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Research Article

Effectiveness Evaluation of the Novel miRNA-based Forensic Age Estimation Strategy for Blood Samples in Forensic Science

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Abstract

Age estimation has always been an important issue in forensic science, which is of great significance in narrowing the scope of suspects and sentencing in the judiciary. As a common test material, bloodstain appear at most judicial case scenes, it's an ideal sample using molecular biology techniques to detect the correlation between miRNA expression profile and age and establish an age estimation model, which is of great significance for developing more convenient age estimation methods. We constructed an age estimation strategy using miR-330-5p and miR-324-3p expression levels in 60 blood samples from Han Chinese males in 20-69, and used 40 blood samples in 20-69 and 32 bloodstains form 30-49-year-old Han Chinese males stored within 8 years to explored the adaptability of the strategy by simulating different forensic practice conditions. Our age estimation strategy has preliminarily solved the forensic practice of using miRNA expression levels in bloodstains stored at dark and room temperature within six months for age estimation, but the effect is not ideal for bloodstains with unfavorable external environments or PCR inhibition conditions.

Keywords: Blood samples; DNA; Forensic science; miRNA

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Introduction

The rapid development of molecular biology technology has also been widely applied in forensic science, among which DNA detection and research techniques have become the cornerstone of forensic detection. However, RNA molecules have been neglected for many years due to their unstable structure and susceptibility to degradation [1]. Until the research that the European DNA Profiling Group (EDNAP) collaborated with numerous forensic laboratories in 2011 revealed message RNA (mRNA) has application value in fluid stains recognition in forensic science [2], leading to a wave of research on microRNA (miRNA), circular RNA (circRNA), long non-coding RNA (lncRNA) and PIWI-interacting RNA (piRNA) in the forensic academic community, which provide new methods for forensic fluid identification, age estimation, cause-of-death analysis and monozygotic twins identification [1].

Age estimation has always been a major research direction in the field of forensic science. In forensic investigations, age estimation is of great significance in narrowing the search scope for suspects and determining whether suspects have undergone judicial sentencing. At present, the accurate conventional morphological methods for age estimation measure bone and teeth, but compared to body fluid evidence, skeleton evidence appears very infrequently [3-5]. Therefore, new research attempts to use molecular biology to analyze body fluid, detecting DNA methylation markers, telomerase length and mitochondrial DNA mutations for age inference, although these methods are expensive and complex to operate [6,7]. Through massive parallel sequencing of 220 Han Chinese male and female venous blood samples aged 20-69, there miRNA profile showed that miR-330-5p and miR-324-3p were closely related to age, and 210 blood samples stored for less than 8 years were used to verify that their gene expression levels were negatively correlated with age [4,8], thus we aims to construct an age estimation by analyzing miR-330-5p and miR-324-3p of 60 Han Chinese male blood samples. Meanwhile, we will conduct multiple simulation experiments to simulate the adverse situations that bloodstains may encounter in forensic practice, explore the applicability of this age prediction model, and hope to complete a new method that can withstand practical testing and use bloodstains for age estimation.

Methods

Collection and processing of blood samples

Collect 60 blood samples from Han Chinese males aged 20-69, 4ml venous blood each. There are five groups, aged 20-29, 30-39, 40-49, 50-59, and 60-69, with 12 cases in each group. All experiments

were evaluated and approved by the Ethics Committee of Shandong First Medical University, and were carried out in accordance with their regulations and guidelines. Blood miRNA in 60 cases are isolated and extracted immediately, and 8 samples were selected from each group, totaling 40 blood samples were smeared on FTA cards, paper, polyester fiber and cotton, and stored in dark place at 20℃ before miRNA isolation.

Construction of age estimation strategy

Extract RNA from a 100μL sample using the miRcute miRNA Isolation Kit (Tianjin, China), dissolve the extracted RNA in 20μL RNase-free water and use NanoDrop ND-1000 spectrophotometer to detect its quantity and purity, and store the purified RNA at -80℃ until use. Reverse transcription was performed using miRcute Plus miRNA First-Strand cDNA Synthesis Kit (Tianjin, China) and miR-324-3p and miR-330-5p primers. Using 2μL diluted cDNA as a template, real-time PCR was performed using miRcute miRNA qPCR Detection Kit (Tianjin, China) and 7500 RT-PCR Detection System (Applied Biosystem, USA), with 10ng of total RNA in 20μL PCR volume. Calculate the Cq values for each reaction, and the observed Ct values were used for analysis $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of miRNA in blood samples. After linear correlation analysis, the correlation between miR-330-5p and miR-324-3p with age was calculated using | r |>0.4 as the standard.

Stimulate on-site sampling materials

Select 8 FTA card Han Chinese male bloodstains sample for each group that stored for 2 weeks, 6 months, 3 years, and 8 years, as well as 8 fresh blood samples from Han Chinese males aged 30-49, to simulate the duration of bloodstain storage. Blood samples smeared on paper, polyester, and cotton were applied to extract RNA. Eight bloodstain samples were taken from 40 fresh blood FTA cards each and divide into 8 groups to simulate different storage environments. Three groups of the temperature control condition was stored at room temperature (20℃), 4 ℃ and -20 ℃, separately in a dark place for 24 hours. Three groups of UV irradiation time control condition were stored at room temperature in a dark place, with UV irradiation for 30 minutes and UV irradiation for 60 minutes. And before RNA extraction, the last two groups were washed with 75% alcohol or 10% sodium alkybenzenesulfonic acid detergent for 30 seconds. Dilute the RNA extracted from FTA card bloodstain to 0.5x, 0.1x, and 0.01x to simulate different miRNA concentrations. Then 200umol/L Hb, 0.1mmol/L Indigotin, 0.1ng/L Human acid, and 0.8mmol/L EDTA were added separately to the RNA extracted from the FTA card bloodstain to simulate the situation of encountering different PCR inhibitors during the evidence collection process.

Results

Construction and validation of miRNA-based age estimation strategy

RNA extraction, reverse transcription, and RT-PCR detection were performed on 60 blood samples, and age estimation model based on SVM, Tree, Linear Regression, Random Forest, AdaBoost and other methods was initially established using Orange (version: 1.0) software. The model was validated using the Leave-one-out method, and it was found that the age estimation model established using AdaBoost algorithm had the smallest error, which was 4.43 years old (Table 1).

Algorithm	MSE	RMSE	MAE	R2
Adaboost	52.33333333	7.234178138	4.433333333	0.734997243
Random Forest	56.29095751	7.502730004	4.804154762	0.714956836
Tree	66.33842593	8.144840448	5.619444444	0.664078998
SVM	121.7349667	11.033357	9.404501442	0.383564934
Linear Regression	147.5670762	12.14771897	10.84229957	0.252757668

Table 1: Leave-one-out method validate age estimation modle. Adaboost has the best fitting and smallest error as 4.43 years old.

Stimulating different storage times for age estimation

The results of extracting RNA from FTA card bloodstains in different storage times for age estimation show that strategy fitted by AdaBoost algorithm has a good evaluation effect on samples stored for less than 6 months, but performs poorly on aged bloodstains stored for longer times (Table 2).

Storage time	MSE	RMSE	MAE
Fresh	32.875	5.734	5.375
2 weeks	42.375	6.51	5.375
6 months	45.75	6.764	5
3 years	81.625	9.035	7.625
8 years	72.625	8.522	6.875

Table 2: Age estimation in different storage times. Bloodstains stored less than 6 months have positive estimation significance.

Simulating different on-site sampling materials for age estimation

Extracting RNA from samples smeared on paper, polyester fiber, and cotton simultaneously. AdaBoost algorithm model found that cotton had the best detection performance, while paper and polyester fiber had acceptable results, but the error was relatively large (Table 3).

Material	MSE	RMSE	MAE
Paper	63.625	7.977	7.125
Polyester fiber	76.500	8.746	7.250
Cotton	49.125	7.009	5.875

Table 3: Age estimation with different on-site sampling materials. Cotton has the best estimation. RNA extracted from paper and polyester fiber have larger error.

Stimulating different on-site environments for age estimation

In the experiments simulating the impact of different on-site environments on FTA card bloodstains, the age evaluation results of the AdaBoost algorithm model showed that low temperature and UV irradiation would affect the age evaluation results. The lower the temperature, the longer the duration of UV irradiation time, the lower the accuracy of age evaluation, and even make the evaluation results invalid. Chemical washing like 75% ethanol and 10% sodium alkylbenzenesulfonic acid detergent can also cause damage to the sample, leading to significant bias in age estimation results (Table 4).

Conditions	Group	MSE	RMSE	MAE
Temperature	Control	63.625	7.977	7.125
	4°C	170.625	13.062	11.125
	-20°C	860	29.326	26
UV irradiation time	Control	63.625	7.977	7.125
	30 min	282	16.793	13
	60 min	463.625	21.532	19.625
Washing	75% ethanol	301.625	17.367	15.375
	10% sodium alkylbenzene-sulfonic acid detergent	256.875	16.027	15.125

Table 4: Age estimation in different on-site environments. Low temperature, UV irradiation, and chemical reagent washing can all affect the age estimation results. The greater the intensity of damage, the larger the age estimation error.

Stimulating different diluted sample for age estimation

The cDNA extracted from FTA card was diluted with 0.5x, 0.1x, and 0.01x, and all dilutions showed good age estimation results, confirming that this model can detect at least 0.15ng of RNA samples with high sensitivity, and the error between the diluent and the original solution has little effect on age estimation (Table 5).

Dilution	MSE	RMSE	MAE
0.5x	49.125	7.009	5.875
0.1x	32	5.657	5.5
0.01x	27	5.196	5

Table 5: Different cDNA concentrations for age estimation. Good age assessment performance is demonstrated when the dilution factor is within 100 times.

Stimulating different PCR inhibitors for age estimation

The addition of different PCR inhibitors to the cDNA solution extracted from FTA card bloodstain resulted in significant deviations in the age estimation fitted by the AdaBoost algorithm, rendering it meaningless for evaluation. The experiment shows that the model is only suitable for blood stains in general situations and is not for difficult blood stain samples (Table 6).

PCR inhibitor	MSE	RMSE	MAE
200umol/L Hb	172.5	13.134	11.25
0.1mmol/L Indigotin	548.875	23.428	22.125
0.1ng/L Humic acid	115	10.724	9.75
0.8mmol/L EDTA	184.1125	13.569	12.375

Table 6: Different PCR inhibitors for age estimation. All inhibitors seriously affect age estimation results.

Discussion

MiRNA is a kind of small non-coding RNA, which is composed of 22-24 nucleotides. It has the function of post-transcriptional regulation and is widely expressed in tissues and body fluids, with strong stability and tissue specificity. As a transcription factor, miRNA regulates many physiological and pathological processes such as cell proliferation, differentiation, and cancer development. Hanson et al. first applied miRNA in the field of forensic science, confirming its potential in body fluid identification. Subsequently, more and more studies have found that miRNA also has significant application value in personal feature identification, time related estimation, and cause of death analysis [8,9]. Aging is an important and complex physiological process in the human body, which is regulated by a large number of miRNAs. Studies have shown that the expression patterns of miRNA in tissues and body fluids are age-related. For example, miR-496 regulates human aging through mTOR, miR-223 and miR-130a control FLNB, and ZNF274 regulates MAPK signaling T cell receptor, which may be age-related [10].

With the development of bioinformatics, machine learning and medical data analysis have achieved increasing success in the fields of medical imaging, big data analysis, and disease diagnosis [11,12]. We actively develop and apply bioinformatics technology in the field of forensic science using molecular biology techniques, and successfully establish an age prediction model. For data with weak correlation, compared with neural networks and SVM, the AdaBoost algorithm does not need to calculate irrelevant features and is less susceptible to overfitting problems, improving accuracy and execution time [8]. In this experiment, the AdaBoost algorithm is also the best strategy for age estimation. Therefore, the AdaBoost algorithm is used in experiments simulating practical applications for age estimation.

By simulating the impact of different situations on bloodstain evidence and testing the accuracy and practicality of the AdaBoost algorithm miRNA-based age estimation model, we found that the model has a good evaluation effect on bloodstains stored at room temperature and for less than six months in dark. It can be applied to bloodstain samples extracted from paper, polyester fiber, and cotton, with cotton being the best. The age estimation model has high sensitivity and can detect samples containing 0.15ng RNA. However, this model also has limitations, as it only provides effective age estimation for undamaged bloodstain samples and is not suitable for bloodstains that have undergone low temperature, UV irradiation, and chemical reagent washing, as well as it is not suitable for bloodstains doped with hemoglobin, indigo, EDTA, or sample material spoilage.

In this experiment, both the construction of the age estimation model and the simulation experiment were conducted using Han Chinese male specimens to eliminate the influence of gender on age estimation. Nevertheless, only miR-324-3p and miR-330-5p miRNAs were selected to construct the age estimation strategy, with only 60 samples included. The less number of miRNAs evaluated in the model and the small sample size make the model more limited. In the future, we hope to further explore age related miRNAs and other ncRNAs through in-depth research, including more male blood samples for analysis and construct a more comprehensive age estimation strategy.

Ethics approval

The study was Ethics Committee of Shandong First Medical University (approval number 2022-729).

Conflict of interest

The authors declare that they have no conflict of interest.

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References

1. Song B, Qian J, Fu J (2023) Research progress and potential application of microRNA and other non-coding RNAs in forensic medicine. Int J Legal Med 138: 329-350.

2. Ingold S, Dørum G, Hanson E, Ballard D, Berti A, et al. (2020) Body fluid identification and assignment to donors using a targeted mRNA massively parallel sequencing approach - results of a second EUROFORGEN / EDNAP collaborative exercise. Forensic Sci Int Genet 45: 102208.

3. Correia Dias H, Manco L, Corte Real F, Cunha E (2021) A Blood-Bone-Tooth Model for Age Prediction in Forensic Contexts. Biology (Basel) 10: 1312.

4. Fang C, Zhou P, Li R, Guo J, Qiu H, et al. (2023) Development of a novel forensic age estimation strategy for aged blood samples by combining piRNA and miRNA markers. Int J Legal Med 137: 1327-1335.

5. San-Millán M, Rissech C, Turbón D (2017) New approach to age estimation of male and female adult skeletons based on the morphological characteristics of the acetabulum. Int J Legal Med 131: 501-525.

6. Jylhävä J, Pedersen NL, Hägg S (2017) Biological Age Predictors. EBioMedicine 21: 29-36.

7. Correia Dias H, Cunha E, Corte Real F, Manco L (2020) Age prediction in living: Forensic epigenetic age estimation based on blood samples. Leg Med (Tokyo) 47: 101763.

8. Fang C, Liu X, Zhao J, Xie B, Qian J, et al. (2020) Age estimation using bloodstain miRNAs based on massive parallel sequencing and machine learning: A pilot study. Forensic Sci Int Genet 47: 102300.

9. Wang J, Zhang H, Wang C, Fu L, Wang Q, et al. (2022) Forensic age estimation from human blood using age-related microRNAs and circular RNAs markers. Front Genet 13: 1031806.

10. Li Y, Wang Z, Ishmael D, Lvy Y (2023) The potential of using non-coding RNAs in forensic science applications. Forensic Sci Res 8: 98-106.

11. Giardina E, Ragazzo M (2021) Special Issue “Forensic Genetics and Genomics”. Genes (Basel) 12: 158.

12. Margagliotti G, Bollé T (2019) Machine learning & forensic science. Forensic Sci Int 298: 138-139.

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