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Absence of association between cyclin D1 (*CCND1*) G870A polymorphism and age of onset in hereditary nonpolyposis colorectal cancer

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Abstract

CCND1 encodes cyclin D1, which plays an important role in the G_1 to S phase transition of the cell cycle. A common polymorphism (c.G870A) increases alternate splicing. Hereditary nonpolyposis colorectal cancer (HNPCC) is caused by mutations in mismatch repair (MMR) genes, mainly *MSH2* and *MLH1*, and shows a wide range in the age of its onset (AO), suggesting the existence of other modifying genetic factors. To date, two studies have investigated the association between *CCND1* G/A variation and AO in HNPCC with contradictory results in 86 and 146 MMR mutation carriers, respectively. To clarify the role of the *CCND1* G/A variation in HNPCC, we performed a study in 406 individuals carrying exclusively clear cut pathogenic mutations in *MSH2* or *MLH1*. We did not observe a significant difference in genotype frequencies of affected and unaffected mutation carriers and healthy controls. A significant association between *CCND1* genotypes and AO was found neither in the global comparison (log-rank, $P=0.2981$; Wilcoxon, $P=0.2567$) nor in a multivariate Cox regression analysis (hazard ratios 1.111, 95%CI 0.950–1.299, $P=0.188$ and 1.090, 95%CI 0.868–1.369, $P=0.459$ for the additive and dominant

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effect, respectively). We conclude, that the *CCND1* G870A sequence variation is not a genetic modifier of the phenotype of HNPCC.

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1. Introduction

Cyclin D1 (encoded by *CCND1*) is a major regulator of the cell cycle, playing a key role in the G_1 to S phase transition. *CCND1* harbours a common G/A sequence variation at the last nucleotide of exon 4, increasing alternate splicing. *CCND1* mRNA is alternately spliced to give two simultaneously occurring transcripts a and b. The regularly spliced transcript a is spliced between exons 4 and 5 and contains both exons, while the unspliced transcript b reads into intron 4 and is lacking exon 5. Transcripts arising from the 870A allele are less likely to be regularly spliced than those from the 870G allele [1]. Both transcripts encode proteins that contain amino acids 55–161, thought to be responsible for the cyclin D1 function. However, transcript b is lacking exon 5, encoding a PEST destruction box responsible for the rapid turnover of the protein. Therefore, transcript b was thought to have a longer half-life than transcript a [1]. Recently, Solomon et al. found that cyclin D1b is not significantly more stable than cyclin D1a, rather is a poor effector of RB phosphorylation/inactivation and disrupts contact inhibition in a manner distinct from cyclin D1a [2]. It has been suggested that the *CCND1* 870A allele is associated with susceptibility to various tumours, including sporadic colorectal adenoma [3], and sporadic colorectal cancer [4,5], familial colorectal cancer [6], squamous cell carcinoma of the head and neck [7], squamous cell carcinoma of the upper aerodigestive tract [8], urinary bladder cancer [9], sporadic renal cell carcinoma [10], prostate cancer and benign prostatic hyperplasia [11], but not colorectal cancer associated with HNPCC [6], breast cancer [12,13], and esophageal squamous cell carcinoma [14].

CCND1 is overexpressed in about a third of colonic adenocarcinomas [15]. A role for cyclin D1 in colorectal carcinogenesis was further suggested since, expression of anti-sense *CCND1* cDNA suppresses the growth of cancer cells in nude mice [16]. Autosomal dominantly inherited hereditary nonpolyposis colorectal cancer (HNPCC), one of the most common cancer susceptibility syndromes, is caused by germline mutations in the DNA mismatch repair (MMR) genes, mainly *MSH2* and *MLH1* [17], and shows a wide range in its age of onset (AO) ranging from 16 to 90 years [18]. To date, two studies have investigated the association between *CCND1* G870A variation and AO in HNPCC with contradictory results: Kong et al. analysed 86 mutation carriers (49 CRC cases and 37 unaffected mutation carriers), most of them non-hispanic white Americans, with truncating ($n=46$, 53.5%) or missense ($n=40$, 46.5%) mutations in *MSH2* or *MLH1,* and found that subjects with *CCND1* AA and AG genotypes developed CRC on average 11 years earlier. They were 2.46 times more likely to get cancer during any interval than those with GG genotype (hazard ratio 2.46, 95% confidence interval 1.16–5.21, $P=0.019$) [19]. In contrast, Bala and Peltomaki did not observe a correlation between *CCND1* genotypes and AO in 146 Finnish affected carriers, most of them ($n=138$, 94.5%) carrying a truncating mutation in *MLH1* (in most cases) or *MSH2* [20]. To clarify the role of the *CCND1* G870A variation in HNPCC, we performed a study on 406 individuals carrying exclusively mutations in *MSH2* or *MLH1*, predicted to be pathogenic due to their nature as protein truncating small insertions/deletions, large genomic rearrangements, nonsense or splice site mutations.

2. Materials and Methods

2.1. Samples

We studied 406 mutation carriers consecutively registered at the university hospitals of the German

HNPCC Consortium in Bonn, Dresden, Munich-Regensburg, Heidelberg, Duesseldorf, and Bochum. Bethesda criteria [21] were applied as inclusion criteria. In addition, six patients with young age of onset of colorectal cancer or with a family history suspicious of HNPCC who did not meet any of the Bethesda criteria were included. Five of these met the revised Bethesda criteria [22]. The inclusion criteria, as well as the clinical and molecular data of our registry are described in detail elsewhere [23]. From these, 315 individuals had a CRC as their first tumour presentation, and 91 were unaffected mutation carriers. All 406 subjects described here had a germline mutation in either *MSH2* (168 CRC cases and 62 healthy carriers) or *MLH1* (147 cases and 29 carriers), predicted to be pathogenic due to their nature as protein truncating small insertions/deletions, large genomic rearrangements, nonsense or splice site mutations. To control for the false inclusion of missense mutations of unknown pathogenic significance, we completely excluded carriers of such mutations. Two hundred and forty-five anonymous healthy blood donors from the Dresden Regional Blood Center served as controls. The compiled data analysis of the affected and unaffected MMR mutation carriers, and the controls are summarized in Table 1.

2.2. Methods

For genotyping of *CCND1* G870A sequence variation we used genomic DNA isolated from peripheral blood leukocytes. PCR (Taq-polymerase, Perkin Elmer Applied Biosystems, Weiterstadt, Germany) was performed as previously described [24] using the same primers as Kong et al. [19] (5′-GTGAAGTTCATTTCCAATCC-3′ [sense] and 5′-TTGGCACCAGCCTCGGCATTTC-3′ [antisense]). We performed independent single base sequencing of guanine and adenine as described by Kristensen et al. [25]. PCR-products were sequenced using the Thermo Sequenase Fluorescent Cycle Sequencing kit and Automated Laser Fluorescence (A.L.F. express) sequencing devices (both Amersham Pharmacia Biotech, Freiburg, Germany). For sequencing we used the Cy5-labeled sense primer and reagents including only guanine or adenine as didesoxynucleotide from the Thermo Sequenase Fluorescent Cycle Sequencing kit.

2.3. Statistical analysis

The AO was analysed using the Kaplan–Meier method. The log-rank and Wilcoxon tests were applied to compare the AO between genotype groups. Cox regression analysis assessed the effects of gender, affected MMR gene and *CCND1* genotype (separating additive and dominant effects) on AO. The Chi-Square test evaluated the homogeneity of genotype frequency distributions among the MMR mutation carriers and the healthy controls. To check the Hardy–Weinberg equilibrium, the expected genotype frequencies in all groups were calculated from the observed allele frequencies according to the Hardy–Weinberg equation. Expected genotype frequencies were compared with the observed genotype frequencies by means of the χ^2 test. *P*-values below 0.05 were considered significant. SPSS 10.0.7 (SPSS Inc., Chicago, IL, USA) was used for all analyses. Power was calculated for the Cox model using PASS 2002 (NCSS, Kaysville, Utah, USA), assuming the sample size of our current study and the hazard ratio of 2.46

Table 1
Compiled data analysis of MMR mutation carriers and controls

	MMR mutation carriers with CRC	Unaffected MMR mutation carriers	Healthy controls
All	315	91	245
Sex			
Male	197	42	113
Female	118	49	132
MMR gene mutated			
MSH2	168	62	
MLH1	147	29	
Median age [years] of onset of CRC (range)	38 (13–75)	33 (18–70)[a]	36 (18–65)[a]
Criterion[b]			
Amsterdam I/II	189	57	
BG 1997[c]	120	34	
BG 2004[d]	5		
None	1		

BG 1997, Bethesda Guidelines; BG 2004, Revised Bethesda Guidelines.
[a] Age.
[b] In unaffected mutation carriers criterion in their respective families.
[c] Bethesda Guidelines criteria 2–7.
[d] Only individuals who did not met Bethesda Guidelines.

Table 2
CCND1 G/A sequence variation genotype distribution among cases and controls and median age of onset of the cases

Group	*n*	GG	GA	AA		GA or AA	
		n (%)	*n* (%)	*n* (%)	*P* value[a]	*n* (%)	*P* value[b]
Controls	245	73 (29.8)	121 (49.4)	51 (20.8)		172 (70.2)	
All MMR[c] mutation carrier	406	141 (34.7)	188 (46.3)	77 (19.0)	0.43	265 (65.3)	0.19
Unaffected	91	31 (34.0)	44 (48.4)	16 (17.6)	0.69	60 (66.0)	0.45
With CRC[c]	315	110 (34.9)	144 (45.7)	61 (19.4)	0.44	205 (65.1)	0.20
Median AO[c] (years)	38	39	38	37		38	

[a] Chi-Square test of genotype distribution in mutation carriers vs. controls.
[b] Chi-Square test of genotype distribution in mutation carriers vs. controls (GA and AA combined).
[c] MMR, mismatch repair gene; CRC, colorectal cancer; AO, age of onset of CRC.

found by Kong et al. for the AA/AG genotype versus the GG genotype.

3. Results

We did not observe a significant difference in genotype frequencies of affected and unaffected mutation carriers and healthy controls (Table 2), and no significant deviations from the Hardy–Weinberg equilibrium were detected ($P=0.8$, $P=1.0$, and $P=1.0$, respectively). Genotype frequencies in all groups were in accordance with those previously reported in large series from a European consortium, the United States, UK, and Finland [1,3,6,20]. In the 315 CRC patients, the median AO was 39 years for the GG individuals, 38 years for the heterozygotes GA, and 37 years for the AA individuals (Table 2).

We did not find a significant association between *CCND1* genotypes and AO in the global comparison (log-rank, $P=0.2981$; Wilcoxon, $P=0.2567$, Fig. 1: Kaplan–Meier analysis). Multivariate Cox regression analysis revealed that male individuals and *MLH1* mutation carriers had a significantly increased risk to develop cancer during any interval (hazard ratios 1.295, 95%CI 1.029–1.631, $P=0.028$ and 1.274, 95%CI 1.018–1.592, $P=0.034$, respectively), while the *CCND1* genotype was not a risk factor (hazard ratios 1.111, 95%CI 0.950–1.299, $P=0.188$ and 1.090, 95%CI 0.868–1.369, $P=0.459$ for the additive and dominant effect, respectively). Using the same approach as Kong et al. [19], we combined AG and AA genotypes: the median AO for the AG+AA individuals was 38 years, and the AO was not significantly different between GG (median AO 39 years) and AG+AA individuals neither in the log-rank ($P=0.1232$)

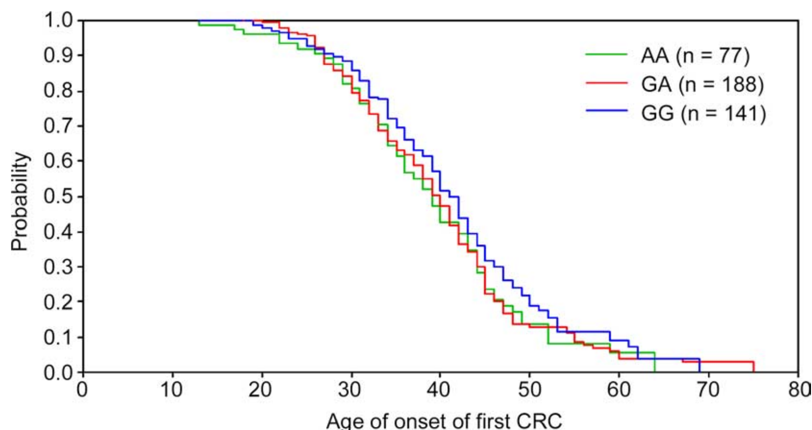

Fig. 1. Kaplan–Meier analysis of age of onset of colorectal carcinoma grouped by *CCND1* genotype ($n=406$). *y* axis, probability of not having developed CRC at given age.

nor in the Wilcoxon test ($P=0.1040$), nor in multivariate Cox regression analysis (hazard ratio for AG+AA 1.218, 95%CI 0.965–1.537, $P=0.097$). In addition, no significant association between *CCND1* genotypes and AO was observed in the subgroup of mutation carriers affected with CRC in the global comparison (log-rank, $P=0.4330$; Wilcoxon, $P=0.3817$) and the multivariate Cox regression analysis in this subgroup (hazard ratios 1.094, 95%CI 0.936–1.280, $P=0.260$ and 1.043, 95%CI 0.830–1.311, $P=0.718$ for the additive and dominant effect, respectively).

4. Discussion

Although the molecular basis of HNPCC are germline mutations in MMR genes, there is no explanation for the wide range of phenotypes observed, e.g. the wide variety in age of onset of CRC. Thus, other environmental or genetic factors are thought to modify the HNPCC phenotype. The first genetic modifier, reported to be associated with AO in HNPCC in white Americans (49 affected and 37 unaffected MMR mutation carriers) was the *CCND1* G870A sequence variation [19], whereas Bala and Peltomaki [20] failed to detect any significant association in a larger Finnish HNPCC population (146 affected carriers).

We analysed 406 consecutively registered MMR mutation carriers (315 affected and 91 unaffected), thus our study had >99.9% power to detect a significant effect of the *CCND1* genotype on AO, assuming an effect size as estimated by Kong et al. [19]. We did not observe any significant association between *CCND1* genotypes and the age of disease onset. The results did not change using the same approach as Bala and Peltomaki [20] regarding only the 315 affected MMR mutation carriers. We concur with Bala and Peltomaki [20] that the 870A allele of cyclin D1 is not associated with a lower AO in MMR mutation carriers. There may be several reasons for the discordance between our findings and those of Kong et al. [19]. First, our series was about five times larger and, unlike the affected carriers studied by Kong et al. [19], in all our groups no significant deviations of observed genotype frequencies from the Hardy–Weinberg equilibrium were detected, therefore, a bias seemed unlikely. Second, in our series only carriers with truncating mutations in MMR genes were included, whereas about half of the individuals (40 of 86, 46.5%) studied by Kong et al. [19] carried missense mutations, the pathogenicity of which is often unclear. Third, a significant fraction of individuals (37 of 86, 43%) studied by Kong et al. [19] were unaffected MMR mutation carriers. Yet the cause of the incomplete penetrance of HNPCC is not known. Therefore, the inclusion of healthy MMR mutation carriers may affect the influence of various genotypes on the age of disease onset. The fraction of unaffected mutation carriers in our series was much lower (91 of 406, 22.4%). In addition, we obtained the same results regarding only the 315 affected MMR mutation carriers. Fourth, although all populations analysed so far [19,20, this study] were of Caucasian origin, ethnic or environmental variation may confound the results. For example, in the Finnish study most patients carried *MLH1* mutations (141 of 146, 96.6%) and most of them ($n=97$) were carriers of a common Finnish founder mutation (3.5-kb genomic deletion affecting exon 16) [20]. The majority of individuals studied by Kong et al. [19] were *MSH2* mutation carriers (57 of 86, 66.3%), whereas in our series the numbers of *MLH1* and *MSH2* mutation carriers were almost equal (43.3 and 56.7%, respectively). Notably, carriers of the Finnish founder mutation have common ancestry and share geographic origin, thus forming a population as homogeneous as possible. The results in this subgroup were the same as in the pooled series, further supporting the reliability of these data [20].

Furthermore, we did not observe significant differences in genotype frequencies among the affected and unaffected MMR mutation carriers and controls, suggesting that the *CCND1* G870A variation is not involved in tumour development. This finding is in accordance with previous studies that failed to detect an association between the occurrence of HNPCC caused colorectal cancers and the *CCND1* G870A variation [6,20].

In summary, we conclude that the *CCND1* G870A sequence variation is not a genetic modifier of the phenotype of HNPCC. Other genetic modifiers have to be considered. For example, very recently a common sequence variation in the tumour suppressor gene *p53* (p.R72P) was found to be significantly

associated with AO in HNPCC [26,27]. Further, studies are needed to identify modifying factors of HNPCC phenotype, which may contribute to a more detailed risk assessment in MMR mutation carriers.

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Appendix

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