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Family History of Colorectal Cancer in *BRAF* p.V600E mutated Colorectal Cancer Cases

Running Title: *BRAF* p.V600E mutated CRC and Family history of CRC

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ABSTRACT

Background: Previous reports suggest that relatives of CRC-affected probands carrying the *BRAF* p.V600E mutation are at an increased risk of colorectal (CRC) and extracolonic cancers (ECCs). In this study, we estimated the association between a family history (FH) of either CRC or ECC and risk of CRC with a *BRAF* p.V600E mutation.

Methods: Population-based CRC cases (probands; aged 18-59years at diagnosis), recruited irrespective of family cancer history, were characterised for *BRAF* p.V600E mutation and mismatch repair (MMR) status. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using multivariable logistic regression.

Results: The 690 eligible probands demonstrated a mean age at CRC diagnosis of 46.9±7.8years, with 313 (47.9%) reporting a FH of CRC and 53 (7.7%) that were *BRAF*-mutated. Probands with *BRAF*-mutated, MMR-proficient CRCs were less likely to have a FH of CRC than probands that were *BRAF*-wildtype (OR=0.46, 95%CI=0.24-0.91; p=0.03). For probands with a *BRAF*-mutated CRC, the mean age at diagnosis was older for those with a CRC-affected first- or second-degree relative (49.3±6.4 years) compared with those without a FH (43.8±10.2 years; p=0.04). The older the age at diagnosis of CRC with the *BRAF* p.V600E mutation, the more likely these probands demonstrated a FH of CRC (OR=1.09 per year of age; 95%CI=1.00-1.18; p=0.04).

Conclusions: Probands with early-onset, *BRAF*-mutated and MMR-proficient CRC were less likely to have a FH of CRC than probands that were *BRAF*-wildtype.

Impact: These findings provide useful insights for cancer risk assessment in families and suggest that familial or inherited factors are more important in early onset, *BRAF*-wildtype CRC.

INTRODUCTION

Colorectal cancer (CRC) is a major cause of morbidity and mortality globally(1), being the third most common cancer worldwide and the fourth most common cancer cause of death. CRC has a strong familial component with 10-20% of cases attributed to having a family history of the disease, depending on the age at diagnosis(2, 3). However, only 2-5% of all CRC arise in the setting of the highly penetrant inherited syndromes, namely Lynch syndrome (caused by mutations in the *MLH1*, *MSH2*, *MSH6*, and *PMS2* genes), and the adenomatous and hamartomatous polyposis syndromes (mutations in the *APC*, *MutYH, STK11, SMAD4, BMPR1A*, and *PTEN* genes)(4). An estimated 30 to 50% of the excess genetic risk of CRC associated with a family history cannot, as yet, be attributed to mutations within the known CRC-predisposing genes(5). Determining molecular markers that are associated with, or can identify, an increase in risk for the relatives of CRC cases is a plausible first step in unravelling the remaining hereditary component of CRC.

The *BRAF* c.1799T>A p.V600E somatic mutation (hereafter referred to as *BRAF* p.V600E or *BRAF*-mutated) is present in approximately 10-20% of all CRC tumors and 30-75% of CRC mismatch-repair-deficient (MMR-deficient) tumors that demonstrate high levels of microsatellite instability (MSI-H) (6, 7). *BRAF* p.V600E mutation is strongly associated with widespread DNA methylation (CpG island methylator phenotype or CIMP) and tumor development via the serrated neoplasia pathway(7-9). As such, the *BRAF* p.V600E mutation is rarely seen in MMR-deficient CRCs that develop via the adenoma-carcinoma pathway as a result of germline mismatch repair (MMR) gene mutations (Lynch syndrome) (10). To date, the clinical utility of finding a *BRAF* p.V600E mutation has been to exclude Lynch syndrome in CRCs that demonstrate loss of the MLH1 and PMS2 proteins by immunohistochemistry

(IHC). Though multiple reports suggest that *BRAF* p.V600E mutation is a predictor of poor prognosis in MMR-proficient CRC (11-14), such testing is not currently in routine use.

In addition to its occurrence in CRCs from individuals with no family history of CRC (7), the *BRAF* p.V600E mutation is frequently observed in the CRCs from multiple relatives within families with serrated neoplasia predispositions such as Jass syndrome (15, 16) and serrated polyposis(17). Previous studies have demonstrated a positive association of family history of both CRC and extracolonic cancers (ECCs) with risk of a *BRAF*-mutated CRC (11, 18, 19). These studies included CRC-affected individuals with a broad range of ages at diagnosis. In the population, the familial relative risks for CRC are highest when one or more first-degree relatives are diagnosed with CRC under the age of 50 years, with the relative risk only slightly reduced when CRC is diagnosed between 50-59 years of age (20). Therefore, in this study, we investigated associations of a family history of CRC and ECCs with the *BRAF* p.V600E mutation status of CRC using probands with CRC diagnosed before 60 years of age.

MATERIALS and METHODS

Study Sample

Population-based incident CRC cases (probands) diagnosed in Victoria between 1997 and 2007 were recruited to the Australasian Colorectal Cancer Family Registry (ACCFR) (21). Of these, we identified 959 probands with primary adenocarcinoma of the colon or rectum (ICD-O-3 codes C180-C189, C199, and C209)(22) during two recruitment periods. Phase I recruitment of CRC patients diagnosed between 1997 and 2001 included all patients with a CRC diagnosed between 18 and 44 years of age and 50% of cases with CRC diagnosed between the ages of 45-59 years. Phase II recruitment of CRC patients diagnosed between 2001 and 2006 included all patients with a CRC diagnosed between 18 and 49 years of age.

Recruitment of probands to the ACCFR was *not* dependent on family history. All first- and second-degree relatives (FDR and SDR) of the proband, and all FDRs of additional CRC-affected family members were recruited where possible. Written informed consent was obtained from all participants to collect a blood sample and tumor pathology materials (tumor blocks and diagnostic slides). This study was approved by the Human Research Ethics Committees of all participating institutions.

Family History of CRC and Extracolonic cancers

Information on personal and family history of CRC and ECCs (defined as any cancer history in first- and/or second-degree relatives), was obtained from completion of a baseline questionnaires completed at recruitment and verified, where possible using pathology reports, medical records, cancer registry reports, and/or death certificates. Probands and relatives were either actively or passively followed-up every 4 to 5 years from initial enrolment, including updating information on the number, sex, and birthdates of relatives (parents, siblings, and children), their cancer history, vital status, and, if deceased, date of death by linkage to tumor registries and death indices. All cancers, except for non-melanoma skin cancers, were recorded with dates of diagnosis. The present study was based on all available baseline and follow-up data such that 49% of all reported CRCs in relatives were confirmed by pathology report, hospital or clinic record, death certificate, or cancer registry.

CRC Pathology Review

Primary CRC tissue from the Jeremy Jass Memorial Tissue Bank was available for 819 of the probands for pathology review and molecular characterisation. CRCs were reviewed by specialist GI pathologists for site, tumor grade, tumor margin, presence of mucinous component, peritumoral lymphocytes, Crohn's-like lymphocytic reaction, tumor-infiltrating

lymphocytes, and synchronous CRC. In probands with synchronous CRCs, one CRC was randomly selected where both were available for testing. Tumors from the ileo-caecal junction through the caecum, ascending colon, hepatic flexure, and transverse colon were grouped as right-sided (proximal) colon cancers (ICD-O-3 codes C180, C182, C183, and C184). Tumors in the splenic flexure (C185), descending (C186), sigmoid colon (C187) and recto-sigmoid junction (C199) were classified as left-sided (distal) colon cancers, with tumors in the rectum (C209) considered as a third distinct group.

Molecular Characterisation

Probands CRCs were characterised for MMR-deficiency by MSI using a ten-marker panel and/or by immunohistochemistry (IHC) for the four MMR proteins as has been previously described (23-25). Tumors were described as: 1) MMR-deficient if they were MSI-H and showed loss of expression of one or more of the MMR proteins by IHC; or 2) MMR-proficient if tumors were MSS (microsatellite stable) or MSI-L (low-level MSI) or showed stable expression of all four MMR proteins by IHC. CRCs where both MSI and MMR IHC testing was completed (486/819, 59.3%), demonstrated 99.8% concordance between MSI and MMR IHC results (one CRC was discordant and excluded), therefore, CRCs were categorised as MMR-proficient or -deficient using results from either MSI or IHC testing or both. In addition, tumors demonstrating loss of the MLH1 protein by IHC (with or without the loss of PMS2) were characterised for methylation of the *MLH1* promoter using the MethyLight assay as previously described (26, 27). Probands with CRC that demonstrated MMR-deficiency through loss of expression of one or more of the MMR proteins by IHC and/or had 30% or more of the markers show instability (MSI-H) underwent germline mutation testing (Sanger sequencing and MLPA) (21, 24). MMR mutation testing was performed as previously described (21, 27-30). All probands were screened for mutations in

the *MUTYH* gene as previously described (31). Probands identified with Lynch Syndrome or *MUTYH* mutation carriers by genetic testing were excluded from the analysis. Individuals with CRCs that demonstrated loss of MMR protein by IHC but did not have an identified MMR-gene mutation and were also negative for both the *BRAF* p.V600E mutation (*BRAF*-wildtype) and *MLH1* promoter methylation were considered probable Lynch syndrome cases and were also excluded from the study. The final CRC cases included in this study were either 1) MMR-proficient as determined by either MMR IHC or MSI testing or 2) demonstrated loss of MLH1 and PMS2 proteins by IHC and were *MLH1* methylated and/or *BRAF* p.V600E mutated.

A fluorescent allele-specific PCR assay was used to detect the somatic T>A mutation at nucleotide 1799 (c.1799T>A p.V600E) in exon 15 of the *BRAF* gene as has been previously described (32).

Statistical Analysis

Unconditional logistic regression was performed to estimate odds ratios (ORs) and their 95% confidence intervals (CIs) for the associations between predictor variables (including family history of CRC or ECCs) and CRC with *BRAF* p.V600E mutation after adjusting for sex and age at diagnosis. Compared with *BRAF*-wildtype CRCs (the referent group), associations were assessed for MMR-proficient and MMR-deficient *BRAF*-mutated CRCs. The association between family history of CRC in FDR and SDR (yes/no) and age at diagnosis of CRC (per year) was assessed in probands with *BRAF*-mutated CRCs after adjusting for sex. The mean age at diagnosis of CRCs were compared using student's *t*-test and ANOVA between males and females, subjects with MMR-deficient and MMR-proficient tumors, those with or without FH of CRC, and those with or without FH of ECC for *BRAF*-wildtype and

BRAF-mutated CRC cases separately. All individuals with missing data for any variable were excluded from the analysis. All statistical analyses were conducted using Stata 11.0 (StataCorp. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP; 2009).

RESULTS

A total of 959 eligible probands were recruited to the ACCFR. Of these, primary CRC tissue was available for molecular characterisation for 819 probands (85.4%). Mutation testing identified 55 probands with a germline mutation in one of the MMR-genes, 4 with biallelic *MutYH* mutations and 11 with monoallelic *MutYH* mutations, all of whom were excluded from analysis. As noted, probands with CRCs demonstrating loss of MSH2 and MSH6, or solitary loss of MSH6 or PMS2 by IHC without an identified MMR-gene mutation or that could not have germline testing performed (no blood-derived DNA), as well as probands with MLH1- and PMS2-deficient CRCs that were *BRAF*-wildtype and without evidence of *MLH1* promoter methylation were also excluded from the study as these findings strongly suggest the presence of an undetected germline mutation (total number of MMR-deficient suspected Lynch syndrome probands excluded=59). The characteristics of the final sample set comprising 690 probands are described in Table 1, where 343 (49.7%) were female. The average age at diagnosis of CRC was 46.9 ± (standard deviation, SD) 7.8 years with a range between 18 and 60 years.

The *BRAF* p.V600E mutation was present in CRCs from 53 of 690 probands (7.7%); 44 of these (83%) were MMR-proficient. Seventeen percent of the 53 *BRAF*-mutated CRCs demonstrated MMR-deficiency (9/53) compared with 0.8% (5/637) of *BRAF*-wildtype CRCs (OR=38.73, 95%CI=11.48-130.67, p<0.001; Table 1). Overall, there was no statistically significant difference in age at diagnosis of CRC between *BRAF*-mutated and *BRAF*-wildtype CRCs (Table 1). However, probands with MMR-proficient, *BRAF*-mutated CRC had an earlier age at diagnosis compared with those with a *BRAF*-wildtype CRC (44.5 ± 9.2 years versus 47.1 ± 7.7 years), and the OR per year of age at diagnosis was OR=0.96 (95%

CI=0.92-0.99, p=0.02) (Table 1). In contrast, the association of age at diagnosis for MMR-deficient, *BRAF*-mutated CRCs (51.4 years ± 8.6 years) compared with *BRAF*-wildtype CRC (47.1 ± 7.7 years; p=0.09) demonstrated an OR per year of age at diagnosis of 1.10 (95% CI=0.99-1.22, p=0.08) (Table 1).

Compared with *BRAF*-wildtype CRC, *BRAF*-mutated CRCs were more likely to have MMR-deficient CRCs (OR=38.73, 95%CI=11.48-130.67, p<0.001), *MLH1* promoter methylation (OR=27.42, 95% CI=3.18-236.08; p=0.003), high-grade CRCs (OR=4.12, 95%CI=2.28-7.44; p<0.001), an infiltrative margin (OR=1.99, 95%CI=1.09-3.62; p=0.02), and increased levels of tumor infiltrating lymphocytes (TILs; p=0.003) (Table 1). Compared with *BRAF*-wildtype CRC, MMR-deficient *BRAF*-mutated CRCs were more likely to be in the right colon (OR=6.01, 95%CI=1.46-24.71; p=0.01), have Crohns-like reactions (OR=9.46, 95%CI=2.15-41.66; p=0.003) and TILs (p<0.001) (Table 1). Compared with *BRAF*-wildtype CRC, MMR-proficient *BRAF*-mutated CRCs were more likely to be high grade (OR=4.38, 95%CI=2.30-8.32; p<0.001) and have an infiltrative margin (OR=2.86, 95%CI=1.46-5.61; p=0.002). We observed no evidence of associations between *BRAF*-mutated CRCs and the presence of a mucinous component, peritumoral lymphocytes or synchronous CRCs, even after stratification by MMR status (Table 1).

In this study group, approximately one third of probands with a *BRAF*-mutated CRC reported having a FDR or SDR with CRC (18/53), therefore, we analysed the probands' age at diagnosis and tumor MMR status in order to assess the association between a family history of CRC and the odds of a *BRAF*-mutated CRCs stratified by these features. The mean age at diagnosis of a *BRAF*-mutated CRC was statistically significantly greater for probands with a family history of CRC (49.3 ± 6.4 years) than for probands without a family history of CRC

(43.8 ± 10.2years; p=0.04) (Table 2). The odds of having family history of CRC in FDR and SDR was 9% higher per year of age in probands with *BRAF*-mutated CRCs (OR=1.09; 95% CI=1.00-1.18; p=0.04). In comparison, the odds of having a family history of CRC in FDR and SDR was 2% higher per year of age in probands with *BRAF*-wildtype CRCs (OR=1.02; 95% CI=1.00 – 1.04; p=0.04), where the mean age at diagnosis for probands with a *BRAF*-wildtype CRC with and without a family history of CRC was 47.7 years ± 7.1 years versus 46.5 ± 8.1 years (p=0.04) (Table 2).

Of all 690 probands, 160 (23.2%) had at least one FDR with CRC and 331 (48%) had at least one FDR or SDR with CRC. Sixty-five probands had both a FDR and SDR with CRC (9.4%), and only one of these had a *BRAF*-mutated CRC (1/65; 1.5%). Compared with probands with a *BRAF*-wildtype CRC, probands with a *BRAF*-mutated CRC were less likely to have a FDR with CRC (OR=0.41, 95%CI=0.17-0.99; p=0.05) or FDR or SDR with CRC (OR=0.55, 95%CI=0.30-1.00; p=0.05). The inverse relationship between family history of CRC and *BRAF* p.V600E mutation was evident for MMR-proficient CRCs (OR=0.46, 95%CI=0.24-0.91; p=0.03) but not for MMR-deficient CRCs (OR=1.20, 95%CI=0.32-4.53; p=0.79) (Table 3).

There was no evidence that the occurrence of ECCs in FDRs or SDRs was associated with having a *BRAF*-mutated CRC (Table 4). Stratification by MMR status did not reveal any significant associations between family history of ECC and *BRAF*-mutated CRC (Table 4). There was no evidence of difference in mean age at diagnosis of CRC in probands with and without a family history of ECCs (Table 2).

DISCUSSION

BRAF p.V600E testing has demonstrated considerable efficacy in its triage of MLH1-deficient CRC into Lynch syndrome and non-Lynch syndrome classes (33). The implication of a positive *BRAF* p.V600E mutation test is that the family concerned were not harbouring a MMR-gene mutation and, therefore, did not require any additional surveillance over and above routine recommendations. However, recent evidence has suggested that the relatives of individuals with a *BRAF* p.V600E mutated CRC are at an increased risk of CRC and possibly ECCs (16, 18, 19, 34). This was also observed for the relatives of individuals with a *MLH1* promoter hypermethylated CRC (35), a tumour feature highly correlated with the *BRAF* p.V600E mutation. In addition, multi-case families, with a predisposition to develop advanced serrated lesions (polyps and CRC) and a high frequency of CRC with the *BRAF* p.V600E mutation and variable levels of MSI (MSI-V), have been described(16). In a study of 11 such families, we have shown that 7 demonstrated linkage to the same region of chromosome 2q32.2-q35 (15) supporting a genetic predisposition to develop serrated neoplasia.

Population-based studies have supported the concept of a familial predisposition associated with *BRAF*-mutated CRC. Samowitz *et al* (11) observed that microsatellite stable, *BRAF* p.V600E mutated CRCs were more likely to have a family history of CRC compared with *BRAF*-wildtype CRCs (OR=4.23, 95% CI=1.65-10.84). CRCs with the *BRAF* p.V600E mutation were also shown to be statistically significantly increased in families with both CRC and ECCs when compared with families affected only with CRC (17.5% vs. 3.5%, p=0.009) (19). Recently, Wish *et al* (18), observed an elevated risk of CRC for FDRs of index patients with CRC demonstrating MSI-H and the *BRAF* p.V600E mutation (HR = 2.49; 95% CI =1.57- 3.93) and with MSS and the *BRAF* p.V600E mutation (HR = 1.64; 95% CI =1.01-

2.66) compared with FDRs of index patients with MSS, *BRAF*-wildtype tumors. Together, these three studies support an association between a family history of CRC and an increased risk of the *BRAF*-mutated CRC.

In contrast, our current study of CRC diagnosed before age 60 years from the Australasian Colorectal Cancer Family Registry showed that family history of CRC was not associated with an increased risk of the *BRAF* p.V600E mutation in CRC, even after stratification by MMR status. Instead, our results demonstrated that a FDR or SDR with CRC was associated with a *lower* risk of early-onset, *BRAF*-mutated CRC. One potential explanation for the discrepancy in findings between this study and the previous reports is the difference in the mean age at diagnosis of the CRC cases. One of the key findings in our study was that the mean age at diagnosis of a *BRAF*-mutated CRC was statistically significantly older (p=0.04) for probands with a family history of CRC (49.3 years ± 6.4 years) compared with probands without a family history of CRC (43.8 years ± 10.2 years) and that the odds of having a family history of CRC (FDR or SDR) were 9% higher per year (P = 0.04) for probands with a *BRAF*-mutated CRC. The ACCFR recruited population-based cases diagnosed at age of 60 years or younger (65% diagnosed before 50 years of age), and therefore, probands with a *BRAF*-mutated CRC were necessarily young, with a mean age at diagnosis of 45.7 ± 9.4 years. In contrast, probands with a *BRAF* p.V600E-mutated CRC described in the study by Wish et al, (18), had a mean age at diagnosis of 61.5 years ± 7.3 years for MSS CRC and 66.2 years ± 6.4 years for MSI-H CRC (overall cohort mean age at diagnosis of 59.9 years). Similarly, in the study of Samowitz et al, study (11), 85% of probands with a *BRAF*-mutated, MSS CRC were older than 55 years of age at diagnosis and 97.5% of probands with a *BRAF*-mutated, MSI-H CRC were older than 55 years of age at diagnosis. Taken together, our data

and these studies suggest that a family history of CRC may be more common in older persons with a *BRAF* p.V600E-mutated CRC.

Alternatively, hereditary factors may be more pronounced in early-onset CRC cases that are *BRAF*-wildtype, as supported by the inverse association between family history of CRC and the *BRAF* p.V600E mutation in this study. If we considered the alternative analytic approach with the *BRAF* p.V600E mutated CRC cases as the reference group, the risk of having a FDR with CRC in this early-onset CRC study would be increased 2.5 fold for the *BRAF*-wildtype CRC cases. This is consistent with *BRAF* p.V600E mutations in early-onset CRC cases being caused by factors that are not correlated or shared by relatives; i.e. *BRAF* p.V600E mutation in early-onset CRC is a marker for non-inherited CRC risk. Almost half of the probands with early-onset, *BRAF*-wildtype CRC reported a FDR or SDR with CRC suggesting that these cases may be influenced by more highly penetrant genetic factors resulting in a more prevalent familial clustering of CRC. In comparison, only a third of the probands with early-onset *BRAF*-mutated CRC reported a FDR or SDR with CRC. A further study comparing the incidence of a family history of CRC in *BRAF* p.V600E mutated cases to that of the general population may provide evidence for any elevated risk of CRC in relatives above that of the general population without the potential confounding influence of early-onset CRC cases that are *BRAF*-wildtype. There is some evidence that this alternate approach would identify an elevated risk of CRC, and possibly ECCs, in relatives of probands with a *BRAF*-mutated CRC based on the findings of a recent study that demonstrated an increased risk of CRC as well as stomach cancer and possibly ovarian and liver cancer in relatives of CRC cases with methylation of the *MLH1* gene promoter(35). The overlap between *MLH1* methylation and *BRAF* p.V600E mutation is reported to be up to 75% in all CRC(10, 36), and lends further

support to heritable factors that influence the risk of CRC developing via the serrated neoplasia pathway.

Of the probands in this study with a *BRAF* p.V600E-mutated CRC, only 31.7% reported having a FDR or SDR with CRC, suggesting that there is potential heterogeneity of familial risk within *BRAF*-mutated CRCs. Stratifying by MMR status provided evidence that probands with MMR-proficient, *BRAF*-mutated CRC were less likely to have a family history of CRC. In contrast, there was no evidence of MMR-deficient, *BRAF*-mutated CRCs associated with a family history of CRC. The identification of further markers of an increased risk of CRC in relatives is needed; histopathologic features that differ between *BRAF*-mutated CRC with and without a family history of CRC may be useful. However, this was beyond the scope of this study because of the small number of *BRAF*-mutated CRC cases but it warrants further investigation in a larger study.

Recently, lower levels of methylation or hypomethylation of the LINE-1 repetitive DNA element was shown to be associated with a family history of CRC(37-39) and with earlier-onset CRC(38, 40). Previous studies have established an inverse association between LINE-1 hypomethylation and CRC characterised by low levels of CIMP (CIMP-low/negative), MSS and *BRAF*-wildtype(38, 41). Together, the results from our study and those previously reported support a sub-group of CRC that is likely to be enriched for a family history of CRC and would be molecularly defined by *BRAF*-wildtype, MSS, CIMP-low/negative, and LINE-1 hypomethylation, presenting at an earlier age at diagnosis and further highlights the importance of molecular pathologic epidemiological studies in CRC(42).

The frequency of *BRAF*-mutated CRCs was 7.7% in this study, with only 17% of these tumors demonstrating MMR-deficiency. This finding is in contrast to previous studies that have described both a higher frequency of the *BRAF* p.V600E mutation in CRC, between 10-17% of CRCs tested in large cohort or population-based studies, and a higher proportion of *BRAF*-mutated CRCs having MMR-deficiency, reporting between 42-52% in these same studies (11, 18, 43). The discrepancy in frequency of both these features between previous studies and ours is likely due to differences in the mean age at diagnosis of the probands, as both the *BRAF* p.V600E mutation and MMR-deficiency as a result of *MLH1* promoter methylation are strongly associated with increasing age at diagnosis (44). In support of this, we observed probands with MMR-deficient, *BRAF*-mutated CRCs to be statistically significantly older than probands with MMR-proficient, *BRAF*-mutated CRCs, a finding also consistent with a previous report (11).

The strength of this study is that it is a population-based cohort of incident early-onset CRC cases who have been well characterised for molecular and genetic indications of Lynch syndrome and *MUTYH* mutations. Family history was collected in a systematic manner and attempts were made to validate reports from medical records, cancer registration, and death certification. However, the small number of *BRAF* p.V600E-mutated CRCs (n=53) in this study, particularly those that were MMR-deficient, represents a limitation of the study.

In conclusion, we identified a novel inverse association between a family history of CRC and early-onset *BRAF* p.V600E-mutated CRCs. The previous study cohorts that identified a significantly increased risk of CRC for relatives were substantially older at diagnosis than this study cohort. Further, our findings suggest that relatives of early-onset, *BRAF*-mutated and MMR-deficient CRC cases do not require additional surveillance. However, despite a

large number of total incident cases, these results should be interpreted with caution as the numbers of *BRAF*-mutated CRCs that were MMR-deficient, were relatively low. We did observe that, despite an inverse relationship between *BRAF*-mutated CRC and family history in the youngest-onset probands, family history prevalence was significantly associated with increasing age at diagnosis when the proband had a *BRAF*-mutated CRC. Therefore, relatives of older patients presenting with a *BRAF* p.V600E-mutated CRC may be at an increased risk of CRC, as has been suggested by the results of Levine et al, (35). Larger studies are needed to explore this risk, stratified by age. We found no evidence that probands with a *BRAF* p.V600E-mutated CRC were more or less likely to report a family history of ECC than were those with *BRAF* wild-type CRC. These data provide useful insights into cancer risk assessment and heterogeneity within families and should facilitate colonoscopic screening for those with an increased risk of CRC.

Table 1

				All			MMR-proficient			MMR-deficient		
		Total	*BRAF* wildtype (ref)	*BRAF* mutated	OR(95%CI)***	p-value	*BRAF* mutated	OR(95%CI)***	p-value	*BRAF* mutated	OR (95%CI)***	p-value
All		690	637	53			44			9		
Gender	Female	343	319	24	ref		18	ref		6	ref	
	Male	347	318	29	1.27 (0.71-2.24)^	0.41	26	1.58 (0.84-2.97)^	0.15	3	0.43 (0.11-1.77)	0.24
Age at CRC diagnosis (SD)		46.96 (7.81)	47.06 (7.66)	45.66 (9.41)	0.98 (0.94-1.01)#	0.18	44.48 (9.21)	**0.96 (0.92-0.99)#**	**0.02**	51.44 (8.59)	1.10 (0.99-1.22)	0.08
Molecular Results												
MSI/IHC	MMR proficient	676	632	44	ref							
	MMR deficient (MLH1/PMS2 loss)	14	5	9	**38.73 (11.48-130.67)**	**<0.001**						
MLH1 methylation	no	36	34	2	ref		0			2	ref	
	yes	12	5	7	**27.42 (3.18-236.08)**	**0.003**	0			7	**27.42 (3.18-236.08)**	**0.003**
	untested	642	598	44			44			0		
Pathology												
Tumor location	rectum	238	224	14	ref		13	ref		1	ref	
	left-sided colon*	274	254	20	1.27 (0.63-2.59)	0.50	18	1.25 (0.60-2.62)	0.55	2	1.79 (0.16-19.95)	0.64
	right-sided colon**	169	151	18	1.98 (0.95-4.13)	0.07	12	1.46 (0.64-3.30)	0.37	6	**8.50 (1.00-72.14)**	**0.05**
	unknown	9	8	1			1			0		
	left-sided/rectum	512	478	34	ref		31	ref		3	ref	
	right-sided	169	151	18	1.73 (0.94-3.17)	0.08	12	1.26 (0.64-2.58)	0.48	6	**6.01 (1.46-24.71)**	**0.01**
	unknown	9	8	1			1			0		

Grade of Tumor	Low Grade (Well + moderate)	551	522	29	ref		23	ref		6	ref	
	High Grade (Poor + undifferentiated)	121	98	23	**4.12 (2.28-7.44)**	**<0.001**	20	**4.38 (2.30-8.32)**	**<0.001**	3	3.33 (0.79-14.03)	0.10
	unknown	18	17	1			1			0		
Mucinous	no	628	582	46	ref		39	ref		7	ref	
	yes	50	44	6	1.76 (0.71-4.37)	0.22	4	1.38 (0.47-4.09)	0.56	2	3.44 (0.68-17.42)	0.14
	unknown	12	11	1			1			0		
Tumor Margin	pushing	372	350	22	ref		15	ref		7	ref	
	infiltrating	235	209	26	**1.99 (1.09-3.62)**	**0.02**	25	**2.86 (1.46-5.61)**	**0.002**	1	0.24 (0.03-1.97)	0.18
	unknown	83	78	5			4			1		
Peritumoral lymphocytes	no	359	337	22	ref		21	ref		1	ref	
	yes	270	244	26	1.68 (0.93-3.05)	0.09	19	1.30 (0.68-2.50)	0.43	7	7.78 (0.93-64.97)	0.06
	unknown	61	56	5			4			1		
Crohn-like Lymphocytes	no	519	481	38	ref		35	ref		3	ref	
	yes	105	95	10	1.33 (0.64-2.76)	0.45	5	0.70 (0.26-1.83)	0.46	5	**9.46 (2.15-41.66)**	**0.003**
	unknown	66	61	5			4			1		
Tumor infiltrating lymphocytes	no	552	516	36	ref		35	ref		1	ref	
	mild	93	82	11	**1.89 (0.92-3.87)**	**0.003^^**	7	1.18 (0.50-2.77)	0.81	4	**26.70 (2.91-244.59)**	**<0.001 ^^**
	marked	15	11	4	**5.20 (1.57-17.24)**		0			4	**230.89 (22.11-2411.36)**	
	unknown	30	28	2			2			0		
Synchronous CRC	no	624	574	50	ref		41	ref		9		

	yes	9	8	1	1.26 (0.15-10.38)	0.83	1	1.39 (0.17-11.60)	0.76	0		
	unknown	57	55	2			2			0		

*left colon included splenic flexure, descending colon, sigmoid colon and rectosigmoid junction
**right colon included caecum through transverse colon
***adjusted for sex and age at diagnosis
^adjusted for age at diagnosis
#adjusted for sex, OR per year of age
^^p-trend

All ORs were adjusted for age and sex, except the odds ratios for age at diagnosis of colorectal cancer and sex
Right colon = from ileo-caecal junction to splenic flexure
Left colon = descending colon and sigmoid colon

- OR could not be retrieved due to low number (due to missing values)

Table 2

		All				*BRAF*-wildtype				*BRAF*-mutated				
		mean	**SD**	**n**	**t-test p value**	**mean**	**SD**	**n**	**t-test p value**	**mean**	**SD**	**n**	**t-test p value**	**ANOVA p value[#]**
Sex	**Female**	46.04	7.98	343		45.92	7.79	319		47.75	10.21	24		
	Male	47.86	7.54	347	**0.002**	48.21	7.36	318	**0.0002**	43.93	8.48	29	0.14	**0.0003**
MMR	**proficient**	46.87	7.80	676		47.04	7.68	632		44.48	9.21	44		
	deficient	51.00	7.09	14	**0.05**	50.20	3.83	5	0.36	51.44	8.59	9	**0.042**	**0.039**
FDR or SDR CRC	**no**	46.19	8.38	359		46.45	8.14	324		43.77	10.21	35		
	yes	47.79	7.05	331	**0.007**	47.70	7.09	313	**0.039**	49.33	6.35	18	**0.04**	**0.008**
FDR or SDR ECC	**no**	46.54	8.14	70		46.77	7.42	65		43.60	15.79	5		
	yes	47.00	7.77	620	0.64	47.10	7.69	572	0.74	45.88	8.73	48	0.61	0.56

[#]ANOVA compared means from *BRAF*-wildtype and *BRAF*-mutated groups per category

Table 3

				ALL			MMR-proficient			MMR-deficient		
		All	*BRAF* wildtype	*BRAF* mutated	OR (95% CI)*	p-value	*BRAF* mutated	OR (95% CI)*	p-value	*BRAF* mutated	OR (95% CI)*	p-value
FDR with CRC	no	530	483	47	ref		39	ref		8	ref	
	yes	160	154	6	**0.41 (0.17-0.99)**	**0.05**	5	0.43 (0.16-1.11)	0.08	1	0.34 (0.04-2.76)	0.31
SDR with CRC	no	454	414	40	ref		35	ref		5	ref	
	yes	236	223	13	0.62 (0.32-1.19)	0.15	9	0.50 (0.23-1.07)	0.08	4	1.43 (0.38-5.43)	0.60
FDR or SDR with CRC	no	359	324	35	**ref**		31	ref		4	ref	
	yes	331	313	18	**0.55 (0.30-1.00)**	**0.05**	13	**0.46 (0.24-0.91)**	**0.03**	5	1.20 (0.32-4.53)	0.79
FDR or SDR with CRC	no	359	340	35	ref		31	ref		4	ref	
	FDR alone	95	91	5	0.53 (0.20-1.40)	0.20	4	0.50 (0.17-1.46)	0.20	1	0.79 (0.09-7.29)	0.84
	SDR alone	171	162	12	0.73 (0.36-1.42)	0.34	8	0.56 (0.25-1.25)	0.15	4	2.01 (0.49-8.22)	0.33
	both FDR and SDR	65	65	1	0.15 (0.02-1.12)	0.06	1	0.18 (0.02-1.33)	0.10	0		

* adjusted for sex and age at diagnosis
≠ per person

FDR with CRC = family history of first-degree relatives with colorectal cancer
SDR with CRC = family history of second-degree relatives with colorectal cancer
FDR or SDR with CRC = family history of first- or second-degree relatives with colorectal cancer

Table 4

				ALL			MMR-proficient			MMR-deficient		
		All (n=690)	*BRAF* wildtype (n=637)	*BRAF* mutated (n= 53)	OR (95%CI)*	p-value	*BRAF* mutated (n=44)	OR (95%CI)*	p-value	*BRAF* mutated (n=9)	OR (95%CI)*	p-value
FDR with ECC	no	225	205	20	ref		18	ref		2	ref	
	yes	465	432	33	0.82 (0.45-1.50)	0.53	26	0.76 (0.39-1.45)	0.40	7	1.48 (0.30-7.36)	0.63
SDR with ECC	no	150	139	11	ref		7	ref		4	ref	
	yes	540	498	42	1.06 (0.53-2.11)	0.87	37	1.47 (0.64-3.37)	0.37	5	0.33 (0.09-1.28)	0.11
FDR or SDR with ECC	no	70	65	5	ref		4	ref		1	ref	
	yes	620	572	48	1.08 (0.41-2.81)	0.88	40	1.11 (0.38-3.22)	0.85	8	0.92 (0.11-7.54)	0.94
FDR or SDR with ECC	no	70	65	5	ref		4	ref		1	ref	
	FDR alone	80	74	6	1.03 (0.30-3.57)	0.96	3	0.63 (0.13-2.95)	0.56	3	2.95 (0.29-30.07)	0.36
	SDR alone	155	140	15	1.30 (0.45-3.77)	0.62	14	1.43 (0.45-4.57)	0.55	1	0.53 (0.03-8.69)	0.66
	both FDR and SDR	385	358	27	0.99 (0.36-2.67)	0.98	23	1.06 (0.35-3.19)	0.92	4	0.70 (0.08-6.46)	0.75

* adjusted for sex and age at diagnosis
≠ per person
FDR with ECC = family history of first-degree relatives with extracolonic cancers
SDR with ECC = family history of second-degree relatives with extracolonic cancers
FDR or SDR with ECC = family history of first- or second-degree relatives with extracolonic cancers

Table Legends

Table 1. Associations between personal and pathological characteristics of colorectal cancer cases and the *BRAF* p.V600E mutation

Table 2. The mean age at diagnosis (and standard deviation) by sex, mismatch repair status, and family history of colorectal cancer or extracolonic cancer for *BRAF*-wildtype and *BRAF* p.V600E-mutated cases.

Table 3. Associations between family history of colorectal cancer in first- and second-degree relatives and colorectal cancer cases with a *BRAF* p.V600E mutation

Table 4. Associations between family history of extracolonic cancers in first- and second-degree relatives and colorectal cancer cases with a *BRAF* p.V600E mutation

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Abbreviations: DNA: deoxyribonucleic acid, MMR: mismatch repair, OR: Odds ratio, 95% CI: 95% Confidence Intervals, MSI: microsatellite instability; MSS: microsatellite stable, CRC: colorectal cancer, ACCFR: Australasian Colorectal Cancer Family Registry, IHC: Immunohistochemistry, FDR: first-degree relative, SDR: second-degree relative.

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