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No Association Between Genetic Polymorphisms in *NAD(P)H Oxidase p22^{phox}* and *Paraoxonase 1* and Colorectal Cancer Risk

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Abstract. *Background: Impaired metabolism of ingested toxic or carcinogenic compounds is a postulated mechanism underlying colorectal cancer (CRC). Furthermore, it is suggested that reactive oxygen species (ROS) may play a role in human cancer development. Polymorphic variations in NAD(P)H oxidase p22^{phox} and paraoxonase 1 (PON1) enzyme activities may alter superoxide production or the rate of chemical metabolism, respectively, and this may influence the risk for CRC. Therefore, this study was designed to determine whether the distribution of polymorphisms in NAD(P)H oxidase p22^{phox} and PON1 genes was different in sporadic CRC patients versus healthy controls. Materials and Methods: The study participants (365 cases and 354 controls) were all of Caucasian origin. NAD(P)H oxidase p22^{phox} H72Y, and PON1 L55M and Q192R polymorphisms were genotyped by polymerase chain reaction, eventually followed by restriction-fragment-length-polymorphism analyses. Results: Comparison of CRC patients and controls revealed no significant differences in genotype distributions or allele frequencies for polymorphisms in the NAD(P)H oxidase p22^{phox} and PON1 genes. Investigation of potential associations between the variant NAD(P)H oxidase p22^{phox} or PON1 alleles and the clinical characteristics, tumour location or tumour stage, also did not reveal statistically significant associations. Conclusion: Variant genotypes of NAD(P)H oxidase p22^{phox} and PON1 do not contribute to the susceptibility to CRC.*

The aetiology of colorectal cancer (CRC) remains elusive. It is estimated that up to 10% of CRC cases can be attributed to hereditary factors of high penetrance (1), leaving approximately 90% so-called sporadic CRC cases, which may be attributed to diet (2), lifestyle factors (3, 4) and genetic factors of low penetrance (5). Genetic predisposition to CRC may involve polymorphic variations in genes encoding for detoxification enzymes. Genetic variations in these enzymes may alter the conversion rate of toxic/carcinogenic compounds ingested by food, medication or lifestyle habits (*e.g.* smoking), which subsequently might influence the levels of these compounds in the colonic lumen or mucosa, possibly altering the risk for CRC. In addition, it is suggested that reactive oxygen species (ROS) may also play a role in human cancer development (6-8). ROS may induce cellular changes characteristic of those produced by known carcinogens (7). Phagocytosis by polymorphonuclear leukocytes results in the release of ROS, referred to as the respiratory burst, which plays an important role in the host defence against certain micro-organisms. However, excessive generation of ROS by these phagocytes may cause harm to surrounding tissue and this may influence the risk for CRC (9).

The NAD(P)H oxidase enzyme is involved in the production of large quantities of superoxide during the respiratory burst of activated phagocytes. However, not only phagocytes, but also non-phagocytic cells, such as endothelial cells, vascular smooth muscle cells, cultures of transformed colonic epithelial cells or primary colonic epithelial cells, may produce superoxide (10, 11). In non-phagocytic cells, superoxide has been suggested to act as a regulator of genes involved in proliferation, apoptosis and inflammation (12). NAD(P)H oxidase is a membrane-bound enzyme complex, which consists of a transmembrane electron transporting component, comprising a catalytic subunit cytochrome b_{558}, consisting of gp91^{phox} and p22^{phox} (13). In the *p22^{phox}* gene a C242T polymorphism has been identified that substitutes histidine by tyrosine at codon 72 (11). This may result in 72H homozygotes with normal enzyme activity, and heterozygotes or 72Y homozygotes both with diminished enzyme activity (14). These variations in *NAD(P)H oxidase p22^{phox}* genotypes

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eventually could lead to different amounts of superoxide in the colonic lumen or mucosa, which actually may influence the risk for CRC.

Paraoxonase 1 (PON1) is an esterase that is widely distributed among tissues such as liver, kidney and intestine, but also is present in blood plasma, where it is associated with high-density lipoproteins (HDL; 15, 16). The PON family (PON1-3) enzymes are hydrolases with a broad substrate specificity (17). The PON1 protein was identified first and, therefore, has been most studied. PON1 is a phase I detoxification enzyme that hydrolyzes organophosphates, such as the insecticides paraoxon and chlorpyriphos, and nerve agents, such as sarin or soman (18). It also hydrolyzes aliphatic lactones, like dihydrocoumarin and homocysteine-thiolactone (19, 20). Furthermore, PON1 inactivates lipoxidation derivatives of low-density lipoprotein (LDL) (15, 21, 22). In summary, PON1 can offer protection against toxic environmental agents, as well as endogenous products of oxidative stress. In the *PON1* gene two common functional polymorphisms, L55M and Q192R, have been described, both of which may affect serum paraoxonase activity. *PON1-55L* is correlated with higher PON1 activity and mRNA levels than *PON1-55M* (23, 24), possibly caused by a decreased stability of the PON1-55M protein (25). The Q192R polymorphism results in substrate-dependent differences in the kinetics of hydrolysis of various substrates. The *PON1-192R* allele is associated with a higher activity to hydrolyze paraoxon, whereas the efficiency to hydrolyze sarin, diazoxon or lactones was lower compared with the *PON1-192Q* allele (18, 26). In addition, the Q192R polymorphism also alters the ability of the enzyme to protect LDL from oxidation, the *PON1-192Q* allele being most protective (27). Since PON is a HDL-associated enzyme, many studies have investigated the relationship between *PON1* polymorphisms and coronary heart disease (28) and atherosclerosis (29). Although PON is also known to play a role in the detoxification of toxic/carcinogenic compounds and, therefore, may influence susceptibility to cancer, data on its association with cancer are rare. Kerridge *et al.* (30) were the first to demonstrate an association between *PON1* polymorphisms and cancer in humans: the homozygous variant *PON1-192R* genotype was significantly more often present in non-Hodgkin's lymphoma cases compared with control subjects. Furthermore, Akcay *et al.* found lower PON serum levels in patients with pancreatic (31) or gastric cancer (32) than in healthy controls, which suggests that PON may play a role in the aetiology of gastrointestinal cancer.

Polymorphic variations in NAD(P)H oxidase $p22^{phox}$ and PON1 enzymes may alter superoxide production or conversion rates of toxic/carcinogenic compounds, respectively, and this may influence the risk for CRC. Therefore, this study was designed to determine whether sporadic CRC patients have another distribution of polymorphisms in *NAD(P)H oxidase* $p22^{phox}$ and *PON1* genes as compared to healthy controls.

Table I. *Clinical characteristics of patients with sporadic colorectal cancer and controls.*

Characteristics	Group	Controls (%)	CRC patients (%)
N		354	365
Age (years; mean ± SD)		43±13	64±11[a]
Gender			
	Male	144 (40.7)	209 (57.3)[a]
	Female	210 (59.3)	156 (42.7)
Location tumour[b]			
	Caecum		40/307 (13.0)
	Ascending colon		22/307 (7.2)
	Transverse colon		17/307 (5.5)
	Proximal		88/319 (27.6)[c]
	Descending colon		8/307 (2.6)
	Sigmoid colon		84/307 (27.4)
	Rectosigmoid junction		41/307 (13.4)
	Rectum		95/307 (30.9)
	Distal		231/319 (72.4)[c]
Stage tumour[d]			
	Dukes' A		7/287 (2.4)
	Dukes' B		101/287 (35.2)
	Dukes' A/B		110/293 (37.5)[e]
	Dukes' C		79/287 (27.5)
	Dukes' D		100/287 (34.8)
	Dukes' C/D		183/293 (62.5)[e]

[a]Controls *versus* CRC patients, $p<0.0001$.
[b]For 58 CRC cases, no information on the exact location of the tumour is available.
[c]For some patients only proximal or distal location was reported in the medical files.
[d]For 78 cases of CRC, no information about the exact stage of the tumour is available.
[e]For some patients, only stage Dukes' A/B or C/D was reported in the medical files.

Materials and Methods

Patients and control subjects. The selection of patients and controls has been described previously (33). Briefly, the sporadic CRC group consists of 365 patients (209 males, 156 females; mean age 64±11 years) and for comparison a control group of 354 healthy subjects (144 males, 210 females; mean age 43±13 years) was recruited by advertisement in a local paper. All subjects studied were Caucasians of Dutch origin. Relevant data of the patients and controls are summarized in Table I.

DNA isolation. DNA was extracted from either whole blood (276 cases and 354 controls) or normal colorectal mucosa (89 cases), as

Table II. *Distribution of p22^{phox} and PON1 genotypes in patients with colorectal cancer and controls.*

Gene	Genotype	Controls (%)[a]	CRC patients (%)[a]	Crude OR (95%CI)[b]	Adjusted OR (95%CI)[c]
p22^{phox}	H/H	169/336 (50.3)	177/365 (48.5)		
	H/Y	137 (40.8)	148 (40.5)		
	Y/Y	30 (8.9)	40 (11.0)	1.1 (0.80-1.4)	1.2 (0.80-1.8)
PON1-55	LL	140/352 (39.8)	139/364 (38.2)		
	LM	162 (46.0)	166 (45.6)		
	MM	50 (14.2)	59 (16.2)	1.1 (0.79-1.4)	1.1 (0.73-1.6)
PON1-192	QQ	158/295 (53.6)	180/354 (50.8)		
	QR	120 (40.7)	150 (42.4)		
	RR	17 (5.8)	24 (6.8)	1.1 (0.82-1.5)	1.1 (0.76-1.7)

[a]In both the CRC and control group there are some missing data because of insufficient amount of DNA or unsuccessful PCR.
[b]Genotypes were combined on the basis of an expected phenotype-genotype relationship (variant genotypes with reduced or enhanced enzyme activity *versus* genotypes with normal enzyme activity) and crude OR were calculated. Starting from paraoxon as substrate, phenotype-genotype relationships for the PON1 polymorphisms were estimated (23).
[c]OR adjusted by age and gender were calculated.

described previously (33). Cases were classified according to Dukes' stages (A, B, C, D) and according to location of the tumour in the large intestine as either proximal (caecum, ascending or transverse) or distal (descending, sigmoid, rectosigmoid junction or rectum).

Genotyping. The genetic polymorphisms in *p22^{phox}* and *PON1* genes were analyzed using polymerase chain reaction (PCR) followed by restriction-fragment-length-polymorphism (RFLP) analyses. Detection of the H72Y substitution in the *p22^{phox}* gene was based on the method described by Inoue *et al.* (11). In short, the forward and reverse primers used were: 5'-TGC TTG TGG GTA AAC CAA GGC CGG TG-3' and 5'-AAC ACT GAG GTA AGT GGG GGT GGC TCC TGT-3', respectively. The PCR conditions were 5 min at 95˚C, then 35 cycles of 30 sec at 95˚C, 40 sec at 58˚C, and 40 sec at 72˚C, and finally an elongation step at 72˚C for 5 min. Digestion of the PCR product with the restriction enzyme *Rsa*I was followed by separation on 2% agarose. A 348-bp fragment indicates for the *72H*-allele, whereas fragments of 188- and 160-bp are indicative for the *72Y*-allele. The polymorphisms in the *PON1* gene corresponding with amino acid substitutions at the codons 55 and 192 were determined according to Humbert *et al.* (34). The forward and reverse primers used for detection of the L55M substitution were: 5'-GAA GAG TGA TGT ATA GCC CCA-3' and 5'-TTT AAT CCA GAG CTA ATG AAA GCC-3', respectively. The PCR conditions were 4 min at 95˚C, then 35 cycles of 30 sec at 95˚C, 1 min at 56˚C, and 1 min at 72˚C, and finally an elongation step at 72˚C for 7 min. Digestion with *Nla*III resulted in a non-digested 170-bp PCR product indicative for the *55L*-allele and 126- and 44-bp fragments for the *55M*-allele. For detection of the Q192R substitution, we used the forward primer: 5'-TAT TGT TGC TGT GGG ACC TGA G-3' and reverse primer: 5'-CAC GCT AAA CCC AAA TAC ATC TC-3'. Except for the annealing temperature, which was 54˚C for 1 min, similar PCR conditions were used as described for the detection of the L55M substitution. Digestion with *Alw*I yielded a non-digested 99-bp fragment indicative for the *192Q*-allele and 66- and 33-bp fragments for the *192R*-allele. Digested PCR products of the two *PON1* polymorphisms were separated on 3% agarose and visualized using ethidium bromide. At each PCR run, sterile H_2O was run in parallel with genomic DNA samples and served as a negative control for amplification.

Statistical analyses. Differences between the characteristics of patients and controls were analyzed with the Chi-square test and *t*-test. A *p*-value below 0.05 was considered significant. All genotypes investigated among controls were tested to find out whether they were distributed according to the Hardy-Weinberg equilibrium. Chi-square statistics were used to test for differences in genotype distribution and allele frequencies between the two study groups. Odds ratios (OR) with 95% confidence interval (95% CI) were calculated by logistic regression analyses, taking into account confounding factors such as age and gender. Finally, the Spearman rank coefficient of correlation was calculated for the association between the two *PON1* polymorphisms. All statistical analyses were performed using SPSS (version 12.0; SPSS Inc., Chicago, USA).

Results

Descriptive statistics of CRC patients and controls are given in Table I. The mean age of the CRC patients (64±11 years) was higher compared to that of the control group (43±13 years; $p<0.0001$). Subsequently, there was a statistically significant difference in gender between the CRC patients and healthy controls, with more female subjects in the control group ($p<0.0001$).

For all polymorphisms investigated, the distribution of the allele frequencies among the control subjects was tested and found to be in Hardy-Weinberg equilibrium. No differences in genotype distribution were observed for the

Table III. *Allele frequencies of p22phox and PON1 in patients with colorectal cancer and controls.*

Alleles	Controls (number/total)[a]	CRC patients (number/total)[a]	Crude OR (95% CI)	Adjusted OR (95% CI)[b]
p22phox 72H	0.71 (475/672)	0.69 (502/730)		
p22phox 72Y	0.29	0.31	1.1 (0.87-1.4)	1.1 (0.93-1.3)
PON1 55L	0.63 (442/704)	0.61 (444/728)		
PON1 55M	0.37	0.39	1.1 (0.87-1.3)	1.1 (0.94-1.2)
PON1 192Q	0.74 (436/590)	0.73 (510/708)		
PON1 192R	0.26	0.27	1.1 (0.86-1.4)	1.1 (0.91-1.3)

[a]In both the CRC and control group there are some missing data because of insufficient amount of DNA or unsuccessful PCR.
[b]OR adjusted by age and gender were calculated.

Table IV. *Allele frequencies of p22phox and PON1 with respect to tumour location and tumour stage in patients with colorectal cancer.*

Gene	Proximal CRC (number/total)[a]	Distal CRC (number/total)[a]	CRC Dukes' A/B (number/total)[a]	CRC Dukes' C/D (number/total)[a]
p22phox 72H	0.67 (117/176)	0.70 (321/462)	0.70 (154/220)	0.68 (250/366)
p22phox 72Y	0.33	0.30	0.30	0.32
PON1 55L	0.63 (111/176)	0.59 (273/460)[b]	0.61 (134/220)	0.64 (235/366)
PON1 55M	0.37	0.41	0.39	0.36
PON1 192Q	0.71 (120/168)	0.74 (331/450)	0.72 (157/218)	0.73 (257/354)
PON1 192R	0.29	0.26	0.28	0.27

[a]There are some missing data because of insufficient amount of DNA or unsuccessful PCR.
[b]OR adjusted by age and gender were calculated. *PON1-55*: Distal CRC 1.1 (0.98-1.3).

three polymorphisms investigated between CRC patients and controls (Table II). The allele frequencies also showed no statistical differences between the two study groups (Table III).

We also investigated potential associations between allele frequencies of the detoxification enzymes and clinical characteristics of the patients, such as tumour location and tumour stage, and the results are summarized in Table IV. These data only reveal a borderline association of the variant *PON1-55M* allele with distal CRC, age- and gender-adjusted OR 1.1, 95% CI 0.98-1.3. No relationship was found between allele frequencies of the investigated enzymes and tumour stage.

Finally, the co-occurrence of the *PON1-55* and *PON1-192* polymorphisms was investigated. A negative correlation was found in both patients (r_s = -0.29, $p<0.01$) and controls (r_s = -0.37, $p<0.01$). Thus, the *PON1-55M* and *PON1-192Q* polymorphisms, both associated with low enzyme activity, are usually linked on one allele. This combination of *PON1* polymorphisms was not distributed differently between the CRC patients and healthy controls.

Discussion

It is generally accepted that inter-individual differences in genetic factors of low penetrance and environmental exposures may influence the risk for CRC (35). Polymorphisms in genes encoding for detoxification enzymes may be of importance in susceptibility to toxic or carcinogenic environmental chemicals (5, 36). In addition, there is increasing evidence that ROS may also play a role in human cancer development (6-8). To investigate the role of

NAD(P)H oxidase and paraoxonase enzymes in colorectal carcinogenesis, we determined the frequencies of *NAD(P)H oxidase p22^{phox}* and *PON1* gene polymorphisms in healthy controls and patients with sporadic CRC. The observed frequencies of the variants of *p22^{phox}* and *PON1* in our control population are in full agreement with the corresponding data from several other studies (14, 17, 30, 37, 38).

Perner *et al.* (10) concluded that superoxide, released in epithelial cells isolated from the normal human colon by NAD(P)H oxidases expressing the p22^{phox} subunit, can contribute to the maintenance of the normal colonic barrier. Knowing this, it may be hypothesized that polymorphisms in *NAD(P)H* oxidase *p22^{phox}* can contribute to an increased risk of colon cancer, but the results of this study do not confirm this hypothesis. The *p22^{phox}-72Y* homozygotes, however, may have only a diminished and not a complete loss of enzyme activity (14), and this reduced enzyme activity may still be sufficient to maintain the normal colonic barrier.

Since it has been demonstrated that polymorphisms affecting amino acid substitutions at positions 55 and 192 are associated with marked alterations of PON1 serum concentrations (23, 24), and PON1 serum levels were reported to modulate susceptibility towards other gastrointestinal cancers (31, 32), we investigated the relationship between *PON1* polymorphisms and CRC. No differences were observed for genotype distributions and allele frequencies between patients and controls, except for a trend towards a slightly increased risk for distal CRC in individuals bearing the *PON1-55M* allele. The *PON1-55M* variant is associated with a reduced enzyme activity (23, 24), which could mean that the conversion rate of carcinogenic compounds is decreased and that these compounds may accumulate in the colonic lumen. Nevin *et al.* (16) reported that the *PON1* genotype accounts for 76% of the variation in serum PON enzyme activity level. In addition, PON1 serum levels are modulated by disease state, dietary, lifestyle and environmental factors and, therefore, may vary up to 13-fold between individuals (17, 39). Moreover, human studies characterizing the *PON1* polymorphisms have indicated the importance of estimating the PON1 status (*i.e.* genotype and phenotype taken together) rather than genotyping alone (40). Unfortunately, no serum was available from the majority of our patients, so we were unable to measure serum PON1 enzyme activities.

Our findings have to be viewed in the perspective of potential limitations. Odds ratios can only be calculated correctly when confounding factors, such as age, gender, diet and other lifestyle factors, are taken into consideration. In this study, we observed a statistically significant difference in age and gender between the CRC patients and controls. By including both age and gender in the logistic regression analyses, we corrected the calculated odds ratios for differences in these factors. This is necessary because the younger control subjects, in comparison to the CRC patients, have had a shorter time of exposure to carcinogens and, thus, at the moment they reach the age of the CRC patients, some of them may also have developed CRC. It would have been preferable to better match the control and patient populations, but in practice this was very difficult to realize. In this study, no information was available on the dietary habits, alcohol use and smoking patterns of both patients and controls, which may also be confounding factors. Possibly some of the low-penetrance genes investigated here only contribute to CRC in combination with (some of) these dietary or lifestyle factors.

In conclusion, no association was found between polymorphic variations in *NAD(P)H oxidase p22^{phox}* and *PON1* genes and the risk for sporadic CRC.

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