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The relationship between microsatellite instability in colorectal adenocarcinoma and tumor budding and histopathological parameters

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Abstract. – OBJECTIVE: This study aims to investigate the correlation between the presence of microsatellite instability (MSI) and tumor budding, as well as their relationship with histopathological parameters in patients diagnosed with colorectal adenocarcinoma.

PATIENTS AND METHODS: The study encompassed patients who underwent curative surgery to treat colorectal cancer. These patients were classified into groups based on their MSI status. The International Tumor Budding Consensus Conference (ITBCC) 2016 guidelines were utilized to identify tumor budding. Demographics, clinical data, tumor budding, and histopathological attributes were assessed across study groups.

RESULTS: The study analyzed 268 patients, out of which 32 (11.9%) were identified as having MSI. Microsatellite Stable (MSS) patients were placed in Group 1, and those with MSI were classified into Group 2. The average age was lower in Group 2 compared to Group 1 (55.9 years *vs.* 61.4 years, *p*=0.034). Tumor localizations in the caecum (5.9% *vs.* 18%) and the ascending colon (11.9% *vs.* 25%) were more prevalent in Group 2 (*p*=0.019). The occurrence of tumor budding (75% *vs.* 62.5%, *p*=0.133) and the budding degree in those with tumor budding were comparable between the groups. Poorly differentiated tumors were more prevalent in Group 2 (5.5% *vs.* 25%, *p*=0.001). Additionally, the tumor diameter was larger in Group 2 (3.58 cm *vs.* 4.35 cm, *p*=0.007).

CONCLUSIONS: MSI is a significant biomarker, possessing diagnostic, prognostic, and predictive value in colorectal cancer (CRC). Understanding the connection between MSI and tumor budding in CRC may provide clinicians with insights to enhance patient management.

Key Words:
Colorectal cancer, Microsatellite Instability (MSI), Tumor budding, Prognosis, Histological type.

Introduction

According to GLOBOCAN 2020 data (available at: https://gco.iarc.fr/today/home) published by the International Agency for Research on Cancer (IARC), colorectal carcinoma (CRC) is the third most frequently diagnosed cancer worldwide, ranking second among women and third among men–and stands second in terms of cancer-related deaths. The incidence and mortality rates are significantly higher in males than females. It is estimated that 1,931,590 individuals (10% of total cases) were diagnosed with colorectal cancer in 2020, leading to 935,173 deaths (9.4% of total cancer deaths)[1].

With advancements in technology and the advent of novel techniques, the discovery of new biomarkers that can guide diagnosis, treatment, and disease subtyping has become easier. Recent years have seen a surge in cancer research in literature focusing on unearthing new biomarkers, especially for CRCs. The incidence of CRC is on the rise, and despite advancements in early diagnosis and treatment, there is still room for improvement in treatment success.

Microsatellite instability (MSI) is associated with microsatellite repeats, of which there are approximately half a million in the human genome[2]. Found in 10-15% of all carcinomas[3], MSI results from the instability of short DNA sequences known as microsatellite repeats during DNA replication due to defects in DNA mismatch repair (MMR) genes. The resulting widespread alteration in repeated sequences is termed "MSI" and signifies a defective DNA mismatch repair system. This route to CRC development is termed the MSI pathway. Carcinomas arising from this

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pathway exhibit distinct histomorphological features compared to those arising from other pathways.

Tumor budding refers to the presence of single tumor cells or small cell clusters of four cells or fewer along the tumor's invasive margin[3,4]. It morphologically reflects the epithelial-mesenchymal transition (EMT) process, triggered by various genetic and epigenetic factors and influenced by the tumor microenvironment. Molecules released from the tumor microenvironment stimulate EMT[4-6].

There have been limited studies to date in literature investigating the molecular underpinnings of tumor budding. However, it has been suggested[7] to be linked to pathways involved in colorectal carcinogenesis, such as mutations in the *adenomatous polyposis coli* (*APC*) gene and MSI. High tumor budding is directly proportional to APC gene mutation and inversely proportional to MSI. Additionally, tumors with high-level MSI reportedly[8] exhibit low rates of tumor budding due to the presence of intense peritumoral lymphocytic infiltration and tumor-infiltrating lymphocytes.

This study aims to investigate the relationship between microsatellite instability (MSI), tumor budding, and histopathological parameters in patients with colorectal carcinoma.

Patients and Methods

This study involved patients who underwent surgery for colorectal cancer between May 2020 and January 2022. Patient data, including age, sex, and clinical stage, were obtained from patient charts and digital records, while macroscopic data, such as tumor localization and size, were retrieved from digital pathology reports. Patients diagnosed with Lynch syndrome were excluded from the study.

Patients were classified according to their MSI status and assessed across various parameters such as demographic data, tumor marker levels, neoadjuvant therapy status, surgical procedures, tumor localization, histopathological diagnosis, tumor diameter, tumor grade, depth of tumor invasion, presence of lymphovascular and perineural invasion, peritumoral lymphocytic response, tumor budding, Crohn's-like lymphocytic reaction, total lymphocytic score, pathological stage, and lymph node metastasis status.

MSI Assessment

MSI was inferred from a loss of reaction (negative) to one of the four markers (MLH1, MSH2, MSH6, PMS2) in immunohistochemical studies, while the absence of loss of expression in these markers (presence of nuclear staining in tumor cells) indicated Microsatellite Stable (MSS).

Immunohistochemical Staining

To evaluate DNA MMR gene expression in the colorectal carcinoma groups, immunohistochemical studies were conducted using MLH1, MSH2, MSH6, and PMS2 antibodies. Sections (four micrometers thick) cut from the array blocks were transferred onto poly-L-lysine-coated slides. Following deparaffinization in a Ventana automated staining device (Ventana Medical Systems-Roche ABD, Basel, Switzerland), the sections were stained using the streptavidin-biotin-peroxidase method, with the Ventana DAB Kit serving as the antibody primer. Colon samples were used as control tissues for all antibodies (MLH1, MSH2, MSH6, PMS2). Tumor cells with positive nuclear staining were included in one group, and those with negative staining were in the second group for the evaluation of the immunohistochemical expressions of all antibodies.

In line with the College of American Pathologists (CAP) 2018 Colorectal Carcinoma Reporting Protocol, the definition from the 2016 International Tumor Budding Consensus Conference (ITBCC) was employed to identify tumor budding — the presence of single tumor cells or small clusters of up to four cells or fewer at the tumor's invasive margin[3]. The peritumoral lymphocytic reaction was defined[9] as a lymphocytic reaction encircling the tumor's invasive margin's stroma. The pathological disease stage for each case was determined based on the 8th edition of the TNM Classification[10].

The study was carried out as part of a doctoral dissertation in the Department of Molecular Oncology at the Health Sciences University.

Statistical Analysis

Statistical analysis of the study data was conducted using SPSS Statistics (Version 25.0, IBM Corp., Armonk, NY, USA). Categorical data were presented as numbers and percentages, and continuous measurements as mean and standard deviation (median and minimum-maximum values where required). The Chi-square test was used for comparing categorical variables. A Shapiro-Wilk test was employed to ascertain whether the study

parameters were normally distributed. Parameters with a normal distribution were evaluated using an Independent Samples *t*-test, and those without a normal distribution were evaluated using a Mann-Whitney U test. In all analyses, the level of statistical significance was set to an alpha of 0.05.

Results

The study included 268 patients, of which 32 (11.9%) were identified as having MSI. Patients with Microsatellite Stable (MSS) tumors were classified as Group 1, while those with Microsatellite Instability (MSI) tumors were classified as Group 2. Immunohistochemical staining studies revealed a 6% loss of expression for MLH1, 5.2% for MSH2, 6% for MSH6, and 6.7% for PMS2.

The mean age was lower in Group 2 than in Group 1 (55.9 years *vs.* 61.4 years, p=0.034). Tumors localized in the cecum (18% *vs.* 5.9%) and the ascending colon (25% *vs.* 11.9%) were more prevalent in Group 2 (p=0.019). Among the preoperative laboratory parameters, platelet counts were higher in Group 2 (360 $\times 10^3$/microL *vs.* 299$\times 10^3$/microL, p=0.005), while the mean CA 19-9 was higher in Group 1 (144 U/mL *vs.* 11.5 U/mL, p=0.014). These findings are summarized in Table I.

Aligned with tumor localization, the rate of right hemicolectomy was higher in Group 2 than in Group 1 (50% *vs.* 22.5%, p=0.004), as shown in Table II.

The incidence of tumor budding (62.5% *vs.* 75%, p=0.133) and the degree of tumor budding were similar across both groups. In terms of tumor grade, poorly differentiated tumors were more common in Group 2 than in Group 1 (25% *vs.* 5.5%, p=0.001). The distribution of T, N, and M stages was similar in both groups. The incidence of lymphovascular invasion was comparable between the two groups (62.5% *vs.* 70.8%, p=0.340). The rate of perineural invasion was

Table I. Demographic and clinical data.

	Group 1 MSI absent (n = 236)	Group 2 MSI present (n = 32)	p
Gender, n (%)			0.944[a]
Male	149 (63.1)	20 (62.5)	
Female	87 (36.9)	12 (37.5)	
Age (mean ± SD)	61.4 ± 13.7	55.9 ± 3.4	**0.034*,[b]**
Neoadjuvant therapy status, n (%)	39 (16.5)	2 (6.3)	0.130[a]
Type of admission, n (%)			
Emergency	40 (16.9)	2 (6.3)	0.118[a]
Elective	196 (83.1)	30 (93.8)	
Localization, n (%)			**0.019*,[a]**
Appendix	1 (0.4)	-	
Cecum	14 (5.9)	6 (18.8)	
Ascending colon	28 (11.9)	8 (25.0)	
Hepatic flexure	8 (3.4)	2 (6.3)	
Descending colon	7 (3.0)	-	
Rectosigmoid	39 (16.5)	-	
Rectum	68 (28.8)	6 (18.8)	
Sigmoid colon	42 (17.8)	6 (18.8)	
Splenic flexure	19 (8.1)	4 (12.5)	
Transverse colon	10 (4.2)	-	
CRP	25.0 ± 2.7	30.9 ± 6.2	0.079
Hemoglobin, gr/dL	11.6 ± 1.9	11.3 ± 0.6	0.392
Albumin, gr/dL	39.8 ± 5.5	40.6 ± 0.8	0.718
Neutrophil, mm^3/L	5.2 ± 2.9	4.6 ± 1.6	0.903
Lymphocyte, mm^3/L	1.71 ± 0.8	1.85 ± 0.9	0.135
Platelet, mm^3/L	299.5 ± 128.4	360.1 ± 139.8	**0.005****
CEA	39.4 ± 10.5	4.84 ± 5.2	0.181
CA 19-9	144.0 ± 77.9	11.5 ± 1.9	**0.014***

*p < 0.05; **p < 0.001; [a]: Chi-square; [b]: Independent Samples t-test; CRP: C-reactive protein, CEA: Carcinoembryogenic antigen, CA: Carbohydrate antigen.

Table II. Operative data.

	Group 1 MSI absent (n = 236)	Group 2 MSI present (n = 32)	p
Operation, n (%)			
Anterior resection	30 (12.7)	-	0.004**[a]
APR	18 (7.6)	2 (6.3)	
Low anterior resection	96 (40.7)	8 (25.0)	
Right hemicolectomy	53 (22.5)	16 (50.0)	
Left hemicolectomy	21 (8.9)	6 (18.8)	
Subtotal colectomy	12 (5.1)	-	
Total colectomy	6 (2.5)	-	
Operation type, n (%)			
Open	152 (64.4)	24 (75.0)	0.477[a]
Laparoscopic	47 (19.9)	4 (12.5)	
Robotic-assisted	37 (15.7)	4 (12.5)	
Mortality	8 (3.4)	2 (6.3)	0.423[a]
Postoperative length of hospital stay (mean±SD)	8.63 ± 0.4	7.40 ± 0.9	0.100[b]

*$p < 0.05$; **$p < 0.001$; [a]: Chi-square; [b]: Mann-Whitney U test; APR: abdominoperineal resection.

higher in Group 1 than in Group 2 (50.4% *vs.* 25%, $p=0.007$). The tumor diameter was larger in Group 2 than in Group 1 (4.35 cm *vs.* 3.58 cm, $p=0.007$). These results are detailed in Table III.

Discussion

The molecular phenotype of colorectal carcinoma is determined by a combination of demographic, clinical, and histopathological characteristics, alongside biological behavior, prognosis, and response to treatment. The current study was designed to assess the relationship between microsatellite instability (MSI) and tumor histology, pathological stage, and clinical outcomes in patients with colorectal carcinoma. In our sample, MSI incidence was found to be 11.9%. Additionally, we noted an association of MSI with several factors, including right-sided tumor localization, younger patient age, elevated platelet count, low levels of tumor markers, low tumor grade, reduced perineural invasion, heightened Crohn's-like lymphoid reaction, and increased tumor diameter.

Several pathways have been identified in the pathogenesis of colorectal carcinomas, among which the MSI pathway stands out[11]. This pathway is characterized by a mutation in one or multiple mismatch repair (MMR) genes. MSI was detected in 11.9% of patients in our study, a figure slightly below the general average. The likelihood of MSI varies depending on the stage of the disease, presenting a higher incidence in early stages (approximately 20% in stages I and II, and 12% in stage III), and a lower incidence in the metastatic setting[12]. Furthermore, a lower incidence of MSI is also observed in tumors located in the rectum and left colon. This might be due to some patients being overlooked during MSI determination *via* immunohistochemical methods. Given these factors, the lower MSI incidence in our study can be attributed to the advanced stage of the disease in our patient population, as well as the presence of tumors located in the left colon and rectum. Taking into account that MSI prevalence varies broadly between 9% and 28% due to numerous variables and limitations, the proportion of cases with MSI identified in our study appears to be consistent with existing literature[13,14].

When considering the mechanisms of colon cancer development, different processes are at play between the right and left colon. The significant variation observed in tumor localization suggests that the mechanisms of right and left colon cancer could differ at the genetic level. Right-sided cancer mainly stems from mutations in tumor-related genes caused by replication errors, while left-sided cancer is primarily related to mutations in oncogenes and loss of heterozygosity (LOH) in tumor suppressor genes. This indicates that dMMR/MSI-H predominantly plays a role in the development of right colon cancer[15,16]. In our study, we detected differences in groups in terms of tumor location. Consistent with the literature, we found in the MSI group that tumors were more frequently located in the right colon.

Table III. Pathological data.

	Group 1 MSI absent (n = 236) n (%)	Group 2 MSI present (n = 32) n (%)	p
Presence of tumor budding	177 (75.0)	20 (62.5)	0.133[a]
In patients with tumor budding, (n = 197)			0.118[a]
Low	100 (56.5)	8 (40)	
Intermediate	49 (27.7)	10 (50)	
High	28 (15.8)	2 (10)	
Mucinous histology	39 (16.5)	8 (25.0)	0.237[a]
Mixed type pathology	18 (7.6)	2 (6.3)	0.781[a]
Grade			**0.001****[a]
Poor	13 (5.5)	8 (25.0)	
Intermediate	195 (82.6)	22 (68.8)	
Good	28 (11.9)	2 (6.3)	
T stage			0.529[a]
0	1 (0.4)	-	
1	8 (3.4)	-	
2	19 (8.1)	2 (6.3)	
3	123 (52.1)	22 (68.8)	
4A	72 (30.5)	6 (18.8)	
4B	13 (5.5)	2 (6.3)	
N stage			0.780[a]
0	101 (42.8)	18 (56.3)	
1	1 (0.4)	-	
1a	40 (16.9)	4 (12.5)	
1b	36 (15.3)	6 (18.8)	
1c	7 (3.0)	-	
2a	23 (9.7)	2 (6.3)	
2b	27 (11.4)	2 (6.3)	
3	1 (0.4)	-	
M1 stage	31 (13.1)	2 (6.3)	0.266[a]
Lymphovascular invasion	167 (70.8)	20 (62.5)	0.340[a]
Perineural invasion	119 (50.4)	8 (25.0)	**0.007****[a]
Crohn's-like lymphoid reaction	18 (7.6)	6 (18.8)	**0.039***[a]
Tumor perforation	30 (12.7)	6 (18.8)	0.347[a]
Tumor diameter (mean±SD)	3.58 ± 1.6	4.35 ± 1.5	**0.007****[b]
Number of removed lymph nodes (mean±SD)	32.4 ± 23.6	31.3 ± 15.9	0.579[b]
Number of malignant lymph nodes (mean±SD)	2.67 ± 0.4	1.50 ± 0.4	0.277[b]

*$p < 0.05$, **$p < 0.001$; [a]: Chi-square; [b]: Mann-Whitney U test.

Tumor size is defined as the maximum tumor diameter obtained from pathology reports of resected patients, and its prognostic significance has been demonstrated in many solid tumors in literature. Liang et al[17], in their study on 180 colorectal cancer cases in stages 1-3, found the median and interquartile range of the maximum tumor diameter in the MSI group to be 6.0 (4.0; 7.0) cm and in the MSS group to be 4.5 (3.5; 5.5) cm, a significant difference (p<0.001). In our study, the tumor diameter was similarly higher in the MSI group, a fact which is challenging to associate solely with the MSI status. We think the inhomogeneous distribution of tumor location could have influenced this result.

Tumor development is a multi-stage process where the immune response plays a significant role. Local anti-tumor immune defense mechanisms determine the formation and organization of the tumor microenvironment, with the composition and proportion of inflammatory cells in this area affecting the quality of the inflammatory response. Many studies on colorectal carcinomas in the literature have found that not only the tumor-infiltrating lymphocytes but also the Crohn's-like lymphocytic response is associated with MSI[18]. The Crohn's-like lymphocytic response can enhance local immunity, potentially aiding the immune response against cancer by presenting more neoantigens. In our series, consistent with the literature, as expected, the Crohn's-like lymphocytic response was higher in the MSI group.

Studies[19,20] evaluating the association between microsatellite instability and tumor budding in CRC have reported high tumor budding rates and poor prognosis in those with microsatellite stability. The relatively low-grade tumor budding in patients with microsatellite instability can be attributed to the intense peritumoral lymphocytic infiltration and the predominance of tumor-infiltrating CD8+ lymphocytes in such tumors. It has also been suggested[19] that a relatively low tumor budding could explain the generally good prognosis in this group of patients. In a study of 458 patients with CRC, Wright et al[21] reported high tumor budding in the MSS group (44.4% *vs.* 15.7%, $p<0.0001$), and the difference remained significant even in stage II and III tumors. In both groups, tumor budding became more frequent with increasing tumor stage, although the rate was significant only in the MSS group. In a large cohort of 833 cases presenting MSI status in detail, Anderson et al[22] reported a lower risk of tumor budding with MSI tumors (12.1% *vs.* 87.9%, $p<0.0001$). Similarly, Van Wyk et al[23] associated MSS with a high rate of tumor budding in their series. In a study conducted by Denčić et al[24] involving patients with stage 2-3 colorectal cancer, high-grade tumor budding was identified as the most important determinant of dMMR in a univariate logistic regression analysis ($p<0.001$).

In a series of 258 patients with stage-2 colorectal cancer, Kevans et al[25] used a staining method for mismatch repair proteins MLH1 and MSH2 and identified MLH1 deficiency in 11% and MSH2 deficiency in approximately 1% of the cases. Overall, 12% of the cases in the study were identified with MSI, and high tumor budding was more common in those with MSI than in those with MSS (48% *vs.* 26%, $p=0.087$). In their series, high tumor budding was more common in MLH1-positive cases than in MLH1-negative cases, where low or absent tumor budding was more common. Conversely, MSH2-positive cases often exhibited high tumor budding.

In a multicenter study involving 59 centers, Karlberg et al[26] divided patients into four groups based on MSI status and the development of metastasis. The mean number of tumor buds was 7±6, with a median of 5 and a range of 0-35, while the mean number of tumor buds was 8±7.3 with a median of 5.5 and a range of 0.26-31.3 in the dMMR subgroup, and 6.4±5.17 with a median of 5 and a range of 0.69-34.9 in the pMMR subgroup. There was a high level of agreement between the investigators ($p<0.001$, r: 1.0; ICC: 0.99). When the cut-off value for tumor budding and MMR status was set to 5, the rate of high-grade tumor budding was significantly higher in the dMMR/met+ group (72%) than in the dMMR/met- group (39%) ($p=0.009$). Furthermore, the rate of high-grade tumor budding was higher in the dMMR/met+ group (72%) than in the pMMR/met+ group (40%) ($p=0.01$). The comparison of the other groups yielded no significant difference when using this cut-off point. When the cut-off point was set to 10, the rate of high-grade tumor budding was significantly higher in the dMMR/met+ group (45%) than in the dMMR/met- group (21%) ($p=0.047$). When this high cut-off point was used, the rate of high-grade tumor budding was 19% in the pMMR/met- group ($p=0.012$) and 17% in the pMMR/met+ group ($p=0.02$). Regardless of the cut-off point used in their study, no significant survival advantage was identified in terms of tumor budding.

The tendency for high-grade tumor budding in pMMR tumors is considered to be caused by mutations in the APC gene and the activation of the Wnt pathway. In a study by Shinto et al[27], lower β-catenin expression was identified in the tumor buds of dMMR tumors than in those of pMMR tumors, suggesting that the Wnt signaling pathway is not the only mechanism involved in tumor budding. In a study conducted by Lugli et al[19], immunostaining for cytokeratin 22 in patients with CRC revealed intratumoral budding to be strongly associated with peritumoral budding, which was linked to poor prognosis in those with high intratumoral budding, regardless of MMR status. High-grade peritumoral budding is often related to urokinase plasminogen activator receptors, the overexpression of matrilysin and matrix metalloproteinases 2 and 9, and the loss of syndecan-1, CD44, CD44v6, EpCAM, and CD166. Tumor buds exhibit strong nuclear staining alone for β-catenin, accompanied by a loss of membranous E-cadherin expression and the overexpression of laminin5γ2. A phenotype exhibiting tumor budding is often associated with APC mutation and the activation of the Wnt pathway.

Many factors related to the epithelium and mesenchyme are involved in the tumor budding phenomenon. Although tumor budding was low in the MSI group, the findings of the present study reveal no statistical significance. While the relatively small number of patients may contribute to this, there are many factors at a molecular level that are yet to be explained that could have been involved.

Limitations

The present study has some limitations, the first of which is its single-center retrospective study design and the resulting potential for selection bias. To avoid selection bias, all consecutive patients were enrolled in the study population after undergoing routine MSI tests for patients undergoing surgery due to CRC. Secondly, the effects of chemotherapy were disregarded in the present study. Furthermore, we were unable to gather sufficient evidence of the prognostic significance of MSI due to the lack of long-term oncological outcomes.

MSI is an important biomarker with diagnostic, prognostic, and predictive significance in CRC, and thus, testing for MSI status is of critical importance in patients with CRC. It is recommended for all patients with newly diagnosed CRC. MSI was the first cancer-type agnostic biomarker to be approved by the FDA for the selection of patients with any advanced solid cancer for pembrolizumab therapy, independent of histology. Reports on the outcomes underline the important role of MSI in the selection of drug therapies for patients who no longer respond to chemotherapy. Considering the available data and the results of the present study, the authors conclude that detecting the presence of MSI is pivotal in predicting tumor behavior, clinical disease course, and prognosis, especially in detecting differences in tumors at a similar stage but indicating a different prognosis. Only in this way can an individualized treatment plan be created for the treatment of cancer.

Conclusions

Assessments grounded in molecular and genetic characteristics are vital to pinpoint these distinctions, and it is critical to clarify any yet unexplored molecular biological mechanisms concerning the impact of microsatellite instability on prognosis. Future investigations should aim to characterize these tumors more comprehensively and potentially leverage the current findings to direct the development of novel treatment strategies. This includes identifying the mechanisms behind immunotherapy resistance and enhancing therapies for tumors with microsatellite stability.

Conflict of Interest

The authors declare that they have no conflict of interests.

Ethics Approval

The study was conducted following the Helsinki Declaration and was approved by the Ethics Committee of Basaksehir Çam and Sakura City Hospital, Istanbul, Turkey (approval number: KAEK/2021.08.157).

Informed Consent

All subjects involved in the study provided informed consent.

Data Availability

The data associated with the paper are available from the corresponding author upon reasonable request.

Authors' Contribution

Ugur Topal contributed significantly to study conception and design, data interpretation, and manuscript writing; Pinar Gulcan contributed significantly to data acquisition, resources finding, and manuscript writing; Sercan Yuksel contributed significantly to study conception, study design and manuscript writing; Zafer Teke and Hasan Bektas contributed significantly to study supervision and study design; Mustafa Duman contributed significantly to manuscript writing, critical revision of the manuscript for important intellectual content and project administration; and in the final version of the manuscript was read and approved by all authors.

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