

ORIGINAL ARTICLE

BRAF mutation in colorectal carcinomas with signet ring cell component

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ABSTRACT

Objective: Signet ring cell carcinoma is a rare subtype of colorectal carcinoma (CRC) with an associated BRAFV600E mutation. We investigated frequencies of *BRAF* mutation in 28 CRCs containing variable signet ring cell component and their relation with clinicopathologic parameters.

Methods: According to the presence of signet ring cell component, tumors were categorized into groups as follows: 0%–9%, 10%–24%, 25%–49%, and >50%. Genomic DNA was isolated and analyzed for *BRAF* V600E gene mutation by polymerase chain reaction-restriction fragment length polymorphism. Eleven of 28 cases (39.3%) showed *BRAFV600E* mutation, which was also confirmed by Sanger sequencing. To elucidate the importance of existence of signet ring cell component at the molecular level, we separated cases into two groups with cut-off levels of 10% and 50%, which pertain to percentages of signet ring cells.

Results: Seven of 19 cases (36.8%) under the threshold of 50% and four of nine cases (44.4%) over this threshold value demonstrated *BRAF* mutation. Three of 7 cases (42.8%) featuring <10% signet ring cell component and eight out of 21 cases (38.1%) showing >10% were *BRAF* mutated.

Conclusions: *BRAF* mutation must be closely associated with the presence of malignant signet ring cells regardless of their percentages.

KEYWORDS

BRAF; mutations; colon cancer; signet ring cell

Introduction

Signet ring cell carcinoma (SRC) is a variant of colorectal carcinoma (CRCs) and defined by the presence of >50% of tumor cells with large mucin vacuole, which abundantly fills the cytoplasm, resulting in compression and eccentric displacement of the nucleus. However, conventional or other specific variants of CRC may also contain variable proportion of signet ring cells with less than 50% of tumor cells. To date, many studies have examined molecular characterizations of SRC-CRC and indicated that most SRC-CRCs share variable molecular alterations, such as high-degree microsatellite instability (MSI-H), frequent CpG island methylator phenotype, and increased methylation level of long interspersed nucleotide element-1¹⁻⁶. Frequent *BRAF* V600E mutation is also one of the most remarkable molecular abnormalities^{2,3,5,6}. However, to the best of our knowledge,

limited information is available on *BRAF* status in CRCs with less than 50% SRC component, because only several studies assessed the status of *BRAF* mutation in such tumors^{2,4,6}.

In this study, we investigated frequencies of *BRAF* gene mutation in 28 cases of sporadic CRCs with various amounts of SRC component and their relationship with clinicopathologic parameters. Percentage values of 50% and 10% for SRC components are comprehensible and sufficient for most practicing pathologists and will provide a good starting point for stratifying cases according to changes at the molecular level for further statistical analysis and interpretation.

Material and methods

Case selection

We retrospectively selected 28 cases of primary and sporadic colorectal adenocarcinomas with variable proportions of SRC among files of the Department of Pathology of Gulhane Military Medical Academy in Ankara. All patients underwent radical surgery and received a close clinical follow-up at 3 months to 144 months after surgery. None of the patients

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showed history of neoadjuvant chemotherapy or radiotherapy. Tissues were fixed in 10% neutral-buffered formalin, processed routinely, embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E). We reevaluated archival slides of each case to confirm their diagnosis and determine percentages of SRC components, which were scored and categorized into groups as: 0%–9%, 10%–24%, 25%–49%, and >50%.

The design of this study was approved by the local ethical committee of Gulhane Military Medical Academy in Ankara.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing

Multiple 4 µm sections were obtained from the most representative blocks of each case. Genomic DNA was manually isolated by using a DNA extraction kit (PureLink Genomic DNA Mini Kit, Invitrogen, Carlsbad, CA, USA) following manufacturer's protocol.

The previously described PCR-RFLP was employed for detection of *BRAF* V600E. To scan *BRAF* V600E mutation, a 237 bp fragment of exon 15 of the *BRAF* gene was amplified using primers: forward 5'-GCTTGCTCTGATAGGAA AATGAG-3' and reverse 5'-GTAACCTCAGCAGCA TCTCAGG-3'. Amplification was carried out in a 50 µL total reaction volume containing 2 mM of dNTPs, 10 pmol each of forward and reverse primers, 2.5 U Taq DNA polymerase, 10× PCR buffer, and 50 ng genomic DNA by using PCR System (CLP Apollo ATC401 Thermalcycler, Apollo Instrumentation, San Diego, CA, USA). PCR cycling conditions were as follows: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s, and final extension step at 72 °C for 5 min. Then, amplified PCR products (237 bp) were digested with restriction endonuclease TspRI (restriction site = NNCASTGNN) (New England Biolabs Beverly, MA, USA) and incubated at 65 °C for 5 h. Undigested and digested PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized using an ultraviolet illuminator (Gel Logic 200 Imaging System, Eastman Kodak Company, Rochester, NY, USA). The 174, 87, or 33 bp fragment alone indicated wild-type *BRAF*, whereas the 204 and 33 bp bands indicated presence of V600E mutation.

We also performed further DNA direct sequencing on all samples to confirm reliability of PCR-RFLP results. Single-pass sequencing was performed on each template using the forward primer. Fluorescence-labeled fragments were purified from unincorporated terminators by ethanol

precipitation. Subsequently, samples were resuspended in distilled water and subjected to electrophoresis in a DNA sequencer (ABI Prism 3730XL DNA Sequencer, Applied Biosystems, Perkin Elmer Corporation, Foster City, USA) in RefGen Ltd.Co. (Ankara University, Technopolis, Ankara, Turkey).

Statistical analysis

All analyses were performed using SPSS for Windows version 17.0 (SPSS, Chicago, IL, USA). Descriptive statistics were presented as mean, minimum-maximum, frequency, and percentage. Chi-square test was used to compare categorical variables. Mann-Whitney U test was used to compare continuous variables. Survival analysis was carried out by Kaplan-Meier method. Cox regression analysis was also performed to determine predictors on overall survival. A *P*-value of < 0.05 was considered statistically significant.

Results

Mean age of patients totaled 51 years (range: 21–81 years) with a male-to-female ratio of 3.7. Overall, most SRC-CRCs were located in the right colon (46.5%) and rectum (35.7%). According to the T (tumor), N (node), and M (metastasis) staging (TNM) system of colorectal cancer staging, five cases were in stage II (17.9%), 16 in stage III (57.1%), and 7 in stage IV (25.0%). SRC components reached 0%–9% in seven cases (25%), 10%–24% in nine (32.1%), 25%–49% in three (10.8%), and >50% in nine cases (32.1%). In the study group, 19 cases (67.9%) showed <50% SRC component, and the remaining nine cases (32.1%) exhibited SRC component of >50%. SRC component totaled <10% in seven cases (25%), whereas 21 cases (75%) contained >10% of SRCs. In total, regardless of SRC percentages, 11 cases (39.3%) showed *BRAF* mutation, and the remaining 17 (60.7%) were wild types (**Figure 1**). Results of DNA sequencing were all consistent with those of PCR-RFLP (**Figure 2**). Locations of mutant and wild-type tumors were nearly similar to each other. **Table 1** summarizes detailed clinicopathologic features and mutation status.

All SRC-CRCs were presented as pT3 (7 cases, 25%) or pT4 (21 cases, 75%) tumors. No SRC-CRC was observed in pT1 or pT2 stage. Although the mutant group featured higher percentage (9 cases, 81.8%) of pT4 tumors than the wild-type group (12 cases, 70.6%), the difference was not statistically significant. A total of 22 out of 28 cases (78.6%) showed metastasis to at least one regional lymph node. Most cases (19 cases, 67.9%) presented four or more lymph node

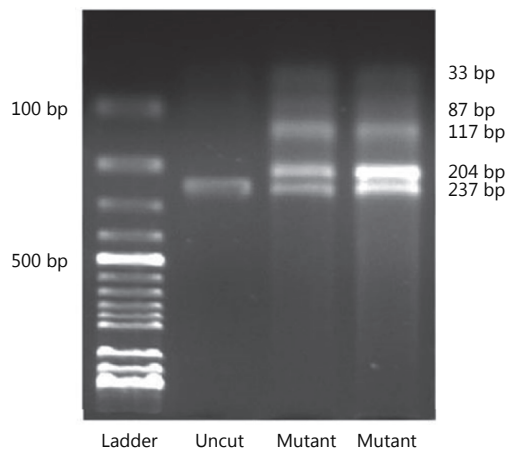


Figure 1 Gel image demonstrates the presence of BRAFV600E mutation in two cases.

A: Wild-type

B: c.1799T>A



Figure 2 Representative electropherograms showing wild-type sequence (A) and mutation (c. 1799T>A) identified in *BRAF* gene (B). Red arrowheads indicate respective nucleotide changes.

metastases (pN2) in the study. Three (10.7%) of SRC-CRCs exhibited 1 to 3 lymph node metastases (pN1). No lymph node metastasis occurred in 6 cases (21.4%). No statistically significant difference was found between percentages of SRC component and clinicopathologic parameters of *BRAF* wild-type and mutant cases. As for categorization of cases using 10% and 50% cut-off points for SRC component, we did not observe statistically significant differences involving *BRAF* mutation status between <10% and >10% groups and <50% and >50% groups. No any relation exists with patients' age, sex, tumor location, or pTNM. When using the 50% cut-off, 7 of 19 cases (36.8%) under the threshold and 4 of 9 cases

(44.4%) over the threshold demonstrated *BRAF* mutation. However, *BRAF* mutation was detected in 3 out of 7 cases (42.8%) with <10% SRC and 8 of 21 cases (38.1%) showing >10%.

Mean follow-up of disease measured 31.7 months (range: 3–144 months). Eleven cases of SRC-CRCs died because of the disease. At the end of follow-up, 5 among 11 patients died (45%) with *BRAF* mutant SRC-CRCs, whereas 6 of 17 cases died because of the disease (35%) in wild-type group of *BRAF* SRC-CRCs. Median survival totaled 12 months in *BRAF* mutant SRC-CRCs and 95 months in wild-type *BRAF* SRC-CRCs. Five-year survival reached 45.0% in cases with *BRAF* mutation and 50.6% in the group with wild-type *BRAF* (Figure 3). When we adjusted age, gender, percentage of signet ring component, and stage and after Cox regression analysis, we reached no statistically significant increased risk in *BRAF* mutant group compared with the *BRAF* wild-type [hazard ratio (HR)=3, 40; 95% confidence interval (CI)=0, 71–16, 29; $P=0.121$] (Figure 4).

Discussion

BRAF is one of the serine/threonine-protein kinases and is a proto-oncogene encoded by the *BRAF* gene, which is localized to human chromosome region 7p34^{7,8}. *BRAF* is a member of the *RAF* gene family and participates in the RAS-RAF-MEK-ERK mitogen-activated protein kinase signaling pathway, which mediates cellular responses to serious growth signals⁹. Activating somatic mutations of *BRAF* were detected in various tumors, including melanoma, papillary thyroid carcinoma, non-Hodgkin's lymphoma, and adenocarcinoma of the lungs^{10,11}. In 90% of mutated cases, thymine is substituted by adenine at nucleotide 1799. This occurrence leads to substitution of valine (V) by glutamate (E) at codon 600 (V600E) in the activation segment⁹. *BRAF* frequency varies; this alteration is shown in nearly all cases of hairy cell leukemia (~100%), cutaneous melanomas (66%), melanocytic nevi (80%), and papillary thyroid carcinoma (30%–70%), and adenocarcinoma of lung (3%)¹⁰⁻¹⁴.

BRAF mutation in several subtypes of CRCs has been reported in the range of 5% to 22%^{10,15-18}. *BRAF* mutation in CRC occurred more commonly in cases with MSI-H but less frequently in patients with germline mutations in one of the mismatch repair genes than in sporadic MSI-H tumors^{15,18}. Mutations of *BRAF* are associated with MLH1 promoter methylation in sporadic CRC¹⁹. Both SRC-CRC and mucinous CRCs are also associated with frequent *BRAF* mutation compared with nonmucinous CRC^{2,4,6,20,21}.

SRC-CRC is associated with extremely poor prognosis

Table 1 Clinicopathologic features and BRAF status in CRCs with variable signet ring cell component

BRAF status	BRAF mutant n=11 (%)	BRAF wild-type n=17 (%)	Total n=28 (%)	P
Sex				0.613
Male	9 (81.8)	13 (76.5)	22 (78.6)	
Female	2 (18.2)	4 (23.5)	6 (21.4)	
Age, mean	48.7±20.9	45.6±20.7		0.705
Location				0.997
Right colon	5 (45.4)	8 (47.1)	13 (46.5)	
Left colon	2 (18.2)	3 (17.6)	5 (17.8)	
Rectum	4 (36.4)	6 (35.3)	10 (35.7)	
SRC component (%)				0.957
0%–9%	3 (27.3)	4 (23.5)	7 (25.0)	
10%–24%	3 (27.3)	6 (35.3)	9 (32.1)	
25%–49%	1 (9.1)	2 (11.8)	3 (10.8)	
≥50%	4 (36.4)	5 (29.4)	9 (32.1)	
pTNM				0.538
T3	2 (18.2)	5 (29.4)	7 (25.0)	
T4	9 (81.8)	12 (70.6)	21 (75.0)	0.309
N0	2 (18.2)	4 (23.5)	6 (21.4)	
N1	2 (18.2)	1 (5.9)	3 (10.7)	
N2	7 (63.6)	12 (70.6)	19 (67.9)	0.581
M0	8 (72.7)	13 (76.5)	21 (75.0)	
M1	3 (27.3)	4 (23.5)	7 (25.0)	
Stage				0.893
II	2 (18.2)	3 (17.6)	5 (17.9)	
III	6 (54.5)	10 (58.9)	16 (57.1)	
IV	3 (27.3)	4 (23.5)	7 (25.0)	

SRC, Signet-ring cell; pTNM, Pathological TNM.

compared with classical ones. BRAFV600E mutation has also been identified as poor prognostic marker in patients with metastatic disease²². To date, many studies investigated molecular characterizations of SRC-CRCs and showed that most SRC-CRCs share various molecular alterations, including MSI-H, high frequency of CpG island methylator phenotype, high methylation level of long interspersed nucleotide element-1, and frequent BRAF mutation^{1,6}. However, to our knowledge, only a few studies assessed the status of BRAF mutation in CRCs with varying SRC components, and they are insufficient for defining such entity as SRC-CRC^{2,4,6}.

Kakar et al.⁴ reported that BRAF mutations occur more frequently in SRC-CRCs compared with conventional CRCs. They discovered that frequency of BRAF mutation in SRC-CRC reached 33.3% (9 out of 27 cases) but totaled 15.7% (9 out of 57 cases) in CRC. Ogino et al.⁶ noted that BRAF mutation exhibited a significant adverse effect in a large cohort of more than 600 CRC patients, and CRC with variable SRC component presented more frequent (28%) (9 out of 32 cases) BRAF mutations compared with that without any SRC component (8.6%) (30 out of 348 cases). In this study, frequency of mutation reached 22% in SRC-CRC (tumors with >50% signet ring cell component) and 30% in

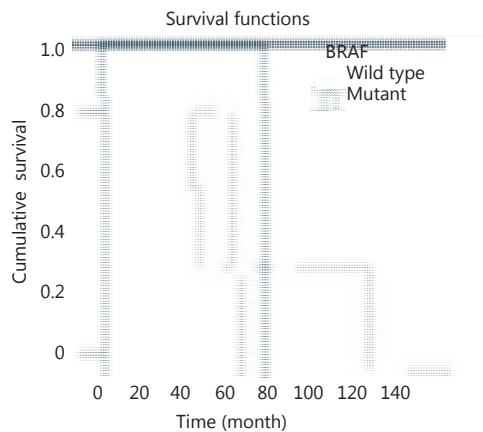


Figure 3 *BRAF* mutation status and 5-year survival in signet ring carcinoma.

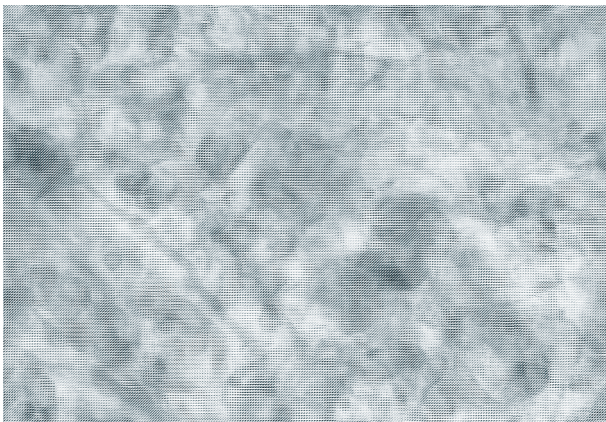


Figure 4 Histopathology shows CRC with signet cell component (H&E staining, 200 \times).

CRC with less than 50% SRC component⁶. Inamura et al.² detected association of high proportion of SRC component with frequent *BRAF* mutation. The same researchers observed that frequency of *BRAF* mutation amounted to 35% (6 out of 17 cases) and 33% (38 out of 114 cases) in SRC-CRC and CRC, respectively, with SRC component (less than 50%). Kadowaki et al.²³ investigated 811 CRCs and showed that *BRAF* mutation was associated with shorter survival and independent of MSI status. *BRAF* mutation frequency totaled 39.3% in CRC containing any SRC component. Our findings agree with results of the abovementioned studies. However, our frequency of *BRAF* mutation in CRC with more than 50% of SRC component is relatively higher than those in other previously reported studies (44.4%). This discrepancy may be related to geographical and epidemiologic differences. In our study, the 5-year survival reached 45% in

cases with *BRAF* mutation and 50.6% in wild-type group. After adjustment of age, gender, stage, and percentage of SRC component, *BRAF* mutation was considered not a statistically significant independent predictor. However, hazard ratio was 3.40 times higher in *BRAF* mutant cases compared with wild-type ones. Thus, *BRAF* mutation status can be an important survival factor. Lack of statistically significant result can be attributed to the limited number of cases included in our study.

We also investigated significance of extent of SRC component in *BRAF* mutation. Frequency of *BRAF* mutation was higher in tumors with >50% SRC component than those with <50% SRC component (44.4% vs. 36.8% for >50% vs. <50%, respectively). However, a significant number of cases (approximately one third of cases) in tumors with <50% SRC component were *BRAF* mutant. *BRAF* mutation was also frequent in CRCs with <10% SRC component (42.8% vs. 38.1% for <10% vs. >10%, respectively). These findings strongly indicate that *BRAF* mutation status is not linked with percentage of SRC, and *BRAF* mutation not only shows frequently in SRC-CRCs but also in tumors featuring minute population of SRC component. Unfortunately, the number of cases in this group (<10% SRC component) was limited. Thus, the possibility of *BRAF* mutation in CRC with minor component of SRC should not be considered negligible. Studies on larger series of CRC with focal SRC can further clarify the relation of *BRAF* mutation status in such tumors. SRC-CRC and CRC with lesser SRC component have been associated with shorter patient survival and are independent of other clinicopathologic and multiple molecular features^{2,5,20}. Most CRCs containing any proportion of SRC were in advanced stage (stage III and IV; 82.1%). When percentages of SRC component and other clinicopathologic parameters (age, sex, location, and outcome) were compared, no statistically significant differences were observed between groups with <50% and >50% SRC and groups with <10% and >10% SRC.

In conclusion, although frequency of *BRAF* mutation was higher in tumors with >50% SRC component, a significant number of tumor cases (approximately one third of cases) with <50% SRC component were also *BRAF* mutant. CRCs with a minor (<10%) SRC component presented *BRAF* mutation rates similar to those of tumors with >10% and >50% SRC. The presence of SRCs in CRCs in minor component (regardless of its percentage) can be an important predictor for *BRAF* mutation in these tumors. However, the small number of cases poses a serious limitation in our study. Larger studies must be conducted to explore the relation

between *BRAF* mutation and CRCs with <10% and >10% SRC.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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