

Polymorphisms of cell cycle regulator genes *CCND1* G870A and *TP53* C215G: Association with colorectal cancer susceptibility risk in a Malaysian population

MOHD NIZAM ZAHARY^{1,2}, ABDUL AZIZ AHMAD AIZAT¹,
GURJEET KAUR³, LEE YEONG YEH⁴, MAYA MAZUWIN⁵ and RAVINDRAN ANKATHIL¹

¹Human Genome Centre, School of Medical Sciences, University of Science Malaysia Health Campus, Kubang Kerian, Kelantan 16150; ²School of Diagnostic and Biomedicine, Faculty of Health Sciences, Sultan Zainal Abidin University, Kuala Terengganu, Terengganu 21300; ³Institute for Research in Molecular Medicine, University of Science Malaysia, Minden, Penang 11800; Departments of ⁴Medicine and ⁵Surgery, School of Medical Sciences, University of Science Malaysia Health Campus, Kubang Kerian, Kelantan 16150, Malaysia

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Abstract. Colorectal cancer (CRC) occurs as a more common sporadic form and a less common familial form. Our earlier analysis of germline mutations of mismatch repair genes confirmed only 32% of familial CRC cases as Lynch syndrome cases. It was hypothesized that the remaining familial aggregation may be 'polygenic' due to single nucleotide polymorphisms (SNPs) of low penetrance genes involved in cancer predisposition pathways, such as cell cycle regulation and apoptosis pathways. The current case-control study involving 104 CRC patients (52 sporadic and 52 familial) and 104 normal healthy controls investigated the contribution of the SNPs cyclin D1 (*CCND1*) G870A and tumor protein p53 (*TP53*) C215G in modulating familial and sporadic CRC susceptibility risk. DNA was extracted from peripheral blood and the polymorphisms were genotyped by employing a polymerase chain reaction-restriction fragment length polymorphism method. The association between these polymorphisms and CRC susceptibility risk was calculated using a binary logistic regression analysis and deriving odds ratios (ORs). The A/A variant genotype of *CCND1* and G/G variant genotype of *TP53* exhibited a significantly greater association with the risk of sporadic CRC [*CCND1*: OR, 3.471; 95% confidence interval (CI), 1.443-8.350; P=0.005. *TP53*: OR, 2.829; CI, 1.119-7.152; P=0.026] as well as familial CRC susceptibility (*CCND1*: OR, 3.086; CI, 1.270-7.497;

P=0.019. *TP53*: OR, 3.048; CI, 1.147-8.097; P=0.030). The results suggest a potential role of the SNPs *CCND1* G870A and *TP53* C215G in the modulation of sporadic and familial CRC susceptibility risk.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer among males and the second most common type among females worldwide, accounting for 746,000 and 614,000 cases in 2012, respectively (1). The incidence of CRC is rapidly increasing in developing countries, including Malaysia, where it ranks as the second most common cancer in men and women, with a total of 2,246 cases diagnosed in 2007 (2,3). CRC occurs as a more frequent sporadic form and a less frequent familial form. Familial aggregation of CRC cases results from the inheritance of germline mutations of mismatch repair (MMR) genes. Previously, from a cohort of CRC patients, we identified 68 suspected Lynch syndrome cases. However, an analysis of germline mutations of the MMR genes, *MLH1*, *MSH2*, *MSH6* and *PMS2*, confirmed only 32% of these CRC cases as Lynch syndrome cases (4). This indicated the existence of additional genetic susceptibility factors that account for familial risk and which remain to be elucidated. It was hypothesized that MMR-non-mutated familial aggregation may be largely 'polygenic' due to single nucleotide polymorphisms (SNPs) of low penetrance genes involved in cancer predisposition pathways, including cell cycle regulation and apoptosis.

Cyclin D1 protein, encoded by *CCND1*, is a key regulator of the cell cycle which modulates the transition from G1 to S phase via cyclin-dependent kinases (CDKs) during cell division (5). However, the overexpression of cyclin D1 due to genetic variation may disrupt cell cycle control and potentially induce the development of cancers (6). A common polymorphism of *CCND1*, G870A, has been widely investigated in numerous case-control studies for its association with different types of cancer, including sporadic CRC,

Correspondence to: Professor Ravindran Ankathil, Human Genome Centre, School of Medical Sciences, University of Science Malaysia Health Campus, Jalan Raja Perempuan Zainab I, Kubang Kerian, Kelantan 16150, Malaysia
E-mail: rankathil@hotmail.com

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familial CRC, squamous cell carcinoma of the head and neck, urinary tract bladder cancer and prostate cancer, in various populations (7-10).

Tumor protein p53, encoded by the *TP53* gene, plays a major role in regulating cell cycle progression, DNA repair, cellular growth and apoptosis (11,12). As an important tumor suppressor gene, the encoded protein acts to suppress tumorigenesis and control the cell cycle checkpoint and apoptosis under physiological stress. A common variation in *TP53*, the C to G substitution at codon 72, has been demonstrated to alter the normal function of p53. The change of amino acid from arginine to proline in a proline-rich region of the protein may affect the role of p53 in apoptosis (13). To date, a small number of case-control studies have been conducted to investigate the potential association of the *CCND1* G870A and *TP53* C215G polymorphisms with CRC susceptibility risk in various populations (14,15). However, no reports are available from Malaysian populations. Therefore, these two SNPs were selected as candidates for investigation, and the current case-control study was undertaken to investigate the genotype frequencies of *CCND1* G870A and *TP53* C215G polymorphisms and determine their role in modulating familial and sporadic CRC susceptibility risk in Malaysian subjects.

Materials and methods

Study subjects. Research conducted at the Malaysian Ministry of Health hospitals in the present study was approved by the Research and Ethics Committee of the School of Medical Sciences, University of Science Malaysia (USM; Kubang Kerian, Malaysia) and the National Institutes of Health (registration ID: NMRR-08-711-1866). Subjects were recruited from various hospitals in Malaysia, including Hospital USM, Hospital Sultanah Bahiyah (Alor Setar, Malaysia), Hospital Raja Perempuan Zainab II (Kota Bharu, Malaysia) and Hospital Queen Elizabeth (Kota Kinabalu, Malaysia) between March 1, 2008 and February 28, 2011. This case-control study involved 208 study subjects, comprising 52 histopathologically confirmed sporadic CRC patients, 52 familial CRC patients and 104 healthy normal controls. Initially, 68 families with suspected Lynch syndrome [also known as hereditary nonpolyposis colorectal cancer (HNPCC)] were identified based on Bethesda Guidelines (16): i) CRC and age <50 years; ii) presence of synchronous or metachronous colorectal or other HNPCC-associated tumors, regardless of age; iii) CRC with microsatellite instability-positive morphology and age <60 years; iv) CRC and one or more first-degree relatives with CRC or other HNPCC-related tumors, with one of the cancers occurring <50 years of age; or v) CRC and two or more first- or second-degree relatives with CRC or other HNPCC-related tumor (regardless of age), including endometrial, stomach, ovarian, cervical, esophageal, leukemia, thyroid, bladder, ureter and renal pelvis, biliary tract, small bowel, breast, pancreas, liver, larynx, bronchus, lung and brain cancers (glioblastoma), sebaceous gland adenomas and keratoacanthomas. Personal and demographic details of the patients, including family history of CRC, were collected and recorded. CRC patients with a strong family history of CRC among first- or second-degree relatives were subjected to a detailed pedigree analysis. Pedigrees of the 68 suspected Lynch syndrome

families were prepared, and these patients were subjected to protein expression and germline mutation analysis of MMR genes. Of these, 16 cases in which germline mutations of any of the 4 MMR genes, *MLH1*, *MSH2*, *MSH6* or *PMS2*, were identified were excluded from the present study, and the remaining 52 cases were included as familial CRC cases. Cases with known familial adenomatous polyposis, ulcerative colitis or Crohn's disease, or any other previous malignancy as stated in the pathology reports were also excluded. For comparison, 52 histopathologically confirmed sporadic colorectal cancer patients and 104 normal controls were also included in the present study. Controls were normal healthy individuals who visited Hospital USM for problems unrelated to cancer and were aged 30-65 years. The control subjects were biologically unrelated to the patients and were cancer-free. Epidemiological data was collected from patients using a pre-structured questionnaire comprising questions on socio-demographic status, physical status, dietary factors, occupation, tobacco/alcohol habits, previous illness and radiation exposure.

Genotyping of *CCND1* G870A and *TP53* C215G polymorphisms. Blood samples (3 ml) were collected from study participants after obtaining informed consent. Genomic DNA was extracted from blood samples using a QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). Genotyping of *CCND1* G870A and *TP53* C215G polymorphisms was conducted using polymerase chain reaction (PCR)-restriction fragment length polymorphism. Regions in *CCND1* and *TP53* containing the polymorphic site were amplified using the following primers: *CCND1* G870A forward, 5'-GTTATGTTTGAGTCAACAGTGG-3', and reverse, 5'-TCTAGGAGCAGTGGAAGAAG-3' (product length, 574 bp); and *TP53* forward, 5'-TCAAACATCCTGTCCCTACT-3', and reverse, 5'-CTGCGATTAAAGGCTGTGGA-3' (product length, 458 bp). PCR was performed in a final volume of 25 µl, consisting of 2 mM MgCl₂, 1X GeneAmp PCR Buffer II, 0.2 mM dNTPs, 0.4 µM of each forward and reverse specific primers, 4 ng/µl of template DNA and 1 U of AmpliTaq Gold DNA Polymerase (reagents purchased from Applied Biosystems Life Technologies, Foster City, CA, USA). PCR was conducted in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) and the conditions were as follows: Pre-denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C (*CCND1* G870A) or 56°C (*TP53* C215G) for 30 sec, and extension at 72°C for 30 sec; and a 72°C final extension step for 5 min. Amplicons were then detected by gel electrophoresis on a 2% agarose gel. Following amplification, PCR products containing the *CCND1* G870A or *TP53* C215G polymorphic sites were digested using *BsrI* and *BstUI* restriction enzymes (New England Biolabs Inc., Ipswich, MA, USA) at 65°C for 1 h or 60°C for 1 h, respectively. For *CCND1* G870A, the G allele was not cleaved by *BsrI* and thus yielded a single fragment (574 bp), whereas the A allele was cleaved and therefore produced two fragments (388 and 186 bp); the heterozygous genotype yielded three fragments (574, 388 and 186 bp). Genotypes were categorized as homozygous wild type (G/G), heterozygous (G/A) or homozygous variant (A/A) based on the fragment sizes, as shown in Fig. 1. For the *TP53* C215G polymorphism, the C allele was cleaved by *BstUI* and thus yielded two fragments (321 and 137 bp) whereas the G

allele was not cleaved and produced a single fragment (458 bp), and the heterozygous genotype yielded three fragments (458, 321 and 137 bp). The genotype was categorized as homozygous wild type (C/C), heterozygous (C/G) and homozygous variant (G/G) based on the fragment sizes (Fig. 2).

Statistical analysis. The χ^2 test was used to compare the frequency distribution of *CCND1* G870A and *TP53* C215G genotypes in sporadic CRC patients, familial CRC patients and controls. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using a binary logistic regression method (SPSS software version 18; SPSS, Inc., Chicago, IL, USA) to assess the risk association between *CCND1* G870A and *TP53* C215G polymorphisms and sporadic CRC and familial CRC. All statistical tests were two sided and $P < 0.05$ was considered to indicate statistical significance.

Results

A total of 104 CRC patients (52 sporadic CRC and 52 familial CRC patients) and 104 normal controls were recruited. Of the 52 sporadic CRC patients, 28 were male and 24 were female, with a mean age (\pm standard deviation) of 60.31 ± 11.29 years. The 52 familial CRC patients comprised 30 males and 22 females, with a mean age of 45.62 ± 10.60 years. The normal controls comprised of 50 males and 54 females with a mean age of 49.62 ± 10.78 years (Table I). The frequency distribution of SNP genotypes was evaluated after genotyping the 208 study subjects. The genotype frequencies of *CCND1* G870A and *TP53* C215G polymorphisms in CRC patients (sporadic CRC and familial CRC cases together and separately) and normal controls are shown in Table II.

The association of *CCND1* G870A and *TP53* C215G polymorphisms with CRC susceptibility risk was analyzed using a binary logistic regression analysis. ORs were calculated relative to subjects, with the wild type G/G genotype of *CCND1* and the C/C genotype of *TP53* used as references. The A/A variant genotype of *CCND1* (OR, 3.471; CI, 1.443-8.350; $P = 0.005$) and G/G variant genotype of *TP53* (OR, 2.829; CI, 1.119-7.152; $P = 0.026$) were revealed to be significantly associated with the risk of sporadic CRC (Table III). Notably, these variant genotypes of *CCND1* (A/A) and *TP53* (G/G) also exhibited a significantly greater association with the risk of familial CRC relative to the wild type genotypes (*CCND1*: OR, 3.086; CI, 1.270-7.497; $P = 0.019$. *TP53*: OR, 3.048; CI, 1.147-8.097; $P = 0.030$) (Table IV).

Discussion

The incidence of CRC has been increasing rapidly, and the disease has become one of the leading causes of cancer-related mortality worldwide in recent years. As a result, large numbers of research studies have been directed towards the investigation of CRC, particularly with regard to its etiology, prevention and treatment (17-19).

Colorectal carcinogenesis is a complex, gradual and multistep process involving various factors (20,21). The protein encoded by *CCND1*, cyclin D1, is involved in colorectal carcinogenesis in its role as one of the key regulatory proteins of the cell cycle transition from G1 to S phase (5,22). *CCND1* G870A (Pro241Pro)

Table I. Distribution of gender and age among colorectal cancer cases and controls.

Variable	Colorectal cancer		Control (n=104)
	Sporadic (n=52)	Familial (n=52)	
Gender, n			
Male	28	30	50
Female	24	22	54
Age, years			
Range	36-83	26-72	27-72
Mean \pm SD	60.31 ± 11.29	45.62 ± 10.60	49.62 ± 10.78

SD, standard deviation.

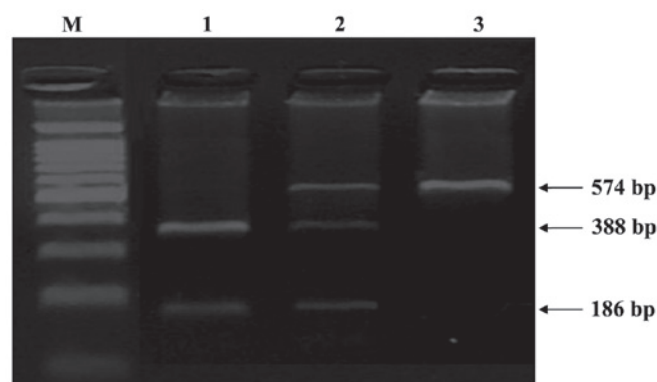


Figure 1. Representative gel electrophoresis of polymerase chain reaction-restriction fragment length polymorphism analysis of *CCND1* G870A. Lanes: M, 100 bp marker; 1, homozygous variant genotype (388 and 186 bp); 2, heterozygous genotype (574, 388 and 186 bp); 3, homozygous wild type genotype (574 bp).

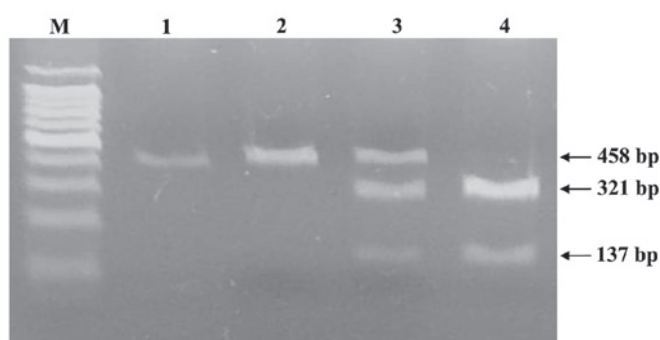


Figure 2. Representative gel electrophoresis of polymerase chain reaction-restriction fragment length polymorphism analysis of *TP53* C215G. Lanes: M, 100 bp marker; 1 and 2, homozygous variant genotype (458 bp); 3, heterozygous genotype (458, 321 and 137 bp); 4, homozygous wild type genotype (321 and 137 bp).

is a silent variant in which the alteration of amino acids does not occur. However, as this variation is in the last nucleotide of exon 4, it may lead to the alternative splicing of *CCND1* mRNA. Both alleles are capable of producing two different transcripts, a normal splicing exon 4 and exon 5, known as 'transcript a',

Table II. Genotype frequencies of *CCND1* G870A and *TP53* C215G polymorphisms in colorectal cancer cases and normal controls.

Polymorphism	Controls, n (%)	Total cases		Sporadic CRC		Familial CRC	
		n (%)	P-value	n (%)	P-value	n (%)	P-value
<i>CCND1</i> G870A							
G/G	36 (34.6%)	20 (19.2%)	0.017 ^a	10 (19.2%)	0.047 ^a	10 (19.2%)	0.047 ^a
G/A	40 (38.5%)	33 (31.7%)	0.309	15 (28.8%)	0.236	18 (34.6%)	0.640
A/A	28 (26.9%)	51 (49.1%)	0.001 ^a	27 (52.0%)	0.002 ^a	24 (46.2%)	0.016 ^a
<i>TP53</i> C215G							
C/C	33 (31.7%)	27 (26.0%)	0.358	15 (28.9%)	0.713	12 (23.1%)	0.261
C/G	57 (54.8%)	43 (41.3%)	0.052	19 (36.5%)	0.031 ^a	24 (46.2%)	0.308
G/G	14 (13.5%)	34 (32.7%)	0.001 ^a	18 (34.6%)	0.002 ^a	16 (30.7%)	0.010 ^a
Total	104 (100%)	104 (100%)		52 (100%)		52 (100%)	

^aP<0.05 vs. controls, statistically significant.

^aP<0.05 vs. controls, statistically significant.Table III. Association of *CCND1* G870A and *TP53* C215G polymorphisms with sporadic CRC susceptibility risk.

Polymorphism	Sporadic CRC, n	Controls, n	OR (95% CI)	P-value
<i>CCND1</i> G870A				
G/G	10	36	1.000 (Ref.) ^a	-
G/A	15	40	1.350 (0.539-3.381)	0.522
A/A	27	28	3.471 (1.443-8.350)	0.005 ^b
<i>TP53</i> C215G				
C/C	15	33	1.000 (Ref.) ^a	-
C/G	19	57	0.733 (0.329-1.634)	0.446
G/G	18	14	2.829 (1.119-7.152)	0.026 ^b
Total	52	104		

^aGenotype served as reference category; ^bP<0.05, statistically significant. CRC, colorectal cancer; OR, odds ratio; CI, confidence interval.Table IV. Association of *CCND1* G870A and *TP53* C215G polymorphisms with familial CRC susceptibility risk.

Polymorphism	Familial CRC, n	Controls, n	OR (95% CI)	P-value
<i>CCND1</i> G870A				
G/G	10	36	1.000 (Ref.) ^a	-
G/A	18	40	1.620 (0.662-3.963)	0.374
A/A	24	28	3.086 (1.270-7.497)	0.019 ^b
<i>TP53</i> C215G				
C/C	12	33	1.000 (Ref.) ^a	-
C/G	24	57	1.103 (0.488-2.496)	0.806
G/G	16	14	3.048 (1.147-8.097)	0.030 ^b
Total	52	104		

^aGenotype served as reference category; ^bP<0.05, statistically significant. CRC, colorectal cancer; OR, odds ratio; CI, confidence interval.

and an alternative transcript, 'transcript b', lacking exon 5, the exon comprising a PEST domain responsible for the degradation of cyclin D1 protein (23,24). Therefore, it has been reported

that the variant A allele may produce a cyclin D1 protein with an increased half life, and the resulting accumulation of the protein may promote cell proliferation (25).

According to Fearon and Vogelstein (26), colorectal carcinogenesis involves a number of genetic changes that include mutation of certain critical genes, such as *TP53*, and these mutations accumulate during the progression from normal epithelium to carcinoma. *TP53* is one of the most extensively studied genes due to its crucial role in regulating the cell cycle, apoptosis, inhibition of angiogenesis and cellular senescence (27). Mutations or variations in *TP53* may completely diminish the function of the p53 protein, and thus may promote cell proliferation and carcinogenesis. The amino acid substitution caused by the C215G SNP may alter the conformation of the protein and, therefore, its ability to bind to elements in target genes, and may affect the stability and interaction of the protein with the other proteins (28,29). Previous studies have described functional differences that are caused by these two alleles in *TP53*. For example, Pim and Banks (30) reported that the wild type G (Arg72) allele is more efficient in promoting apoptosis compared with the variant C (Pro72) allele. A similar finding also has been observed by Marin *et al* (31), who reported that the mutant p53 protein exhibited a reduced efficacy during apoptosis compared with the Arg allele protein. Thus, these studies provide evidence that variation in *TP53* may lead to abnormal protein function and contribute to cell proliferation and cancer.

The present study investigated the genotype frequencies of *CCND1* G870A and *TP53* C215G polymorphisms and the potential association with susceptibility risk in Malaysian CRC patients. The results revealed that the variant genotypes of *CCND1* (A/A) and *TP53* (G/G) have a significantly higher risk association with sporadic CRC relative to the homozygous wild type genotypes (OR, 3.471 and 2.829, respectively; $P < 0.05$; Table III). In addition, the variant genotypes of the two SNPs exhibited a significantly higher risk association with familial CRC compared with the wild type genotypes (OR, 3.086 and 3.048, respectively; $P < 0.05$; Table IV). The results suggest that individuals with the A/A genotype of *CCND1* G870A and G/G genotype of *TP53* C215G have a three-fold higher risk for CRC development compared with individuals possessing the G/G and C/C wild type genotypes.

The association of the *CCND1* G870A polymorphism with CRC susceptibility risk has been reported previously in a number of studies (32-34). The present results are in concordance with studies conducted by Hong *et al* (14) and Jiang *et al* (35), in which the A/A genotype of *CCND1* G870A was found to be associated with CRC susceptibility risk in Singaporean and Indian patients (OR, 2.4 and 1.56, respectively). Another Indian case-control study conducted in a population of Kashmiri ethnicity also identified an association between the variant genotype of *CCND1* G870A and risk of CRC, with a two-fold increase in OR relative to the wild type genotype (34). Previous meta-analyses involving numerous case-control studies also demonstrated an association between *CCND1* G870A polymorphism and CRC susceptibility risk (32,33), suggesting that the *CCND1* 870A allele may be a low-penetrant risk factor for CRC.

On the other hand, conflicting results have been reported on the possible association between *TP53* C215G polymorphisms and CRC susceptibility. The G/G variant genotype was found to be associated with an increased risk of CRC in

Japanese, Korean and Indian Kashmiri populations (36-38). A case-control study involving 444 sporadic CRC patients in China also reported that the G/G variant genotype was associated with an increased risk of CRC (39). In addition, an earlier study conducted by our group (40) revealed that the G/G genotype had a significant risk association with sporadic CRC susceptibility in a Malaysian population (OR, 2.047; CI, 1.063-4.044; $P = 0.013$). By contrast, meta-analyses conducted by Economopoulos *et al* (41) and Dahabreh *et al* (42) did not identify any significant risk association between G/G genotype and CRC susceptibility. Furthermore, Oh *et al* (43) reported that the variant genotype of *TP53* C215G was associated with a decreased risk of CRC.

Notably, in addition to their association with sporadic CRC, the variant genotypes of both SNPs investigated in the present study (A/A in *CCND1* and G/G in *TP53*), exhibited a significant association with the risk of familial CRC (Table IV). However, few other studies have been conducted on the association of these SNPs with familial CRC susceptibility risk, and the results have been contradictory. In a study by Chen *et al* (44), *CCND1* G870A was demonstrated to contribute to the early onset of CRC in cases of Lynch syndrome, whilst the *TP53* C215G SNP was not. In a meta-analysis involving 20 different populations, the variant allele of *CCND1* G870A was found to be significantly associated with an elevated CRC risk (OR, 1.23); however, no association with CRC risk was observed in Lynch syndrome patients (32). Rather than focusing on CRC susceptibility risk, the majority of previous studies have been focusing on the impact of *CCND1* G870A and *TP53* C215G on the age of onset in familial CRC cases, including cases of Lynch syndrome (45,46). Krüger *et al* (47) observed no association between *CCND1* G870A and age of onset of Lynch syndrome among 406 MMR mutation carriers. Conflicting results also have been reported regarding the association of *TP53* C215G polymorphism with the risk of CRC in Lynch syndrome patients. In other studies, the age of diagnosis of CRC in Lynch syndrome patients was not associated with *TP53* C215G genotype (46,48). In the present study, CRC patients identified to harbor MMR mutations were excluded, and the association of the two SNPs with age of onset of CRC in Lynch syndrome patients was therefore not investigated.

In summary, our results provide evidence of the effect of *CCND1* G870A and *TP53* C215G polymorphisms on CRC susceptibility risk. Despite the small sample size, the two SNPs have been observed to contribute to the susceptibility risk of sporadic and familial CRC in a Malaysian population. Individuals with *CCND1* G870A A/A and *TP53* C215G G/G genotypes in particular may have a higher risk for sporadic and familial CRC susceptibility. However, further studies into polymorphisms of other genes involved in pathways associated with sporadic and familial CRC in larger sample size, in addition to functional studies, are required to confirm these findings.

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