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ORIGINAL ARTICLE

N-Acetyltransferase (NAT) 2 acetylator status and age of tumour onset in patients with sporadic and familial, microsatellite stable (MSS) colorectal cancer

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Abstract
Introduction *N*-Acetyltransferase (NAT) 2 is an important enzyme involved in the metabolism of different xenobiotics, including potential carcinogens. Allelic variants of the *NAT2* gene are determined by a pattern of single nucleotide polymorphisms (SNPs) resulting in slow (SA), intermediate (IA) or rapid acetylator (RA) phenotypes and causing the individual differences in the *NAT2* metabolic capacity. To clarify the potential modifying role of the *NAT2* acetylator status in microsatellite stable (MSS) colorectal cancer (CRC), we studied 140 patients with sporadic CRC (group 1) and 69 patients with CRC who met at least one criterion of the revised Bethesda guidelines (group 2).
Observations We did not observe any significant difference in the *NAT2* acetylator status frequency between patients in both groups and 100 healthy controls (P=0.486). Regardless of a younger median age of tumour onset (AO) of 41 years in group 2 patients compared to 64 years in group 1 patients, no significant difference in AO was found between RA and SA status patients in both groups. The median AO in group 1 was 65 years in patients with RA and 63 years with SA status (P=0.065). The median AO in group 2 was 40 years in patients with RA and 42 years with SA status (P=0.814). Multivariate Cox regression analysis revealed that neither the *NAT2* acetylator status (P=0.064 and 0.810, respectively) nor the gender (P=0.165 and 0.918, respectively) was a risk factor for the AO in both groups. These data do not support the hypothesis that the *NAT2* acetylatorship acts as a modifying factor on the AO in sporadic and familial, microsatellite stable CRC.

Keywords *NAT2* · Sporadic colorectal cancer · Familial colorectal cancer · Bethesda criteria · Microsatellite instability

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Introduction

N-Acetyltransferase (NAT) 2 is an essential polymorphic enzyme that is involved in the metabolism of various xenobiotics, including potential carcinogens like aromatic and heterocyclic amines [1]. Allelic variants of the *NAT2* gene (chromosome 8p22), which are determined by a pattern of single nucleotide polymorphisms (SNPs), result in slow (SA), intermediate (IA) or rapid acetylator (RA) phenotypes and cause the individual differences in the *NAT2* metabolic capacity. Whilst the *NAT2**4 allele (wild-type) encodes the RA phenotype, other alleles (*NAT2**5–7 and rare alleles) encode the SA or IA phenotype [2]. Although a recent meta-analysis including 36 studies and more than 13,000 CRC cases showed that the *NAT2* RA

phenotype is associated with an increased CRC risk [3], numerous studies which analysed the role of *NAT2* acetylatorship as a risk factor in CRC patients assessing the exposure to diet and smoking presented controversial results [4–9]. For example, studies by Robert-Thomson et al. [7] and Chan et al. [9] showed an association between the *NAT2* acetylator phenotype and CRC risk whilst studies by Slattery et al. [4, 8], Kampman et al. [5] and Ye and Parry [6] did not verify these results.

Certain alleles in glutathione *S*-transferase [10–16], aldehyde dehydrogenase [17, 18], methylenetetrahydrofolate reductase [19–23], cytochrome P450 [24–27], adenomatous polyposis coli [28, 29], *NAT* 1 [30–32] and 2 [32–38] and Harvey ras-1 variable number tandem repeat alleles [39–41] are considered candidates for risk modification for CRC. In most studies, populations of molecularly unclassified CRC patients were used. One study investigated the role of *NAT2* acetylatorship for AO in primary sporadic CRC and showed a significant association between the *NAT2* SA status and early AO in 275 patients [36]. To clarify the role of *NAT2* acetylator status in patients with molecularly classified CRC, we performed a study in 140 patients with sporadic, microsatellite stable (MSS) CRC and 69 patients with MSS CRC who met at least one criterion of the Bethesda guidelines [42].

Materials and methods

Patients

Peripheral blood samples of 140 Caucasian patients with sporadic, MSS CRC (group 1) and 69 Caucasian patients with MSS CRC who met at least one criterion of the Bethesda guidelines (group 2) were selected for *NAT2* genotyping. All patients were given written informed consent for study participation. One hundred anonymous healthy blood donors from the Dresden Regional Blood Centre served as controls.

Microsatellite analysis

Microsatellite analysis was performed in patients on paired samples of lymphocyte DNA and paraffin-embedded or fresh-frozen tumour tissue as described elsewhere, [43] applying at least five markers of the reference panel and using the definition of MSS CRC [42].

NAT2 genotyping

An assay based on the LightCycler® (Roche Molecular Systems) technique was used to screen for the *NAT2* SNPs that determine *NAT2* allelic variants. Template DNA amplification was performed with real-time PCR, and fluorescence resonance energy transfer technology was applied to facilitate the online melting-curve analysis of oligonucleotide probes bound to the target SNPs. After PCR on the LightCycler, `hybridization probes in combination with the LightCycler DNA Master Hybridization Probes Kit (Roche Diagnostics) were used. PCR primers and hybridization probes were synthesised by TIB MOLBIOL (Berlin, Germany) and METABION (Martinsried, Germany), respectively. Primer and hybridization probe sequences are shown in Tables 1 and 2. PCR was performed in a total volume of 20 μl in glass capillaries. The reaction mixture used in each PCR consisted of 50 ng of genomic DNA, 2 μl of each primer (5 μmol/l), 2 μl of

Table 1 Primers used for PCR

SNP	Primers
C282T	F: 5′-GTCACACGAGGAAATCAAATGC-3′
	R: 5′-TCCTTCCCAGAAATTAATTCTAG-3′
C341T	F: 5′-GTCACACGAGGAAATCAAATGC-3′
	R: 5′-TCCTTCCCAGAAATTAATTCTAG-3′
C481T	F: 5′-TGCATTTTCTGCTTGACA-3′
	R: 5′-GTTGGGTGATACATACACAA-3′
G590A	F: 5′-TGCATTTTCTGCTTGACA-3′
	R: 5′-GTTGGGTGATACATACACAA-3′
A803G	F: 5′-TGCATTTTCTGCTTGACA-3′
	R: 5′-GTTGGGTGATACATACACAA-3′
G857A	F: 5′-TGCATTTTCTGCTTGACA-3′
	R: 5′-GTTGGGTGATACATACACAA-3′

F Forward, *R* reverse

Table 2 Hybridization probes used for SNP detection

SNP	Hybridization probes
282	5′-GGTATTTTTACATCCCTCCAGTTAA-X-3′
	5′-LC 640-ATACAGCACTGGCATGGTTCACCTTCTC-*p*-3′
341	5′-CAGGTGACCACTGACGGCAGGAATTACAT-X-3′
	5′-LC 705-TCGATGCTGGGTCTGGAAGCTCCTCCC-*p*-3′
481	5′-GCATTTTCTGCTTGACAGAAGAGAGAGGA-X-3′
	5′-LC 640-TCTGGTACCTGGACCAAATCAGGA-*p*-3′
590	5′-GACGTCTGCAGGTATGTATTCATAGACTCAAAAT-X-3′
	5′-LC 705-TCAATTGTTCGAGGTTCAAGCGT-*p*-3′
803	5′-AAGAGGTTGAAGAATTGCTGAAAAATATATTTAA-X-3′
	5′-LC 640-TTCCTTGGGGAGAAATCTCGTGC-*p*-3′
857	5′-ACCTGGTGATGAATCCCTT-X-3′
	5′-LC 705-CTATTTAGAATAAGGAACAAAATAAACCCTTG-*p*-3′

X Fluorescein-labelled, *LC 640* LC Red640-labelled, *LC 705* LC 705-labelled

the LightCycler FastStart DNA Master Hybridization Probes Kit reagent, 1.5 μl of each hybridization probe (1.5 μmol/l) and 1.6 μl of $MgCl_2$ (25 mmol/l). After 10 min of initial denaturation at 94°C and 2 min at 95°C, 45 PCR cycles were performed with 5 s of denaturation at 94°C and 20 s (for SNPs 282, 341, 481 and 857) / 15 s (for SNPs 590 and 803) of annealing at 50°C (for SNPs 282 and 341) / 52° C (for SNPs 481, 590, 803 and 857) with a 25-s (for SNPs 282, 341, 481 and 857) / 20 s (for SNPs 590 and 803) extension at 72°C. The PCR and the melting procedures were detected online with the LightCycler. The generated melting curves enabled a clear-cut differentiation between homozygous wild-type (*w*/*w*), homozygous variant (*v*/*v*) and heterozygous wild-type/variant (*w*/*v*) samples as previously described [44].

Although it has been shown that the determination of the SNPs at bp 282 and 341 in Caucasians is sufficient for prediction of the *NAT2* phenotype [45], SNPs at bp 481, 590, 803 and 857 were included in the analysis. The resulting pattern of the SNPs allows the distinction between the *NAT2**4 allele (conferring the RA phenotype) and the group of *NAT2**5 (including alleles A, B and C), *NAT2**6 and *NAT2**7 alleles, all of which confer the SA phenotype. Homo- or heterozygous carriers of the *NAT2**4 allele were phenotypically classified as RA [2].

Statistical analysis

NAT2 acetylator frequencies were compared between patients in groups 1 and 2 and controls using the chi-square test. The AO of CRC was analysed by the Kaplan–Meier (product-limit) method. The log-rank test was applied to compare the age of onset between different groups. Multivariate Cox regression analysis was used to evaluate the independent effect of gender and the *NAT2* acetylator status on AO. *P*-values below 0.05 were considered significant. All statistical analyses were performed using SPSS 10.0.7 (SPSS, Chicago, IL, USA).

Results

NAT2 allele genotype frequencies are shown in Table 3, and frequencies of rapid (RA) and slow acetylators (SA) are shown in Table 4. We did not observe any significant difference in the *NAT2* acetylator status frequency among 140 patients with sporadic, MSS CRC (group 1) and 69 patients with MSS CRC who met at least one criterion of the revised Bethesda guidelines (group 2) and 100 healthy controls (P=0.486).

Stratification of group 2 patients (Table 4) for a positive family history (criteria 1, 3 and 3+4 of the Bethesda guidelines) or a negative family history (criteria 2, 4 and 2+4 of the Bethesda guidelines) revealed no significant difference in *NAT2* acetylator frequencies (P=0.302).

There was no significant difference between the median AO in group 1 patients with RA status (65 years, 95% CI 60–70) and with SA status (63 years, 95% CI 60–66; P=0.065, Fig. 1). The median AO in group 2 was 40 years (95% CI 36–44) in patients with RA and 42 years with SA status (95% CI 39–45; P=0.814, Fig. 2). In addition, the median AO did not significantly differ between group 2 patients. In patients who met criteria 1, 3 and 3+4 of the Bethesda guidelines, the median AO was 43 years, while in patients who met criteria 2, 4 and 2+4 of the Bethesda guidelines, the median AO was 39 years (P=0.199).

Multivariate Cox regression analysis showed that neither the *NAT2* acetylator status (group 1: P=0.064, group 2: P=0.810) nor the gender (group 1: P=0.165, group 2: P=0.918) was the risk factor for the AO in both groups. Regardless of a significantly younger median AO of 41 years in group 2 patients compared to 64 years in group 1 patients (p<0.0001), no significant difference in AO was found between patients with RA and SA status in both groups.

Table 3 Results of *NAT2* genotyping

NAT2 alleles	Group 1 (sporadic), *n* (%)	Group 2 (Bethesda +), *n* (%)	Controls, *n* (%)
*4/*4	5 (3.6)	4 (5.8)	4 (4.0)
*4/*5A	0 (0)	1 (1.4)	1 (1.0)
*4/*5B	32 (22.9)	10 (14.5)	21 (21.0)
*4/*5C	1 (0.7)	1 (1.4)	0 (0)
*4/*6	24 (17.1)	8 (11.6)	17 (15.9)
*4/*7	2 (1.4)	2 (2.9)	1 (1.0)
RA	64 (45.7)	26 (37.7)	44 (44.0)
*5A/*5A	0 (0)	0 (0)	0 (0)
*5A/*5B	1 (0.7)	1 (1.4)	1 (1.0)
*5A/*5C	0 (0)	1 (1.4)	0 (0)
*5A/*6	0 (0)	1 (1.4)	0 (0)
*5A/*7	1 (0.7)	2 (2.9)	0 (0)
*5B/*5B	25 (17.9)	13 (18.8)	17 (17.0)
*5B/*5C	3 (2.1)	0 (0)	0 (0.0)
*5B/*6	31 (22.1)	18 (12.5)	28 (28.0)
*5B/*7	0 (0)	1 (1.4)	1 (1.0)
*5C/*5C	0 (0)	0 (0)	0 (0)
*5C/*6	0 (0)	0 (0)	1 (1.0)
*5C/*7	0 (0)	0 (0)	0 (0)
*6/*6	15 (10.7)	5 (7.2)	7 (7.0)
*6/*7	0 (0)	1 (1.4)	1 (1.0)
*7/*7	0 (0)	0 (0)	0 (0)
SA	76 (54.3)	43 (62.3)	56 (56.0)
Total	140	69	100

Table 4 Data analysis and results of *NAT2* phenotyping in group 1 and 2 patients and controls

	Group 1	Group 2	Controls	*P*-value
All	140	69	100	
RA (%)	64 (45.7)	26 (37.7)	44 (44.0)	0.486
SA (%)	76 (54.3)	43 (62.3)	56 (56.0)	
Sex				
Male (%)	82 (58.6)	25 (36.2)	54 (54.0)	0.009
Female (%)	58 (41.4)	44 (63.8)	46 (46.0)	
Bethesda criterion				
1 (Amsterdam)		6		
2		10		
3		5		
4		37		
2 and 4		1		
3 and 4		10		
Median AO (95% CI)	64 (61–67)	41 (39–43)		<0.0001
In RA	65 (60–70)	40 (36–44)		
In SA	63 (60–66)	42 (39–45)		
P-value	0.0645	0.8135		

RA Rapid acetylators, *SA* slow acetylators

Discussion

Our study is the first study showing that the *NAT2* acetylator status is neither a relevant risk factor for CRC nor a relevant modifier of AO in Caucasian patients with MSS tumours.

Concerning familial forms of CRC, the role of *NAT2* was investigated in hereditary nonpolyposis colorectal cancer [46, 47] and familial adenomatous polyposis coli [48, 49] with controversial results. However, *NAT2* acetylatorship frequencies were analysed in only a few studies regarding the family history or the microsatellite status. In a multicentre study of 1993 colon cancer patients from the United States, no correlation was observed between *NAT2* acetylator status and a positive family history in first-degree relatives for CRC [50]. Although Slattery et al. [51] found a significant interaction between family history of CRC, Western dietary pattern and AO for colon cancer, *NAT2* did not significantly interact with Western diet. A third large case-control study of the same author, published in 2002, showed that the *NAT2* phenotype was not independently associated with microsatellite instability in colon cancer patients [52].

Concerning the role of *NAT2* acetylatorship for AO, one study investigated 275 patients with sporadic CRC and showed a significant correlation between *NAT2* SA status

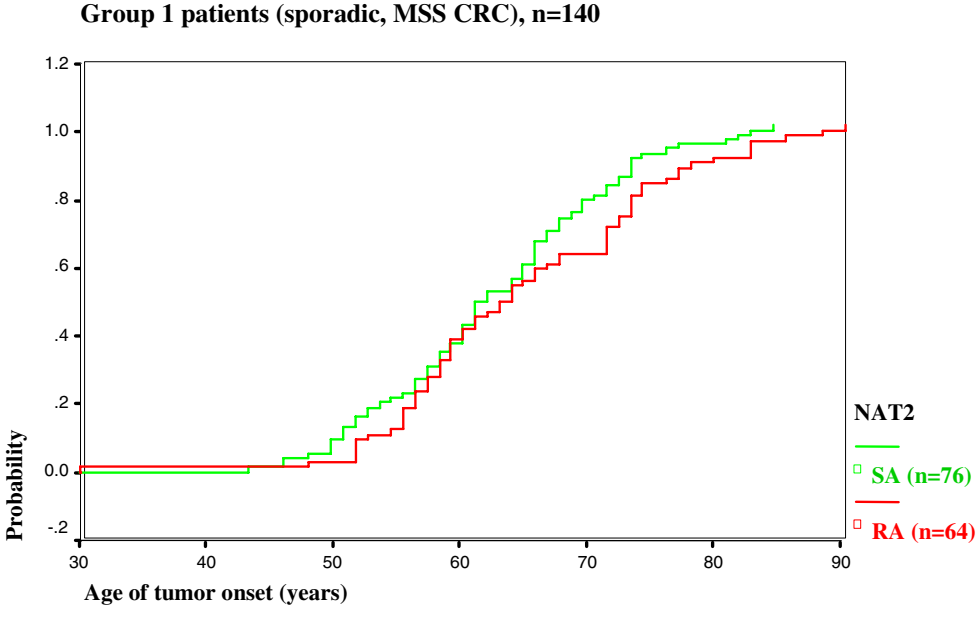

Fig. 1 Kaplan–Meier analysis: age of CRC onset in group 1 patients (sporadic, microsatellite stable colorectal cancer) with rapid (RA) and slow acetylator (SA) status in *NAT2* (*P*=0.0654)

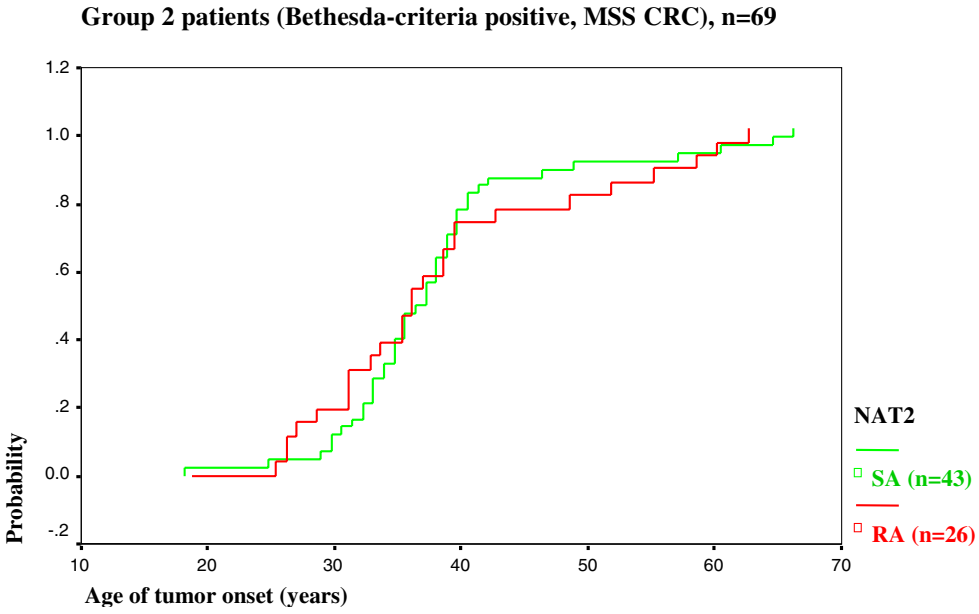

Fig. 2 Kaplan–Meier analysis: age of CRC onset in group 2 patients (Bethesda-criteria positive, microsatellite stable colorectal cancer) with rapid (RA) and slow acetylator (SA) status in *NAT2* (P=0.8135)

and early AO. There was no statistical difference in *NAT2* allele frequencies in patients and controls. However, no information about the microsatellite status of the carcinomas was presented [36]. In our study, which analysed only patients with MSS CRC, no correlation between *NAT2* acetylator status and AO was observed.

Conclusion

In summary, we conclude that the *NAT2* acetylator status is neither a risk factor nor a genetic modifier of AO in patients with sporadic, MSS CRC or in patients meeting at least one criterion of the Bethesda guidelines and MSS CRC. For a more detailed risk assessment in patients with CRC, further correlations should include numerous genetic variants as well as dietary factors in multivariate analyses in large, molecularly and clinically well-characterised populations.

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