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Effect of STAT3 status on RAS & RAF mutation in patients with metastatic colon carcinoma

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Abstract

Aim: Colon adenocarcinomas are the most common gastrointestinal tract malignancy and constitute one of the leading causes of mortality and morbidity worldwide. STAT3 is a transcription factor activated by many cytokines and growth factors and plays a crucial role in cell survival, proliferation, and differentiation. In this research, we aimed to elucidate the molecular properties of colon tumors.

Materials and Methods: A total of 196 patients with metastatic colon tumors whose samples were analyzed for KRAS, NRAS, and BRAF between 2016 and 2022 have been investigated in this retrospective study. The samples were analyzed via polymerase chain reaction (PCR) at our institution. On the formalin-fixed, paraffin-embedded tissue blocks, immunohistochemistry was performed to determine STAT3 expression. Immunohistochemical staining with a fully automated assay was performed on 3-4 µm-thick slices based on manufacturer's instructions. Phospho-STAT3 Antibody(B7):sc-8059 Conc. 0.1mL (1:100) antibodies were used on the Ventana® Benchmark XT (Ventana Medical Systems,Inc,Tucson,AZ,USA). All the slides were examined by a pathologist. STAT3 expression was scored between 0 and 3 points due to staining intensity. No staining was considered as 0, light staining as 1, moderate staining as 2, and strong staining as 3 points.

Results: A total of 196 patients, 79 (40.3%) female and 117 (59.7%) male, were included in the evaluation within the scope of the study. There was no significant difference between STAT3 staining intensities in terms of demographic characteristics. On the contrary, there was a statistically significant relationship regarding tumor grades ($p<0.05$). KRAS mutation was found in 40.8% of the patients (n=80), NRAS mutation was found in 2% (n=4), and BRAF mutation was found in 4.1% (n=8). It was determined that there was a statistically significant relationship between KRAS and STAT3 grades of mutations ($p<0.05$). It is seen that KRAS positivity increases as the STAT3 staining intensity of the patients' increases. There was no statistically significant correlation between other mutation results and STAT3 grades.

Conclusion: In light of the findings obtained from our study and previous literature, it has been determined that routine STAT3 gene screening is a necessity before the treatment is determined.

Introduction

Colon adenocarcinomas are the most common gastrointestinal tract malignancy and constitute one of the leading causes of mortality and morbidity worldwide. STAT3 is a transcription factor activated by many cytokines and growth factors and plays a crucial role in cell survival, proliferation, and differentiation [1]. The gene encoded by STAT3 is located on chromosome 17q21, encoding a 92-kDa protein. STAT3 protein has been detected in many malignant tumors such as breast, head and neck, liver, melanoma, prostate, pancreas, colon cancers, and leukemia [2].

Current studies reveal that activation of this protein contributes to oncogenesis. In addition, oncogenes such as Src and Ras stimulate STAT3. Seven types of STATs have been identified in mammals. K-ras mutations are observed in approximately 15% of all cancers [3]. It is one of the most frequently mutated genes in cancer. The biliary tract, colon, lung, and pancreas are the organs where mutations are most frequently observed. The K-ras gene is located on the short arm of chromosome 12 and is associated with GTP-dependent signal transduction, control of cell growth, differentiation, and life cycle [4]. Mutation

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of the K-ras oncogene results in constitutive activation of this signaling pathway, resulting in unregulated proliferation and impaired differentiation. It has been shown in many studies that anomalies occurring in this gene complex in tumor cells can cause high rates of relapse and a low life span [5].

The epidermal growth factor receptor (EGFR) signaling is elicited by ligand binding, initiating the activation of many downstream signaling cascades, including: RAS-RAF-ERK1/2,c-SRC, and signal transducer and activator oftranscription (STAT) family members [6]. The increased interleukin-6/JAK/STAT3 signaling pathway plays a significant role in the tumorigenesis of colorectum cancers. Cytokine-driven JAK/STAT3 facilitates signal transduction processes, associated with the hyperproliferative and invasive phenotype of colorectal cancer cells [7]. Approximately 3,000 KRAS point mutations have been reported in colorectal cancers. The activating point mutation of the KRAS oncogene at codon 12 (exon 2) is the initiating event in the majority of colorectal cancercases (83%) [8].

STAT3 is thought to have a significant role in tumor invasion and metastasis in different stages of cell growth, differentiation, proliferation, and angiogenesis. Previous literature has elaborated its relationship with prognostic factors such as differentiation, tumor diameter and lymph node metastasis, and tumor location [9]. Current studies reveal that activation of this protein contributes to oncogenesis. In addition, because the STAT3 signaling pathway is a potential drug target, the clinical, pathological, molecular, or prognostic features of STAT3-activated colorectal cancer are essential exploratory areas [10]. It was shown that STAT3 activation might be significant for MEK inhibitor resistance targeting the RAS pathway in K-Ras mutant colon cancer cells. This situation complicates the treatment of patients. Limited publications in the literature related to STAT3 and colon adenocarcinomas include initiation, development, and formation of colorectal cancer [11].Within the scope of this research, we aimed to elucidate the molecular properties of colon tumors.

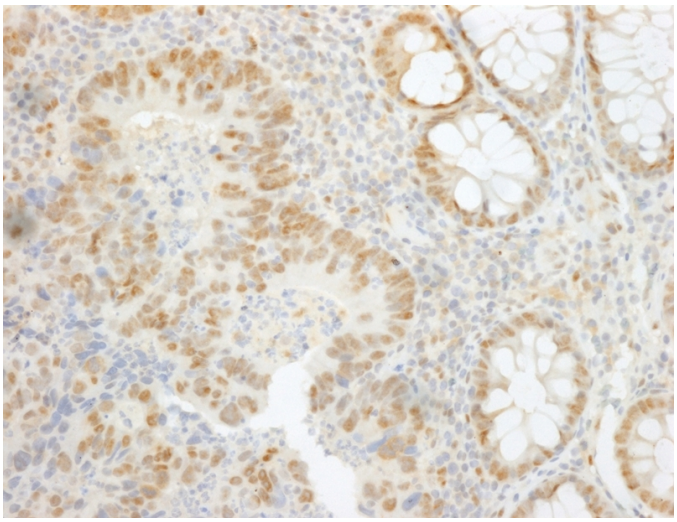

Figure 1. Severe positive staining with STAT 3 in tumor and normal colonic mucosa.

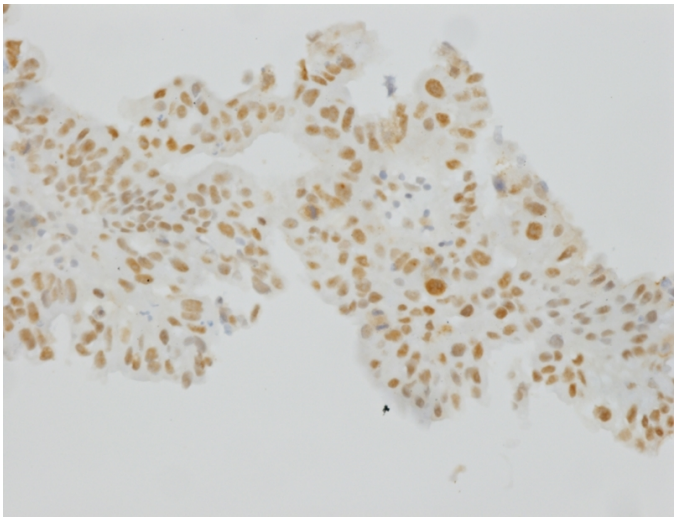

Figure 2. Severe positive staining with STAT 3 in moderately differentiated colon tumor.

Materials and Methods

A total of 196 patients with metastatic colon tumors whose samples were analyzed for KRAS, NRAS, and BRAF between 2016 and 2022 have been analyzed in this retrospective study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Ethics committee approval has been granted from our institution with protocol at 17.04.2023 ((Bezmialem University Non-Interventional Clinical Research Ethics Committee, 2023/79).

For Molecular Pathology tests, we examined slides of patients with metastatic colorectal carcinoma in our pathology laboratory. Preferably, the largest tumor area with preserved integrity without necrosis was sampled and used for PCR.

On the formalin-fixed, paraffin-embedded tissue blocks, immunohistochemistry was performed to determine STAT3 expression. The pathological material was examined by a pathologist to make sure every paraffin-embedded tissue block has sufficient tumor content for immunohistochemical analysis immunohistochemical staining with a fully automated assay was performed on 3-4 µm-thick slices based on manufacturer's instructions. Phospho-STAT3 Antibody(B7):sc-8059 Conc. 0.1mL (1.100) antibodies were used on the Ventana® Benchmark XT (Ventana-Medical Systems,Inc,Tucson,AZ,USA). All the slides were examined by a pathologist. STAT3 expression was scored between 0 and 3 points due to staining intensity. No staining was considered as 0, light staining as 1, moderate staining as 2, and strong staining as 3 points. (Figure 1.2.3.4.5).The results were compared with prognostic factors such as tumor molecular characteristics, lymph node status, grade, location, and diameter.

Statistical analysis

The patient data collected within the scope of the study were counted with STAT3 positively stained cells and quantitative evaluation was performed by IBM Statisti-

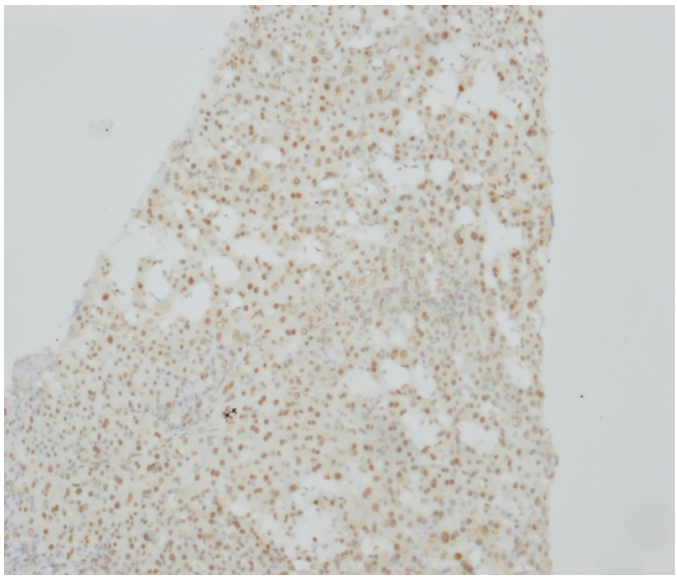

Figure 3. Severe STAT3 staining in poorly differentiated colon tumor.

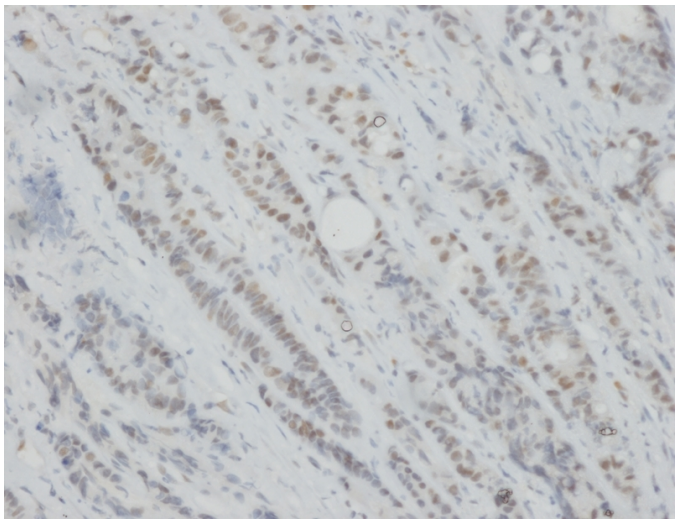

Figure 4. Moderate STAT3 staining in poorly differentiated colon tumor.

cal Package forthe Social Sciences (SPSS) for Windows 23.0 (IBM Corp., Armonk, NY) were analyzed with the package program.Frequency and percentage were given for categorical data, and median, minimum, and maximum descriptive values for continuous data. "Kruskal Wallis H Test" was used for comparisons between groups, and "Chi-square Test" was used to compare categorical variables. The results were considered statistically significant when the p-value was less than 0.05.

Results

A total of 196 patients, 79 (40.3%) female and 117 (59.7%) male, were included in the evaluation within the scope of the study. The distribution of demographic and clinical findings of the patients according to their STAT3 grades is given in Table 1. No significant difference has been detected between STAT3 staining intensities regarding demographic characteristics when the table is examined. On the contrary, there was a statistically significant relationship in terms of tumor grades ($p<0.001$).

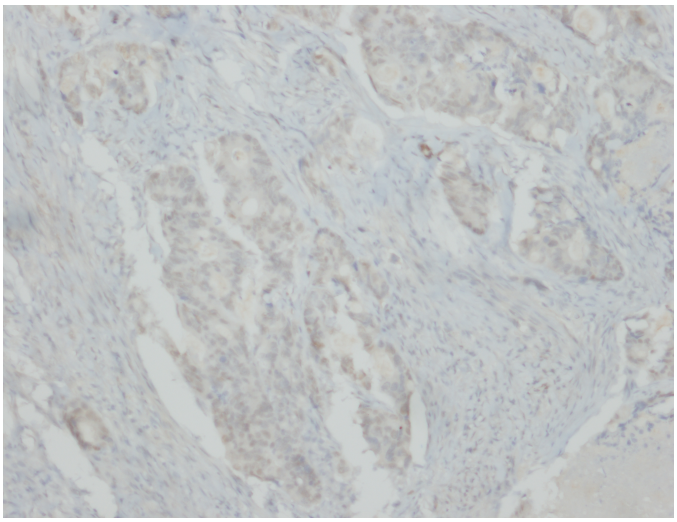

Figure 5. Weak STAT3 staining in moderately differentiated colon tumor.

The distribution of mutation types according to the STAT3 grades of the patients is given in Table 2. KRAS mutation was found in 40.8% of the patients (n=80), NRAS mutation was found in 2% (n=4), and BRAF mutation was found in 4.1% (n=8).

It was determined that there was a statistically significant relationship between KRAS and STAT3 grades of mutations ($p<0.003$). It is seen that KRAS positivity increases as the STAT3 staining intensity of the patients' increases. There was no statistically significant correlation between other mutation results and STAT3 grades.

Discussion

Three different signaling pathways activated by EGFR have been identified. These signal pathways: RAS/RAF/MAP (Mitogen-activated protein) kinases are Jak/STAT and PI3K/Akt signaling pathways. Activation of these signaling pathways triggers cellular activities associated with cell proliferation, metastasis, tumor invasion, antiapoptotic signal transduction, resistance to chemotherapy, angiogenesis, and establishment of cell-cell connections. Tumor-specific molecular markers are used to identify suitable patients for targeted therapy in colorectal carcinomas [12]. Considering that targeted therapy is becoming increasingly common in tumor groups such as breast cancer and lung cancer with significant morbidity and mortality, in tumor treatment and prognostic evaluations, the use of molecular markers to guide treatment selection in colorectal carcinomas and classification of cases accordingly is extremely important and necessary [13].

RAS mutation status has a significant role in treatment decisions for colorectal cancer patients. Considering the molecular basis of EGFR-targeting agents, EGFR blockade at the receptor level cannot stop downstream signal activation in tumors with RAS mutations and active RAS protein because the active RAS protein provides signal activation independent of EGFR. For this reason, RAS mu-

Table 1. Distribution of demographic and clinical characteristics of the cases by STAT3 density.

	STAT3 Staining Intensity					P-value
	Total (n=196)	Negative (n=46)	Weak (n=38)	Moderate (n=63)	Severe (n=49)	
	n (%) or Median (Min-Max)	n (%) or Median (Min-Max)	n (%) or Median (Min-Max)	n (%) or Median (Min-Max)	n (%) or Median (Min-Max)	
Age (years)	61 (17-88)	59 (37-88)	59 (17-83)	62 (23-82)	62 (23-80)	0.934
Gender						0.853
Male	117 (59.7)	29 (63)	21 (55.3)	39 (61.9)	28 (57.1)	
Woman	79 (40.3)	17 (37)	17 (44.7)	24 (38.1)	21 (42.9)	
Tumor Location						0.463
Right column	73 (37.2)	18 (39.1)	14 (36.8)	24 (38.1)	17 (34.7)	
Left column	77 (39.3)	17 (37)	13 (34.2)	30 (47.6)	17 (34.7)	
Rectum	46 (23.5)	11 (23.9)	11 (28.9)	9 (14.3)	15 (30.6)	
Lymph node						0.552
Negative	91 (46.4)	21 (45.7)	16 (42.1)	27 (42.9)	27 (55.1)	
Positive	105 (53.6)	25 (54.3)	22 (57.9)	36 (57.1)	22 (44.9)	
Grade						**<0.001**
Poorly differentiated	**62 (31.6)**	**6 (13)**	**5 (13.2)**	**23 (36.5)**	**28 (57.1)**	
Moderately differentiated	**112 (57.1)**	**28 (60.9)**	**28 (73.7)**	**36 (57.1)**	**20 (40.8)**	
Well-differentiated	**22 (11.2)**	**12 (26.1)**	**5 (13.2)**	**4 (6.3)**	**1 (2)**	
Tumor Diameter (cm)	5 (1.5-12)	5 (2-9)	5.1 (1.5-11)	5 (2.5-12)	5.1 (2.5-11)	0.291

tation analysis is performed before anti-EGFR treatment is applied to the patient, and tumors with wild-type RAS genes (not carrying mutations) are accepted as candidates suitable for treatment [14].

The RAS -MAPK(mitogen-activated extracellular signal-regulated kinase) signaling pathway is used by the cell's various hormones, growth factors, and differentiation factors. This signaling pathway begins with activating the RAS protein, and a kinase cascade occurs through the RAF, MEK, and ERK proteins, respectively. For the RAS protein to become active, it must be embedded in the membrane after posttranslational modification [15]. When the cell receives a growth signal, it transforms into RAS – GTP. The active RAS initiates the mitogenesis process by activating the RAF protein. While the RAS protein is important in normal tissue proliferation, differentiation, and senescence signals, the RAS protein formed by the mutant gene is a potential oncogenic protein effective in cancer. Activating KRAS mutations have been identified in 17 – 25% of all human tumors and 30 – 40% of colorectal tumors. The BRAF protein is another molecule stimulated by the RAS protein and takes part in the RAS-MAPK signaling pathway. BRAF mutations have been reported in 8% of all human tumors and 5 – 12% of colorectal tumors [16].

To initiate anti-EGFR antibody therapy in metastatic colon cancer patients, determining the KRAS/NRAS and BRAF mutation status must be determined according to the National Comprehensive Cancer Network (NCCN) guideline. According to the ASCO guideline, KRAS exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) regions should also be screened for mutations as they have predictive importance in patients with metastatic colorectal carcinoma [17-19]. In this study, we have found that KRAS mutation was found in 40,8% of the patients, NRAS mutation was found in 2%, and BRAF mutation was found in 4,1% as mutation types according to the STAT3 grades. It was determined that there was a statistically significant relationship between KRAS and STAT3 grades of mutations. It is seen that KRAS positivity increases as the STAT3 staining intensity of the patients' increases.

STAT3 is a transcription factor activated by many cytokines and growth factors and plays a crucial role in cell survival, proliferation, and differentiation. It is a member of the JAK/STAT signaling pathway [20]. STAT3 was first identified as a DNA binding factor released from hepatocytes stimulated with interleukin-6 (IL-6). STAT3 activation is one of the critical signaling pathways in SRC that makes cells resistant to apoptosis. Its activated form protects tumor cells from apoptosis and supports cell proliferation by regulating genes encoding antiapoptotic and proliferative proteins such as Bcl-xL, Mcl-2, Fas, cyclin D1, survivin, and c-Myc. STAT3 protein has been detected in many malignant events such as breast, head and neck, liver, melanoma, prostate and pancreatic cancers, and leukemia [21].

Briefly, colorectal cancer is a multifactorial genetic disease that occurs in somatic cells due to the accumulation of mutations that provide the cell with the advantage of continuous growth and escaping apoptosis. Metastatic colorectal cancer treatment involves surgical intervention and metastasectomy [22]. In cases where surgical intervention is insufficient, anti-EGFR treatment using various com-

Table 2. Distribution of mutation types according to the STAT3 staining intensities of the cases.

	STAT3 Staining Intensity					P-value
	Total (n=196)	Negative (n=46)	Weak (n=38)	Moderate (n=63)	Severe (n=49)	
	n (%)	n (%)	n (%)	n (%)	n (%)	
KRAS						**0.003**
Negative	**116 (59.2)**	**36 (78.3)**	**26 (68.4)**	**31 (49.2)**	**23 (46.9)**	
Pozitive	**80 (40.8)**	**10 (21.7)**	**12 (31.6)**	**32 (50.8)**	**26 (53.1)**	
A146V	1 (1.3)	0 (0)	0 (0)	0 (0)	1 (3.1)	
G12A	6 (7.5)	1 (10)	1 (8.3)	3	1 (3.1)	
G12C	13 (16.3)	0 (0)	4 (33.3)	4	5 (15.6)	
G12D	23 (28.8)	4 (40)	3 (25)	9	7 (21.9)	
G12R	1 (1.3)	0 (0)	0 (0)	0 (0)	1 (3.1)	
G12S	1 (1.3)	0 (0)	0 (0)	1	0 (0)	
G12V	21 (26.3)	2 (20)	1 (8.3)	11	7 (21.9)	
G13D	7 (8.8)	1 (10)	1 (8.3)	3	2 (6.3)	
K117N	4 (5)	0 (0)	2 (16.7)	1	1 (3.1)	
Q61H	2 (2.5)	1 (10)	0 (0)	0 (0)	1 (3.1)	
Q61R	1 (1.3)	1 (10)	0 (0)	0 (0)	0 (0)	
NRAS						0.699
Negative	192 (98)	46 (100)	37 (97.4)	61 (96.8)	48 (98)	
Pozitive	4 (2)	0 (0)	1 (2.6)	2 (3.2)	1 (2)	
A146T	1 (25)	0 (0)	0 (0)	0 (0)	1 (100)	
G12D	1 (25)	0 (0)	1 (100)	0 (0)	0 (0)	
G13V	1 (25)	0 (0)	0 (0)	1 (50)	0 (0)	
Q61K	1 (25)	0 (0)	0 (0)	1 (50)	0 (0)	
BRAF						0.072
Negative	188 (95.9)	46 (100)	37 (97.4)	61 (96.8)	44 (89.8)	
Pozitive	8 (4.1)	0 (0)	1 (2.6)	2 (3.2)	5 (10.2)	
V600E	8 (100)	0 (0)	1 (100)	2 (100)	5 (100)	

binations of EGFR monoclonal antibodies is applied as a standard approach. EGFR monoclonal antibodies bind to EGFR, preventing sustained activation of growth factor. However, mutations in the RAS gene, a member of the MAPK pathway in which EGFR is induced, spontaneously activate the signal pathways downstream in the signal transduction cascade. 90% of the mutations in the RAS gene are KRAS mutations [23, 24]. Therefore, KRAS mutation has occurred.

In some cases, there is no response to the treatment of agents that block EGFR receptors on the cell surface. In this case, a different chemotherapy regimen, VEGF monoclonal antibody bevacizumab, treats tumor nutrition and invasion using various combinations. Therefore, RAS mutation screening should be performed on the patient before the chemotherapy regimen is determined [25].

Conclusion

Various studies have shown that the presence of STAT 3 in Ras-positive metastatic colon carcinomas may cause resistance to anti-EGFR therapy and may be an indicator of poor prognosis [9, 10,11]. In the light of the findings obtained from our study and previous literature, it has been determined that routine STAT3 screening is a necessity before treatment is determined in RAS positive colon adenocarcinomas.

Abbreviations

ASCO : American Society of Clinical Oncology

CRC : colorectal cancer

EDRF : epidermal growth factor receptor

GTP : guanosine triphosphatase

JAK : Janus kinase

MAP : mitogen activated protein

MEK : mitogen-activated extracellular signal-regulated kinase

NCCN : National Comprehensive Cancer Network

PCR : polymerase chain reaction

SPSS : Statistical Package for the Social Sciences

STAT3 : signal transducer and activator of transcription 3

Ethical approval

This study was approved by the Bezmialem University Non-Invasive Clinical Research Ethics Committee (2023/79).

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