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Determination of blood stains exposed to environmental conditions using miRNA biomarkers

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Abstract

The fact that DNA cannot be used for identification in some cases due to the small amount of sample or degradation due to environmental conditions increases the importance of RNA. In this direction, studies are carried out on the use of miRNA markers in the detection of body fluids in forensic sciences. The focus of the study is the identification of the miRNA biomarkers hsa-miR-203a-3p and hsa-miR-451a, especially in bloodstains exposed to environmental conditions. The samples that were created by the modeling method were kept in different environmental conditions for 45 days and then miRNA markers were investigated. As a result of the analysis, hsa-miR-451a expression was observed in the samples kept at -20°C and +4°C and in sealed bags, while hsa-miR-203a-3p expression was not observed. This study proposes the use of RNA and especially miRNA instead of DNA in forensic sciences. In conclusion, this study points to the importance of using miRNA profiling in forensic science. In particular, the identification of bloodstains taken from the crime scene and exposed to various environmental conditions with the miRNA biomarker hsa-miR-451a may be an important step in future forensic science applications. Since this study was carried out as a master's thesis, it was studied with a small sample group, and the results were intended to form a basis for future studies. Increasing diversity with larger sample groups in future studies is scientifically valuable.

Keywords: MicroRNAs, identification, blood, body fluids, forensic sciences, degradation, biomarkers

Introduction

Identification from body fluid samples obtained from the crime scene is the most fundamental issue in forensic science [1,2]. Today, there are chemical, enzymatic and immunological biological body fluid determination tests, and these tests have high sensitivity but limited specificity [3,4]. Blood fluid is generally used in identification analyses. Blood is an essential tissue circulating in the body and accounts for 8% of body weight. While most of the blood consists of plasma, the rest consists of blood cells. Leukocytes are cells with a nucleus, so they contain DNA. These cells perform many essential functions of the body, they are also used in DNA analysis for use in identification and forensic science [5]. Erythrocytes and platelets are nucleated cells and do not contain DNA [6].

Blood, which is frequently encountered at crime scenes, can be mistaken for substances such as red ink or fruit juices, which have similar properties. In detecting blood-like stains at the scene, it is necessary to determine whether these stains are actually blood [7-9]. To detect blood stains in forensic analysis, different methods such as luminescence methods, chemical methods, and immunological methods are used [9-11].

Bloodstain Detection Methods Consist of Two Stages

Preliminary Diagnostic Tests: These are quick and easy-to-use tests. It generally has high sensitivity but can sometimes give

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false positive results. The important thing is that these tests do not negatively affect the DNA [9,12-15].

Confirmation Tests: Used to confirm samples determined as positive. It is more specific and applied under laboratory conditions. It minimizes false positive results with its high specificity. The purpose of these tests is to prevent unnecessary tests, to ensure that forensic laboratories work more effectively, and to identify samples to be tested for DNA among the evidence taken from the scene. [12,13,17-20].

DNA profiling in forensic science works on the basis of amplification of autosomal short tandem repeats (STR) by multiplex PCR method [21]. However, this system may sometimes be insufficient in samples of degraded DNA and trace amounts. In such cases, the use of messenger RNA (mRNA), a type of RNA that is resistant to degradation due to its small size, has been considered for analysis, but it has been determined that these molecules are not resistant to harsh environmental conditions. For this reason, studies have begun on miRNAs, which are smaller in size and more durable than mRNAs [22].

MicroRNAs (miRNAs) are endogenous, single-stranded, non-translated RNA types that are approximately 22-25 nucleotides long [23]. These RNAs inhibit posttranscriptional gene expression by binding to mRNA. This causes protein translation to be inhibited or mRNA to be fragmented. Approximately 60% of the activity of protein-coding genes in mammals is thought to be controlled by miRNAs. miRNAs have been studied as potential markers in the characterization of body fluids [24]. MiRNAs fulfill their functions by recognizing target genes that are complementary to their nucleotide sequences. With the formation of the RISC complex, miRNAs bind to mRNA through base pairing. This may result in interruption of protein translation of that gene or degradation of the mRNA. Just as a single miRNA can regulate the reading of many mRNAs, it is known that an mRNA can be read by more than one miRNA. As a result of these interactions, it is estimated that miRNAs direct approximately half of the protein-coding genes. MiRNAs are involved in the regulation of gene expression pathways in cellular processes such as embryonic development, cell division and differentiation. It is thought that in pathological conditions, there may be an increase or decrease in the gene expression levels of miRNAs. Therefore, there are studies on miRNAs in the diagnosis and treatment of many diseases, including cancer, neurodegenerative diseases and metabolic disorders. It is accepted that the existence of these diseases is associated with miRNAs [25-29].

mRNA markers have been proposed for the identification of body fluids in forensic science. However, miRNAs was thought to be more suitable for forensic use due to its higher stability. Some miRNAs have been found to show tissue-specific expression. The small size of miRNAs makes them more resilient to environmental factors, giving them an advantage over mRNAs in degraded forensic samples. Hanson et al. stated that the expression levels of certain miRNAs are different in different body fluids and that these miRNAs may be potential markers in the identification of body fluids [22,23,30,31]

The aim of the study is to determine the effect of hsa-miR-451a and hsa-miR-203a-3p miRNA biomarkers on blood and the change in their expression levels after keeping them under different environmental conditions for 45 days. This study also aims to develop a new method to be used in forensic medicine and to improve existing DNA profiling methods.

Material and Methods

Working Group and Sample Preparation

12 healthy volunteers over the age of 18 were included in the study. An equal number of male and female participants were selected to ensure gender balance. The study received approval from Istanbul University-Cerrahpaşa Ethics Committee (approval ID number: A-54 / dated: 02Mar2021). Peripheral venous blood samples were collected into 2 ml EDTA tubes from volunteers who signed the informed consent form. Samples were stored in a +4°C refrigerator. Cotton denim fabrics were washed with water only, dried and cut into appropriate sizes. Fabrics were stained with blood samples and allowed to dry for 24 hours.

Different environmental conditions were simulated to evaluate their effects on stained fabrics (Table 1). Fabric samples were exposed to the following conditions:

Table 1. Sample groups according to different environmental conditions

Sample code	Environmental condition
A (A1/A2/A3)	Burying in the soil (45 days)
B (B1/B2/B3)	Keeping in the refrigerator at -20°c (45 days)
C (C1/C2/C3)	Keeping in +4°C refrigerator (45 days)
D (D1/D2/D3)	Soaking in lake water (45 days)
E (E1/E2/E3)	Soaking in sea water (45 days)
F (F1/F2/F3)	Keeping in a closed plastic bag (45 days)
W (W1/W2/W3)	Washing in the washing machine at 60°c + drying at room temperature for 24 hours
X (X1/X2/X3)	Washing in the washing machine at 20°c + drying at room temperature for 24 hours
Y (Y1/Y2/Y3)	Soaking in fully concentrated bleach for 24 hours + drying at room temperature for 24 hours
Z (Z1/Z2/Z3)	Waiting for 24 hours in bleach diluted in 1/10 + drying at room temperature for 24 hours

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Blood samples were stained on denim fabrics washed with water and left to dry for 24 hours. These stained fabrics were soaked in full concentrated household bleach (n=3) and 1:10 diluted household bleach (n=3) concentrations for 24 hours. Another modeling method was to wash the stained fabrics in a home washing machine in the pre-wash program at two different temperatures, hot (60°C) (n=3) and cold (20°C) (n=3), at the same spin setting for the same time.

As for environmental conditions, firstly, the soil collected by removing the grass layer from within the provincial borders of Istanbul was placed in an open plastic container with a depth of 15 cm x 15 cm x 15 cm, and the stained fabric (n=3) was placed in the middle of this soil sample and kept for 45 days.

Secondly, the stained fabric sample (n=3) was placed in approximately 1 liter of salt water taken from the Marmara Sea and kept with its mouth open for 45 days.

The third environmental condition was that the stained fabric sample (n=3) was placed in approximately 1 liter of fresh water taken from Küçükçekmece Lake and kept open for 45 days.

Finally, as another environmental condition, the stained fabric samples (n=3) were kept in a sealed plastic bag in an airtight manner for 45 days. The stained fabric samples were stored in -20°C (n=3) and +4°C (n=3) refrigerators for 45 days.

Blood on fabrics exposed to the environmental conditions determined for examination was transferred to sterile swaps and used for isolation. First, RNA isolation from the samples was performed using the phenol-chloroform method. Methods involving column or spin were not preferred within the framework of the literature research based on the miRNA size being very small and resistant to environmental conditions. cDNA was created from the isolated total RNAs. Gene expressions of miRNAs were analyzed by RT-qPCR using primers specific for the relevant miRNAs.

For singleplex reactions, the method specified in the Mir-X miRNA First-Strand Synthesis and TB Green kit was used to create a 10 µL total reaction [32].

The PCR cycle was carried out under specified conditions. (Denaturation 10 s at 95°C; qPCR x 40 cycles 5 s at 95°C, 20 s at 60°C; Dissociation Curve 60 s at 95°C, 30 s at 55°C, 95° 30 sec at C). The delta Ct method was used for the quantitative evaluation of the data obtained.

RNA Isolation and Analysis

Blood samples on the fabric were absorbed into cotton swabs using the transfer method, and these swabs were used for analysis. 1 ml purezol was added to the tubes and the tubes were centrifuged at the highest speed for 45 seconds. Thus, with the help of the beads in the tube, the cells in the tissue and liquid were homogeneously disintegrated. Then, purezol reagent was added and a pink color was observed. The solution was left at room temperature for 5 minutes. Then, 200 µl chloroform was added to the tubes and waited for another 5 minutes. After this stage, RNA, DNA and protein were obtained. Centrifugation was performed in the centrifuge device at 12,000 g at +4 C for 20 minutes. At the end of centrifugation, 3 phase formation was observed.

PHASE 1 (Aqueous phase): Contains RNA. (colorless),

PHASE 2: Contains DNA. (white),

PHASE 3 (Organic phase): Contains protein. (red).

For RNA isolation, the colorless phase was transferred to a new tube. 500 µl isopropanol (used to purify RNA from DNA and other impurities) was added to the new tube and incubated for 10 minutes at room temperature. The supernatant was discarded by centrifugation at 12,000 g at 4 C for 10 minutes. 1 ml of 75% Ethanol was added to the precipitate formed. It was centrifuged again at 7,500 x g for 5 minutes at +4 C. At the end of centrifugation, the supernatant was discarded and ethanol was evaporated at 57°C. 50-100 µl RNAse-free water was added to the remaining precipitate and pipetted.

Obtaining cDNA; Since miRNAs are very small and not polyadenylated, miRNAs cannot be obtained by RNA preparation methods. For miRNA analysis, polyadenylation is required in cDNA synthesis. For this purpose, a cDNA library was created. This analysis was performed on the Mir-X miRNA First-Strand Synthesis and TB Green qRT-PCR device. The method briefly includes the following steps: For reverse-transcriptase polymerase chain reaction (RT-PCR), add 5 µl mRQ Buffer (2x), 3.75 µl RNA sample (0.25–8 µg), 1 to each miRNA into a 0.2 ml RNase-free tube, with a total volume of 10 µl. 25 µl mRQ enzyme was added. In a thermal cycler, the tube was incubated at 37°C for 1 hour, followed by at least 5 hours at 85°C to inactivate the enzymes. cDNA was prepared by adding 90 µl ddH2O to make the total volume 100 µl.

Expression Analysis; 9 µl ddH2O, 12.5 µl TB Green Advantage Premix (2X), 0.5 µl ROX Dye (50X), 0.5 µl miRNA-specific primer (10 µM), 0.5 µl mRQ 3' Primer (10 µM) for a total volume of 25 µl.) and 2.0 µl cDNA was added (Table 2).

Quantitative analysis; The snRNA U6 gene was used as a control parameter. The miRNA itself was used as the specific miRNA 5' primer in accordance with the kit protocol. Then, the reaction is prepared with TB Green Premix, ROX dye, 3' primer from the kit and synthesized cDNA, with a total volume of 25 (µl). The forward primers used for microRNAs are given in the table below. Reverse primers are universally included in the kit (Table 3).

Delta Ct (Cycle threshold, ΔCt) method is used to quantify miRNA expression. U6 gene was used as the reference gene (housekeeping gene). The same reaction is prepared for U6 and run according to the specified kit procedure. The calculation was made according to the formula $\Delta Ct = Ct(U6) - Ct(miRNA)$.

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Statistical analysis; The data obtained as a result of the analysis were analyzed with the IBM SPSS Statistic Version 25 program. Differences between groups in terms of microRNA averages were evaluated using one-way analysis of variance (ANOVA) and post-hoc Tukey test, taking into account the correlation between miRNAs. The statistical significance level was determined as $p<0.01$. Compared to the sample group kept at +4 degrees, the difference between the other groups was found to be statistically significant. ($p<0.01$)

Table 2. Referenced miRNA biomarkers

miRNA	Mature miRNA sequence	miRbase code
hsa-miR-203a-3p	5' GUGAAAUGUUUAGGACCACUAG 3'	MIMAT0000264
hsa-miR-451a	5' AAACCGUUACCAUUACUGAGUU 3'	MIMAT0001631

Table 3. Universal miRNA Primers

MiRNA	Primer
miR-203a-3p	-GTGAAATGTTTAGGACCACTAG
miR-451a	-AAACCGTTACCATTACTGAGTT

Results

The environmental conditions evaluated for hsa-miR-203a-3p and hsa-miR-451a in this study are as follows; Burying in the Soil (45days), Keeping in the Refrigerator at -20°C (45 Days), Keeping in +4°C Refrigerator (45 Days), Soaking in Lake Water (45 Days), Soaking in Sea Water (45Days), Keeping in a Closed Plastic Bag (45 Days), Washing in the Washing Machine at 60°C + Drying at Room Temperature for 24 Hours, Washing in the Washing Machine at 20°C + Drying at Room Temperature for 24 Hours, Soaking in Fully Concentrated Bleach for 24 Hours + Drying at Room Temperature for 24 Hours, Waiting for 24 Hours in Bleach Diluted in 1/10 + Drying at Room Temperature for 24 Hours. Comparative information of miRNA expression levels and Ct values of each studied sample is given in Table 4. Comparison of Hsa-Mir-451a Δct in all Samples is given in Figure 1.

Table 4. Comparison of determined miRNA Expression levels and Ct values

Environmental condition	Sample code	hsa-miR-203a-3p			hsa-miR-451a		
		Ct	ΔCt	av ΔCt	Ct	ΔCt	av ΔCt
Burying in the soil (45 days)	A1	29.73	-10.28	-11.56	29.68	-10.23	-10.5933
	A2	33.08	-12.78		32.83	-12.53	
	A3	32.65	-11.62		30.05	-9.02	
Keeping in the refrigerator at -20°c (45 days)	B1	36.64	-15.78	-18.2033	9.49	11.37	10.28
	B2	35.14	-23.83		4.98	6.33	
	B3	36.36	-15		8.22	13.14	
Keeping in +4°C refrigerator (45 days)	C1	35.31	-17.27	-15.9633	8.72	9.32	11.33667
	C2	35.62	-15.36		7.94	12.32	
	C3	35.29	-15.26		7.66	12.37	
Soaking in lake water (45 days)	D1	32.07	-9.24	-8.86667	34.91	-12.08	-11.83
	D2	31.63	-10.7		34.21	-13.28	
	D3	30.57	-6.66		34.04	-10.13	
Soaking in sea water (45 days)	E1	30.37	-13.94	-11.8967	33.76	-17.33	-13.3733
	E2	29.32	-13.28		28.28	-12.24	
	E3	30.99	-8.47		33.07	-10.55	
Keeping in a closed plastic bag (45 days)	F1	36.47	-15.28	-14.99	7.35	13.84	13.71333
	F2	36.92	-15.17		7.43	14.32	
	F3	36.59	-14.52		9.09	12.98	
Washing in the washing machine at 60°c + drying at room temperature for 24 hours	W1	39.54	-9.97	-10.71	24.9	4.67	3.65
	W2	38.73	-11.42		24.04	3.27	
	W3	39.1	-10.74		25.35	3.01	
Washing in the washing machine at 20°c + drying at room temperature for 24 hours	X1	38.19	-11.25	-10.5833	20.08	6.86	7.723333
	X2	38.09	-11.9		18.59	7.6	
	X3	39.74	-8.6		22.43	8.71	
Soaking in fully concentrated bleach for 24 hours + drying at room temperature for 24 hours	Y1	38.65	-8.56	-7.89	29.05	1.04	3.15
	Y2	38.86	-7.22		26.38	5.26	
	Y3	N/A	N/A		N/A	N/A	
Waiting for 24 hours in bleach diluted in 1/10 + drying at room temperature for 24 hours	Z1	37.28	-11.11	-11.3133	20.25	5.92	4.62
	Z2	39.27	-12.52		22.88	3.87	
	Z3	37.96	-10.31		23.58	4.07	

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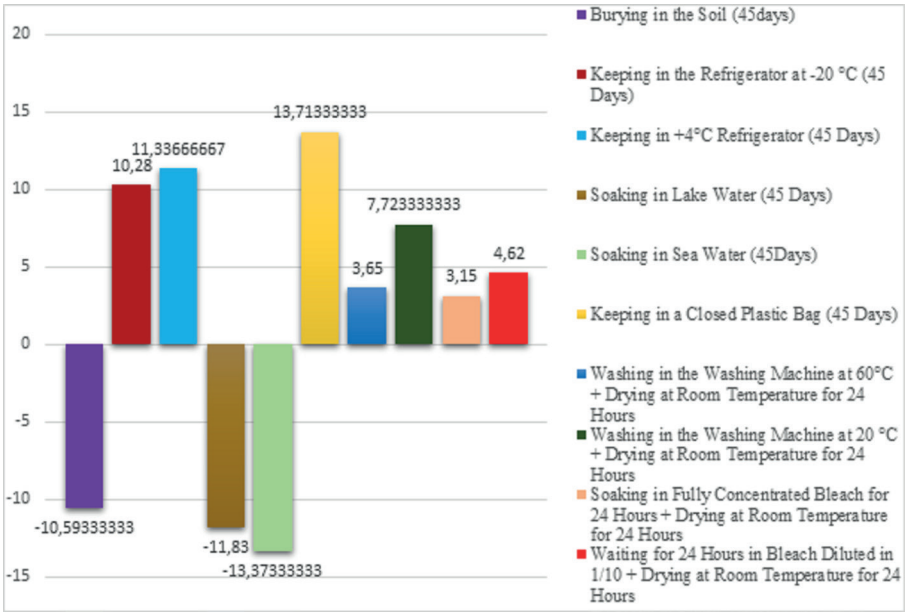

Figure 1. Comparison of Hsa-Mir-451a Δct in all samples

Discussion

Several RNA biomarkers identified by recent studies have been shown to have great potential in cell type determination and body fluid identification [33,34]. No detailed research has been conducted on the expression profile and stability of miRNAs in blood spot samples. Due to technical limitations, the entire miRNA profile could not be studied, but specific miRNA expressions were examined. When past studies on miRNA sequencing method in blood spot samples are examined, the results show that although a small amount of body fluid-specific miRNA is detected, miRNA profiles are successfully obtained. Compared to the direct blood sample, miRNAs detected in the blood spot sample are less [35,36].

Within the scope of this study, the classical phenol-chloroform method was primarily tried for RNA isolation. However, the insulation was not successful because the denim fabric absorbed the insulation liquid. Thereupon, changes were made to the method and samples were taken using the blood transfer method from the fabric. When various studies are examined, the Hsa-miR-203a-3p gene is generally seen in cervix, esophagus and epithelial cells. It is concluded that the hsa-miR-203a-3p gene is not expressed in any sample group and that this gene is not expressed in blood tissue and will not be mentioned in the rest of the discussion [37-39]. Since the sample group closest to laboratory conditions was the sample group kept at +4 degrees, statistical analysis values were determined by comparing the other groups with the sample group at +4 degrees. There are differences between groups. ($p<0.01$)

It is observed that there is no hsa-miR-451a gene expression in the first sample group, which we kept buried in the ground for 45 days (group A) ($p<0.01$). Biological samples mixed with soil are often contaminated with materials that cause a threat to DNA profiling. It is known that garden soils in Turkey are rich in humus and contain abundant microorganisms . The reason why we could not obtain the expression of hsa-miR-451a genes in group A samples may be due to the organic structure of the soil damaging the genetic material of the blood [40].

In the second and third sample groups (Groups B and C), the samples were kept at +4 and -20 degrees for 45 days. It was observed that the hsa-miR-451a gene expression in these samples was significantly higher than the other sample groups ($p<0.01$). In the study published by Fang et al. in 2019, miRNAs could be easily detected in the sample kept for five months, and it was stated that degradation was minimized when stored under normal temperatures. In the study published by Zhao et al. in 2020, they stated that the stability of blood spot samples exposed to various temperatures was preserved more at low temperatures and miR-451a could be determined, which supports the result of our study. The data from the study conducted by Williams et al. also supports the study conducted by Fang et al. in 2019, and more than 600 miRNAs were identified. It has been determined that miRNAs are stable under laboratory conditions due to their small size. This stability shows that it is quite suitable for use in forensic science for the determination of body fluids. It has been stated that this stability varies depending on the waiting time of the samples. According to the results of this study, low miRNA expression in the blood spot sample may be associated with degradation. In the study conducted by Zhao et al. in 2020, blood samples were dried on glass sheets and kept at -20 degrees, +4 degrees and various conditions. The hsa-miR-451a gene was detected in blood samples kept at -20 and +4 degrees and its expression level was statistically significant. ($p<0.01$)

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It was observed that the color of the blood stains in sample groups D (Soaking in Lake Water) and E (Soaking in Sea Water) changed and took on a color close to green. It can be seen that the water surface is covered with a gelatinous layer. It appears that the hsa-miR-451a gene is not expressed in the analyzed samples. It is known that high amounts of salt accumulate around lakes and seas according to Schlick et al., 1994, since genetic materials are more sensitive to aqueous and salty environmental conditions, their degradation rate is high. It may cause degradation in the amount and structure of genetic material. Water, salt and microbial contamination in the environment are highly degradative for RNA. Due to these environmental conditions, hsa-miR-451a gene expression could not be obtained from highly degraded RNA.

In the samples of another group, group F (kept in a closed plastic bag), the expression of the hsa-miR-451a gene is seen to be statistically significant ($p<0.01$). The genetic material was well preserved in a sealed bag at room temperature. After dripping blood onto the fabric, the fabric was left to dry at room temperature for 24 hours, and then the plastic bag was immediately emptied of air as much as possible and stored in a clip-top bag. It is thought that the sample group does not degrade since its contact with external factors is minimized and it is dried.

When the blood stained samples (W, X groups) washed in the washing machine at different temperature are examined, the blood stains on the samples are still visible after washing. In this study, it is seen that hsa-miR-451a is not expressed in group X (20 °C Washing in Cold Setting + 24 Hours Drying at Room Temperature) and in group W (60°C Washing in Warm Settings + 24 Hours Drying at Room Temperature). In the study conducted by Mayes et al. in 2019, miR-451a could not be detected in the sample group washed at cold setting (15°C) and machine dried. ($p<0.01$)

In addition, according to the study of Fang et al. in 2018, miRNA levels (Y and Z) decreased significantly in samples exposed to high oxidant substances. In this study, supporting these results, hsa-miR-451a gene expression could not be detected in samples exposed to bleach. ($p<0.01$) The reason for this is thought to be largely degraded genetic material. Layne et al., conducted a study similar to this study in 2019. In their study of samples washed with bleach and a washing machine, they suggested that miRNA levels decreased significantly but were detectable. However, as a result of this study, it is seen that the hsa-miR-451a gene is not expressed in groups W, X, Y, Z. According to the study of Gilad et al., degradation and aberrant miRNA expression are significantly associated with relative expression and GC content. It has been observed that miRNAs with lower GC content are degraded more slowly. miRNA analyzes are determined based on relative expression level rather than absolute expression levels. This may be associated with the different degradation rates of miRNAs being upregulated in the blood spot sample. Studies have shown that more than a hundred new miRNAs have been identified and two of them were found in both liquid blood samples and blood spot samples. This development is a candidate to be a very important and routinely usable analysis method, as blood samples are taken directly from the suspects and sometimes need to be compared with the blood spot sample. Multi-step procedures must be performed to investigate the stability of miRNAs and to determine whether they are expressed fluid-specifically in the body. miR-451a is also expressed in blood, as reported in many studies. In the study conducted by Layne et al., there was no significant difference in the amount of RNA between samples exposed to environmental conditions and control samples.

Conclusion

Although the presence of the hsa-miR-451a gene in blood tissue was determined by its expression levels in studies, the hsa-miR-451a gene could not be detected in degraded blood samples within the scope of this thesis study. However, although these studies are still in their early stages, miRNAs are thought to show serious potential in the identification of body fluids. Therefore, additional detailed studies are needed to make miRNAs a standard and reliable method that can be used in the field of forensic sciences [33,34] miRNA stability was seriously affected in the strong oxidant and freeze-thawing method. In previous studies, it was stated that healthy miRNA analysis could not be performed on samples treated with strong oxidant and freeze-thawing methods, and that samples should be protected from these conditions. These results support the results of this study.

In conclusion, the results of our study support the feasibility of using miRNA in the identification of body fluids in the field of forensic sciences. However, there are many issues that need to be clarified regarding the use of miRNAs in this field. Although some of the candidate miRNAs used in the studies have the ability to distinguish body fluids, it will be possible to identify stronger candidates by determining the expression profiles of many miRNAs. In addition, the potential effects of healthy or unhealthy tissues and body fluids should also be investigated in future studies. In addition, more comprehensive studies on larger samples are needed to prove the benefit of using miRNA in the analysis of spoiled samples, which are frequently encountered in forensic cases. Various methods and different devices are used in the studies that can give different results from the samples. Therefore, there is a need for a standard procedure optimized for use in the field of forensic science. Despite all this, miRNAs undoubtedly have great potential in the field of forensic sciences and are promising as a practical new technique suitable for routine use in forensic laboratories as biomarkers in the identification of body fluids. Since this study was carried out as a master's thesis, it was studied with a small sample group, and the results were intended to form a basis for future studies. Increasing diversity with larger sample groups in future studies is scientifically valuable.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
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Ethical Approval
After obtaining institutional review board approval in Istanbul University-Cerrahpasa, Cerrahpasa Medicine Faculty Clinical Research Ethics Committee (approval ID number: A-54 / dated: 02Mar2021).

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