

**Cyclin D1 rare variants in UK and French multiple adenoma
and early onset colorectal cancer patients**

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Short title: Rare variants in *CCND1* and colorectal cancer risk

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Abbreviations: CCND1, cyclin D1; HNPCC, hereditary non-polyposis colorectal cancer; FAP, familia adenomatous polyposis; MAP, *MYH*-associated polyposis; OR, odds ratio; CI, confidence interval; PoBI, People of the British Isles; UV, ultraviolet; TEAA, triethylammonium acetate; UTR, untranslated region; HSF, human splicing finder; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; GWAS, genomewide association studies

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Novelty and impact: This paper reports the analysis of genetic variation within the *CCND1* gene as a candidate for colorectal cancer, with a focus on rare and low frequency variants. Our findings indicate that rare variants most probably play a significant role in the pathogenesis of the disease.

Abstract

We have examined the influence that rare variants and low frequency polymorphisms in the cancer candidate gene *CCND1* have on the development of multiple intestinal adenomas and the early onset of colorectal cancer. Individuals with <100 multiple polyps and patients with colorectal cancer diagnosed before 50 years of age were recruited in the UK and screened for sequence changes in the coding and regulatory regions of *CCND1*. A set of more than 800 UK control individuals was genotyped for the variants discovered in the cases. Variants in the promoter, intron-exon boundaries, and untranslated regions (UTRs) of the *CCND1* gene had higher frequencies in cases than in controls. Five of these variants were typed in a set of French multiple adenoma and early onset patients and these also had higher allele frequencies than UK controls. When pooled together, variants with frequencies lower than 1% conferred an increased risk of disease that was significant in the multiple adenoma group. Most variants had a putative functional effect when assessed *in silico*. We conclude that rare variants of *CCND1* are risk factors for colorectal cancer, with considerably larger effects than common polymorphisms, and as such should be systematically evaluated in susceptibility studies.

Introduction

The *CCND1* gene is located on chromosome 11q13 and encodes the protein cyclin D1 that regulates cell-cycle progression from G1 to S phase during cell division via its interactions with the cyclin dependent kinases¹. Increased cyclin D1 expression has been reported as an early event in colorectal tumourigenesis² and has been observed in other cancer types including prostate, breast, lung and endometrial carcinomas^{3, 4}. Prior work has shown, however, that *CCND1* is not essential for the development of colorectal cancer, although it may act as a modifier of disease severity⁵.

Most association studies of *CCND1* have so far focused on the common and functionally significant G870A (P241P, rs9344) polymorphism, which affects splicing by eliminating a donor site at the end of exon 4. However, results correlating this polymorphism with cancer risk have been inconsistent. A recent meta-analysis of 60 published case-control studies has shown that, overall, individuals with the GA or AA genotype exhibited a 1.1 to 1.2-fold increased risk of developing cancer compared to individuals with the GG genotype⁶. With respect to colorectal cancer in particular, subjects with hereditary nonpolyposis colorectal cancer (HNPCC) who carry the rs9344 polymorphism have been reported to acquire the disease at an earlier age⁷⁻⁹. Also, carriers of the A allele appear to be more frequent among individuals who developed non-syndromic colorectal cancer before the age of 60¹⁰, subjects with familial colorectal cancer^{11, 12}, and affected women^{13, 14}. Other reports, however, do not subscribe to rs9344 genotype being a modifier of the colorectal cancer phenotype^{15, 16}.

Given the low odds ratios (OR) associated with common variants, such as rs9344, we have argued that genetic risk factors underlying complex diseases are more likely to be due to functionally relevant rare variants with moderate penetrance that will considerably increase susceptibility and will, therefore, sometimes justify prophylactic interventions¹⁷⁻¹⁹. We defined rare variants as those having higher frequencies than rare severe effect clearly familial mutations but lower frequencies than polymorphisms. Thus, rare variants will generally be in the frequency range between 0.1% and 1%. We consider low frequency variants to be those with frequencies between 1 and 5%, which are not normally used in standard association studies. Following the strategy we previously proposed¹⁹, we have screened the regulatory and coding regions of *CCND1* in individuals with multiple adenomas and patients with early onset colorectal cancer recruited in UK clinics. A few of the variants found this way were examined in a group of similarly ascertained French patients. We sought

to assess the impact that collections of variants with gene frequencies lower than 1%, and between 1% and 5%, have on the onset and progression of colorectal cancer, whether there are population-based differences in the impacts, and what role, if any, the rs9344 polymorphism plays in the pathogenesis of this disease.

Materials and Methods

Subjects

The UK patient group consisted of 112 individuals with 3 to 100 histologically proven synchronous or metachronous adenomatous polyps¹⁸ and 44 individuals with colorectal cancer diagnosed before 50 years of age. Thirty-eight individuals with early onset disease were obtained through the VICTOR clinical trial, a Phase III double-blind placebo controlled study of rofecoxib in Dukes stage B or C colorectal cancer patients following potentially curative therapy, while the remaining six were recruited through the John Radcliffe and Churchill hospitals' gastrointestinal clinics. With the exception of one Black Caribbean and one Indian individual, ethnic origin was White British for all UK patients for whom information was available. Non-white individuals were excluded from further analysis. No patient fulfilled the criteria for familial adenomatous polyposis (FAP), autosomal recessive *MYH*-associated polyposis (MAP), or hereditary nonpolyposis colorectal cancer (HNPCC) on clinical grounds¹⁸. Some of these patients had already been screened for germline mutations in the *APC* and *MYH* genes during previous studies^{20, 21}.

In addition, we collected samples from 131 French patients, 75 with multiple adenomas and 56 with early onset colorectal cancer, who were recruited in the Department of Digestive Surgery at the Hôpital Saint-Antoine in Paris. All patients who underwent a colectomy or total colectomy for colorectal cancer or polyposis were selected for the

study *a priori*. Those diagnosed with colorectal cancer before age 50 or with more than three polyps detected after 2005, were referred for a consultation with the geneticist. Immunohistochemical staining to determine loss of expression of the genes *MLH1* and *MSH2* and microsatellite status was performed for all patients with early onset colorectal cancer. Sequencing of the entire *MYH* and *APC* genes was carried out in patients with multiple adenomatous polyps. Only patients with no evidence of HNPCC, MAP or FAP were included in the current study. No ethnic identification was available for the French patients.

All UK and French cases had histological confirmation of adenomatous polyps, but the precise number of polyps was not determined for all of them. For 24 UK and 14 French adenoma patients only “multiple” was recorded.

Controls comprised 866 individuals collected in 10 different regions across the UK as part of the People of the British Isles (PoBI) study (see website link below), and were unselected with respect to disease status.

Blood samples from cases and controls, and clinicopathological information from patients, were collected with individual informed consent and local ethical committee approvals.

DNA extraction and processing

Genomic DNA for patient samples was extracted from peripheral venous blood using standard techniques. The PoBI control blood samples were transported at room temperature to the laboratory, where the peripheral blood lymphocytes were separated under sterile conditions²² within two days of collection. DNA was prepared from the 10ml blood residue remaining after sterile separation using either magnetic beads (GeneCatcher™, Invitrogen, Carlsbad, CA, USA) or spin columns (Qiagen or MN? **Bruce*still needs to be checked.**). DNA

concentration was determined using Pico Green²³ and normalised for genotyping to 25ng/ul. Samples from UK cases underwent whole genome amplification due to limited volumes and amounts of genomic DNA. We used the Repli-g Mini kit (Qiagen, Valencia, CA, USA) which implements a multiple displacement amplification reaction to generate up to 10 ug of DNA per 50 ul reaction from a starting amount of at least 10 ng of genomic template. Genomic DNA from French cases and UK controls was used for genotyping.

PCR amplification

All exons, 5'UTR, 3'UTR, intron-exon boundaries and about 1.5 kb of the *CCND1* promoter were screened, covered by a total of 21 PCR fragments (Supplementary Table 1). DNA amplification was carried out in 50 ul reactions with a final concentration of 1X PCR Gold buffer, 200 uM dNTPs and 0.5 uM of each primer. Two units of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 1.5-2.5 mM of MgCl₂ and 20-50 ng of genomic or whole genome amplified DNA were used per reaction. Cycling conditions basically consisted of an initial denaturing step at 95°C for 10 min, followed by 35 cycles at 95°C, 25 sec; annealing temperature, 35 sec; 72°C, 30 sec; and a final elongation step at 72°C for 5 min. 2% agarose gels were run to verify the successful amplification of each fragment.

Mutation analysis

Mutation screening in UK patients was performed using a WAVETM DNA fragment analysis dHPLC system with UV detection (Transgenomic, Inc., Omaha, NE, USA). Temperature gradients were designed using the WaveMakerTM software (Transgenomic, Inc.) to obtain fragment melting profiles. PCR products, of sizes between 234 bp and 522 bp, were denatured for 5 minutes at 94°C, then gradually reannealed by decreasing the temperature to 25°C for

30 minutes to form homo and/or heteroduplexes. PCR products were subsequently eluted through an acetonitrile gradient at 0.9 ml/min over 6.8 min, at one or two different temperatures, selected according to their melting profiles. The column mobile phase consisted of buffer A, a 0.1 M triethylammonium acetate (TEAA) solution at pH 7, and buffer B, a 0.1 M TEAA solution containing 25% acetonitrile at pH 7. The retention time of the eluate was registered by the UV detector at 260 nm. Under these conditions, invariant DNA fragments elute as a single peak, whereas variant fragments, which contain mixtures of homoduplex and heteroduplex DNAs, elute as two to four peaks or as a single peak with a shoulder.

DNA sequencing and genotyping

Cases with heteroduplex peaks, and several individuals with only homoduplex peaks were sequenced to identify the genetic variants. Each PCR product was purified following the EXO-SAP protocol and submitted to the WIMM central facility for direct sequencing. Whenever possible, both strands were sequenced. Sequences were analyzed using Sequence Scanner version 1 (Applied Biosystems) and compared with *CCND1* GenBank entries NM_053056 and NT_167190.

All newly discovered variants were verified by genotyping the UK cases. Controls were then genotyped for variants identified and validated in patients. Two variants (*CCND1*-3 and 30) were genotyped in a subset of only 222 PoBI controls. Because the screening was not fully successful for exon 2 and the distal 5' upstream fragment, we selected twelve additional *CCND1* variants from dbSNP ($\leq 3\%$ minor allele frequency in HapMap European population, when reported) located in these regions, and typed them in both cases and controls. French patients were genotyped for a subset of five variants: two promoter variants (*CCND1*-3, 7),

one 5'UTR variant (CCND1-19), one coding SNP (CCND1-21) and one 3'UTR SNP (CCND1-30). Genotyping of all variants, including rs9344, was done using the Sequenom MassArray technology, namely matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and the iPLEX Gold assay.

Statistical analysis

Hardy-Weinberg equilibrium was tested for in controls using a chi-square goodness-of-fit test. Variant differences between cases and controls were assessed using Fisher's exact test. Two sided p-values below 0.05 were considered statistically significant. Heterogeneity between UK and French patients, and multiple adenoma and early onset cases, was evaluated with a contingency table approach implemented in the program PowerMarker version 3.25²⁴. Odds ratios and 95% confidence intervals (95% CI) were estimated with the software package SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Linkage disequilibrium patterns across *CCND1* were examined using the program Haploview version 4.1²⁵ and by counts in 2x2 tables and their statistical assessment.

Functional *in silico* analysis of *CCND1* variants to identify changes in transcription factor binding and alternative splicing sites was carried out using the web programs AliBaba 2.1 and Human Splicing Finder (HSF)²⁶, respectively. The effect of 5'UTR and 3'UTR variants was analysed using the online database UTRdb. Additionally, we used the GenEpi toolbox²⁷ to examine conservation across species, as well as disruption of microRNA target sequences in the 3'UTR.

Results

The characteristics of both sets of patients and controls are summarised in Table 1. All *CCND1* variants were in Hardy-Weinberg equilibrium in controls. A list of all variants, either first identified by dHPLC and or selected from dbSNP, is given in Supplementary Table 2, and patient and control gene frequency data are given in Supplementary Table 3.

Stratifying the cases into multiple adenoma and early onset groups (see Table 2) suggested that there might be some differences in the variant frequencies between the UK and French groups, notably for the *CCND1*-3 variant. Heterogeneity test results showed, however, that, with the exception of *CCND1*-3 ($p=0.04$, no multiple testing correction), there were no significant between group differences in allele frequencies per locus. This justifies combining the sets of patients for an assessment of overall association patterns, for those variants typed in both groups of patients.

Rare, low frequency and common variants

Overall 20 variants were typed in UK cases and controls, of which 10 were detected through mutation analysis and 10 were selected directly from dbSNP. Four additional variants were found by dHPLC screening but could not be validated because of technical issues with the Sequenom assays (*CCND1*-23, 24, 25, 26). Similarly, two further dbSNP variants were examined in patients and were not subsequently analysed in controls because they could not be successfully typed (*CCND1*-5, 6). There were three common variants detected, with gene frequencies equal to or higher than 10% in the HapMap European population (CEU), one of which was rs9344 (*CCND1*-21, 23, 30). The other two were not included in the analysis. Another common polymorphism located in exon 5, rs7177, was excluded as well. Of all the variants successfully genotyped in patients and controls five sites were invariant in both and these were also excluded from analysis (*CCND1*-1, 2, 11, 14, 16). Three variants were

monomorphic only in controls (CCND1-19, 22, 28), and one was invariant only in cases (CCND1-15), and these were included in the analysis, which therefore involved 14 variants in total, including rare, low frequency and common variants. Six out of the 14 variants uncovered by dHPLC screening of the UK samples were not present in dbSNP (CCND1-17, 18, 22, 24, 25, 26). Three variants (CCND1-13, 20 and 29) had reported CEU gene frequencies between 3 and 5%, and two variants (CCND1-16, 27) had CEU gene frequencies lower than 1% (see Supplementary Table 2 for more details about the variants detected, genotyped and subsequently analysed).

Association analysis

In an analysis with relatively small numbers it is not expected that individual variants will show significant effects unless they are associated with large ORs. Nevertheless, one rare variant (CCND1-7) did give a significant p value for the difference between UK cases (combining the early onset and multiple adenoma sets) and controls ($p=0.03$; OR 3.8, 95% CI 1.2-12.1). Aggregating all French and UK cases produced a borderline significant result for this variant ($p=0.06$; OR 2.8, 95% CI 1.0-8.1; Table 3). When we examined all rare variants together there was a significant increase in risk ($OR \geq 2$) for the UK multiple adenoma group as well as for all UK cases (Table 4). There was also an increase in risk for the early onset group considered on its own, but it did not reach significance presumably because of the small sample size.

There is strong linkage disequilibrium (LD) in the region between CCND1-10, 13 and 20 ($r^2 > 0.8$, for each pair of variants) with the minor alleles all present in one haplotype (ATT), except for a single patient who carried the haplotype ATC. Therefore, these lower frequency variants were combined and analysed as a single haplotype instead of independent variants.

The aggregation of the CCND1-10/13/20 haplotype with the other nine variants with frequencies equal to or less than 5%, in the analysis, yielded lower ORs and a non-significant effect (Supplementary Table 4), as did a separate test on only the low frequency (1% to 5%) variants (Supplementary Table 5).

Functional analysis

We investigated the potential functional consequences of variants using *in silico* approaches. Promoter variants were examined for changes in transcription factor binding sites based on the matrices compiled by the program AliBaba 2.1. Several variants affect Sp1 sites (i.e. CCND1-7, 10, 17) whereas CCND1-3 alters a CACCC box, which is recognised by transcription factors of the Sp/Krüppel-like Factor (Sp1/KLF) family. CCND1-13 creates a Cdx2 site. CCND1-18 has no apparent effect on transcription factor binding sites. For variants within the coding region we examined the creation and/or elimination of alternative splicing sites. The rare C allele at CCND1-22 is predicted by the software tool HSF to disrupt an acceptor site, generate a splicing enhancer site and also break up a splicing silencer site. In addition, it is well-known that the common variant rs9344 A allele modifies a splicing donor site and predisposes for an alternatively spliced transcript of the cyclin D1 protein (cyclin D1b)²⁸. In transcript b no splicing occurs at the exon 4-intron 4 boundary and exon 5 is missing¹. Most variants found in patients' 3'UTRs are reported to be within conserved blocks, as are the majority of the variants in the promoter regions and the exons. However, no 3'UTR variant altered a miRNA target site. There are no changes in the 5'UTR caused by CCND1-19 according to UTRdb, but HSF indicates the potential creation of a splicing enhancer site (see Table 5 for a summary of the variants' properties).

rs9344 G/A and rare variants

The frequency of the rs9344 A allele was higher in UK and French cases as compared to controls, although not significantly so (Supplementary Table 3). French early onset cases showed the highest frequency (0.53), which was, however, comparable to that reported for the CEU population. UK samples had lower frequencies than CEU, in agreement with findings for the British 1958 Birth Cohort (frequency of A allele = 0.44).

Linkage disequilibrium analysis using Haploview indicates that rs9344 does not appear to be in strong LD with any rare or low frequency variant in the UK or the French samples, and the correlation with the haplotype harbouring the variants CCND1-10, 13 and 20, is also fairly weak ($r^2=0.02$). However, examination of 2x2 tables reveals that LD between CCND1-10/13/20 haplotype and CCND1-21 is nevertheless highly significant (Supplementary Table 6). Given that it has been demonstrated that this polymorphism is not independently predictive of cancer and that additional events may be necessary to induce cyclin D1b production²⁸, we examined whether the presence of rare variants along the *CCND1* gene is in any way associated with genotype at this position. We found that UK and French patients who carry at least one rare variant are more likely to also carry the rs9344 G allele than cases who do not harbour rare variants. A similar finding was obtained in controls although the effect was somewhat smaller (Table 6). We believe, however, that this association is much more likely to be due to the effect of LD than to an actual relationship with disease.

Discussion

We have screened the coding and regulatory regions of the oncogene *CCND1*, a well-known candidate for colorectal cancer susceptibility, in about 150 UK individuals with multiple intestinal polyps or early onset colorectal cancer. Fourteen variants were selected for analysis, including nine rare variants (MAF < 1%), four lower frequency variants (MAF: 1-

5%), and one common variant (MAF > 5%). Three of the rare variants had not been reported elsewhere (CCND1-17, 18, 22) and only CCND1-18, was detected in more than one affected subject. Three of the rare variants were not found in a set of over 800 UK controls (CCND1-19, 22, 28). Four variants were also typed in a set of French patients with similar disease phenotypes to those of the UK cases. One of these variants (CCND1-19) was not present in any of the French cases. These results demonstrate that current catalogues of genetic variation are not exhaustive enough to represent low frequency variation among patients, which are also often likely to be population specific.

Our findings show that, when all the rare variants are combined, there is a significant increase in the risk of colorectal disease with an OR of about 2. The effect appears to be stronger for developing multiple adenomas than for early onset disease. In one case, CCND1-7, the effect was significant even for a single variant, with an OR greater than 3 for all UK cases and greater than 2 when French and UK cases are collectively tested. There were not enough data from the French population to show such significance at a comparable OR. When the data for all variants with frequencies lower than 5% (i.e. rare and low frequency variants) were combined the association was not significant. This suggests, as might be expected, that the lower the frequency of a disease associated variant, the higher the OR is likely to be.

Nearly all the variants analysed have putatively recognisable functional effects or lie within a conserved sequence block. However, no functional effects have yet been directly confirmed experimentally. CCND1-7 is a putative regulatory variant that alters two Sp1 binding sites. The Sp1 sites in the *CCND1* promoter are highly conserved²⁹ and are required for the transcriptional activation of *CCND1* following mitogenic stimulation^{30, 31}.

It is interesting to note that, out of the eleven variants with MAF < 5% identified in our study by chromatographic screening, eight are located in non-coding regions of the gene, six

of them in the 3'UTR. It has recently been established that genomic modifications of the *CCND1* 3'UTR in mantle cell lymphoma tumors produced mRNAs with truncated 3'UTRs that have considerably longer half lives than those of the full length mRNAs³². These transcripts are shorter versions of cyclin D1a and are not the alternatively spliced cyclin D1b isoform. Genetic changes that generate such transcripts include deletions of part of the 3'UTR or point mutations that create novel polyadenylation signals³². Wiestner and colleagues believe that these alterations are somatic, although they did not examine germline DNA. Other authors^{33, 34} have also suggested the involvement of 3'UTR changes, including polymorphisms and rare deletions, as a cause of increased expression of *CCND1* in cancer. In addition, loss of microRNA target sites as a result of 3'UTR shortening can lead to pathogenic over expression of the protein³⁵. In our *in silico* analysis, however, none of the variants studied was suggested to modify a microRNA binding sequence.

We have also explored the putative relationship between a common variant that has been repeatedly associated with colorectal cancer, however inconsistently, and the presence of rare variants with potentially functional consequences. It could be the case that common and rare variants in the same gene or in different genes along the same pathway interact and modify each other's effects in order to produce the phenotype^{19, 49}.

A search for possible combined effects of the common variant rs9344 and one or more of the rare or low frequency variants is almost certainly likely to be confounded by weak LD between rs9344 and these variants, and so would require a much larger sample size. This is indicated by the very significant, but low level of, LD between rs9344 and the *CCND1*-10/13/20 haplotype.

Our analysis of variation at the *CCND1* gene has added to the evidence for the importance of rare variants as determinants of disease susceptibility. It has also shown how

a moderate size study of rare variants in a candidate gene can reveal effects that are of clearly greater biological significance than very much larger whole genome association studies (GWAS) of common variants. Thus, in these large case-control association studies only variants with frequencies higher than 5% are examined and the vast majority of significantly associated variants have been shown to give rise to very modest risk increases, generally with ORs not more than about 1.2. The case for rare variants has now been extensively discussed, both from observed data^{17, 19, 36-38} and on the basis of theoretical considerations^{39, 40}. More recent studies have demonstrated additional rare variant influences on the pathogenesis of a variety of complex diseases and traits such as type 1 diabetes, colorectal cancer, plasma lipoprotein levels and neurological disorders⁴¹⁻⁴⁸.

Screening candidate genes in groups of patients for germline variation is the first step to unravelling rare variation, a step that is already being made much easier by the increasing accessibility of next-generation sequencing technologies. However, functional studies of the most interesting variants must follow closely. Cyclin D1 is a regulator of the entrance into the G1 phase of the cell cycle and has been considered a promising predictive and prognostic biomarker for a number of cancers. Nevertheless, there have been few assessments of *CCND1* levels of variation, with studies mostly focusing on the analysis of the functional polymorphism rs9344. More exhaustive studies, that include rare and low frequency variants as well as evaluate regulatory regions, are necessary if *CCND1* is to be effectively used in the clinic.

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URLs

AliBaba 2.1 <http://www.gene-regulation.com/pub/programs/alibaba2/index.html>

British Birth Cohort <http://www.b58cgene.sgul.ac.uk/index.php>

dbSNP <http://www.ncbi.nlm.nih.gov/projects/SNP/>

GenEpi toolbox http://genepi_toolbox.i-med.ac.at/

Human Splicing Finder (HSF) <http://www.umd.be/HSF/>

People of the British Isles study (PoBI) www.peopleofthebritishisles.org

UTRdb <http://utrdb.ba.itb.cnr.it/>

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