Molecular pathology of colorectal cancer predisposing syndromes

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CHAPTER 8

Homozygosity for a *CHEK2**1100delC mutation identified in familial colorectal cancer does not lead to a severe clinical phenotype

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Homozygosity for a *CHEK2**1100delC mutation identified in familial colorectal cancer does not lead to a severe clinical phenotype

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Abstract

It has recently been suggested that the frequency of the germline *CHEK21100delC mutation is higher among breast cancer families with colorectal cancer, although the mutation does not seem to be significantly associated with familial colorectal cancer. Five hundred and sixty-four familial colorectal tumours were studied for expression of CHEK2 using tissue microarrays and an antibody against the NH_2-terminal SQ regulatory domain of the CHEK2 protein. Normal colonic tissue from patients whose tumours showed loss of CHEK2 expression was investigated further using fragment and sequence analysis for the presence of a *CHEK2**1100delC mutation and five other (R117G, R137Q, R145W, I157T, and R180H) known germline variants in *CHEK2*. Twenty-nine tumours demonstrated loss of expression for CHEK2. Analysis of matched normal colonic tissue from these patients revealed germline *CHEK2**1100delC mutation in three cases. In two of these, the mutation was heterozygous but, interestingly, the third patient proved to be homozygous for the deletion, using six different primer pair combinations. None of the other tested germline variants were identified. No *CHEK2**1100delC mutations were found in patients whose tumours stained positive. Homozygosity for the *CHEK2**1100delC mutation appears not to be lethal in humans. No severe clinical phenotype was apparent, although the patient died from colonic carcinoma at age 52 years. This observation is in line with recent knockout mouse models, although in the latter, cellular defects in apoptosis and increased resistance to irradiation seem to exist. It is also concluded that CHEK2 protein abrogation is not caused by the *CHEK2* germline variants R117G, R137Q, R145W, I157T, and R180H in familial colorectal cancer.**

Keywords: TMA; familial colorectal cancer; HNPCC; CHEK2

Introduction

CHEK2 on chromosome 22q is the human homologue of the yeast Cds1 and Rad53 G2 checkpoint kinases. The Chek2/Rad53/Cds1 family of proteins identifies DNA damage in eukaryotic cells [1]. Pseudo-genes of exons 10–14 of *CHEK2* are found on chromosomes 15 and 16 and, with lower homology, on chromosomes 2, 10, 13, X, and Y [2,3]. The protein truncating mutation *CHEK2**1100delC, present in exon 10 of the functional gene on chromosome 22q, abolishes the kinase function of CHEK2 [4,5]. The role of the *CHEK2**1100delC and other germline variants has been well studied in breast cancer. The 1100delC allele has been claimed to be a low penetrance susceptibility allele for breast cancer and carriers appear to have a two-fold increase in breast cancer risk [6]. CHEK2 protein is abrogated or reduced to a large extent in breast tumours of heterozygous *CHEK2**1100delC mutation carriers [7–9]. The incidence of the 1100delC mutation has been suggested to be higher among breast cancer families with colorectal cancer than in those without colorectal cancer, identifying a hereditary breast and colorectal cancer (HBCC) phenotype [10]. Recently, the incidence of the *CHEK2**1100delC mutation in familial and non-familial colorectal cancer (CRC) patients was determined to be 1.3% and 2.9%, respectively, which is not significantly higher than the 1.1–1.4% frequency with which this allele is found in the healthy European population studied so far. With an estimated range of 1.3–1.6%, this frequency seems similar in the Dutch population [11]. These results suggest that the *CHEK2**1100delC mutation may not be significantly associated with familial colorectal cancer or with colorectal cancer risk in the population, although a very

low penetrance effect on colorectal cancer could not be excluded [12,13].

In addition to the 1100delC mutation, other germline variants in *CHEK2* have been identified among families with cancer, only two of them with known reduced (R145W) or absent (1422delT) catalytic activity [5]. Mis-sense variants R117G, R137Q, and R180H have been detected with an increased incidence in affected individuals from breast cancer families. Tumours with these mutations have been demonstrated to show loss of the mutant allele, suggesting a mechanism for tumour genesis other than loss of the wild-type allele [14]. R145W was identified in a sporadic colon cancer cell line (HCT15), and I157T and 1422delT have been identified in Li–Fraumeni syndrome variants [15]. I157T has also been detected with an increased frequency in several tumour types including breast cancer [8,13], prostate cancer [13,16–18], and thyroid cancer cases [13], although the variant appears to exhibit wild-type activity [5].

To evaluate the frequency of the *CHEK2**1100delC mutation in a well-defined familial colorectal cancer cohort, and to study the possible role of five *CHEK2* germline variants (R117G, R137Q, R145W, I157T, and R180H) in abrogation of the CHEK2 protein, we used tissue microarrays (TMAs) and examined CHEK2 protein expression in tumours with immunohistochemistry. Patients with loss of the protein were investigated further at the molecular level with fragment and sequencing analysis.

Materials and methods

Patients

To protect the information on each patient analysed, protect patient privacy, and prevent misuse of data, we acted according to the national code for working with patient data. In The Netherlands, all patient-related data used for research are protected by the Code for Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences: www.fmwv.nl/gedragscodes/goedgebruik/CodeProperSecondaryUseOfHumanTissue.pdf.

Five hundred and sixty-four Dutch cases recorded as familial CRC [397 microsatellite-stable (MSS) familial CRC, 140 microsatellite-instable (MSI-H) (suspect) hereditary non-polyposis colorectal cancer (HNPCC), and 27 familial adenomatous polyposis (FAP)] were used for the study. The 564 tumours were located respectively in the caecum (61), left colon (24), transverse colon (11), right colon (70), sigmoid (57), recto-sigmoid (29), and rectum (87): in 225 cases, the location was not specified. In addition, two cases, one with a rectal adenoma and one with two colon adenomas, were included.

Tissue microarray (TMA) construction

Triplicate tissue cores from tumour areas, selected by a pathologist (HM) on the basis of a haematoxylin and eosin (H&E)-stained slide, were taken from each specimen (Beecher Instruments, Silver Springs, MD, USA). The punches, which had a diameter of 0.6 mm, were arrayed on a recipient paraffin wax block, using standard procedures [19].

Immunohistochemistry and evaluation

Staining of CHEK2 was performed with anti-CHEK2 (clone DCS 270.1, 1 : 100; Novocastra Laboratories Ltd, UK). Clone DCS 270.1 localizes within the NH_2-terminal SQ regulatory domain of CHEK2 [20]. Sections from the constructed tissue arrays were transferred to glass slides using a paraffin sectioning aid system (Instrumedics Inc, Hackensack, NJ, USA). Next, tissue sections were dewaxed three times in xylene for a total of 15 min and subsequently rehydrated. Antigen retrieval was performed by boiling in 10 mM citrate buffer (pH 6.0) for 10 min using a microwave oven, after which the sections were cooled in this buffer for at least 2 h at room temperature. After rinsing in demineralized water and phosphate buffered saline (PBS), the tissue sections were incubated with the primary antibody diluted in 1% (w/v) PBS/bovine serum albumin overnight at room temperature. Sections were washed in PBS and endogenous peroxidase was blocked in 0.03% hydrogen peroxide PBS for 20 min, washed with PBS, and incubated with biotinylated rabbit anti-mouse (1 : 200; DAKO, Glostrup, Denmark) for 30 min, washed again with PBS, and incubated with streptavidin–biotin complex (1 : 100; DAKO) for 30 min. Sections were washed and developed in 3,3′-diaminobenzidine tetrahydrochloride substrate solution containing 0.002% hydrogen peroxide for 10 min. The sections were then counterstained with haematoxylin, dehydrated, cleared in xylene, and mounted with pertex. Microscopic analysis was done by a pathologist (HM). CHEK2 expression was scored positive or negative in tumour nuclei. In the majority of negative cases, no internal positive stromal and inflammatory control cells could be identified, including the three cases in which a *CHEK2**1100delC mutation was eventually identified. This might be explained by the fact that CHEK2 expression is lower in stromal and inflammatory cells than in epithelial cells and by the fact that in our hands using TMA, staining is often somewhat weaker in comparison with whole-slide analysis.

DNA isolation

Genomic DNA from normal colon (89 cases plus two affected family members described in the results) and colorectal tumour (6 cases) tissue was extracted from paraffin wax-embedded material as described previously by de Jong *et al* [21].

PCR and sequencing of the *CHEK2**1100delC mutation

PCR for the *CHEK2**1100delC mutation was performed as described previously by Cleton-Jansen *et al*

[22]. Mixtures of 24 µl of de-ionized formamide, 1 µl of TAMRA 500 size standard (Applied Biosystems Inc, Foster City, CA, USA), and 1 µl of PCR product were each run on an ABI 310 Genetic Analyzer (Applied Biosystems Inc) for 20 min with run profile GS STR POP 4 (1.0 ml) C and analysed with Gene Scan Analysis *3.1*. The *CHEK2**1100delC mutation is characterized by the generation of a PCR product that is one base shorter than the control sample. To confirm this mutation, sequence analysis was performed. Furthermore, alternative primers were designed to confirm the *CHEK2**1100delC mutation and to exclude technical problems caused by possible polymorphisms in the primer annealing site. Primers rv4 and rv5 were chosen on the basis of the mismatches that they have at the 3′ end with the pseudo-genes (Figure 1 and Table 1). Afterwards, sequencing of the PCR products was performed at Base Clear LABSERVICES and analysed with chromas 1.5.

PCR and sequencing of polymorphisms R117G, R137Q, R145W, I157T, and R180H

PCR was carried out in a total reaction volume of 12 µl, containing the same chemicals as used for the *CHEK2**1100delC mutation PCR and 10 pmol of the primer pairs as described in Table 1. The following PCR conditions were used in the Gene Amp 9700 thermocycler (Applied Biosystems Inc): initial denaturation step 5 min at 96 °C, followed by 33 cycles of 45 s at 94 °C, 1.5 min at 60 °C, and 45 s at 72 °C; thereafter, a final elongation step of 7 min at 72 °C was performed. Afterwards, sequencing analysis was performed.

Results

Twenty-three microsatellite-stable (MSS) familial CRC cases and six MSI-H (suspect) HNPCC cases showed loss of CHEK2 expression in their tumours by TMA immunohistochemistry (IHC) (Figure 2). In the majority of negative cases, no internal positive control cells could be identified.

The *CHEK2**1100delC mutation was present in normal tissue from three of these 29 cases (Figure 2). Sixty of the 475 cases with positive nuclear CHEK2 staining in tumour cells were used as controls and no *CHEK2**1100delC mutations were identified in these cases ($p = 0.011$). Two cases were heterozygous for the *CHEK2**1100delC germline mutation, while one case proved to be homozygous for the mutation with both fragment and sequence analysis. The homozygous status for the *CHEK2**1100delC mutation was also confirmed by five alternative primer pair combinations to exclude a possible polymorphism in the primer annealing site or amplification of pseudo-genes (Table 1 and Figure 1). Pedigree analysis for this homozygous case (case 01 272) is shown in Figure 3. The index case died at the age of 52 years with

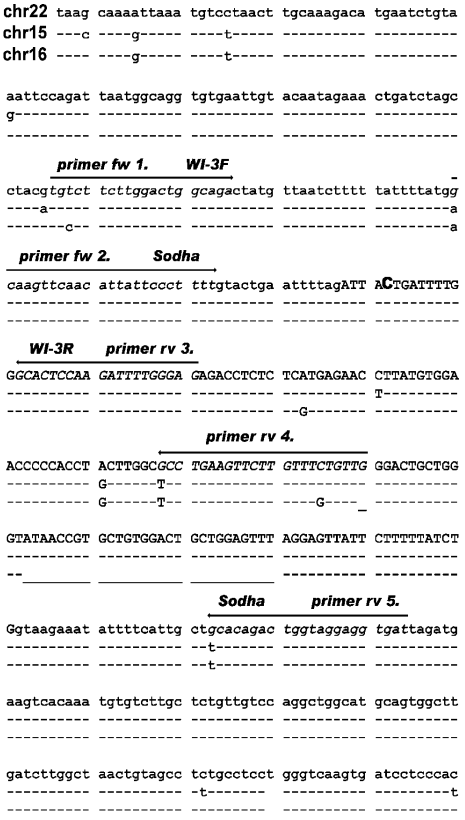


Figure 1. Detection of the *CHEK2**1100delC mutation in DNA extracted from archival paraffin wax-embedded tissue. Identification of the *CHEK2**1100delC mutation and other *CHEK2* variants in cases that stained negative for CHEK2 by IHC as well as analysis of positive staining control cases. *CHEK2* exon 10 (containing the *CHEK2**1100delC) on chromosome 22q is shown and compared with pseudo-genes containing the same region on chromosomes 15 and 16. The pseudo-genes on chromosomes 2, 7, 10, 13, X, and Y are not shown since the homology is limited. Sequence differences between *CHEK2* and the pseudo-genes are noted by the indicated nucleotide positions. The underscore in chromosome16 means that these specific nucleotides are not present on this chromosome. The different primers are indicated above the sequences, as well as in Table 1

metastatic disease from a sigmoid carcinoma. The mother (rectal adenoma at age 69) is heterozygous for the *CHEK2**1100delC mutation; the brother (two colon adenomas at age 45) has no *CHEK2**1100delC mutation. The father could not be tested. All the tumours tested in this pedigree were MSS with normal positive nuclear expression for the mismatch repair proteins MLH1, PMS2, MSH2, and MSH6, indicating mismatch repair proficiency. To exclude the involvement of a base excision repair defect, the mutational hotspots of *MYH* (Y165C, G382D, and P391L) [23] were shown to be absent in the mother and two affected sons tested (data not shown). Also,

Table 1. Different primers used for the identification of six *CHEK2* germline variants including I100delC

CHEK2 mutation	Exon	Forward primer		Reverse primer	Sequencing Primer
I100delC nested	10	fw* 1	TGT CTT CTT GGA CTG GCA GA	rv‡ 5 ATC ACC TCC TAC CAG TCT GTG C	—
				rv 3 GTT TGT TCT CCC AAA ATC TTG GAG TGC	TGT CTT CTT GGA CTG GCA GA
ALT† I 1100delC	10	fw 1	TGT CTT CTT GGA CTG GCA GA	rv 3 GTT TGT TCT CCC AAA ATC TTG GAG TGC	TGT CTT CTT GGA CTG GCA GA
ALT 2 I 100delC	10	fw 1	TGT CTT CTT GGA CTG GCA GA	rv 4 CAA CAG AAA CAA GAA CTT CAG GC	TGT CTT CTT GGA CTG GCA GA
ALT 3 I 100delC	10	fw 1	TGT CTT CTT GGA CTG GCA GA	rv 5 ATC ACC TCC TAC CAG TCT GTG C	TGT CTT CTT GGA CTG GCA GA
ALT 4 I 100delC	10	fw 2	GCA AGT TCA ACA TTA TTC CCT	rv 4 CAA CAG AAA CAA GAA CTT CAG GC	CAA CAG AAA CAA GAA CTT CAG GC
ALT 5 I 100delC	10	fw 2	GCA AGT TCA ACA TTA TTC CCT	rv 5 ATC ACC TCC TAC CAG TCT GTG C	ATC ACC TCC TAC CAG TCT GTG C
R117G	2	—	ATT CAA CAG CCC TCT GAT GC	— GCA GTG GTT CAT CAA AGC AA	ATT CAA CAG CCC TCT GAT GC
R137Q	2	—	TTG CTT TGA TGA ACC ACT GC	— TCC ATT GCC ACT GTG ATC TT	TTG CTT TGA TGA ACC ACT GC
R145W	2	—	TTG CTT TGA TGA ACC ACT GC	— TCC ATT GCC ACT GTG ATC TT	TTG CTT TGA TGA ACC ACT GC
I157T	3	—	TTG CTT TGA TGA ACC ACT GC	— TCC ATT GCC ACT GTG ATC TT	TTG CTT TGA TGA ACC ACT GC
R180H	3	—	ATC ACA GTG GCA ATG GAA CC	— CTC CCA AAG TGC TGG GAT TA	ATC ACA GTG GCA ATG GAA CC

* Forward primer.
† Alternative primer combination.
‡ Reverse primer.

the typical somatic *K-RAS2* mutations described in *MYH*-defective tumours were not found [24] (data not shown). The two heterozygous *CHEK2**1100delC cases proved to be MSS tumours, one from a 63-year-old female (a left-sided colon carcinoma without lymph node metastasis; case 01 033) and the other from a 53-year-old male (rectal carcinoma without lymph node metastasis; case 00 207). LOH analysis was performed to analyse the wild-type allele in the latter two cases (Figure 2). A control case with positive immunohistochemical staining for CHEK2 in tumour nuclei (case 00 076) showed only wild-type 121 base-pair allele fragments in tumour as well as in normal colon DNA, as expected. The second heterozygous case showed loss of the wild-type allele of *CHEK2* in the tumour, while the first case did not show any LOH. Re-evaluation of the CHEK2 staining in the latter case showed that although strikingly diminished, there was a remnant of positive staining in the tumour nuclei, compared with control cases.

In addition, five other *CHEK2* germline variants (R117G, R137Q, R145W, I157T, and R180H) were examined in the 29 patients; none of them was identified.

Discussion

In this study, we analysed 564 tumours from patients with familial colorectal cancer for abrogation of the CHEK2 protein and examined the patients with abrogation for the *CHEK2**1100delC mutation and five other germline variants of *CHEK2*, R117G, R137Q, R145W, I157T, and R180H.

The homozygous *CHEK2**1100delC mutation that we identified has not been described previously in humans, but in view of the 1.1–1.4% allele frequency of *CHEK2** 1100delC in the general European population and the 1.3–1.6% frequency in the Dutch population [11], homozygous status should be encountered in about 1/10 000 individuals. Although the patient identified in this study died from colon cancer, no severe syndrome seemed clinically apparent. It is likely that another gene defect is responsible for this family's colorectal tumours (a brother does not carry the variant but has already had four polyps at an early age), although we have ruled out HNPCC and MYH-associated polyposis.

The fact that *Chek2* −/− knockout mice seem to appear normal is in line with our observation. However, the phenotype of *Chek2*-deficient mice is dominated by increased resistance to irradiation and by defects in apoptosis [25]. Hirao *et al* showed, on the basis of *Chek2* −/−mice, that *Chek2* is not essential for somatic growth, fertility, or immunological development [26]. Manipulated HCT-116 human colon carcinoma cells carrying a homozygous deletion for *CHEK2* yielded no defective phenotype with respect to p53, G1 or G2 cell-cycle arrest and apoptosis [27]. Whether these data suggest that *CHEK2* has a complementary or even redundant function in

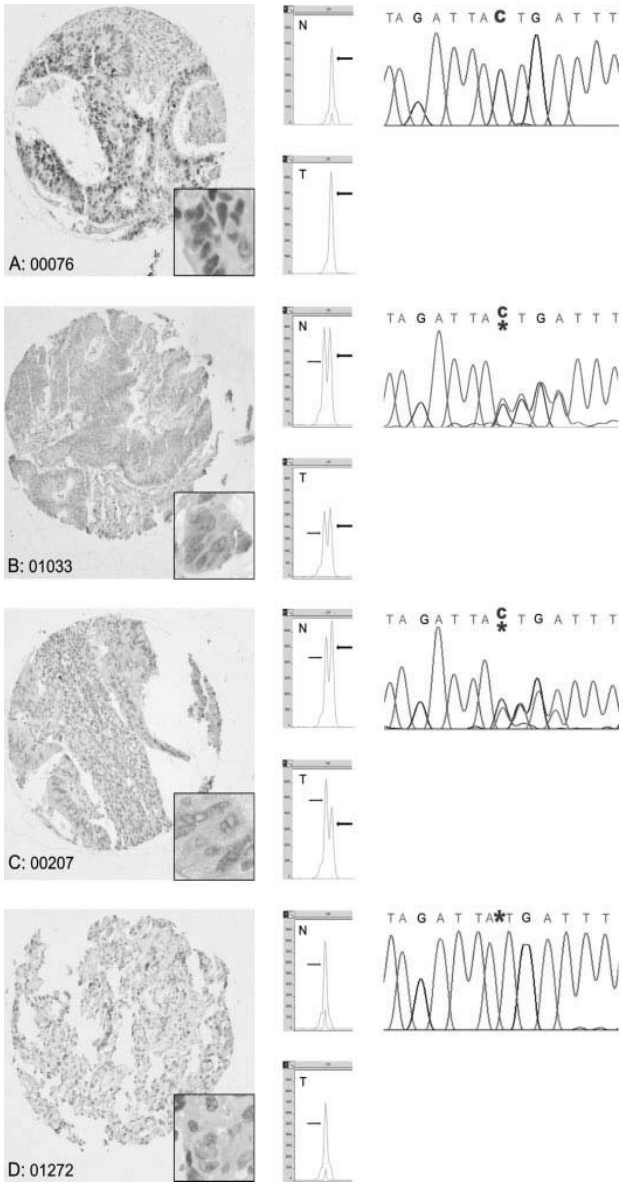
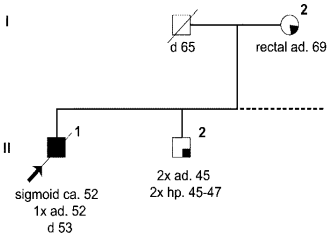


Figure 2. Three familial colorectal cancer cases with a *CHEK2**1100delC mutation and one control case. The *CHEK2**1100delC mutation (disease allele) was characterized by a PCR product that was one base shorter than the control sample. Mutation sequence analysis was performed to confirm this. (A) Patient (00 076) without mutation; positive immunohistochemical staining with CHEK2 antibody (magnification 50× and 200×). The wild-type alleles (thick arrows) are found in normal and tumour DNA and sequence analysis shows an 1100 C wild-type sequence in both alleles. (B) Patient (01 033) with a heterozygous 1100delC mutation; the tumour cells from this patient were initially scored as negative immunohistochemically. Re-evaluation showed some residual brown staining. In normal and tumour DNA, the wild-type (thick arrow) and mutant (thin arrow) allele appears, indicative of retention of the wild-type allele in the tumour. (C) Patient 00207 with heterozygous 1100delC mutation; staining for CHEK2 is negative. In normal DNA, the wild-type (thick arrow) and disease allele appear; LOH of the wild-type allele is present in tumour DNA. (D) Patient (01 272) homozygous for *CHEK2**1100delC; CHEK2 staining is negative. Only the mutant allele is present in normal and tumour DNA amplified with six different primer combinations (Table 1 and Figure 1); sequence analysis confirmed the homozygous *CHEK2**1100delC mutation



		MYH						IHC 4 MMR proteins			
Patient	*CHEK2* 1100delC	Y165C	G382D	P391L	Histology	*K-RAS2*	MSI-analysis	MLH1	PMS2	MSH2	MSH6
I-2	mut/wt	wt	wt	wt	ad.	wt	MSS	+	+	+	+
II-1	mut/mut	wt	wt	wt	ca.	wt	MSS	+	+	+	+
II-2	wt/wt	wt	wt	wt	ad.	wt	MSS	-	-	-	-

Figure 3. Pedigree of the family of the index patient (01 272). ad. = adenoma; hp. = hyperplastic polyp; ca. = carcinoma; d = age at time of death. Different analyses are shown schematically. mut = mutant; wt = wild type. MSI analyses were performed using markers recommended by Boland *et al* [31]. MMR = mismatch repair

human colon cells remains to be established. This finding is especially intriguing in view of the fact that *CHEK2**1100delC mutation is associated with familial breast cancer and is also strongly associated with bilateral breast cancer [6,7,28,29].

Overall, we identified only a low percentage of cases that exhibited abrogation of CHEK2 protein staining and actually carried the *CHEK2**1100delC mutation in our familial colorectal cancer cohort. The range of possible frequencies of this abnormality is 0.5% (3/564) to 3.4% (3/89). Sixty cases with positive staining were analysed genetically; if the number of positive staining cases were increased, the upper range would become much lower than 3.4%. This is in line with the observations of Kilpivaara *et al* [12], who identified *CHEK2**1100delC mutation in 1.3% of familial colorectal cancer cases. Furthermore, based on the results of our control group with positive staining (half of which showed weak positive intensity of staining), and the studies of Vahteristo *et al* [7] and Oldenburg *et al* [9], it is not likely that we missed many *CHEK2**1100delC mutation carriers by selecting cases on the basis of protein expression. The contribution of *CHEK2**1100delC mutation to the risk of multiple colorectal adenomas and carcinomas has been studied by Lipton *et al* [30]. Their data and a recent study by Cybulski *et al* [13] suggest that the 1100delC mutation is not associated with an increased risk for colorectal cancer.

None of the five other known germline variants in *CHEK2* (R117G, R137Q, R145W, I157T, and R180H) were identified and are thus not an explanation for the abrogation of CHEK2 staining. In breast cancer, it has already been shown that in cases with the I157T variant, the tumours stain positively for CHEK2 [8]. However, the protein stability of CHEK2 mutant R145W is questionable, considering its reduced kinase activity [5]. It is still possible that the unexplained negative staining for CHEK2 in some of the remaining cases is an artefact, although other causes such as promoter hypermethylation and the involvement of other components of the pathway(s) regulating the expression of CHEK2 protein have been suggested [9]. In two other studies, the percentage of cases with unexplained negative staining seems to be in the same range [7,9].

Taking our data together, we found that only a low percentage of patients whose tumours exhibited abrogation of CHEK2 protein staining actually carried the *CHEK2**1100delC mutation. Homozygosity for *CHEK2**1100delC appears not to be lethal in humans, although subtle molecular defects cannot be excluded. We conclude that CHEK2 protein abrogation is not caused by the germline variants R117G, R137Q, R145W, I157T, and R180H in familial colorectal cancer.

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