sporadic cases. Moreover, no comparable studies have been reported on a French population, which highlights the interest and originality of our cohorts, as genetic predisposition is definitively a population-specific phenomenon. In addition, we were especially careful about the quality of the recruitment, whether this was the clinical criteria for patients (every doubtful familial case was excluded systematically) or the homogeneity of both cohorts (i.e., matching as well as possible for age, sex, and geographic origins). We are aware that a weak point of our recruitment resides in the imbalance between patients and controls of over 70 years old; however, simulations of OR calculations on population samples showed that this imbalance did not seem to alter our estimations of CRC risk.

As mentioned in Results, response rate to the questionnaire on life habits was very good, which was translated by a high statistical significance of the results we obtained for the univariate analyses of environmental factors (P < 0.001 for many analyses). These results were consistent with previous descriptions of environmental risk factors in other epidemiologic studies (30-35). Thus, physical activity, fruit, dairy products, and wholemeal bread appeared as protective factors regarding the risk of CRC. However, above all, the most striking result concerned the high consumption of red meat (more than five times a week), which we found associated with a sizable increase in CRC risk (OR, 3.414; 95% CI, 1.93-6.04) compared with a more reasonable consumption (four times a week at the most). Of course, this observation would be moderated by the fact that individuals with this habit constitute only a minor group within both cohorts (69 individuals; i.e., 3.2% of the whole study population). Nevertheless, the procarcinogenic role of red meat is no longer a novelty because it has already been reported several times in the literature and it has been biologically explained by the metabolism of red meat into deleterious poly cyclic aromatic hydrocarbons and heterocyclic aromatic amines (36). In any case, the size of the two study cohorts leads us to believe that the number of frequent red meat eaters—although they are small in number—is representative of the local French population we examined. Therefore, the results we obtained may be a little overestimated but they reflect a true tendency.

In the same way, certain other results deserve to be discussed here. For example, the observation of a reduced risk of CRC in patients with dyslipidemia was unexpected. As dyslipidemia was taken into account only in patients requiring medical therapy, we may assume that most of them were treated with inhibitors of hydroxy-3-methylglutaryl CoA reductase (also called statins), a widely prescribed class of lipid-lowering agents. This may be a confounding factor as these agents exert a documented protective effect against CRC (37). As regards the lack of effect relating to fish and vegetables, this can be ascribed to an excessively poor representation of the “high consumer categories” for these two covariates. Despite
Public Health communications on the virtues of these two products against cancer, they have failed to become essential elements in today’s French diet. In contrast, the absence of influence of tobacco on CRC risk is not that surprising because its deleterious effect was essentially described in lung and upper digestive tract cancers (38, 39). Moreover, the participants’ answers may have been distorted, notably in patients for whom tobacco consumption appears to be a deliberate risk habit. The same remark could be applied to alcohol consumption, the relative protective effect of which rather contradicted the data reported in the literature and is illogical in terms of detoxification. As regards covariate “cooking methods,” the absence of significant results can be correlated to the relatively low response rate (88.2%), which certainly translates the difficulty encountered by the participants to choose a single answer definitively representative of their habits. Indeed, this problem probably comes from the formulation of the question.

The results of the analysis of multiple environmental factor combinations turned out to be extremely logical. Frequent eating of meals rich in fruit, vegetables, dairy products, and wholemeal bread appeared as the most effective combination against CRC. Yet, the relatively weak difference found between single or combined environmental factors underline a barely detectable additive effect or even an independent effect of these covariates on carcinogenesis in the study population.

For the genetic part of our work we focused on six SNPs of four CYP genes that all belong to phase I of the xenobiotic detoxification metabolic pathway, and that are not localized on the same chromosome. We therefore expected to find an additive or rather interactive effect not due to real haplotypes but to variations in the activity of putative interdependent encoded enzymes. According to our bibliographical meta-analysis of previous association studies, five of the six SNPs of CYP genes that we analyzed had already been found to be associated with a rise in risk for colorectal adenomas or cancer, whether alone or in interaction with an environmental factor. In general, the increased risk had been correlated to allelic variants increasing the activity of encoded enzymes, whether for SNPs c.−163A>C (rs762551) in CYP1A2 (13, 19-21, 40), g.−1053C>T (rs2031920) and g.−1293G>C (rs1056836) in CYP2E1 (14, 15), c.1294C>G (rs1056836) in CYP1B1 (17), or c.430C>T (rs1799853) in CYP2C9 (16, 18). However, we were unable to reproduce these results in our cohorts, as we found no association with modulation of CRC risk for any of the six single SNPs analyzed independently or together with environmental factors in our lifestyle questionnaire. An explanation for the divergence with prior studies probably lies in the smaller populations tested: For instance, for CYP1A2 c.−163A>C, 94 individuals (OR, 3.7; 95% CI, 1.3-10.7) in one study (20) and 377 cases/326 controls in yet a second more comprehensive study (OR, 1.53; 95% CI, 1.3-10.7) in another study (21). The absence of significant results can be correlated to the relatively low response rate (88.2%), which certainly translates the difficulty encountered by the participants to choose a single answer definitively representative of their habits. Indeed, this problem probably comes from the formulation of the question.

On the other hand, analyses of multiple-SNP combinations showed a strong increase in the intrinsic risk of excessive red meat consumption for three different combinations of allelic variants, ATGCCG, CCGCGC, and ATGCCGT, found in 31.6%, 13.4%, and 4.4% of the study individuals respectively (see Table 4). As opposed to these three allelic variant combinations, two other frequent ones, ATGCCG (22.1%) and CCGCGC (9.8%), appeared “neutral” as they barely enhanced the basal CRC risk due to red meat consumption alone (see Table). Interaction analyses with other environmental factors revealed that, among the combinations above, ATGCCG was the one that predisposed the most to CRC, as it was the only one found to be additionally associated with a perceptible increase in the risk of CRC in obese individuals (~14.4% of the study population). This last observation is a good example of gene-environment interaction: In a way, the genetic background seems to exacerbate the procarcinogenic effect of the environmental factor, because, in our cohorts, obesity alone was not found to be associated with an increased CRC risk as reported in the literature (41). On the other hand, genetic expression seems to be influenced by environmental factors, as independent analysis of the ATGCCGT combination showed no association with CRC risk. Interestingly, obesity was shown to increase expression and thereby oxidative activities of CYP enzymes, especially CYPIE2, which exacerbates the procarcinogenic effect of xenobiotics (42, 43).

A hypothesis for the marked interaction of combination ATGCCGT with obesity and red meat consumption would be that the allelic variants composing this combination contribute to the simultaneous enhancement of the metabolic activities of CYP1A2, CYP2E1, CYPIB1, and CYP2C9 enzymes. Yet, if we turn from previous works the theoretical combination leading to the greatest enzymatic activity of these four enzymes, we obtain ATGCCG (i.e., a combination we found not to be associated with an effect on CRC risk in our cohorts). In fact, the first four allelic variants of this combination perfectly match the observed ATGCCGT combination and they correspond to allelic variants of CYP1A2 (c.−163A and c.1548T) and of CYP2E1 (g.−1293G and g.−1053C) that have been shown to induce an increased enzymatic activity in vitro, and which have been found to be associated with an increase in CRC risk by interacting with red or processed meat (14, 19, 20, 44). This first part of the combination should be associated with an elevated production of polycyclic aromatic hydrocarbons and heterocyclic aromatic amines, and thereby to an increased cancer risk. In the same way, the “neutral” effect on CRC risk observed for combination CCGCGC can be explained as the result of the concomitant decreased activity of CYPIA2 and increased activity of CYP2E1. In fact, the divergence between observed and theoretical predisposing hexanucleotide combinations resides in the last two variants corresponding to c.1294C>G of CYPIB1 and c.430C>T of CYP2C9. Akllflu et al. (45) showed that the allelic variant c.1294C>G of CYPIB1 increases enzymatic activity, but, to date, it has only been found to be associated with lung and head-and-neck cancers (46-48). The allelic variant c.430C>T of CYP2C9 was found to be associated with a decrease in CRC risk (16). However, the size of the population studied in this report was small and the significance of these results may be disputable. Therefore, this divergence between theory and observation does not discredit the predisposing bearing on CRC that we found for the ATGCCGT combination. On the contrary, it illustrates the difficulty in extrapolating a general biological model from the combination of independent analyses. This contrasts with environmental factors for which theory perfectly matched observations. It is precisely the very notion of dependence or rather interactivity, between enzymes of the same pathway in this case, which creates all the complexity of the susceptibility phenomenon.

In conclusion, we observed three combinations of CYP allelic variants that strongly predispose to CRC by interacting and enhancing the intrinsic predisposing potential of excessive red meat consumption. One of these combinations, ATGCCGT, has a particularly strong effect on CRC risk and also interacts positively with obesity, another known promoting factor of CRC. Our present work illustrates the need to go beyond the analysis of single SNPs proposed in most of the current studies on genetic predisposition for complex diseases like cancer. Studies on predisposition to colorectal and prostate cancer have already started to show the power of combative approaches (49-52). In addition, our present data highlight the very interest of analyzing SNP combinations in connection with environmental risk factors to a better appreciation of the complexity of in vivo events accounting for cancer susceptibility.

We are aware that our approach can be considerably improved. The most significant results presented here relate indeed to a small subgroup of the cohort [69 great red meat consumers (3.2%) of 2,144 individuals], and they would need to be reproduced and confirmed on larger cohorts. In addition,
investment of a more comprehensive panel of SNP's would certainly lead to even more conclusive observations. Therefore, we now plan to examine additional polymorphic genes involved in the metabolic pathway of xenobiotic detoxification, such as genes of glutathione S-transferases, UDP-glucuronosyltransferases, or sulfotransferases. Nevertheless, in the present work, we deliberately reduced the number of studied SNPs in a didactical perspective to better illustrate the phenomenon of interaction between genes.

Although our combinative approach contains room for improvement, we believe that it is more meaningful than association studies dealing with one risk factor at a time. The identification of SNP combinations modulating CRC risk cannot be sufficiently precocious if environmental context is a first step toward the determination of genetic profiles of susceptibility, which should enable the establishment of personal nutritional recommendations for patients.

Acknowledgments

We thank all the patients, control individuals, and physicians who participated in this work and made it possible.

References


Combinations of Cytochrome P450 Gene Polymorphisms Enhancing the Risk for Sporadic Colorectal Cancer Related to Red Meat Consumption

Sébastien Küry, Bruno Buecher, Sébastien Robiou-du-Pont, et al.


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