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Association of KRAS Gene and microRNA-124-3p in Sporadic Colorectal Tumours

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Abstract

Aim: To reveal the exonic and 3'UTR sequences of KRAS, TP53, APC, BRAF, PIK3CA genes in sporadic colorectal tumors and to investigate the clinical relevance of 3'UTR variations in miRNA profiles. **Methods:** In the study, the exonic and 3'UTR sequences of five genes in 12 sporadic colorectal tumors were extracted by next generation sequencing. In tumors with variation in the 3'UTR region, the changes caused by the variation in the miRNA binding profile were detected. The expression profile of these miRNAs in colorectal and other solid tumors compared to normal tissue was determined. Pathway analysis was performed to determine which signaling pathways miRNAs affect. **Results:** Case-10 in our study was wild type KRAS and received cetuximab treatment and developed drug resistance. In this case, it was concluded that the expression of KRAS increased and tumorigenesis progressed due to miRNAs that do not bind to this region due to variations in the 3'UTR region. Among these miRNAs, hsa-miR-124-3p was found to have decreased expression in colorectal tumors and to be associated with the ECM-receptor interaction pathway. **Conclusion:** Variations in the 3'UTR regions of genes critical in the process of carsinogenesis are associated with drug resistance and the process of tumorigenesis.

Keywords

Colorectal Tumours, Drug Resistance, Personalised Medicine, microRNA-124-3p

1. Introduction

According to 2020 data, colorectal cancers (CRC) are the 3rd most common type of cancer with approximately 2 million new cases per year worldwide and the

2nd most common type of cancer with approximately 1 million deaths per year [1]. Approximately 20% of patients with colorectal cancer have metastatic disease at the time of diagnosis and the 5-year survival rate in these patients remains at 14%. For this reason, in recent years, a significant part of cancer research has focused on new treatment approaches in metastatic colorectal cancers, and with the developments achieved in the last 20 years, overall survival times have increased from 10 months to more than 20 months [2]. 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. It is now known that many factors leading to cancer initiation and progression are associated with genetic aberrations in oncogenes and tumor suppressor genes [3]. Many studies have been conducted on the prognostic value of many molecular markers in CRC and some molecular biomarkers have been included in the treatment processes in the light of the data obtained from these studies. RAS mutations predict lack of efficacy for agents targeting EGFR. KRAS mutations involving codon 12 or 13 are found in 12% - 75% of CRC. However, it has been associated with poor prognosis in most studies. A third marker with potential use as a prognostic and predictive factor is BRAF mutations [4] [5]. According to The Catalogue Of Somatic Mutations In Cancer (COSMIC) database, the most frequently mutated genes in rectum adenocarcinoma and colon adenocarcinoma histologically are KRAS, TP53, APC, BRAF, PIK3CA genes [6]. However, CRC is genetically, epigenetically and metabolically controlled by multiple mechanisms.

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 [7] [8]. miRNAs are a type of RNA that function as 22 nucleotide long non-coding RNA molecules and regulate gene expression. These small RNA molecules play important roles in the regulation of various biological functions such as cell survival, cell proliferation, apoptosis, tumour growth and metastasis [9]. Studies have shown that miRNAs regulate gene expression by binding to the 3'UTR regions of target genes and play a role in biological processes such as invasion, metastasis and cell proliferation during carcinogenesis [10]. Genomic variations in the 3'UTR regions of mRNAs cause different phenotypic outcomes by affecting regulatory structures such as miRNA binding sites, poly (A) signalling and RNA binding proteins. These variations are defined as somatic 3'UTR mutations [11]. Somatic 3'UTR variants are more common in solid tumours compared to normal cells. However, it has been reported that variations defined in the 3'UTR regions of genes involved in the carcinogenesis process may be associated with low survival and poor prognosis [12].

In this study, exonic and 3'UTR sequences of *KRAS*, *TP53*, *APC*, *BRAF*, *PIK3CA* genes were performed in 12 sporadic colorectal cancer tumours. In tumours with somatic variation in the 3'UTR region, the changes caused by the

variation in miRNA binding profile were determined by bioinformatic analyses. The expression profile of these miRNAs in colorectal cancer and other solid tumours compared to normal tissue was determined. KEGG pathway analysis was performed to determine which signalling pathways are affected by miRNAs that differ depending on variation.

2. Methods

2.1. DNA Isolation and Sequencing Analyses

The patients included in our study were patients diagnosed with colorectal cancer who were referred to our department for genetic analysis from Selçuk University Medical Oncology Department within the last 1 year (12 cases). DNA isolation from 10-micron sections of paraffin-embedded tissues whose diagnosis was confirmed by pathological examination was performed using the Kapa NGS DNA extraction kit (Roche molecular systems, Inc., Germany). 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). 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.

2.2. miRNA Profile Analysis in Relation to Somatic 3'UTR Mutation

Whether the variants detected in the 3'UTR regions of *KRAS*, *TP53*, *APC*, *BRAF*, *PIK3CA* genes cause changes in the miRNA binding profile was performed using the miRDB bioinformatics tool [13]. This analysis was performed by uploading and analysing the mutated and non-mutated sequences of the 3'UTR regions of the genes of interest to miRDB. The 3'UTR sequences of the genes were obtained from Ensembl genome browser using GRCh38 homo sapiens assembly [14].

2.3. Expression Analysis

To determine the expression level of variation-dependent miRNAs in lung tumours and other solid tumours, dbDEMC was used to analyse The Cancer Genome Atlas (TCGA) and Sequence Read Archive (SRA) datasets [15] [16].

2.4. Pathway Analysis

KEGG pathway analysis of which signalling pathways are affected by variation-dependent miRNAs was performed with DIANA-miRPath v3.0 [17].

3. Results

In the study, 12 sporadic colorectal tumours were examined. Histologically, 6 of these tumours were Rectum adenocarcinoma and 6 were Colon adenocarcinoma. Radiological examinations revealed metastasis in 5 of the tumours. Sequence analysis of both exonic and 3'UTR regions of *KRAS*, *TP53*, *APC*, *BRAF*, *PIK3CA* genes, which are known to be clinically important in these tumours and targeted by various therapeutic agents, were performed. The classification of the detected variants was made according to The American College of Medical Genetics and Genomics (ACMG) and no pathogenic or likely pathogenic mutation was detected in these genes in 2 of the tumours according to ACMG classification. Tumours with pathogenic or likely pathogenic mutations in at least one of *KRAS*, *TP53*, *APC*, *BRAF*, *PIK3CA* genes and tumours with metastasis were shown in Table 1.

No pathogenic or likely pathogenic mutation in the *PIK3CA* gene was detected in any tumour. The highest number of 3'UTR variations was found in *KRAS* gene (18 different variants), whereas no variants were found in *APC* gene (Table 2).

Table 1. Variants detected in *KRAS*, *TP53*, *APC* and *BRAF* genes and metastasis status of tumours.

Tumor ID	Histology	KRAS	TP53	APC	BRAF	Metastasis
1	Rectum Adenocarcinoma					
2	Rectum Adenocarcinoma					
3	Rectum Adenocarcinoma		c.524G > A^{LP} c.400T > C^{LP}			
4	Rectum Adenocarcinoma		c.635_636del^{P}			
5	Rectum Adenocarcinoma				c.1570C > T^{LP} c.1633G > A^{LP}	Positive
6	Rectum Adenocarcinoma		c.637C > T^{P}			
7	Colon Adenocarcinoma	c.35G > A^{LP}	c.430C > T^{P}			Positive
8	Colon Adenocarcinoma		c.743G > A^{LP}		c.1799T > A^{LP}	
9	Colon Adenocarcinoma	c.35G > C^{LP}	c.844C > T^{LP}			
10	Colon Adenocarcinoma		c.524G > A^{LP}		c.1798_1799delinsAA^{LP}	Positive
11	Colon Adenocarcinoma	c.35G > T^{LP}	c.637C > T^{P}	c.3927_3931del^{LP}		Positive
12	Colon Adenocarcinoma			c.583C > T^{LP}		Positive

LP: ACMG klasifikasyonuna göre likely pathogenic variant; P: ACMG klasifikasyonuna göre pathogenic variant.

Table 2. Variants detected in the 3'UTR region of *KRAS*, *BRAF*, *TP53* and *PIK3CA* genes and miRNA changes

Gene	3'UTR variant	Detected cases	miRNA alteration
	chr12:25362556 G->GA	1 - 4, 6 - 12	Same
	chr12:25362556 GA->G	1, 2, 8, 10, 12	Same
	chr12:25362776 C->T	4	Decreased
	chr12:25362776 CA->TG	4	Decreased
	chr12:25362728 T->C	5	Same
	chr12:25362722 A->T	5	Same
	chr12:25362774 A->T	5	Same
KRAS	chr12:25362544 T->G	10	Same
	chr12:25362614 T->G	10	Same
	chr12:25362540 A->C	10	Same
	chr12:25362511 T->G	10	Decreased
	chr12:25362454 A->C	10	Same
	chr12:25362701 T->G	10	Decreased
	chr12:25362618 T->G	10	Same
	chr12:25362535 T->G	10	Same
	chr12:25362613 C->A	12	Same
	chr12:25362471 A->T	12	Increased
	chr12:25362496 T->A	12	Same
	chr7:140434294 TA->T	1-12	Same
BRAF	chr7:140434294 T->TA	1-12	Same
	chr7:140434374 C->T	5	Decreased
	chr7:140434323 T->G	12	Same
	chr7:140434392 T->A	12	Decreased
TP53	chr17:7572918 T->G	10	Increased
	chr17:7572925 T->G	10	Same
PIK3CA	chr3:178952191 A->C	10	Same
	chr3:178952193 A->C	10	Same

The detected 3'UTR mutations and the changes in the number and type of miRNAs targeting this region due to these mutations were revealed. As a result of the analysis, no change was observed in the number and type of miRNA binding to this region because of 3'UTR variations of the *PIK3CA* gene. On the other hand, miRNAs that could not bind to the target site due to 3'UTR variants of *KRAS* and *BRAF* genes were detected (**Table 3**).

In addition, when the expression levels of these miRNAs, which cannot bind to the target site, were examined in colorectal and other solid tumours compared to normal tissue, it was found that the expression level of hsa-miR-124-3p decreased in colorectal cancers and the expression level of other miRNAs did not change (**Figure 1**).

Table 3. miRNAs that cannot bind to the 3'UTR region of *KRAS* and *BRAF* due to variation.

Gene	3'UTR variant	Cases detected	miRNAs
KRAS	chr12:25362776 C->T	4	hsa-mir-4426, hsa-mir-4647
	chr12:25362776 CA->TG	4	hsa-mir-4426, hsa-mir-4647, hsa-mir-4662b
	chr12:25362511 T->G	10	hsa-mir-3908, hsa-mir-6514-3p
	chr12:25362701 T->G	10	hsa-mir-124-3p, hsa-mir-3714, hsa-mir-3910, hsa-mir-506-3p
BRAF	chr7:140434374 C->T	5	hsa-mir-4768-3p
	chr7:140434392 T->A	12	hsa-miR-1252-3p

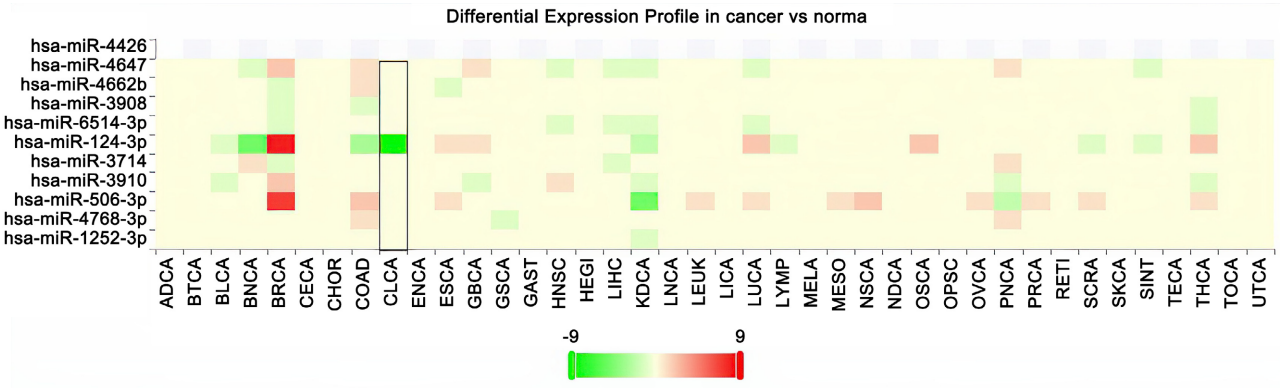

Figure 1. Expression levels of miRNAs that cannot bind to target sites in colorectal and other solid tumours compared to normal tissue.

Furthermore, in the analysis performed to determine which signalling pathways these miRNAs, which could not bind to the target site, were associated with, it was found that hsa-miR-124-3p, which was found to have a low expression level in rectal cancers, was associated with the ECM-receptor interaction pathway (**Figure 2**).

4. Discussion

It has been reported in various studies that 3'UTR sequences, which are the sequences surrounding protein-coding regions in mRNAs, may play a role in many biological processes. The first report on this subject was the rs61764370 variant in the 3'UTR, which disrupts the regulation of the oncogene *KRAS* gene via let-7 and is associated with poor prognosis for multiple cancers [18] [19]. In addition, Kataoka *et al.* found many variations in the 3'UTR region of the *PD-L1* gene in both leukaemias and many solid tumours and reported that these variations cause increased expression of the *PD-L1* gene by altering the 3'UTR region and as a result, cancer cells are able to escape from the immune system [20]. In the present study we performed sequence analysis of the exon regions as well as the 3'UTR regions of *KRAS*, *APC*, *TP53*, *BRAF* and *PIK3CA* genes in 12 sporadic colorectal carcinoma tumors. We found that *KRAS* was the gene with the highest variation in the 3'UTR region. In our study, it was found that 3 of 5 metastasis cases had miRNAs that could not bind due to variations in the 3'UTR

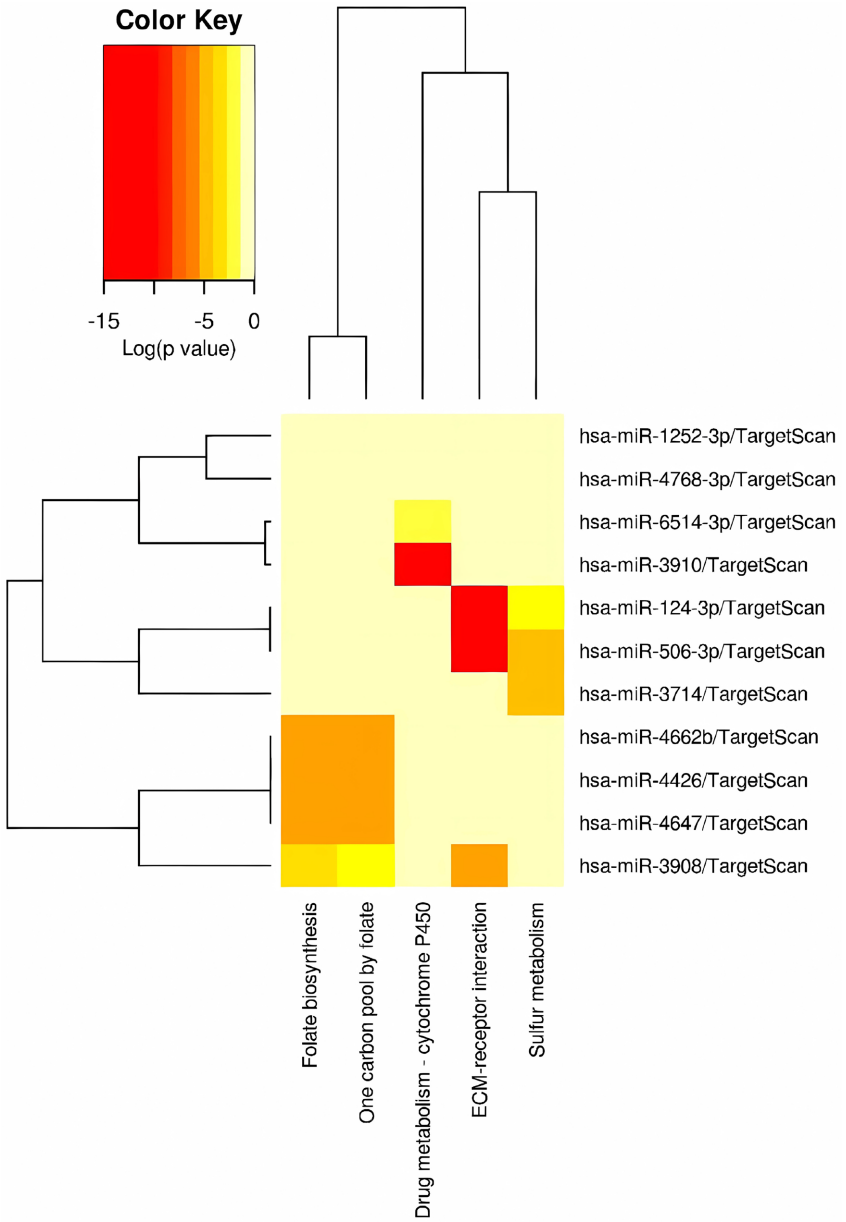

Figure 2. Pathway analysis of miRNAs that cannot bind to the target site.

regions of *BRAF* and *KRAS* genes, which have oncogenic functions in colorectal cancers. This may be explained by the inability of miRNAs to bind to the 3'UTR region of *BRAF* and *KRAS* oncogenes due to variation, resulting in increased expression of these oncogenes and consequently advanced progression. In parallel, KM Smits *et al.* reported that a variant in the 3'UTR region of *KRAS* in colorectal tumours inhibits the binding of let-7 miRNA in colorectal tumours, causing high levels of KRAS oncogenic protein, which may increase colorectal tumourigenesis [21].

Although KRAS mutations play an active role in the carcinogenesis process in colorectal tumours, another study emphasized that increased expression of wild-type *KRAS* has a similar effect on tumour growth. In addition, they re-

ported that KRAS protein expression was positive in approximately 60% of wild-type *KRAS* colorectal tumours and this was due to the inability to bind miRNAs that regulate *KRAS* expression due to the variant in the 3'UTR region [22]. Cetuximab, a monoclonal antibody targeting the extracellular domain of the epidermal growth factor receptor (EGFR), is used as a targeted therapy for *KRAS* wild-type and metastatic colorectal cancers [23]. However, drug resistance develops after a short period of time in patients treated with this treatment protocol and therefore the clinical benefit of the drug remains limited. The mechanisms underlying this condition are not yet known. However, in one of the limited number of studies, it was shown that the resistance mechanism was related to the expression level of *KRAS*. In the same study, it was reported that cells with low *KRAS* expression levels were more sensitive to EGFR inhibition, while cells with high expression levels were significantly more resistant to Cetuximab [24]. In another study conducted in conjunction with this study, they suggested that the increase in *KRAS* expression that causes drug resistance in wild type tumours occurs by amplification mechanism [25]. Another recent study on lung adenocarcinoma tumors showed that patients carrying *KRAS* G12C mutation develop resistance to KRAS inhibitors after a short period of time and that this resistance mechanism is due to the continuous expression of *KRAS* oncogene both in vivo and in vitro models. In addition, the same study reported that not de novo oncogenic mutation was found in tumors resistant to KRAS inhibitors. They also showed *KRAS* amplification in only 2 of 10 patients with KRAS inhibitor resistance (Adagrasib) and only in certain cell lines in cell line studies, although the mechanism of expression has been shown to be by *KRAS* amplification [26].

In our study, in Case 10, Cetuximab treatment was started due to the detection of tumour in the left colon and *KRAS* wild type, but it was found that the tumour did not respond to the treatment after a while and the tumour metastasised. A possible reason for this drug resistance mechanism developed in this patient is that 6 miRNAs (hsa-mir-3908, hsa-mir-6514-3p, hsa-mir-124-3p, hsa-mir-3714, hsa-mir-3910, hsa-mir-506-3p) because of 2 variants detected in *KRAS* region. Failure to bind to the target site of *KRAS* may be due to increased expression of *KRAS*. At the same time, expression analysis of hsa-mir-124-3p, which could not be bound in the patient, showed that it was down-regulated in colorectal tumours and KEGG pathway analysis showed that it was associated with ECM-receptor interaction pathway. Previous studies reported that hsa-mir-124-3p was discovered in 2002 and is conserved and expressed in many species from simple to complex organisms [27]. In subsequent studies, hsa-mir-124-3p was reported to be a potential tumour suppressor gene that regulates the biological behaviour of cancer cells by targeting many important genes in the carcinogenesis process [28] [29]. At the same time, in a study on pre-eclampsia patients, hsa-mir-124-3p was shown to reduce migration and invasion of cells [30]. Studies have revealed that hsa-mir-124-3p is also associated with the mechanism of drug resistance. In chronic myeloid leukaemia cell lines,

hsa-mir-124-3p was shown to be low expressed in the K562-R cell line with imatinib resistance and high expressed in the K562-S cell line without imatinib resistance [31]. Similarly, it was reported that hsa-mir-124-3p was low expressed in sorafenib-resistant thyroid carcinoma cells [32]. In addition to *KRAS* expression in case 10 in which cetuximab resistance was found in our study, another mechanism may have occurred via hsa-mir-124-3p. Although many studies have shown that hsa-mir-124-3p suppresses proliferation, migration and invasion in colorectal cancers, in vivo and in vitro studies have reported that hsa-mir-124-3p increases cellular sensitivity to ionising radiation in the treatment phase of colorectal cancer cells [33] [34].

5. Conclusion

A limitation of this study is that our data from bioinformatic analyses were not validated by functional and additional experimental studies. Since we did not have a sufficient number of sample groups in the study, statistical analysis was not performed. This study needs to be repeated with a larger sample group with both bioinformatics and experimental studies. However, many targeted therapies are currently used in solid tumours, including colorectal cancers, but many patients develop resistance to these drugs after a short period of time. In recent studies, it has been experimentally demonstrated that one of these resistance mechanisms is caused by an increase in the expression of oncogenes, the exact mechanism of which is unknown. In this study, we propose that somatic variants in the 3'UTR region of these genes may be one of the mechanisms of increased expression in oncogenes causing drug resistance. Therefore, the 3'UTR regions of critically important genes and miRNAs targeting this region will be particularly important in the discovery of new therapeutic agents in the coming period.

Compliance with Ethical Standarts

Before the initiation of the study, all participants received an explanation of the procedure and the risks that would later be faced in their participation, and they provided informed consent to participate in this study. During this study, the World Medical Association (WMA) HELSINKI Declaration (and/or World Psychiatric Association HAWAII Declaration Good Clinical Practices) were followed according to the ethics committee of Selçuk University Faculty of Medicine.

Data Availability

The all datasets supporting the findings of this study are available within the article. I accept full responsibility for the conduct of the study, had access to the data, and controlled the decision to publish.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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