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Variant analysis of MiRNA regulatory genes in colorectal cancer

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ABSTRACT

Aims: The aim of this study was to investigate the clinical significance of mutations in *AGO2*, *DICER* and *DROSHA* genes, which are involved in miRNA biogenesis, as well as *TP53*, *KRAS*, *BRAF*, *PI3KCA* and *APC* genes, which are important in the pathophysiology of CRC, and their association with metastasis in patients diagnosed with sporadic colorectal cancer

Methods: DNA isolation was performed by taking 10-micron sections from paraffin-embedded tissue samples of 12 patients diagnosed with CRC and Kapa NGS DNA extraction kit was used for sequence analysis. The purity and concentration of the DNA obtained was measured by Qubit fluoremeter, and NadPrep DNA Universal Library Preparation Kit was used for high quality library preparation. Bioinformatics analyses were performed on the Genomize Seq platform.

Results: In our study, metastasis was detected in 42% of 12 colorectal cancer patients. Mutations in at least two miRNA biogenesis genes were detected in 80% of metastatic patients. In addition, variants detected in miRNA biogenesis regulatory genes and oncogenic genes were summarized according to pathogenicity status according to the American College of Medical Genetics and Genomics (ACMG) classification.

Conclusion: Genes involved in miRNA biogenesis and mutations of clinically relevant genes in CRC have important implications on disease prognosis and response to therapy. Mutations in these genes may be associated with the development of metastases and mechanisms of resistance to treatment and may be potential genetic markers for the development of personalized treatment strategies.

Keywords: Colorectal cancer, miRNA biogenesis, *AGO2*, *DICER*, *DROSHA*

INTRODUCTION

Cancer is a pathological condition caused by uncontrolled growth and division of cells and disruption of the mechanisms that regulate the normal behavior of the cell.[1] The first findings on the cellular changes involved in carcinogenesis were in the field of cancer genetics. It is now known that many factors leading to cancer initiation and progression are associated with genetic aberrations in oncogenes and tumor suppressor genes. Evidence from many years of research suggests that epigenetic changes play an important role in cancer development. In particular, epigenetic factors such as microRNAs (miRNAs) and histone proteins may play a role in the carcinogenesis process as a result of mutations and expression changes in the genes encoding them.[2,3]

miRNAs are a type of RNA that function as non-coding RNA molecules 22 nucleotides in length and regulate gene expression. These small RNA molecules play important roles in regulating various biological functions such as cell survival, cell proliferation, apoptosis, tumor growth and metastasis.[4] The cellular biogenesis of miRNAs is a complex process and starts with the transcription of miRNA genes through the enzyme RNA polymerase II.[5] This process starts with the formation of precursor molecules called pri-miRNA, which contain a hairpin structure.[6] The hairpin structure of the pre-miRNAs is then recognised and cut by *DROSHA* and the DGRCR8 complex, resulting in the formation of a 70-nucleotide pre-miRNA.[7] This pre-miRNA is transported from the nucleus to the cytoplasm by RanGTP/Exportin 5 complex.[8] Pre-miRNA is cleaved in the cytoplasm by the Rnaz III enzyme *DICER* and miRNA doubles of approximately 22 nucleotides are formed.[9] When miRNA duplexes are formed, they interact with the RNA-induced silencing complex (RISC) formed by Argonaute (Ago) proteins. As a result of this interaction, one strand of the 22-nucleotide RNA duplex remains in Ago as mature miRNA, while the other duplex strand is cleaved. RISC directs the single-stranded mature active miRNA to target mRNAs and enables it to take part in post-translational gene regulation.[10]

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Colorectal cancer (CRC) is the third leading cause of cancer death worldwide and is a serious cancer with more than 1.85 million cases and 850,000 deaths annually.[11] It is the second most common cancer in women and the third most common cancer in men in terms of gender.[12] The vast majority of colorectal carcinomas are adenocarcinomas that develop from epithelial cells, accounting for over 90%. Rare types of colorectal carcinomas include neuroendocrine, squamous cell, adenosquamous, spindle cell and undifferentiated carcinomas.[13] According to studies on CRC, *TP53*, *KRAS*, *BRAF*, *PIK3CA* and *APC* genes are among the most frequently studied genes. In addition, these genes are reported as the most frequently mutated genes in CRC cases according to the Catalogue of Somatic Mutation in Cancer (COSMIC) database.[14] Among these genes, *KRAS* has been identified as the most critical oncogenic factor and mutations in this gene have been associated with poor prognosis, undifferentiated tumor, distant metastasis, low survival and recurrence. Currently, patients are treated with combined therapies targeting *KRAS* mutations.[15]

Although research has provided some insights into CRC prognosis, there are still questions about recurrence, metastasis or survival.[16] This suggests that epigenetic factors may also play a role in this process.[17] In a study conducted on recurrent colon cancer tissue, miR-21, miR-106a, miR-155 and miR-200c expression levels were found to be increased compared to the control group, indicating that these miRNAs may be associated with the mechanism of recurrence in colon cancer.[18] In another study on colorectal cancer, low expression of miR-320 and miR-498 was associated with lower survival rates in the disease.[19] Studies in various solid tumors have shown that miR-21 is up-regulated especially in breast, lung, colorectal and pancreatic cancers and acts as an oncogene by targeting multiple tumor suppressor genes involved in cell proliferation, apoptosis and metastasis.[20] In contrast, miR-34a has been reported to play an important role in tumor suppression by targeting *TP53*.[21] According to the literature, miRNAs have been reported to play a role as oncogenes or tumor suppressors in various cancer types, but changes in key molecules involved in miRNA biogenesis have also been associated with the carcinogenesis process.[22] It has been reported that changes in the expression of *DICER* and *DROSHA* are associated with carcinogenesis and mutations in these genes may play a role in this process and cause changes in the expression of miRNAs.[23]

In this study, we performed sequence analysis of *AGO2*, *DICER* and *DROSHA* genes involved in miRNA biogenesis and *TP53*, *KRAS*, *BRAF*, *PI3KCA* and *APC* genes known to have clinical significance in CRC in 12 patients diagnosed with sporadic CRC and investigated the clinical significance of the variants detected.

METHODS

Ethics

The study was carried out with the permission of Selçuk University Faculty of Medicine Ethics Committee. (Date: 13.11.2023, Decision No: E-70632468-050.01.04-639070). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

DNA Isolation and Sequencing Analyses

The patients included in the study were all patients diagnosed with CRC who were referred to the Medical Genetics Department of Selçuk University Medical Oncology Department within the last 1 year. DNA isolation was performed from 10 micron sections of paraffin-embedded tissues obtained from the patients using the Kapa NGS DNA Extraction Kit manufactured by Roche Molecular Systems, Inc. (Germany). Translated with www.DeepL.com/Translator (free version) The purity and concentration of the DNA obtained were measured using a Qubit fluoremeter (ThermoFisher Scientific, USA). To generate a high-quality library from double stranded DNA (dsDNA), we used the NadPrep DNA Universal Library Preparation Kit (Nanodigmbio (Nanjing) Biotechnology Co., Ltd, China), which includes the Library Prep Module and Adapter Primer Module. NAD panels within 5' biotinylated probes, optimised for targeted capture applications in NGS, were used for libraries prepared using the NadPrep DNA Universal Library Preparation Kit (for MGI). In this study, 500 ng of DNA from each library was used for hybrid capture. After these procedures, a single-stranded circular DNA library was prepared using the MGIEasy Circularisation Kit (MGI Tech Co., Ltd, China).

Single-stranded circular DNAs were converted into nanoballs (DNBs) by rolling circle amplification using the DNB SEQ-G50RS high-throughput sequencing kit (MGI Tech Co., Ltd, China). The sequencing cartridge was then prepared, and the DNBs were placed in the DNB tube and inserted into the instrument. Samples were passed through the flow cell in the instrument and sequencing was performed on the DNBSEQ-G50RS instrument (MGI Tech Co., Ltd, China).

Statistical Analysis

Bioinformatic analysis of the data obtained from the study was performed on the Genomize Seq (v8.0.4) platform. The bioinformatic analysis of the data obtained from the study was carried out on the Genomize Seq platform.

RESULTS

In this study, we performed a comprehensive analysis on the miRNA biogenesis genes *AGO2*, *DICER*, *DROSHA* and *TP53*, *KRAS*, *BRAF*, *APC*, *PIK3CA* genes associated

with CRC clinic in the literature in 12 colorectal cancer patients. Our patient group consisted of 7 men and 5 women with a mean age of 66 years. One of the patients (case5) died during the process. The patient who died had metastasis and 5 mutations in *AGO2*, 4 mutations in *DICER*, 4 mutations in *DROSHA*, 1 mutation in *TP53*, 5 mutations in *BRAF* and 2 mutations in *PIK3CA* were detected. In this study, metastasis was detected in 42% of 12 colorectal cancer patients. Mutations in at least two miRNA biogenesis genes were detected in 80% of metastatic patients. No mutation was detected in miRNA biogenesis genes in 5 of 7 patients with no metastasis. In addition, the pathogenicity status of the variants detected in both miRNA biogenesis regulatory genes and oncogenic genes evaluated in our study according to the American College of Medical Genetics and Genomics (ACMG) classification are shown in Table 1-3.

Table 1. Distribution of cases according to the variants detected

CASES	*AGO2*	*DICER1*	*DROSHA*	METASTASIS	ACMG*
Case 1	c.700C>T c.2899C>T			✓	LP**/ VUS***
Case 2		c.2690T>C		✓	VUS
Case 3					
Case 4				✓	VUS
Case 5	c.1325T>C c.1228G>A c.1927G>A c.1861C>T c.2393C>T	c.4453A>T c.358A>T c.4666G>A c.2978C>T c.4426G>A c.2792T>G c.4561G>A c.3766C>T	c.859C>T c.2650G>A c.124C>T c.1555C>T	✓	VUS
Case 6					VUS
Case 7			c.3071A>C		VUS
Case 8					VUS
Case 9					
Case 10		c.5127T>G		✓	LP
Case 11					
Case 12	c.854C>T	c.4019A>C	c.1412C>T	✓	VUS/ VUS/VUS

*Pathogenicity according to SEQ Autopathogenicity Algorithm. VUS+ and VUS++ are new classifications developed by Genomize to let user see how close a variant to being pathogenic, **Likely pathogenic, ***Variant of Uncertain Significance

Table 2. Distribution of cases according to clinical and cultural characteristics

Cases	Gender	Age	Death	Metastasis	Histology
Case 1	Female	85			Colorectal
Case 2	Male	67		✓	Colorectal
Case 3	Female	76		✓	Colorectal
Case 4	Male	67		✓	Colorectal
Case 5	Male	73	✓		Colorectal
Case 6	Male	70		✓	Colorectal
Case 7	Male	49		✓	Colorectal
Case 8	Female	81			Colorectal
Case 9	Female	74			Colorectal
Case 10	Female	43		✓	Colorectal
Case 11	Male	55		✓	Colorectal
Case 12	Male	56		✓	Colorectal

DISCUSSION

CRC stands out as a major health problem, ranking third among cancer-related deaths worldwide.[24] Metastasis and drug resistance are among the main reasons for the high mortality rate and poor prognosis.[25] While 25% of patients show signs of metastasis at the time of initial diagnosis, metastatic spread is observed in 50% of CRC patients. Despite the use of multiple drugs in clinical practice, drug resistance is an important obstacle that cannot be overcome in CRC treatment. Drug resistance is a condition that cancer cells can develop through various epigenetic changes that occur before or during drug treatment. Moreover, epigenetic changes are associated with drug resistance by controlling key signaling pathways such as pro-survival signaling pathways and pro-apoptosis pathways. These mechanisms may help cancer cells to develop resistance to therapy, and epigenetic factors may play important roles in the metastatic progression of the disease.[26,27]

miRNAs can play different roles in various types of cancer. For example, miR-96 can be oncogenic in some cases, while in others it can act as a tumor suppressor. While miR-96 expression levels are increased in lung, prostate, bladder, colorectal and breast cancers,[28] was reported to be decreased in pancreatic cancer. Studies have shown that miR-96 may be effective on cell proliferation, metastasis and apoptosis by binding to the 3'UTR region of *KRAS* G12C mRNA in pancreatic cancer cells.[29] MiRNAs may also play tumor suppressor roles. For example, studies have shown that miR-30b acts as a *KRAS* tumor suppressor in CRC. In addition, decreased expression of miR-143 has been reported to contribute to CRC prognosis through suppression of *KRAS* expression. According to the results observed after treatment, inhibition of miR-143 increased cell proliferation in CRC cells. In contrast, increasing the levels of miR-143 decreased the proliferation capacity of the cells. These observed changes were attributed to the specific binding of miR-143 to the 3' end of *KRAS* mRNA and its inhibition of the activation of the ERK1/2 pathway.[30] Current research emphasizes the importance of miRNAs due to their critical role in CRC progression and points to miRNA biogenesis in this context.

Uncertainty regarding the regulation of *AGO2*, *DICER* and *DROSHA* during carcinogenesis, which are involved in miRNA biogenesis in various cancer types, has emerged as an important factor influencing cancer prognosis. However, the mechanisms of why these genes are up- or down-regulated in certain cancer types are not yet fully understood.[31] This is an important question for understanding the process of carcinogenesis. In limited studies, expression levels of *DICER*, *DROSHA* and *AGO2* genes have been associated with prognosis,

Table 3. Distribution of cases according to variants detected in oncogenic and tumor suppressor genes

Cases	*TP53*	*KRAS*	*BRAF*	*APC*	*PI3KCA*	ACMG*
Case 1			c.1208del c.2144A>G	c.6363_6365del		LP**/VUS
Case 2		c.436G>A	c.443A>C			VUS+/LP/LP
Case 3	c.320A>C c.400T>C		c.2144A>G		c.320A>C	LP/LP/LP/VUS
Case 4 Case 5	c.635_636del c.860A>T		c.2144A>G c.1570C>T c.1593G>T c.1505T>G c.1769T>G c.1592G>T		c.1636C>A c.1633G>A	P/LP/VUS VUS++/LP/LP/LP LP/LP/LP
Case 6	c.637C>T				c.3922A>T	LP/VUS++
Case 7	c.430C>T	c.35G>A			c.882T>G	VUS***/LP/LP
Case 8	c.743G>A		c.1799T>A			LP/LP
Case 9	c.844C>T	c.35G>C	c.1208del			LP/LP/LP/VUS+
			c.443A>C			
Case 10	c.578A>C	c.193A>C	c.1208del	c.573T>G	c.750T>G	P****/P/LP/LP/LP/LP
	c.902del	c.195T>G	c.1799T>A			LP/LP/LP/LP
			c.786A>C			LP/LP/VUS++/LP/LP
			c.770A>C			
			c.1388T>G			
			c.1798G>A			
			c.1589A>C			
			c.1445T>G			
			c.1798_1799delinsAA			
			c.750T>G			
Case 11	c.637C>T	c.35G>A	c.750T>G			LP/LP/LP
Case 12						

*Pathogenicity according to SEQ Autopathogenicity Algorithm. VUS+ and VUS++ are new classifications developed by Genomize to let user see how close a variant to being pathogenic, **Likely pathogenic, ***Variant of Uncertain Significance, **** Pathogenic

survival time and metastasis development. Upregulation of *DICER* expression level in CRC patients has been associated with reduced survival and poor prognosis.[31] The expression level of the *DICER* gene varies for tumors of different histological origin. While ovarian cancer patients have been shown to have reduced expression levels of the *DICER* gene, overexpression of the *DICER* gene in prostate cancer, leiomyosarcomas, CRC and neuroblastoma has been shown to make the tumor more aggressive.[32] This suggests tumor-specific regulation and function of the *DICER* enzyme. Studies in many solid tumors have revealed that mutations in *AGO2*, *DICER* and *DROSHA* genes, which play a role in miRNA biogenesis, may be associated with prognosis.[33-35] In a study, it was reported that rs11786030 and rs2292779 in *AGO2* gene, rs1057035 in *DICER* gene and rs874332 in *DROSHA* gene were associated with survival processes in breast cancer and rs2292779 variant in *AGO2* was associated with poor prognosis.[33] Ke et al.[36] showed that rs1187652 and rs11160231 variants in *DICER* are associated with cancer progression and recurrence in non-muscle invasive bladder cancer. In a study in Wilms tumor, it was reported that mutations in *DROSHA* and *DICER* lead to disruption of miRNA biogenesis, which results in decreased expression of tumor suppressor miRNAs.[37]

A study in metastatic CRC patients emphasized that the rs10719 variant in the *DROSHA* gene and some miRNAs may be a potential biomarker for treatment.[38] When *AGO2*, *DICER* and *DROSHA* genes are analyzed in terms of somatic mutations occurring in CRC, it is seen that they have different mutation profiles. According to the COSMIC database, 36 different mutations have been reported in *AGO2* gene, 55 in *DROSHA* gene and 100 in *DICER* gene in colorectal carcinoma.14 In our study, 6 different mutations in *AGO2* gene, 11 different mutations in *DICER* and 7 different mutations in *DROSHA* were detected in patients with CRC. Among these variants, only the c.1412C>T mutation in *DROSHA* was reported in the COSMIC database.

According to the Cancer Genome Atlas, *TP53*, *KRAS*, *BRAF*, PI3K and *APC* genes are the most important genes with critical importance in the pathophysiology of CRC and *KRAS* has been reported as the most critical oncogene according to COSMIC.[39] A total of 40 different mutations were detected in the clinically relevant genes of colorectal cancer patients examined in our study. *TP53* gene c.400T>C, c.635_636del, c.637C>T, c.430C>T, c.743G>A, c.844C>T, c.578A>C, c.902del, c.637C>T mutations; *KRAS* gene c.436G>A, c.35G>A, c.35G>C

mutations; *BRAF* gene c.1208 del, c.1799T>A, c.1798G>A, c.1798_1799delinsAA mutations; c.1636C>A, c.1633G>A in the *PIK3CA* gene and c.3922A>T mutations in the *APC* gene, previously reported in COSMIC in colorectal cancer cases. G12C mutation (**Figure 1**), a common mutation in the *KRAS* gene, was detected in patient Case.[7] The patient was initially treated with capecitabine and concurrent radiotherapy as neoadjuvant therapy. Despite the treatment, disease progression was observed. In the following period, the patient was switched to a chemotherapy regimen including 5-fluorouracil, oxaliplatin and bevacizumab. After 11 months of this treatment, the disease progressed again and the patient started a new treatment protocol including fluorouracil, irinotecan and aflibercept. However, there was no response to this treatment in the current situation. On the other hand, another patient in the cohort (Case2) was assigned to a treatment regimen including capecitabine and oxaliplatin. No information is yet available on the response of this patient and the efficacy of the treatment is awaited to be evaluated.

In our study, 58% of a group of 7 patients had mutations in at least one of the clinically relevant genes, while no pathogenic mutation was detected in miRNA biogenesis genes. Metastasis was observed in only 2 of these 7 patients. On the other hand, metastasis was observed in 3 of 5 patients with mutations in miRNA biogenesis genes. These results suggest that mutations in miRNA biogenesis genes may be associated with mutations in clinically known important genes and may be associated with metastatic behavior. In the present study, mutations in *AGO2*, *DICER* and *DROSHA* genes associated with miRNA biogenesis were analyzed in a cohort of patients with CRC. In addition, mutations in oncogenic genes *KRAS*, *BRAF*, *PIK3CA* tumor suppressor *TP53*, *APC* genes were also evaluated and their association with metastasis was investigated. The fact that the patient with *KRAS* G12C mutation (case7) did not respond to standard drug therapies suggests that mutations in these biogenesis genes may play an important role by contributing to the mechanism of treatment resistance by decreasing the expression levels of tumor suppressor miRNAs and increasing the activation of mutant *KRAS*. Our findings highlight the potential of these genes as important genetic markers in the development of personalized treatment strategies and management of treatment-resistant colorectal cancer cases.

Limitations

The findings of this study provide an important basis for understanding the association of variants detected in miRNA biosynthesis regulatory genes with CRC patients and their possible prognostic implications. However, limitations of the study include the use of a small cohort of patients and therefore statistical analyses could not be performed. In addition, we performed somatic

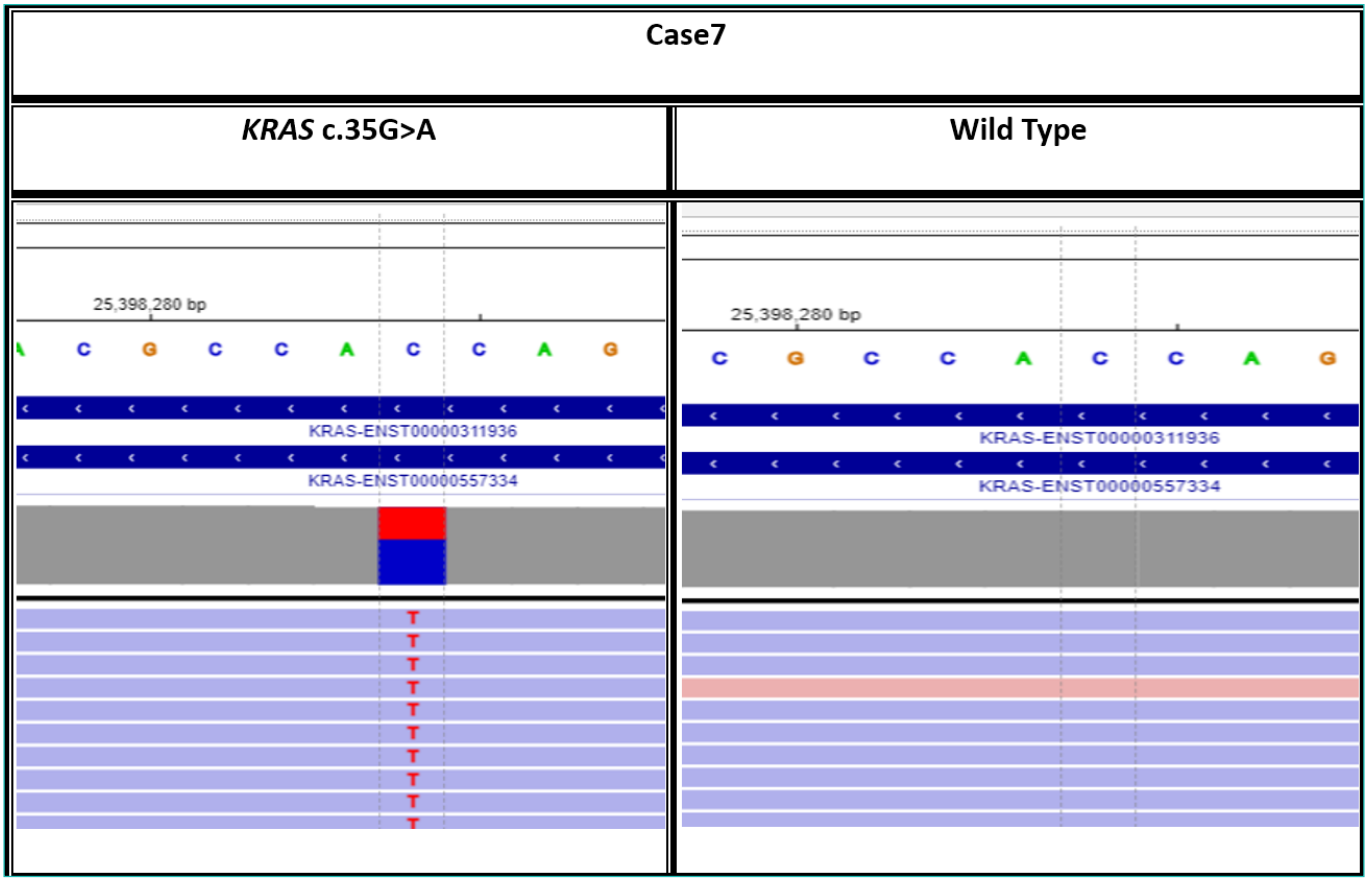

Figure 1. IGV image of *KRAS* G12C mutation

mutational analyses of key genes in miRNA biogenesis but not their expression levels. Therefore, these important findings need to be supported by larger patient groups and detailed molecular analysis.

CONCLUSION

In this study, mutations in *AGO2*, *DICER* and *DROSHA* genes associated with miRNA biogenesis and alterations in *TP53*, *KRAS*, *BRAF*, *PI3KCA* and *APC* genes, genes clinically associated with CRC, were analyzed in sporadic CRC patients. Although pathogenic mutations in miRNA biogenesis genes were not detected in the majority of patients, the presence of mutations in clinically important genes was a finding that may be associated with metastatic behavior of the disease. Our study suggests that mutations in miRNA biogenesis genes are more common in CRC patients with metastatic disease and that these mutations may contribute to metastatic behavior by reducing the expression of tumor suppressor miRNAs. In particular, it has been emphasized that *KRAS* G12C mutation may play an important role in patients showing resistance to standard treatment protocols. Considering the limitations of our study, it is important to support the findings obtained with larger patient groups and functional studies. However, it supports the hypothesis that mutations in miRNA biogenesis genes can be used as potential genetic markers for the development of personalized treatment strategies and management of treatment-resistant CRC patients.

ETHICAL DECLARATIONS

Ethics Committee Approval

The study was carried out with the permission of Selçuk University Faculty of Medicine Ethics Committee. (Date: 13.11.2023, Decision No: E-70632468-050.01.04-639070).

Informed Consent

This study was designed retrospectively and consent forms were also obtained from the patients.

Referee Evaluation Process

Externally peer reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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