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NAD(P)H:quinone oxidoreductase-dependent risk for colorectal cancer and its association with the presence of K-*ras* mutations in tumors

M.J.Lafuente[1], X.Casterad[2], M.Trias[3], C.Ascaso[4], R.Molina[5], A.Ballesta[5], S.Zheng[6], J.K.Wiencke[6] and A.Lafuente[2,7]

[1]Department of Surgery, Hospital Clinic, [2]Department of Pharmacology, School of Medicine, Casanova 143, E-08036, Barcelona, Spain, [3]Department of Surgery, Hospital St Pau, E-08025 Barcelona, Spain, [4]Department of Statistics, School of Medicine and [5]Clinical Chemistry Department, Hospital Clinic, University of Barcelona, E-08036 Barcelona, Spain and [6]Laboratory for Molecular Epidemiology, Department of Epidemiology and Biostatistics, School of Medicine, University of California San Francisco, San Francisco, CA 94143-0560, USA

[7]To whom correspondence should be addressed
Email: alafuente@med.ub.es

NAD(P)H:quinone oxidoreductase (NQO1) is a polymorphic enzyme involved in the detoxification of potentially mutagenic and carcinogenic quinones. The homozygous C609T NQO1 genotype resulting in loss of reductase activity is found in 2–20% of individuals. In the present study, the NQO1-dependent risk for sporadic colorectal cancer (CRC) was studied in 247 incident CRC cases and 296 hospital-based controls recruited during 1996–1997. Four subgroups of cases were studied: (i) all CRCs; (ii) a molecular CRC subgroup (n = 117, cases with molecular tumor analyses); (iii) within the molecular subgroup those tumors with K-*ras* mutations in codon 12 (CRC K12); (iv) within the molecular subgroup those tumors with K-*ras* mutations in codon 13 (CRC K13). The C609T NQO1 genotype was found to be twice as prevalent in all CRC patients (6.8%) compared with controls (3%) and six times more common in the subset CRC K12 (20%). Multivariant analyses in the overall population of 247 cases and 296 controls showed a significant age and gender adjusted risk for CRC associated with the C609T NQO1 genotype (OR 2.9, 95% CI 1.19–6.97; P = 0.01) or with any variant genotype (the low activity allele frequency, i.e. heterozygotes plus homozygotes) (OR 1.41, 95% CI 1.02–1.92; P = 0.03). Within cases of the molecular subgroup (n = 117) the C609T NQO1 genotype was associated with the presence of K-*ras* codon 12 mutation (OR 6.5 95%, CI 1.39–34.9; P = 0.003). Logistic regression showed an age and gender adjusted risk for K-*ras* codon 12 mutant CRC associated with the C609T NQO1 genotype (OR 10.5, 95% CI 2.99–36.7; P = 0.0002) or with any variant NQO1 genotype (OR 2.23, 95% CI 1.23–4.00; P = 0.007) compared with the control group. Genetically determined variations in NQO1 may modify the risk for CRC and these risks may be greatest for tumors containing K-*ras* codon 12 mutations. CRC with K-*ras* codon 12 mutations may represent a distinct and etiologically more homogeneous subtype of the disease, which may be associated with toxicants that are metabolized via a NQO1-dependent pathway.

Abbreviations: BP, benzo[*a*]pyrene; BPQ, quinone metabolites of benzo[*a*]pyrene; C609T NQO1 genotype, homozygous C609T NQO1 genotype; CE, catechol estrogens; CI, confidence interval; CRC, colorectal cancer; CRCK12, colorectal tumors with a mutation in *K-ras* codon 12; CRCK13, colorectal tumors with a mutation in *K-ras* codon 13; NQO1, NAD(P)H:quinone oxidoreductase; OR, odds ratio.

Introduction

Detoxification of xenobiotics is usually linked to liver metabolism, but it can also be mediated by colonocytes of the intestinal tract and may play a role in colorectal cancer (CRC) (1). Among the enzymes present in the intestinal mucosa, NAD(P)H:quinone oxidoreductase (NQO1) (EC 1.6.99.2) carries out the two electron reduction of potentially toxic quinones (2), which are ubiquitous in aerobic plants and animals and are present in the environment as combustion by-products (e.g. automobile exhaust, cigarette smoke and urban airborne particles) (3,4). Diet can also be an indirect source of exposure to these compounds as some pro-carcinogens present in food, such as benzo[*a*]pyrene (BP), can be metabolized in cells to quinone intermediates. Because of their electrophilic nature and very high redox potential, quinones can be highly toxic. Indeed several compounds containing the quinone nucleus are extensively used as cytotoxic antitumor drugs (5). The mechanisms underlying the toxicity of quinones, particularly those important in CRC carcinogenesis and which are under genetic control, are poorly understood.

Recently, a genetic polymorphism in NQO1 involving a C→T nucleotide substitution at base pair 609 has been identified (6), which in homozygous form is associated with non-detectable NQO1 activity (7,8); ~2–20% of individuals carry the C609T NQO1 genotype (9). The prevalence of the low activity allele is highest among Asians and lowest in European populations (9–11). NQO1 has been implicated in lung cancer (10, 12, 13), benzene poisoning (14), leukemia (15,16), cutaneous basal cell carcinoma (17) and urological malignancies (18). *In vitro*, the formation of DNA adducts by quinone metabolites of BP (BPQs) (19) and benzene (20) have been shown to be significantly reduced by NQO1 activity. In addition, NQO1 is also involved in the activation of vitamin K, which is a potent antioxidant (21) and has antimutagenic effects against genotoxic heterocyclic amines from cooked food (22). For these reasons we have examined NQO1 as a candidate susceptibility locus for sporadic CRC in the present hospital-based case–control study.

Because genetic susceptibility involving altered quinone metabolism may influence risk for specific molecular subtypes of CRC, we examined the potential associations of NQO1 with mutations in the K-*ras* gene within a subset of CRC cases whose tumors were collected at surgery. The K-*ras* gene encodes a protein ($p21^{ras}$) belonging to a family of GTP/GDP-binding proteins with GTPase activity and participates in the transduction of mitogenic signals from the membrane to the cell nucleus. Mutated ras proteins have reduced GTPase activity and/or an increased dissociation rate of ras–GDP, leading to a prolonged mitogenic signal (23). K-*ras* mutations are found

in 40–60% of sporadic CRCs (24); ~70% of these occur within codon 12 and the remainder in codon 13.

Materials and methods

Study population

Patients were recruited through the University of Barcelona Hospital Clinic, a 900 bed institution with ~33 000 admissions/year, which serves a source population of ~600 000 persons within Catalonia, Spain. It is a teaching institution and tertiary care facility. During 1996–1997, 275 consecutive sporadic CRC patients undergoing surgery were identified through the Clinic's two surgical services as potential subjects for a hospital-based case–control study of CRC. Patients who were ⩾40 and <90 years old were invited to participate in the study. Eighteen subjects refused to participate, leaving a final total of 247 enrolled Caucasian subjects (131 men and 116 women). A consecutive case series subset of 117 patients (originally 120, but three patients were older than 90) within one surgical service also provided tissue specimens (uninvolved colon and tumor) for the molecular study. The remainder of the CRC group only provided blood samples. Primary tumors were surgically dissected and immediately frozen at –80°C. Patients gave their signed informed consent; all procedures were approved by the Ethical Research Board of the Hospital Clinic. A questionnaire was administered to each patient by an interviewer; the questionnaire elicited demographic information, data on occupation and smoking habits and personal medical history. The primary measure of smoking was the pack year unit (pack year = number of packets of 20 cigarettes per day×number of years). Clinical and pathological data were collected and merged with patient questionnaires. No cases reported a positive family history of early onset CRC or multiple familial cases that might indicate an inherited dominant CRC gene. A modified version of Duke's staging system was used. Surgical records identified eight subsites: cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectosigmoid junction, rectum and anal canal. Proximal tumors were defined as including the right side of the colon and descending colon up to the sigmoid colon.

The control group consisted of consecutively recruited patients presenting to the Trauma service of the Hospital Clinic during the same time period as case accrual. This service deals with ~2000 admissions/year of patients living in the same metropolitan area (Barcelona) as the cases studied. To facilitate sampling, patients whose pre-operatory study (including blood analysis) was performed in the outcare service were excluded. Only patients between ⩾40 and <90 years old were invited to participate in the study, following the same procedure as for cases. The same epidemiological data and blood samples were collected from controls as was done for cases. Most of the control group were admitted for articular hip or knee replacement, pelvis fracture or injury to upper or lower ribs. Controls were excluded if they reported a history of malignancy.

DNA was isolated from blood and tumor specimens using standard methods employing RNase, proteinase K and chloroform/isoamyl alcohol extraction and ethanol precipitation. DNA was quantified by Hoescht 33258 fluorimetry (Hoefer Scientific, San Francisco, CA). All analyses were carried out on coded samples and the analyst was blind to the case/control status.

NQO1 polymorphism

In the PCR analysis the sense (5′-TCCTCAGAG TGGCATTCTGC-3′) and antisense primers (5′-TCTCCTCATCCTGTACCTCT-3′) amplify a 211 bp region including the last 7 bases of exon 5 and the first 204 bases of intron 6 (25). PCR reactions were carried out in a 30 µl volume of 30 mM Tris–HCl (pH 8.3) containing 80 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 0.620 µM each primer and 3.5 U AmpliTaq. Reaction mixtures were preincubated for 3 min at 94°C. The reactions were heated to 94°C for 30 s, cooled to 55°C for 30 s and heated to 72°C for 30 s for 35 cycles, with a final extension of 10 min at 72°C. Amplifications were performed with 1 µg genomic DNA with a Perkin Elmer 9600 cycler. An aliquot of 10 µl of the PCR products was then digested with 25 U *Hin*fI for 4 h at 37°C. *Hin*fI recognizes GANTC and so the transition from wild type -GAA CCT- to -GAA TCT- in the variant creates a new *Hin*fI restriction site. Fragments were separated by electrophoresis through 2% agarose gels containing ethidium bromide (0.5 mg/ml). Controls for the PCR method consisted of known NQO1 heterozygous DNA which was included in each batch of assays. A single high molecular weight PCR product that is resistant to enzyme digestion indicates a homozygous wild-type genotype, two bands correspond to the heterozygote and two lower molecular weight bands correspond to the homozygous C609T NQO1 genotype (C609T NQO1 genotype).

K-ras mutation detection

K-*ras* mutations in codons 12 and 13 were detected by a nested PCR method in which the second round PCR used mismatched primers that introduced restriction sites into products derived from wild-type alleles. Mutations identified by this screening method were then sequenced using dot-blot hybridization or by direct sequencing. First round primers and conditions were taken from Nelson *et al.* (26): sense, 5′-CATGTTCTAATAAGTCACA-3′; antisense, 5′-TCAAAGAATGGTCCTGCACC-3′ (GenBank accession no. L00045). PCR reactions (25 µl volume) were carried out with 50 ng tumor DNA and the following cycling conditions (15 cycles) with a Perkin Elmer 9600 thermocycler: preheating at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, with a final extension at 72°C for 10 min. The second round PCR has been described by Jacobson and Mills (27). An aliquot of 2 µl of the first round PCR product was added to the second round PCR (100 µl volume) and amplified with either a screening primer (sense) for codon 12 (5′-ACTGAATATAAA-CTTGTGGTAGTTGGACCT-3′) or for codon 13 (5′-CTGAATATAAAC-TTGTGGTAGTTCCAGCTGGT-3′). The same antisense primer as used in the first round PCR was used for both codon 12 and 13 screening. PCR was repeated for 35 cycles using the following conditions: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, with a final extension at 72°C for 10 min. After PCR, 16 µl of the second round PCR product was digested with either *Bst*NI (codon 12) or *Pfl*m1 (codon 13) for 4 h. Digests were analyzed using 3.5% agarose gels. PCR products resistant to enzyme digestion indicated the presence of a base pair substitution. For each set of samples a positive control DNA (SW480) containing a codon 12 mutation was also run to provide quality control on the amplification and restriction enzyme digestion. After tumors were identified as having a mutation in codon 12 or 13 they were analyzed using the human *ras MUTALYZER* probe panel and dot-blot hybridization according to the manufacturer's instructions (Clontech, Palo Alto, CA). If the products were very strong, they were sequenced directly. For direct sequencing, the second round products were gel purified (Qiagen, Valencia, CA) and sequenced with the antisense primer using an ABI 373 automated sequencer. To confirm the accuracy of the dot-blot results, a total of 31 samples were directly sequenced and dot-blotted.

Statistical analyses

Data were analyzed using SPSS 6.1.2 (statistical analysis software). Means and standard deviations were computed for continuous variables. Age-adjusted univariate analysis was employed to identify environmental factors associated with CRC and CRC with K-*ras* mutations. Univariate analysis also tested for associations of NQO1 genotypes with either the risk of CRC or CRC with specific K-*ras* mutations. Multivariate analyses (multiple logistic regression) were used to estimate the independent contribution of each variable identified though univariate analysis to the risk of CRC and CRC with K-*ras* mutations. Confidence limits for the adjusted odds ratio were calculated with the associated logistic coefficients and standard errors.

Results

Table I shows the characteristics of CRC patient and control groups. The subset of patients for whom tumor tissue was available (molecular CRC group) was very similar to the overall CRC group in terms of age, sex and smoking patterns. There were more male cases than females in both case groups; the distribution of the sexes was identical to the 53% males

Table I. Characteristics of the CRC patients and the control group

	Controls	All CRC	Molecular CRC[a]
n	296	247	117
Mean age	61.9 ± 11.6	70.4 ± 11.2	71.0 ± 11.1
Women			
n	185/296 (62.5%)	116/247 (46.9%)	56/117 (47.8%)
Smokers *n*[b]	21/185 (11.3%)	4/116 (3.4%)	1/56 (1.7%)
Mean pack years[c]	2.2 ± 7.7	0.8 ± 6.0	0.07 ± 0.5
Men			
n	111/296 (37.5%)	131/247 (53.0%)	61/117 (52.1%)
Smokers *n*	82/111 (73.8%)	95/131 (72.5%)	44/61 (72.1%)
Mean pack years	34.6 ± 35.0	38.7 ± 43.6	35.2 ± 40.6

[a]Molecular CRC is the subgroup of CRC patients that participated in the molecular study.
[a]Number of individuals who smoke/total (%) (smokers plus former smokers).
[b]Pack year = number of packs of 20 cigarettes per day×number of years.

Table II. Clinical-pathological characteristics of CRC patients and distribution of K-*ras* mutations in the molecular CRC group

Variable	All CRC	Molecular CRC	K12 mutation[a]	K13 mutation[b]
n	247	117	35/117 (29.9%)	14/117 (11.9%)
Men	131/247 (53.0%)	61/117 (52.1%)	21/61 (34.4%)	6/61 (9.8%)
Women	116/247 (46.9%)	56/117 (47.8%)	14/56 (25%)	8/56 (14.3%)
Stage[c]				
A	4/236 (1.6%)	2/116 (1.7%)		
B	96/236 (40.2%)	46/116 (39.6%)	18/48 (37.5%)[d]	5/48 (10.4%)[d]
C	67/236 (28.3%)	33/116 (28.4%)		
D	69/236 (29.6%)	35/116 (30.1%)	17/68 (25%)[e]	9/68 (13.2%)[e]
Differentiation[f]				
Moderate to well	191/208 (91.8%)	95/107 (88.7%)	31/95 (32.6%)	11/95 (11.5%)
Poor	17/208 (8.1%)	12/107 (11.2%)	2/12 (16.6%)	2/12 (16.6%)
Location				
Cecum	20/247 (8.0%)	14/117 (11.9%)		
Ascending colon	34/247 (13.7%)	12/117 (10.2%)		
Transverse colon	19/247 (7.6%)	9/117 (7.6%)		
Descending colon	9/247 (3.6%)	5/117 (4.2%)		
Proximal	82/247 (33.1%)	40/117 (35.3%)	13/40 (32.5%)	4/40(10.0%)
Sigmoid colon	87/247 (35.2%)	40/117 (34.1%)		
Rectosigmoid junction	19/247 (7.6%)	12/117 (10.2%)		
Rectum	59/247 (23.8%)	25/117 (21.3%)		
Distal	165/247 (66.8%)	77/117 (65.8%)	22/77(28.5%)	10/77 (12.9%)

[a]Number of patients in the molecular CRC group with K-*ras* mutations in codon 12 [*n*/total (%)].
[b]Number of patients in the molecular CRC group with K-*ras* mutations in codon 13 [*n*/total (%)]. There was no coincidence of mutations in both codons for the same patient.
[c]There are some cases for which we have no clinical information (all CRC 11 cases missing; molecular CRC 1 case missing).
[d]Dukes $<$ C.
[e]Dukes $\geqslant$ C.
[f]There are some cases for which we have no clinical information (all CRC 34 missing; molecular CRC 10 missing).

among incident CRCs reported by the population-based Cancer Registry of Catalonia (Tarragona). The control group, however, contained relatively greater numbers of women. This is most likely due to the hospital-based recruitment of patients seeking medical treatment for hip, pelvic and other fractures, which are more prevalent among post-menopausal women compared with older men. As is typical of an older female cohort in Spain, relatively few women (cases and controls) smoked cigarettes and those that did reported very low numbers of pack years compared with men. The control group was on average ~10 years younger than the CRC group.

Clinical-pathological characteristics of CRC patients are shown in Table II. The overall and molecular CRC groups were very similar in terms of the distributions of Duke's stages A–D, percentage of poor histopathological differentiation and anatomical location of the primary tumor. Approximately 57.6% of the patients had Duke's stage C or D disease and 8.1% presented with tumors classified as poorly differentiated. In addition, men $\leqslant$70 years old more frequently had distal tumors (all CRC 79.4%; molecular CRC 93.1%) and women aged $>$70 years presented with proximal tumors in 46.5 and 62.5% of cases within the overall CRC and molecular CRC groups, respectively.

K-*ras* mutations were observed in 41.8% of all cases; these were located in codon 12 in 29.9% of patients and in codon 13 in 11.9% of patients (Table II). Although the prevalence of K-*ras* mutations was higher in men than in women (34.4 versus 25% mutant at codon 12), the difference was not statistically significant. Poorly differentiated tumors had roughly half the number of codon 12 mutations compared with moderate or well-differentiated tumors, but again the difference was not statistically significant.

To explore potential associations of NQO1 genotype with clinical variables and to compare the overall and molecular groups we show in Table III the distribution of C609T NQO1 genotypes among CRC patients according to several clinical characteristics. The data do not suggest an association of genotype with stage, differentiation or location of the tumor. Considering the small number of cases in some of the subgroups, the range in prevalence of the C609T NQO1 genotype by these variables in the overall CRC group (5.2–11.7%) was similar to that of the molecular CRC group (5.8–16.6%).

Table III. Distribution of homozygous C609T NQO1 genotype[a] in controls and in CRC patients according to clinical characteristics

Variable	Controls	All CRC	Molecular CRC
n	9/296 (3%)	17/247 (6.8%)	10/117 (8.5%)
Less than mean age[b]	3/138 (2.1%)	9/111 (8.1%)	5/57 (8.7%)
More than or equal to mean age[b]	6/158 (3.7%)	8/136 (5.8%)	5/60 (8.3%)
Stage			
Dukes $<$ C		9/100 (9%)	6/48 (12.5%)
Dukes $\geqslant$ C		8/136 (5.8%)	4/68 (5.8%)
Differentiation			
Moderate to well		10/191 (5.2%)	8/95 (8.4%)
Poor		2/17(11.7%)	2/12 (16.6%)
Location			
Proximal		8/82 (9.7%)	5/40 (12.5%)
Distal		9/165 (5.4%)	5/77 (6.4%)

There are some cases for which we have no clinical information
[a]Number of cases with homozygous C609T NQO1 genotype/total (%).
[b]Subgroups $<$ or $\geqslant$ mean age (controls, 61 years; all CRC, 70 years; molecular CRC, 71 years).

Table IV shows the distribution of the C609T NQO1 genotype in the different subgroups studied: controls, all CRC, molecular CRC, molecular CRC patients with K-*ras* mutations

in codon 12 (CRC K12) and molecular CRC patients with K-*ras* mutations in codon 13 (CRC K13). The overall prevalence of the C609T NQO1 genotype in the control group was 3% and the low activity allele frequency was 20.1%. There were no apparent differences in the prevalence of these genotypes when the group was divided into different age and sex strata. Although non-smokers had a higher percentage of the homozygous mutated genotype (3.6%) than smokers (smokers plus former smokers 1.9%), these differences were non-significant. Hence, within controls no associations were indicated between the C609T NQO1 genotype and age, gender or smoking.The C609T NQO1 genotype was found to be twice as prevalent in CRC patients (6.8%) compared with controls (3%) and six times greater in the subset CRC K12 (20%). The low activity allele frequency was 24.2% in the CRC group, 28.2% in the molecular CRC group and increased to 34.2% in the subgroup CRC K12; unadjusted odds ratio (OR) for the C609T NQO1 genotype and case status was 2.36 [95% confidence interval (CI) 097–5.84]. When the groups were stratified by gender a significant risk was observed in women (OR 3.13, 95% CI 1.03–9.80), while in men a lower point estimate was not significant (OR 1.73, 95% CI 0.37–8.96). In addition, within cases there was a positive but non-significant association of K-*ras* mutation (either codon 12 or 13) with the C609T NQO1 genotype (OR 3.47, 95% CI 0.75–18.09), however, there was a significant association of the C609T NQO1 genotype with K-*ras* codon 12 mutation (OR 6.58, 95% CI 1.39–34.9). Because of the unbalanced gender and age distribution of the controls it is especially important to adjust for these factors. In addition, smoking was considered a potential confounder but was so strongly associated with male gender that it was analyzed in models without gender.

Summarized results on the multivariate analysis including age and gender are shown in Table V. In Model 1 NQO1 is considered a recessive trait so that homozygous variants are compared with heterozygous and wild-type individuals. In this model the C609T NQO1 genotype was associated with CRC case status in comparisons, including all patients (OR 2.9, 95% CI 1.19–6.97), only with the subgroup who had K-*ras* codon 12 mutations (OR 10.52, 95% CI 2.99–36.7) or with the subgroup who had any K-*ras* mutation (codon 12 or 13) (OR 5.92, 95% CI 1.88–18.2). In Model 2 of the logistic analyses we considered NQO1 as a dominant trait and compared the homozygous and heterozygous individuals with wild-type individuals. Lower but statistically significant risks were associated with carrying one or two NQO1 variant alleles, including: all patients (low activity allele frequency, OR 1.41, 95% CI 1.02–1.92), only cases with K-*ras* codon 12 mutations (OR 2.23, 95% CI 1.23–4.00,) or cases with any K-*ras* mutations (codon 12 or 13) (OR 2.12, 95%CI 1.27–3.52). Non-significant interactions were observed between NQO1 and sex in both Models 1 and 2. Similar results were obtained in models that included cigarette smoking and not gender (including a non-significant interaction between NQO1 and smoking), however, because the overwhelming number of smokers were men it was not possible to separate out the effects of male gender and smoking. Small sample sizes precluded stratified analyses of smoking within women or men alone.

Other demographic and socioeconomic factors were examined. We tested the potential association of case status with low physical activity, female reproductive history (menopause or total time of fertility, parity or age at first birth), urban versus rural residence, years of formal education and current marital status; none of these variables approached statistical significance in any model. Moreover, no significant association was found between smoking and risk of CRC in males (results not shown).

To provide a descriptive presentation of the mutational spectra of K-*ras* within this group of Spanish CRC patients, we summarized the results of the sequencing data for the

Table IV. The homozygous C609T NQO1 genotype and low activity allele frequency in controls, all CRC and molecular CRC groups

Homozygous C609T genotype	Controls	All CRC	Molecular CRC[a]	CRC K13 mut	CRC K12 mut	CRC K12 non-mut
Women	6/185 (3.2%)[b]	11/116 (9.4%)[b]	5/56 (8.9%)	0/8	3/14 (21.4%)	2/42 (4.8%)
Men	3/111 (2.7%)	6/131 (4.5%)	5/61 (8.1%)	0/7	4/21 (19.0%)	1/40 (2.4%)
All	9/296 (3%)[c]	17/247 (6.8%)[c]	10/117 (8.5%)[d]	0/15[4]	7/35 (20.0%)[d,e]	3/82 (3.6%)[e]
Overall low activity allele frequency	20.1%	24.2%	28.2%	28.5%	34.2%	25.5%

[a]Subgroup of patients for molecular study.
[b]OR = 3.13 (CI 1.03–9.8; $P = 0.02$).
[c]OR = 2.36 (CI 0.97–5.84; $P = 0.03$).
[d]Within (a) K-*ras* mutated tumors (codon 12, K12, or codon 13, K13) versus non-mutated tumors (OR = 3.47, CI 0.75–18.09; $P = 0.06$).
[e]OR = 6.58 (CI 1.39–34.9; $P = 0.003$).

Table V. Summarized results for multivariant analysis of the NQO1-dependent risk for CRC and CRC with mutated K-*ras*

	Controls versus all CRC			Controls versus CRC K12			Controls versus CRC K12 or K13[a]		
	OR	*P*	*CI*	OR	*P*	CI	OR	*P*	CI
Model 1: NQO1	2.90	0.01	1.19–6.97	10.52	0.0002	2.99–36.7	5.92	0.002	1.88–18.2
Model 2: low activity allele	1.41	0.03	1.02–1.92	2.23	0.007	1.23–4.00	2.12	0.004	1.27–3.52

[a]Controls versus CRC with K-*ras* mutations in either codon 12 or 13.
Model 1 was a multivariant analysis that studied the NQO1 genotype in two categories and adjusted by age and sex: homozygous C609T NQO1 genotype versus heterozygous and homozygous wild-type genotype.
Model 2 studied the NQO1 low activity allele considered as a continuous variable (0, wild-type; 1, heterozygous; 2, homozygous mutated) and adjusted by age and sex.
No significant interactions were observed between NQO1 and sex in either Model 1 or 2.

molecular CRC group (Table VI). The most common mutation in K-*ras* results in a Gly→Asp amino acid substitution: 48.4% in codon 12, 100% in codon 13. Mutations leading to Gly→Val substitutions were the second most common mutation. Transitions from G to A occurred in 68% of mutations seen either in codon 12 or 13, transversions of G to T in 27.6% and G to C in only 4.2%. There was no evidence of a trend towards a specific mutation pattern in smokers.

Discussion

Although the base pair alteration leading to loss of enzymatic activity of NQO1 was first discovered within a CRC cell line and later identified as a common polymorphism, the potential risk associated with this allele for CRC has not been studied. In the present investigation we genotyped a relatively large number of incident CRC cases from a single institution and found these patients to have a higher prevalence of the NQO1 low activity allele (both as homozygous and heterozygous traits) compared with a control group selected concurrently from the Trauma service of the same hospital. Also, the association was significant in stratified analyses considering only women in the study. In interpreting our results it is important to note that our choice of method for control selection, although economical, has some drawbacks. The control group from the Trauma service was somewhat younger than the cases and contained relatively more women (62.5%) than the case group (46.9%). Overall, smoking was more common in female controls than in female cases. The rates of smoking were similar among male cases and controls. Consistent with the base population in Catalonia, rates of smoking were markedly higher in men compared with women (cases and controls). The importance of differences in smoking rates in interpreting the observed association of NQO1 in cases and controls is not entirely clear, as smoking has only inconsistently been implicated as a risk factor for CRC (28). It was a secondary aim of the study, however, to examine possible relationships of smoking history with K-*ras* mutations, given that tobacco has been linked with K-*ras* in other cancers (e.g. lung cancer). Nonetheless, a primary consideration is whether the NQO1 genotype could be associated with features of the control group that arise as a consequence of our method of enrollment. We did not find evidence that the control was biased with respect to the NQO1 gene. First, the prevalence of the C609T NQO1 genotype and allele frequencies in controls, although lower than ethnic groups within the USA, was very consistent with multiple reports from Europe and the UK. For example, we observed a prevalence of 3% C609T NQO1 genotypes among our 296 controls; studies in Germany reported a 1.5% (n = 271) (11) and 2.0% (n = 100) (29) prevalence, whereas in the UK the prevalence is 1% (n = 100) (15) and in French Caucasians 3.8% (n = 210) (30); the frequency of the low activity allele in the DNA bank of the Center d'Etude Polymorphisme Humain was 13% (n = 82) (10). Also important is our failure to observe an association of the NQO1 genotype with gender, age or smoking status in controls. Although other unidentified risk factors could potentially bias our study, we examined demographic, socioeconomic and reproductive factors and did not observe any significant difference in these indices between cases and controls. Finally, there is no evidence that the primary medical conditions affecting controls from the Trauma service are associated with genetic variations at the NQO1 locus. Specifically, fractures, hip replacements and other orthopedic conditions have no known or hypothesized connection with quinone metabolism or NQO1. Hence, we conclude that our control group is unlikely to be biased in terms of its genetic background and distribution of NQO1 genotypes, which is the primary focus of this research.

The clinical-pathological characteristics of our CRC group are similar to those reported in a recent population-based case–control study in Majorca, Spain (31) and for other series (32,33).

The association between the C609T NQO1 polymorphism and CRC risk is clear, especially for CRC with a mutation in codon 12 (no association was evident for K-*ras* codon 13), but it weakens when the frequency of the low activity allele is studied. Therefore, toxicological consequences of this polymorphism seem especially relevant in homozygous individuals when there is complete lack of NQO1 activity. Codon 12 is the predominant target for mutations in K-*ras* in CRC, here accounting for 70% of the observed alterations. This is similar to lung cancer, where codon 12 mutations comprise >95% of K-*ras* mutations and are closely linked with cigarette exposure, asbestos and female gender. The relatively lower number of codon 13 mutations in human cancers is not understood, however, the chemical reactivity of mutagens with specific nucleotides within *ras*, the repair efficiency toward different DNA modifications and the resulting mutational spectra have been shown to be highly sequence dependent (34–37). Hence, our *a priori* idea was that NQO1 may affect codons 12 and 13 differentially. In our study of CRC, as in others, G→A transitions are the principal type of K-*ras* mutation. In lung cancer, the primary changes in K-*ras* are G→T transversions, which is consistent with the genotoxic action of compounds found in tobacco smoke such as BP diol epoxide**.** In *in vitro* studies that reported NQO1 inhibition of the mutagenicity of quinone metabolites of BP (BPQs), the mutational spectra of these compounds included G→T transversions, as does that of BP diol epoxide, but G→A transitions were also detected (38).

Other authors have attempted to associate human lifestyle factors, such as smoking and alcohol consumption, with a

Table VI. Sequencing for K-*ras* mutations in the molecular CRC subgroup

Genotype	Patients with K-*ras* mutations[a]	Smokers[b]
Codon 12 (GGT)[c]	33[d]	
Serine (AGT)	2/33 (6.0%)	1/2 (50.0%)
Aspartate(GAT)	16/33 (48.4%)	5/16 (31.2%)
Alanine (GCT)	2/33 (6.0%)	2/2 (100%)
Valine (GTT)	13/33 (39.3%)	5/13 (38.4%)
Codon 13 (GGC)[b]	14	
Aspartate (GAC)	14/14 (100%)	4/14 (28.5%)
G→A[e]	32/47 (68.%)	10/32 (31.2%)
GGT (Gly)→AGT (Ser)	2/32 (6.2%)	1/2 (50%)
GGT (Gly)→GAT (Asp)	16/32 (50.0%)	5/16 (31.2%)
GGC (Gly)→GAC (Asp)	14/32 (43.7%)	4/14 (28.4%)
G→T[e]	13/47 (27.6%)	5/13 (38.5%)
GGT (Gly)→GTT(Val)	13/13 (100%)	
G→C[e]	2/47 (4.2%)	2/2 (100%)
GGT (Gly)→GCT (Ala)	2/2 (100%)	

[a]Number of cases with this mutation/all (%).
[b]Number of cases with this mutation who smoked/all (%).
[c]Wild-type for codons 12 and 13.
[d]Two missing cases in sequencing (one is a smoker).
[e]Wild-type for either codon 12 or 13.

specific K-*ras* mutation pattern in pancreatic tumors (39,40), but evidence is still scant and inconsistent. In other cancers, the relationship of smoking with K-*ras* has not been established (41–43). In our study, K-*ras* mutations were almost equally common in tumors from men and women, although the rates of cigarette smoking were much greater in men. This would tend to indicate that smoking may not be involved in the induction of K-*ras*, but we did not have a sufficient sample size to address this question adequately nor did we collect information on lifetime exposure to environmental tobacco smoke in women, which may have been significant. Anecdotally, we noted that all four C609T NQO1 genotypes from patients who reported being smokers or former smokers had tumors with mutant *K-ras* codon 12 (versus 10/41 smokers with other NQO1 genotypes; unpublished results). However, our study failed to reveal any specific mutational pattern for smokers, which suggests that factors other than cigarette smoke (e.g. environmental, dietary or endogenous factors) may also be involved in the genesis of CRC among these patients.

A number of carcinogens are capable of inducing the K-*ras* base pair substitutions we observed, including BPQs, heterocyclic amines and *N*-nitroso compounds (44), as well as endogenous processes and hormones. Because we found a significant association of NQO1 with CRC in females it is pertinent to point out that the better characterized endogenous quinone pathways involve naturally occurring estrogens (45). The estrogens β-estradiol and estrone are metabolized via two major pathways, 16α-hydroxylation and formation of catechol estrogens (CE), the 2-hydroxy and 4-hydroxy derivatives (46). Generally these two CE are mainly inactivated by *O*-methylation catalyzed by catechol-*O*-methyl transferases, although other possible conjugations of CE, such as glucuronidation and sulfation, may also play a role. If production of these conjugates is incomplete, CE may be oxidized to semiquinones (CE-2,3-semiquinone and CE-3,4-semiquinone) and quinones (CE-2,3-quinone and CE-3,4-quinone). Quinones may be conjugated with glutathione by glutathione *S*-transferases or be reduced to CE by quinone reductase. If these two inactivating processes are incomplete, CE-2,3-quinone reacts with DNA to form stable adducts that remain in DNA unless repaired (47). CE-3,4-quinone, if not inactivated, reacts with DNA forming N7 Gua adducts that represent a tumor-initiating event in a number of human cancers (45). Larger sample sizes will be necessary to consider these additional metabolic pathways and the related gene polymorphisms known to exist in estrogen conjugation and reduction. It is of interest that a recent study reporting an association of microsomal epoxide hydrolase deficiency (exon 3) (48) and CRC found no preference for tumors containing K-*ras* alterations.

The significant association between the C609T NQO1 genotype and the risk for a specific subtype of CRC (K-*ras* codon 12 mutant) highlights once more the need to incorporate more sophisticated methods to subclassify CRC into etiologically more homogeneous groups, perhaps more closely linked to environmental causes of the disease. Such an approach, when integrated with analytical epidemiological designs, may reveal distinct etiological pathways and risk factors for CRC, which is increasing in many parts of the world (including Spain) that have historically experienced low and moderate rates of the disease. Our results also reinforce the notion that NQO1 is an important enzyme involved in protecting cells against natural and xenobiotic quinones and should be studied further for its potential to prevent human cancer. Indeed, chemopreventive agents such as Oltipraz and other dithiol-3-thiones, indoles and isothiocyanates may exert their protective effects against toxicity, mutagenesis and carcinogenesis through their induction of NQO1 activity (49). A note of caution is also indicated from our studies as genetic deficiency affecting the NQO1 locus may increase CRC risk and mitigate against such pharmacological strategies; the C609T NQO1 genotype precludes any benefit that might derive from treatment with inducers of reductase activity, and alternative interventions for genetically susceptible individuals may be needed.

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