Rapid *N*-acetyltransferase 2 imputed phenotype and smoking may increase risk of colorectal cancer in women (Netherlands)

Olga L. van der Hel[1], H. Bas Bueno de Mesquita[2], Lodewijk Sandkuijl[1,3], Paul A.H. van Noord[1], Peter L. Pearson[3], Diederick E. Grobbee[1] & Petra H.M. Peeters[1,*]
[1]*Julius Center for Health Sciences and Primary Care, University Medical Centre, Utrecht, 3508 GA Utrecht, The Netherlands;* [2]*Department of Chronic Diseases Epidemiology, National Institute of Public Health and the Environment, Bilthoven, 3720 GA Bilthoven, The Netherlands;* [3]*Department of Medical Genetics, University Medical Centre, Utrecht, 3508 GA Utrecht, The Netherlands*

Abstract

Objective: The relationship between smoking and colorectal cancer risk and whether such effect is modified by variations in the *NAT2* genotype is investigated.
Methods: In the prospective DOM (Diagnostisch Onderzoek Mammacarcinoom; 27,722 women) cohort follow-up from 1976 until 1987 revealed 54 deaths due to colon or rectal cancer, and follow-up from 1987 to 01-01-1996 revealed 204 incident colorectal cancer cases. A random sample (n = 857) from the baseline cohort was used as controls. Four *NAT2* restriction fragment length polymorphisms (RFLPs) were analysed using DNA extracted from urine samples. Rapid or slow acetylator phenotype status was attributed to individuals.
Results: Smoking may increase the risk for colon cancer (RR = 1.36, 95% CI 0.97–1.92) as well as for rectal cancer (RR = 1.31, 95% CI 0.76–2.25), although not statistically significant. Rapid NAT2 acetylation did not increase colorectal cancer risk, but in combination with smoking the risk was statistically significant increased, compared to women who had a slow NAT2 imputed phenotype and never smoked (RR = 1.56, 95% CI 1.03–2.37). For colon cancer, but not for rectal cancer the increased risk was statistically significant (RR = 1.67, 95% CI, 1.05–2.67 *versus* RR = 1.30 95% CI 0.63–2.68).
Conclusions: Our study points to smoking as a risk factor for colon and rectal cancer and, in addition, especially in women with rapid *NAT2* imputed phenotype.

Introduction

Colorectal cancer is the second most common cancer in women in the Netherlands. Family history, diet, alcohol intake, physical inactivity and hormone replacement therapy have been either implicated or suggested as risk factors [1]. A recently updated review showed that a 30–45 year induction period for smoking has to pass before a relation between smoking and colorectal cancer in women can be observed [2]. Cigarette smoke contains aromatic amines such as 4-aminobiphenyl and heterocyclic amines [3]. In rodents, exposure to these heterocyclic amines is associated with increased colorectal cancer risk [4]. To initiate carcinogenesis these amines require metabolic activation by *N*-acetyltransferase. NAT2 (*N*-acetyltransferase 2) is a polymorphic gene and sequence variants can affect the phenotype, namely NAT2 enzyme activity, stability or substrate affinity. Therefore, individuals can be broadly classified in two different types of NAT2 acetylator phenotype, *i.e.* slow or rapid. The genotypes corresponding to the rapid acetylator phenotype occur at frequencies of approximately 10% in northern Africa, 45% in populations of European descent, 55% in those of

* Address correspondence to: Petra H.M. Peeters, Julius Centre for Health Sciences and Primary Care, University Medical Centre, D01.335, P.O. Box 85500, 3508 GA Utrecht, The Netherlands. Ph.: +31-30-2509363; Fax: +31-30-2505480; E-mail: p.h.m.peeters@jc.azu.nl

American-African descent, and 95% in Japanese [5]. Several studies found an association between rapid NAT2 acetylator genotype and colorectal cancer, whereas other studies did not (reviewed by Brockton et al. [6]). Smoking, particularly in rapid acetylators, may increase risk for colorectal cancer. Population differences in the frequency of both NAT2 rapid acetylator phenotypes and smoking may explain geographical differences in colorectal cancer occurrence as well as inconsistency in studies addressing the relationship between smoking and colorectal cancer. Although colon and rectal cancer share many environmental risk factors and are both found in individuals with specific genetic syndromes, there are some reports that show a stronger relationship between smoking and rectal cancer than for smoking and colon cancer [7].

We set out to study the relationship between smoking and colon and rectal cancer risk particular for women who were rapid acetylators. We included a total of 258 colorectal cancer cases and 857 control persons identified in a large prospective cohort of Dutch women.

Materials and methods

Study subjects

Between 1976 and 1978, all women born between 1911 and 1925, and in 1982 until 1986, all women born between 1932 and 1945, who lived in the city of Utrecht, the Netherlands, were invited for a population-based screening program for the early detection of breast cancer, the so-called 'DOM' (Diagnostisch Onderzoek Mammacarcinoom) project [8]. A total of 27,722 women attended the screening and the participation rate was 70%. Each participant provided an overnight urine sample that was stored at −20 °C.

A mortality registry was set up from 1976 onwards with the co-operation of all general practitioners. From 1987 onwards it became possible to identify developed cancers by using the regional cancer registry, part of the Netherlands Cancer Registry. Follow-up from 1976 until 1987 revealed 56 deaths because of colon cancer and eight deaths because of rectal cancer; follow-up from 1987 to 01-01-1996 revealed 161 incident colon cancer cases and 73 incident rectal cancer cases (fatal and non-fatal). For the present study a total of 298 cases and 1000 controls randomly selected from the DOM baseline cohort were initially available for genotyping. The women were mainly Caucasians. Urine samples of the study population were thawed overnight at room temperature, mixed vigorously, and 50 ml was removed for DNA isolation.

The institutional review board of the University Medical Centre Utrecht for human studies approved our study.

Detection of NAT2 polymorphisms associated with acetylation

The alcohol precipitation method was used for all DNA isolations as described earlier [9]. In brief, after centrifugation, DNA was isolated from the urine pellet by protein precipitation and DNA was precipitated by alcohol. DNA was finally resuspended in 40 μl 10 mM Tris, 1 mM EDTA, pH 7.6 (TE).

NAT2 polymorphisms at positions 341, 590, 803, and 857 were detected by RFLP (restriction fragment length polymorphism) as described earlier [10].

In each experiment three known blood samples were used as controls for PCR as well as for RFLP analyses and the sequence was confirmed by DNA sequencing.

The alleles were determined according to the known nomenclature [11]. $G^{191}T$ is not determined because it is not seen in Caucasian people. A person was considered to be a rapid acetylator if she was heterozygote or homozygote for the alleles NAT2*4 or NAT2*12, otherwise, she was considered as a slow acetylator. All laboratory analyses were performed blind with respect to the case–control status. Association analyses were performed using the putative acetylator phenotypes determined from the NAT2 genotypes.

Questionnaires

Smoking status was assessed at baseline by questionnaire. In this analysis two smoking classes are determined. Women, who reported smoking now or in the past, were classified as ever-smokers. All others were classified as never-smokers. As the number of ever-smokers was small, we were not able to differentiate the ever-smokers into categories according to smoking exposure.

Data analysis

For 10 persons the smoking status was not known and for 169 participants there was insufficient DNA available or the genotyping method failed; this left 258 cases and 857 controls (non-cases) for analysis. As our reference group was a random sample of the total cohort of non-cases, multiplication of the person years in the reference group by 25.9 (the inverse of the sampling fraction) enabled us to analyse the nested case–control approach exactly as a full cohort analysis, in which person years are unbiased estimates of true

person years. Poisson regression models were used for calculating relative risks with slow acetylator or slow acetylator in combination with never smoker as reference category. Robust 95% confidence intervals were calculated with Hubers method [12]. Relative risks are also presented for ever and never smoking women separately. Factors considered for confounding were age at study entry (continuous) and (body mass index BMI) (continuous) and (Hormone Replacement Therapy HRT) (yes/no). We decided to include confounders in the model if exclusion changed the estimate for the association with cancer risk by more than 10%. Since this was the case for age at study entry and BMI, these were included in the final models. Analyses were done for colorectal cancer, and separately for colon (n = 191) and rectal (n = 67) cancer cases. Analyses were performed using SPSS 9.0 and Stata 6.0.

Results

General characteristics of colorectal cases and controls are shown in Table 1. Forty-eight women were identified by the mortality registry to have died of colon cancer and seven of rectal cancer. One hundred and forty-three women were newly diagnosed with colon cancer and sixty women with rectal cancer.

Of the women, 35% smoked in the case group, while 31% smoked in the control group. Smoking increased the risk for colon cancer (RR = 1.36, 95% CI 0.97–1.92), and the effect was the same for rectal cancer (RR = 1.31, 95% CI 0.76–2.25). Both results were statistically non-significant. The RR for total colorectal cancer for women who ever smoked was 1.35 (95% CI 0.99–1.83) (Table 2). Forty-four percent and 42%, respectively, of colorectal cases and controls were assessed as rapid *NAT2* acetylators.

Women who are rapid acetylators showed no significantly increased risk for colorectal cancer (RR = 1.06,

Table 1. Characteristics of the study population

Parameter	Mean (SD)	
	Cases (n = 258)	Controls (n = 871)
Age (years)	55.6 (6.03)	54.0 (6.29)
Height (cm)	163.0 (6.2)	162.5 (6.2)
Weight (kg)	68.6 (10.5)	69.0 (10.8)
BMI	25.8 (3.6)	26.1 (3.9)
Follow-up (years)	12.0 (4.85)	16.0 (4.99)
	Number (percentage)	
Smoking		
Ever (persons)	87 (35%)	270 (31%)
Colon cancer		
Newly diagnosed	143 (55%)	
Death	48 (19%)	
Rectal cancer		
Newly diagnosed	60 (23%)	
Death	7 (3%)	

95% CI 0.81–1.41). The risk was slightly increased for colon cancer (RR = 1.16, 95% CI 0.84–1.59) and slightly decreased for rectal cancer (RR = 0.83; 95% CI 0.49–1.38) (Table 3). Analysis of the combined effect of acetylator status and smoking showed that, in comparison to slow *NAT2* acetylators who never smoked, rapid acetylators who ever smoked were at statistically significant increased risk for colorectal cancer (RR = 1.56; 95% CI 1.03–2.37). The increased risk was found for colon cancer as well as for rectal cancer, but only for colon cancer the result was statistically significant (Colon: RR = 1.67, 95% CI, 1.05–2.67 and Rectum: RR = 1.30, 95% CI 0.63–2.68) (Table 4).

Discussion

The results of our study suggest that smoking could be a risk factor for colon and rectal cancer. This might be more pronounced in women with a *NAT2* genotype for

Table 2. Association between smoking and colon and rectal cancer

Smoking	Cases, n (%)	Controls, n (%)	RR[a] (95% CI)
Colorectal cancer			
Never	162 (65%)	590 (69%)	1.00
Ever	87 (35%)	266 (31%)	1.35 (0.99–1.83)
Colon cancer			
Never	119 (65%)	590 (69%)	1.00 (Ref)
Ever	64 (35%)	266 (31%)	1.36 (0.97–1.92)
Rectal cancer			
Never	43 (66%)	590 (69%)	1.00 (Ref)
Ever	23 (34%)	266 (31%)	1.31 (0.76–2.25)

[a] Adjusted for age and BMI.

Table 3. Associations between *NAT2* genotype and colon and rectal cancer

NAT2	Cases, n (%)	Controls, n (%)	RR[a] (95% CI)
Colorectal cancer			
Slow	146 (56%)	495 (58%)	1.00
Rapid	112 (44%)	362 (42%)	1.06 (0.80–1.41)
Colon cancer			
Slow	104 (54%)	495 (58%)	1.00 (Ref)
Rapid	87 (46%)	362 (42%)	1.16 (0.84–1.59)
Rectal cancer			
Slow	42 (63%)	495 (58%)	1.00 (Ref)
Rapid	25 (37%)	362 (42%)	0.83 (0.49–1.38)

[a] Adjusted for age.

rapid acetylation, in relation to colon cancer (RR = 1.67, 95% CI 1.05–2.67) and possibly also for rectal cancer (RR = 1.30, 95% CI 0.63–2.68).

To understand the relevance of these findings some aspects of the study need to be addressed. Urine samples are used as the source of DNA. The DNA yield from urine is variable and in approximately 10–20% of the samples the amount is not enough to perform genotyping [9]. The percentages of failures of genotyping were equally distributed among cases and controls (13% both). We previously showed that NAT2 genotyping in DNA isolated from blood and urine of the same person, using blood results as a reference standard, resulted in a sensitivity rate of 97%; *i.e.* 97% of subjects who were classified as rapid acetylators based on blood DNA, were also classified as rapid when using their DNA obtained from urine. The specificity rate was also 97%, *i.e.* slow acetylators were classified as slow [10]. Distributions of polymorphisms in cases and controls were both in Hardy–Weinberg equilibrium.

We used DNA genotyping data to predict the phenotype. Prediction of the NAT2 acetylation phenotype from *NAT2* genotypes has some limitations. Hein *et al.* [13] showed that some phenotype misclassification from intermediate to slow phenotype can occur if the incorrect polymorphisms are determined. Therefore, we determined the four polymorphisms, at position 341, 590, 803 and 857. We did not make a distinction between rapid and intermediate otherwise the groups become too small and all presumptive intermediate/rapid subjects were scored as rapid.

We did not find a direct association between the rapid *NAT2* acetylators and colon or rectal cancer risk, which is in concordance with several studies [6, 14–16]. Three studies reported an association between *NAT2* genotype corresponding to rapid phenotype and colorectal cancer for individuals consuming red meat and, presumably, with a higher intake of heterocyclic amine carcinogens [16–18]. There is no data available on meat consumption

for our cohort. Another source of heterocyclic amine carcinogens is cigarette smoke. Four studies reported evidence for an interaction between smoking and acetylation activity in relation to colorectal adenomas or colorectal cancer. In a study of adenomas, Probst Hench *et al.* [19] observed an increased risk in current smokers who were NAT2 rapid acetylators in comparison to never-smokers and slow acetylators (RR = 2.3, 95% CI 1.0–5.1). This was later confirmed by Potter *et al.* [20], who found associations between smoking status and hyperplastic polyps (RR = 4.1, 95% CI 1.4–2.9) and adenomas (RR = 2.0, 95% CI 1.4–2.0), which were slightly modified by rapid NAT2 activity (RR = 4.9, 95% CI 2.6–9.4 and RR = 2.3, 95% CI 1.4–3.9; respectively). Welfare *et al.* [16] reported that cigarette smoking in the past 5 years was not associated with colorectal cancer risk among *NAT2* rapid acetylators, but was associated with a significantly raised risk among slow acetylators (RR = 2.3, 95% CI 1.0–7.3). Slattery *et al.* [21] found an increased colon cancer risk for women who ever smoked and were intermediate/rapid acetylators (OR = 1.4, 95% CI 1.0–1.9) compared to slow acetylators that have never smoked. In our study rapid *NAT2* acetylators combined with smoking increased risk for colon cancer and probably for rectal cancer. Agundez *et al.* [22] observed an increased risk of sigmoid colon cancer, but not rectal cancer, for individuals with genotypes leading to high NAT2 activity. In our study, the numbers become very small if such sub-site analysis is performed.

Follow-up from 1976 until 1987 revealed colorectal deaths and follow-up from 1987 to 01-01-1996 revealed colorectal incidents. Restricting analyses to incident cases only did not materially change our results.

This is a prospective study that studies the role of NAT2 and smoking in colorectal cancer risk. Compared with a case–control study there is no advantage for accuracy of genotyping data, but recall bias of smoking will not distort relative risks in a prospective study.

Table 4. Associations of *NAT2* genotype in combination with smoking status and colon and rectal cancer

Smoking status	NAT2	Colorectal cancer			Colon cancer			Rectal cancer		
		Cases, n (%)	Controls, n (%)	RR[a] (95% CI)	Cases, n (%)	Controls, n (%)	RR[a] (95% CI)	Cases, n (%)	Controls, n (%)	RR[a] (95%CI)
Never	Slow	99 (40%)	341 (40%)	1.00	69 (38%)	341 (40%)	1.00	30 (45%)	341 (40%)	1.00
Never	Rapid	63 (25%)	249 (29%)	0.89 (0.63–1.28)	49 (27%)	249 (29%)	0.98 (0.66–1.47)	14 (21%)	249 (29%)	0.68 (0.35–1.31)
Ever	Slow	42 (17%)	153 (18%)	1.09 (0.72–1.66)	30 (17%)	153 (18%)	1.11 (0.69–1.78)	12 (18%)	153 (18%)	1.05 (0.51–2.17)
Ever	Rapid	45 (18%)	113 (13%)	1.56 (1.03–2.37)	34 (19%)	113 (13%)	1.67 (1.05–2.67)	11 (17%)	113 (13%)	1.30 (0.63–2.68)

[a] Adjusted for age.

A recent review showed that for smoking to be able to cause colorectal cancer a 30–45 year induction period is mandatory [2]. In The Netherlands, women started to smoke after the second world war (1945) and on a larger scale in the 1960s. The induction period of three to four decades would, thus, only be achieved for cases occurring after 1980s or 1990s. The cases in our cohort were diagnosed between 1974 and 1996 and, therefore, for some of these cases the relevant induction period had not passed. We were unable to check this, because data on individual smoking duration and starting age were not available. However, even with our crude classification (ever, never), and limited study size, we were able to observe a statistical significant effect of smoking in smoking in women with rapid NAT2 genotype.

Confounding for age, use of HRT and BMI was taken care of in our analysis. The use of non-steroidal anti-inflammatory drugs (found to protect) was not administered in the DOM cohort. However, since differential prescription according to NAT2 genotype status is unlikely, confounding is unlikely too. Additional confounding by other established risk factors, such as low physical activity or consumption of well-done meat, is also unlikely, but cannot be ruled out.

The *NAT2* gene codes for an enzyme that is a part of a set of metabolic enzymes. Interaction of NAT2 with other enzymes may be very important. Moreover, NAT1 and NAT2 metabolise some of the same procarcinogens. Other genes and/or environmental factors may be relevant and may, in a complex way, be related to the carcinogen exposures and genes evaluated here.

In conclusion, the results of our study suggest that the risk of colon cancer and probably rectal cancer associated with smoking is enhanced in *NAT2* rapid acetylators.

Acknowledgements

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