BRAF V600E Mutation in Colorectal Cancer Is Associated with Right-sided Tumours and Iron Deficiency Anaemia

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Abstract. *Background: BRAF gene encodes a serinethreonine kinase that inhibits the RAS/MAPK intracellular pathway. BRAF mutations occur at an early stage of colorectal cancer and their presence, 10-20% of colorectal cancer (CRC), is usually associated with inferior prognosis. Materials and Methods: From 41 consecutive CRC confirmed referrals from 1,446 suspected cancer cases (mean age=67.99+13.451, male=21, female=20), we retrospectively analyzed collected data from haemoglobin (Hb) and symptoms at presentation, location of tumor and stage of the disease, including lymphovascular invasion (LVI). Gene profile analysis data (KRAS, BRAF) were retrospectively collected and associated with the presentation profile above. Results: There was no significant correlation in presentation Hb levels and eventual disease staging (p>0.05 for all associations). Patients with right-sided tumours were found to have a lower Hb level than patients with either left-sided colonic or rectal tumours. Hb levels were also significantly lower in patients with the BRAF V600E mutation. KRAS status or LVI status did not have a specific correlation with Hb levels. Conclusion: BRAF V600E mutation might be associated with right-sided tumors and subsequently related unexplained iron-deficiency anaemia (IDA) at presentation. This finding may affect the choice of clinical strategy for investigation of unexplained IDA. Further research should be conducted in order to identify and support the potential biological explanation of the findings above.*

Colorectal cancer is the third most common cancer worldwide and there has been a significant progress in understanding its pathogenesis (1). Currently, there are three distinct molecular pathways to explain its complex pathogenesis. The first one is chromosomal instability (CIN) where the aetiology is attributed to numerical (aneuploidy) or structural loss of chromosomes, whereas loss of heterozygosity (LOH) is a classic component of this theory. CIN is linked with several point mutations in genes, which are thought to be involved in the CRC pathway (*APC*, *KRAS*, *BRAF*, *etc*.) (1, 4). The second pathway consists of a fault of the mismatch repair (MMR) system of the DNA and this is defined as microsatellite instability pathway (MSI) (1, 5, 6). MSI is a unique feature of Lynch syndrome (LS), although it was also found in sporadic cancer. Lastly, a newer theory is that of C-phosphate-G (CpG) island methylation (CIMP), which can result in CIMP-high and CIMP-low phenotypes. *BRAF* tends to be associated with CIMP-high tumour phenotype (1, 7, 8).

V-raf murine sarcoma viral oncogene homolog B (*BRAF*) gene is involved as an inhibitor in the RAS/RAF/MAPK pathway (9, 10). Somatic mutations in *BRAF* gene are found in 10-20% of colorectal cancer cases (11) and result in activation of *BRAF* serine-threonine kinase and subsequent up regulation of the RAS/RAF/MAPK signalling pathway (9, 12). This can result in abnormal cellular growth, invasion and metastasis (2).

BRAF V600 E mutation is the most well -studied mutation and occurs in 90% of *BRAF* mutations (12-14). It results in a substitution of glutamic acid for a valine at amino acid position 600 (c.f. 1799T>A) (15, 16).

In terms of *BRAF* mutations and their relationship to other genetic events in CRC pathogenesis, there has been documented an association between *BRAF*-mutant status and sporadic MSI-high tumours (11, 17-19). Lochhead *et al*. state that combined *BRAF* is related with MSI profile through its association with high level CIMP and *MLH1* promoter methylation. The same study concluded that *BRAF*/*MSI* status could be used as a prognostic tool (11).

There are existing data which associate *BRAF* status with certain macroscopic features. Roth *et al*. 3 concluded that *BRAF* mutations are significantly associated with female sex, right-sided location, older age, high grade and MSI-high tumours (20). A recent metanalysis 3 concluded that *BRAF*

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Table I. *Cancer stage in the study patients.*

Stage	Colonic (N, %)	Rectal (N, %)	Total (N, %)
I	4 (15.4%)	4 (26.7%)	8 (19.5%)
II	9 (34.6%)	3 (20.0%)	12 (29.3%)
III	10 (38.5%)	5 (33.3%)	15 (36.6%)
IV	3 (11.5%)	3 (20.0%)	6 (14.6%)
Total	26 (100.0%)	15 (100.0%)	41 (100.0%)

V600E is linked with advanced TNM stage, poor differentiation, mucinous histology, MSI, CIMP, as well as female gender, older age, proximal location and mutL homolog 1 (*MLH1*) methylation (21).

There has been a long discussion about the prognostic and predictive value of *BRAF* mutational profile (22-24). Recent studies associate *BRAF* mutations with poorer prognosis (11, 24) and support their role in the response to chemotherapy (15, 16, 25).

Materials and Methods

All our data derive from the urgent referral ("2 week-wait" or "2WW") Cancer Database. 2WW is a pathway where general practitioners (GP) refer suspected colorectal cancer cases to a specialized unit in the UK. We used as a source of information the original GP referral letter, as well as the Colorectal Clinic letters in order to identify the presenting symptoms of each patient. Biochemistry profile information (haemoglobin (Hb)), histology reports and molecular diagnostics were kept online on Electronic Patients' Records (EPR) database.

We retrospectively identified 41 confirmed CRC cases from 1,446 consecutive referrals for suspected cancer. From those patients, 21 were male (51.2%), 20 female (48.8%). Mean age was 67.99 years and standard deviation (SD) 13.451 years (Figure 1).

Data were collected on clinical presentation profile, *i.e.* changes in bowel habit (CIBH), rectal bleeding (RB), unexplained irondeficiency anaemia (IDA), weight loss, abdominal mass and family history (FH). Moreover, we have focused on the Hb levels at presentation, as well as tumour location and staging profile including lymphovascular invasion (LVI).

KRAS and *BRAF* mutations ware defined using the pyrosequencing technique from extracted tumour DNA. The whole series of those were performed in our Advanced Diagnostics Lab.

Data were analysed on IBM SPSS Statistics for Macintosh (Version 22.0. Armonk, NY: IBM Corp.) using *t*-test and ANOVA test. Finally, cross-referencing of our results was performed using existing data in the literature.

Results

The mean overall initial Hb level was 115.8 (SD=20.4) g/dl, with 10 patients being referred with a diagnosis of IDA (24.4%). Amongst those referred with IDA, the mean Hb was 95.3 g/dl, significantly lower than those not identified as having IDA who had a mean Hb of 124.3 g/dl (*t*-test, $p<0.0001$). Twenty patients were referred with rectal bleeding (48.8%), 26 (63.4%) with a change in CIBH, 6 (14.6%) with weight loss and 1 (2.4%) with a significant family history.

Table II. *Relationships of Hb level to clinical features (ANOVA associations).*

Feature	Hb (g/dl)
Total	115.8
I	116.0
II	114.6
III	120.6
IV	105.7
Right-sided	98.6*+
Left-sided	120.3*
Rectal	122.3+
KRAS wt	116.6
KRAS 12	108.1
KRAS 13	120.4
BRAF wt	123.5*
BRAF 600	89.0*
LVI-negative	115.0
LVI-positive	109.7

Disease stage p>0.05 for all associations with Hb levels; Primary location *p=0.017 (right-sided vs. left sided tumour), +p=0.009 (right-sided vs. rectal tumours' Hb levels); KRAS status p>0.05 for all associations with Hb levels; BRAF status *p=0.009; LVI status p=0.456. LVI: Lymphovascular invasion.

Hb levels were significantly lower in patients referred with rectal bleeding (107.1 g/dl with and 126.3 g/dl without, $p=0.002$). There was no significant difference in Hb level between patients according to CIBH (119.6 g/dl with CIBH and 111.5 g/dl without, $p=0.245$) or abdominal mass (109.3 g/dl with a mass, 118.3 g/dl without, $p=0.323$).

All patients had a confirmed diagnosis of CRC: 10 right-sided (24.4%), 31 left-sided (75.6%), comprised of 16 patients with left-sided colonic (39.0%) and 15 with rectal tumours (36.6%). Overall, there were 26 patients with a colonic primary (63.4%) and 15 (36.6%) with a rectal primary.

There were 8 patients (19.5%) with stage I disease, 12 stage II (29.3%), 15 Stage III (36.6%) and 6 stage IV (14.6%). There was an uneven distribution of patients by stage, with relatively more patients with stage II and III disease than I and IV disease (see Table I and Figure 2, $p=0.003$), which was not altered significantly between those with colonic or rectal primaries (Table II, $p=0.212$). The presence of LVI was available in 36 patients of whom 25 had no LVI (69.4%) and 11 with LVI (30.6%).

Of the 41 patients, *KRAS* status was available in 40, of whom 28 were wild-type (wt, 70.0%), 7 mutation 12 (17.5%) and 5 mutation 13 (12.5%). *BRAF* was available in 24 patients of whom 14 were wt (82.5%) and 3 with V600E mutation (17.6%).

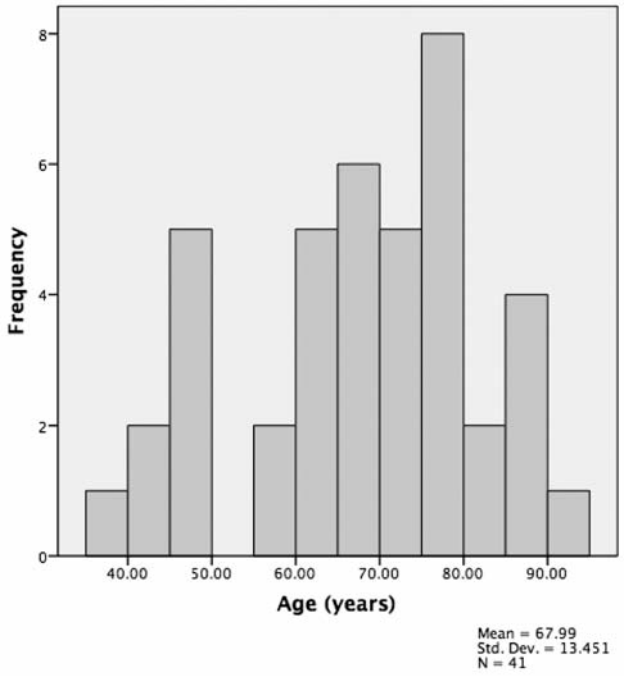

Figure 1. *Age distribution*.

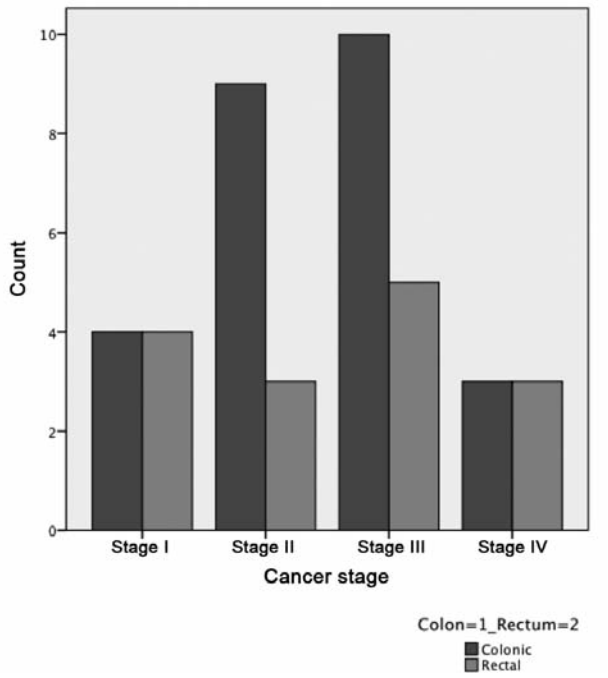

Figure 2. *Cancer stage*.

There was no significant correlation in presentation Hb levels and eventual disease staging ($p>0.05$ for all associations). Patients with right-sided tumours were found to have a lower Hb level than patients with either left-sided ($p=0.017$) or rectal tumours ($p=0.009$). Hb levels were also significantly lower in patients with the *BRAF* V600E mutation ($p=0.009$). *KRAS* status was not found to be associated with Hb levels ($p>0.05$). LVI status was also not found to be linked with Hb levels ($p>0.05$).

Discussion

There has been a long discussion about clinical significance of *BRAF* V600E mutation in colorectal cancer (20, 23, 24). Recent studies conclude that *BRAF* V600E mutation is associated with poorer prognosis (11, 24) and support its role in the response to chemotherapy (10, 16, 26). More specifically, Mao *et al*. state that *BRAF* V600E is associated with resistance to anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies in patients with metastatic CRC and wt *KRAS* (15). Phipps *et al*. (12, 27) conclude that *BRAF* mutational prognostic value can vary, depending on patient and tumour characteristics. Ciombor *et al*. (28) highlight the significance of *BRAF* in terms of prognostic value, as well as a new CRC therapy target.

However, there is also emerging data, which liaise *BRAF* V600E with MSI-high tumours (2). In those cases, there is still a negative effect of the V600E mutation on overall survival (29), although some other researcher still doubt this and support that *BRAF* V600E has a negative prognostic effect only in microsatellite stable (MSS) tumours (30, 31).

BRAF V600E is generally linked in the literature with certain clinicopathological features, *i.e*. female sex and mucinous differentiation (1, 2, 32, 33). There is a well-defined relationship between V600E mutation and generally poorer prognosis; however, no convincing explanation has been given yet.

Our study concludes that there is a well-defined relationship between significantly lower Hb levels and V600E mutant neoplasms comparing to the wt samples. There is no doubt that this could be attributed to the fact that all those patients presented with right-sided tumours and also by the fact that all *BRAF* V600E specimens were advanced-stage tumours (T3b and above). However, this observation could alter the significance of the presence of IDA in the clinical investigations pathway by demonstrating that IDA may signify a higher risk cancer. This may well affect the choice of treatment and would we should look for *BRAF* mutation in unexplained IDA, where colorectal cancer is suspected. Of note is the fact that we could not identify any statistical significance between Hb levels and disease stage (I-IV).

The main question that this study raises is at what stage of the carcinogenesis pathway does *BRAF* interfere. According to the literature, most of the *BRAF* V600E specimens appear to be in advanced stage (20, 21, 32). It is, thus, quite important to establish in which time point *BRAF*

interferes as it appears that this happens on a mature stage in the pathway. Taking into consideration the latter, it is quite important to interpret the potential link between IDA at presentation and *BRAF* V600E.

A recent promising study (33) suggests a multitarget stool DNA test with faecal immunochemical test (FIT) as a non-invasive diagnostic technique for CRC screening. The sensitivity for detecting CRC was 92.3% with DNA testing and 73.8% with FIT detecting *versus* 42.4% with DNA testing and 23.8% with FIT in advanced precancerous lesions. This test does not include *BRAF*. The main question is whether there would be any clinical importance to incorporating *BRAF* screening in these tests, especially in those cases where IDA is noted at presentation. This would help in increasing the sensitivity of these methods and IDA could be used as an alarm point where advanced cancer with mutant *BRAF* is suspected. In terms of precancerous lesions, again, our question regarding the stage of carcinogenesis that *BRAF* interferes with, could be a crucial point in order to improve the sensitivity of this method, as well as stratifying the risk of potential progression to cancer. In other words, if we knew the exact point where V600E mutation appears, then we would be able to tell if a precancerous lesion has a potentially higher risk of progression to cancer.

Moreover, we tried to liaise potential clinical presentation features with the molecular status of *KRAS* and *BRAF*, which are thought to play a significant role in prognosis of CRC, as well as response to neoadjuvant chemotherapy. The main question was whether there would be any unique clinical presentation or histopathology features, which could potentially be liaised with a known mutation of *KRAS* or *BRAF*.

With regards to *KRAS* mutational status, there was no significant relationship between disease stage and *KRAS* status ($p>0.05$). This is in line with literature, as *KRAS* is not an established prognostic marker (20). It is well known that in many centres around the world, *KRAS* can be used as a predictive tool to identify whether a patient would benefit from anti-EGFR chemotherapy agents (16, 33, 35, 36). Nevertheless, despite its well known predictive value, there is no conclusion as to whether *KRAS* status could be used as a prognostic tool, with the vast majority of researchers doubting its significance in terms of defining disease-free survival (33, 35). An interesting point from or results is that there was no direct relationship between Hb levels or clinical presentation and *KRAS* status ($p>0.05$). This could be easily explained by the fact that CRC pathogenesis is quite complex and a single gene is difficult to be linked to specific macroscopic presentation features unless there is an established strong relationship between certain histopathology characteristics and molecular status. Similarly, there was no defined statistical relationship between LVI and *KRAS* mutational status ($p>0.05$), which could be attributed again to its controversial prognostic value.

KRAS mutations tend to appear in earlier stages of the carcinogenesis pathway (1). Contrary to *BRAF* V600 E neoplasms, where there is an established relationship with proximal location, *KRAS*-mutant tumours tend to have a bimodal distribution in the proximal-distal axis and tend to appear more proximally and to the caecum (37).

On the other hand, *BRAF* V600E mutation is well associated with proximally located tumours (32). On our sample, even though it is quite small, there was a well-defined relationship between right-sided neoplasms and V600E mutation.

Nevertheless, there was no defined statistical relationship between LVI and *BRAF* status ($p>0.05$) and this is difficult to comment based on the limitations of our sample. Positive LVI is associated with a slightly lower Hb level comparing to LVI negative (109.7 g/dl *vs*. 115.1 g/dl).

In conclusion, given the complexity of the molecular pathways described, as well as the fact that many molecular agents interfere one each other, it is quite difficult to establish a clear picture for the unique impact of every gene contributing to CRC pathogenesis. Our study concludes by raising a question regarding the stage of carcinogenesis where *BRAF* appears to be mutant. It is quite important to consider whether IDA could also be used as a potential marker of advanced cancer and, given its relationship with proximal tumours and subsequently with *BRAF* V600E mutation, this could be used as an starting point to question whether V600E mutation could be incorporated into DNA screening methods that were recently released. This would be indisputably useful in cases where IDA could potentially be a sign of more advanced disease.

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