Original

Enhancement of miRNA-223-5p and miRNA-30b-5p expression in the saliva of abused children

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Abstract: Child abuse is a serious problem worldwide, and saving children from abuse is an important mission. Child abuse-induced psychological stress triggers diseases such as adjustment disorders and depression. Therefore, there is a strong demand for the practical application of biomarkers that can objectively identify child abuse. However, no objective index or quantitative method for abuse or neglect-induced mental stress has been established. In this study, we compared the expression levels of saliva-derived microRNAs（miRNAs）in abused children with those in non-abused children and assessed their effectiveness as biomarkers for child abuse. Salivary miRNAs were extracted from 52 non-abused children（CG）and 52 children stressed by abuse（SG）. The expression levels were compared using microarray and quantitative reverse transcription-polymerase chain reaction（RT-PCR）. In primary screening, several miRNAs were found to be expressed more than double in SG when compared with CG. In the quantitative RT-PCR analysis, the expression levels of hsa-miRNA-223-5p, hsa-miRNA-181a-5p, and hsa-miRNA-30b-5p in SG were significantly higher than those in CG. Receiver operating characteristic curve showed that three types of miRNAs possessed excellent sensitivity and specificity. Two miRNAs, miRNA 223-5p and miRNA 30b-5p, were considered to be desirable biomarkers because of their extremely high expression levels. We speculate that the expression levels of hsa-miRNA-223-5p and hsa-miR-30b-5p are upregulated during child abuse and hence can be used as biomarkers to determine child abuse-related psychological stress.

Keywords: Child abuse, MicroRNAs, Biomarkers, Psychological stress, Saliva

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Introduction

Child abuse encompasses physical, sexual, or psychological abuse and generally occurs in children aged <18 years. These forms of abuse are recognized as a serious public problem worldwide, according to the United Nations Children's Fund (UNICEF) 1), affecting hundreds of thousands of children every year. However, the exact number of abused children remains unknown. Psychological stress can be caused by various factors and can be harmful to one's health. Harmful experiences and stress experienced by children are among the causes of borderline personality disorder onset and are thought to manifest in adulthood as attention deficit hyperactivity disorder (ADHD), post-traumatic stress disorder (PTSD), etc. 2)-5). In particular, child abuse is defined as psychological and physical stress in children. Therefore, the early detection of child abuse, such as physical, sexual, and psychological child abuse, and the reduction of psychological stress, are important for child and adult health. Furthermore, child abuse victims have been reported to be at high risk of developing obesity, heart disease, and lung disease; therefore, the presence or absence of abuse should be determined earlier 6)-8). However, it is difficult to judge abuse because children are not good at transmitting stress in their own words 9). In addition, the identification of abuse is very difficult in the absence of physical signs. Therefore, the establishment and practical application of objective indicators that can be conveniently applied in the early detection of child abuse, such as biomarkers, is a major goal.

Biomarkers are host-derived components (e.g., proteins and nucleic acids) contained in body fluids and tissues, such as the blood and urine; they are indicators of changes in one's health condition 10)-12). Biomarkers are easy to collect, and some with high specificity reliability 13),14) have already been used as tumor markers and determinants of disease detection and prognosis 15),16). In the saliva, amylase, cortisol, and chromogranin A have been reported as biomarkers induced by stress 17)-19). Although these biomarkers may be used to determine the acute phase of stress, whether they can be used to identify chronic stress remains unclear 20)-22). Therefore, specific biomarkers that can be easily collected and that can objectively determine acute and chronic stress should be discovered. This would enable the early detection of stress due to abuse and could be expected to reduce the number of children being abused.

Saliva is a specimen that can be collected with minimal invasiveness. Recently, microRNA (miRNA) has become a candidate for saliva-derived biomarkers. miRNAs regulate the post-transcriptional expression of many genes 23),24). The expression of miRNAs in psychological stress translates to physiological and genetic stress and regulates the expression of 30%–50% of genes encoding proteins; each mRNA is regulated by multiple miRNAs 25),26). However, many genes under their control have not yet been elucidated. In humans, >2000 types of miRNA are registered in the database (miRBase: www.mirbase.org); however, not all of their functions have been clarified. The usefulness of miRNAs as biomarkers in the plasma for malignancies, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis has been investigated 27)-30).

Currently, a measurement method of miRNA as an indicator of mental stress has been investigated among adults, but not among

children 31),32). In this study, we searched for salivary miRNAs that increased in the presence of child abuse and investigated their potential as biomarkers for the expression of psychological stress in children.

Materials and Methods

Ethics

This study design was approved by the Ethics Committee of the Tsurumi University School of Dental Medicine (Approval no. 1610). After orally explaining the study document, all the parents of participants in the control group and all the guardians of participants in the stressed group signed a consent form for participation in this study.

Clinical specimens

Among 104 participants, 52 were stressed due to parental abuse (SG) aged 6–15 (34 boys, 18 girls, 10.40 ± 2.94 years, mean ± S.D.) and 52 non-abused children (CG) aged 6–15 (24 boys, 28 girls, 10.27 ± 2.88 years, mean ± S.D.), were included in this study. The participants in SG were physically abused by their parents and were protected by the child consultation center of Kanagawa Pref., Japan, whereas the participants of CG were selected from primary and/or junior high school of Kanagawa and Gunma Pref., Japan. SG has been identified as a definite abuse by the government and has been protected in shelters. CG confirmed that there was no abuse from their parents when obtaining informed consent. Salivary samples were collected more than 1 hour after brushing teeth; moreover, samples of individuals after chewing paraffin wax and spitting were collected at the same time as their dental examinations.

In the primary screening, 8 SG aged 7–10 (5 boys, 3 girls, 8.75 ± 1.04 years, mean ± S.D.) and 8 CG aged 7–10 (3 boys, 5 girls, 8.88 ± 1.13 years, mean ± S.D.) were performed.

In the quantitative analysis of miRNAs, 44 SG aged 6–15 (29 boys, 15 girls, 10.70 ± 3.08 years, mean ± S.D.) and 44 CG aged 6–15 (21 boys, 23 girls, 10.52 ± 3.03 years, mean ± S.D.) were performed. Furthermore, 88 subjects were divided into four groups; 29 SG-boys, 15 SG-girls, 21 CG-boys and 23 CG-girls (9.93 ± 2.96 years, 12.20 ± 2.81 years, 10.29 ± 2.78 years and 10.84 ± 3.29 years, mean ± S.D., respectively). The p values between CG-boys and CG-girls, CG-boys and SG-boys, CG-boys and SG-girls, CG-girls and SG-boys, CG-girls and SG-girls, and SG-boys and SG-girls were 0.96, 0.98, 0.24, 0.77, 0.46, and 0.09, respectively.

Extraction and purification of miRNA from the saliva

miRNA was purified from the saliva using the miRNeasy Mini Kit (QIAGEN, Limburg, Netherlands) 33). The quality and quantity of purified miRNA samples were evaluated using Agilent 2100 Bioanalyzer (Agilent, Cheshire, UK). Then, 100 pmol of miRNA from Caenorhabditis elegans (cel-39-3p) was added to samples as external control, respectively 34).

miRNA microarray analysis

miRNA samples from 8 SG and 8 CG were used for primary screening. Then, samples were labeled Cy3 and hybridized to the SurePrint G3 Human miRNA r21 Array Kit (8 × 60 K) using miRNA Labeling Reagent and Hybridization Kit (Agilent). After hybridization, slides were washed and scanned by the Agilent Microarray Scanner G2505C (Agilent). Microarray expression data of 16 samples were analyzed and visualized in the heatmap with the GeneSpring 14.9 (Agilent).

Quantitative real-time PCR (qRT-PCR) analysis

The miRNA from 88 participants has been reverse-transcribed into cDNA using TaqMan Advanced miRNA cDNA Synthesis Kit (ThermoFisher Scientific, Walthan, MA, USA). qRT-PCR was performed using a CFX Connect Real-Time System (Bio-Rad laboratories, Hercules, CA, USA) and SYBRGreen Supermix (Bio-Rad laboratories) with gene-specific primer sets designed from the TaqMan Advanced miRNA assay (ThermoFisher Scientific). miRNA expression levels of target genes were normalized with cel-39-3p, an external control 34), and conducted using the delta-delta Ct method.

Statistical analysis

In all experiments, the difference was analyzed using the Fisher's exact test and the age difference was determined using one-way analysis of variance (ANOVA) and Tukey's test. The statistical analysis for miRNA expression levels among 16 samples used in primary screening was conducted using t-test. The expression levels in qRT-PCR analysis were conducted using one-way ANOVA and Tukey's test, and all values are expressed as mean ± significant differences (S.D.). A receiver operating characteristic (ROC) curve analysis was performed to determine the sensitivity, specificity, and veracity. Differences were considered significant at $p < 0.05$.

Results

miRNA microarray analysis for primary screening

We examined differences of miRNA expression levels among 8 SG and 8 CG with miRNA microarray analysis and illustrated

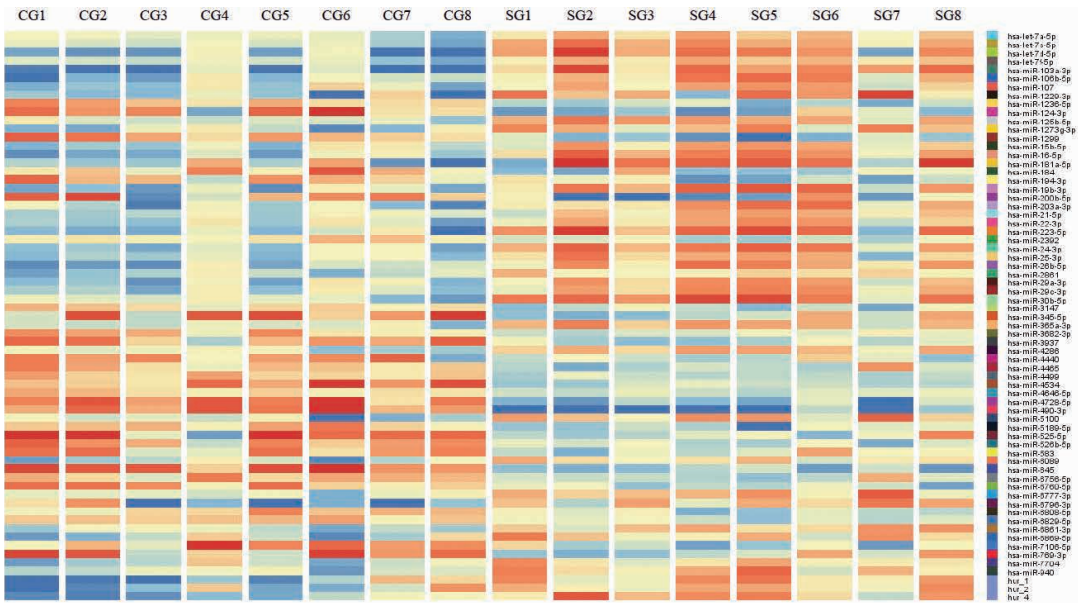

Figure 1. Heatmap of miRNA expression patterns in SG compared with CG. Each row represents a miRNA, and each column represents a subject. CG1 to CG8 indicate CG subjects (n=8) and SG1 to SG8 indicate SG subjects (n=8). Red color indicates upregulation and blue color indicates downregulation of each miRNA expression.

with heatmap (Fig 1). No significant differences were observed between the two groups based on gender and age bias. As a result, six miRNA expression levels (hsa-miR-103-3a, hsa-miR-1229-3p, hsa-miR-181a-5p, hsa-miR-19b-3p, hsa-miR-223-5p, and hsa-miR-30b-5p) were significantly increased in SG than those in CG ($p < 0.05$). Fold changes of those miRNAs were 20.81, 6.27, 6.40, 6.36, 8.22, and 5.55, respectively.

Expression levels of six miRNAs

We compared six miRNA expression levels among 44 SG and 44 CG subjects using the quantitative RT-PCR. In addition, SG and CG were classified into four groups (SG: SG-boys and SG-girls, CG: CG-boys and CG-girls), and their expression levels within gender were compared (Table 1). In SG, expression levels of hsa-miR-181a-5p, hsa-miR-223-5p, and hsa-miR-30b-5p were significantly higher than those in CG (fold change expression ± S.D.; 5.56 ± 0.83, 7.14 ± 1.21, and 6.30 ± 0.90, respectively). The expression levels of hsa-miR-181a-5p, hsa-miR-223-5p, and hsa-miR-30b-5p in each SG group were significantly higher than those of each CG group (fold change expression ± S.D.; boys: 4.64 ± 0.79, 7.29 ± 1.29 and 4.62 ± 0.82, girls: 7.23 ± 1.97, 8.01 ± 2.52, and 9.39 ± 2.40, respectively).

Diagnostic accuracy of three miRNAs in abused children

We used ROC curve analysis to determine the diagnostic accuracy of hsa-miR-181a-5p, hsa-miR-223-5p, and hsa-miR-30b-5p. The sensitivity, specificity, and area under the ROC curve (AUC) in subtotal subjects were as follows: hsa-miR-181a-5p (0.818, 0.795, and 0.8843); hsa-miR-223-5p (0.795, 0.795, and 0.9159); and hsa-miR-30b-5p (0.795, 0.841, and 0.8670) (Fig 2A). Furthermore, we also analyzed the diagnostic accuracy according to gender. The sensitivity, specificity, and AUC in boys and girls were as follows: hsa-miR-181a-5p (boys:0.69, 0.952, and 0.8 92; girls: 0.867, 0.87, and 0.8745); hsa-miR-223-5p (boys:0.759, 0.81, and 0.9217; girls: 0.867, 0.826, and 0.8986); and hsa-miR-30b-5p (boys:0.759, 0.857, and 0.867; girls: 0.867, 0.826, and 0.8703) (Fig 2B and C). The hsa-miR-223-5p had the highest AUC score (0.9159) among the three miRNAs

Table 1. Fold change expression of six miRNAs from the SG group compared to those of the CG group.

	SG	SG-boys	SG-girls
miRNA	Mean ± SD	Mean ± SD	Mean ± SD
103-3a	17.20 ± 10.90	40.16 ± 34.58	9.67 ± 5.73
1229-3p	3.48 ± 2.67	2.90 ± 2.26	0.80 ± 0.60
181a-5p	5.56 ± 0.83*	4.64 ± 0.79*	7.23 ± 1.97*
19b-3p	1.55 ± 0.47	3.86 ± 1.54	0.79 ± 0.19
223-5p	7.14 ± 1.21*	7.29 ± 1.29*	8.01 ± 2.52*
30b-5p	6.30 ± 0.90*	4.62 ± 0.82*	9.39 ± 2.40*

Asterisk indicated significant difference ($p < 0.05$).

and the highest AUC score according to gender (boys: 0.9217 and girls: 0.8986). From the results of the ROC curve, hsa-miR-223-5p was considered to be a desirable biomarker because its expression levels were extremely high; however, the sensitivity and specificity (0.795 and 0.795, respectively) were not sufficient.

Correlation between hsa-miR-223-5p and other two miRNAs

Based on our result, hsa-miR-223-5p had the highest diagnostic accuracy in abused children. However, the sensitivity and specificity of hsa-miR-223-5p (0.795 and 0.795, respectively) indicated moderate accuracy 35). To improve diagnostic accuracy in identifying abused children, we assessed the correlation between hsa-miR-30b-5p or hsa-miR-181a-5p and hsa-miR-223-5p to examine if they could be auxiliary biomarkers. If the expression levels of hsa-miR-223-5p and the other two miRNAs had no correlation with each other in CG, it was presumed that these miRNAs are expressed via a different transcriptional mechanism. We speculated that miRNA expressed in CG without correlation with hsa-miR-223-5p made it a useful biomarker for SG. Positive correlations were observed between hsa-miR-223-5p and hsa-miR-181a-5p in CG and SG ($r = 0.825$; $p < 0.05$ and $r = 0.911$; $p < 0.05$, respectively) (Fig 3A). On the contrary, the expression level of hsa-miR-30b-5p had positive correlation to that of hsa-miR-223-5p in only SG ($r = 0.927$; $p < 0.05$) (Fig 3B). The correlation between hsa-miR-223-5p and hsa-miR-30b-5p was examined within gender. These relationships were significantly associated in SG-boys and SG-girls ($r = 0.938$; $p < 0.05$ and $r = 0.917$; $p < 0.05$, respectively) but not in CG-boys and CG-girls ($r = 0.302$; $p > 0.05$ and $r = 0.374$; $p > 0.05$, respectively) (Fig 3C and D).

Discussion

Childhood abuse and family discord have been known to adversely affect physical and mental health, especially emotional control and emotional behavior, in childhood and adulthood 36)-39). Such harmful experiences and psychological stresses received by children are one of the causes of borderline personality disorder onset such as ADHD, and PTSD 2)-5). In particular, child abuse is defined as the psychological and physical stress experienced

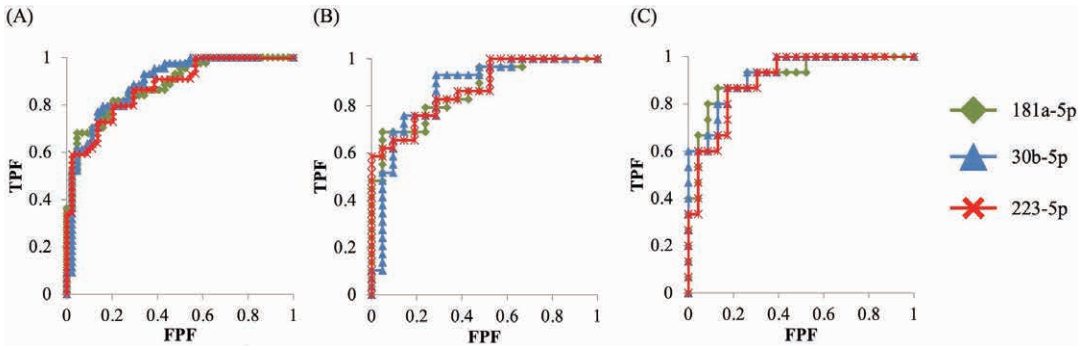

Figure 2. ROC curves for three miRNAs. (A) All subjects (n=88); (B) Boys (n=50); (C) Girls (n=38). Green line, blue line, and red line indicate hsa-miR-181a-5p, hsa-miR-30b-5p, and hsa-miR-223-5p, respectively. TPF: true positive fraction; FPF: false positive fraction.

by children. Therefore, early detection of child abuses, such as physical, sexual, and psychological, negligence of abuses, and reduction of psychological stress are important for childhood and adult health. However, a reliable method for diagnosis of child abuse

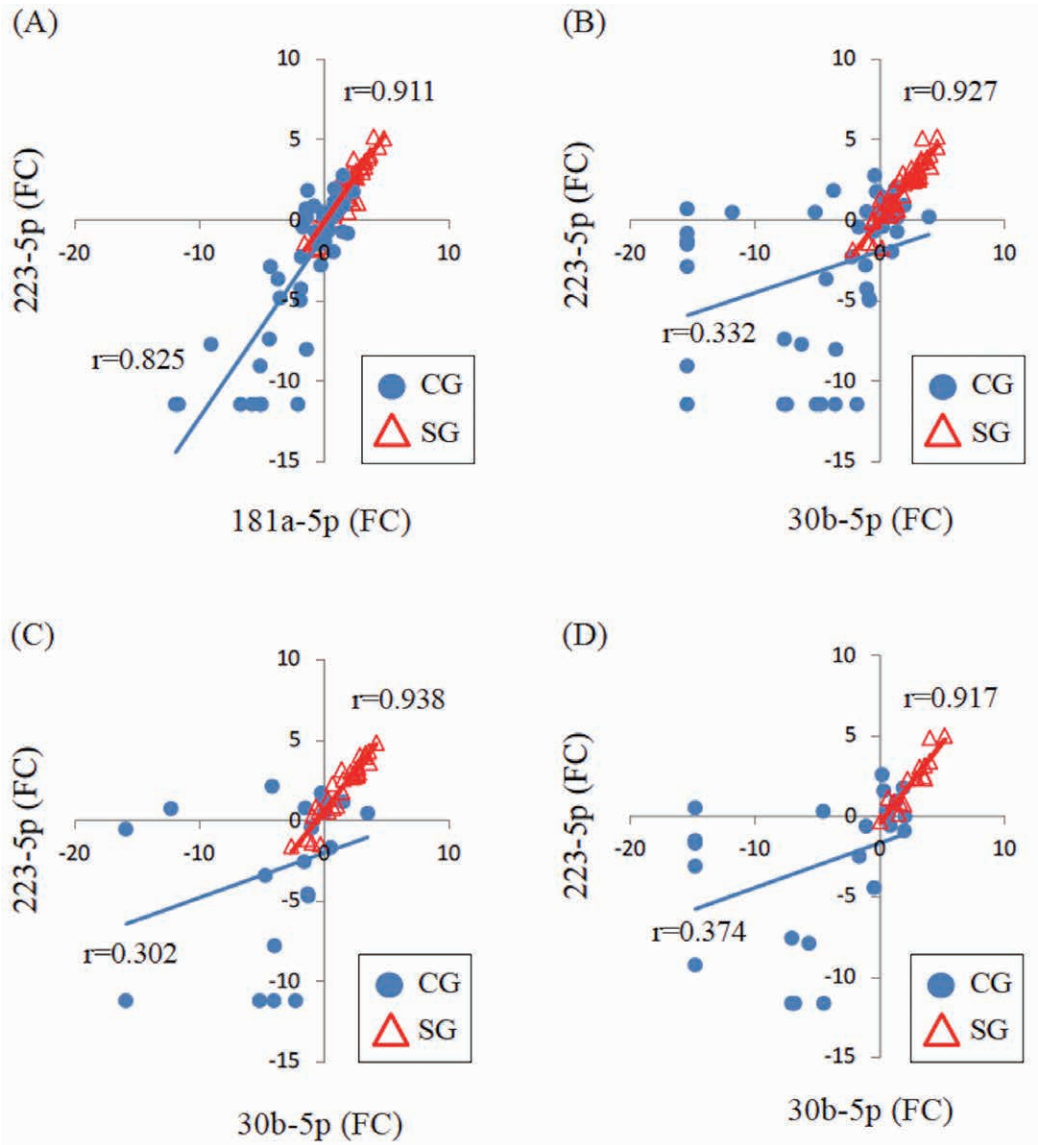

Figure 3. Correlation of hsa-miR-223-5p with hsa-miR-181a-5p and with hsa-miR-30b-5p. Blue circle and red lined triangle indicate CG and SG, respectively. Positive correlation of the expression levels of (A) hsa-miR-223-5p and hsa-miR-181a-5p in CG (n=44, r=0.825) and SG (n=44, r=0.911) ; (B) hsa-miR-223-5p and hsa-miR-30b-5p in CG (n=44, r=0.332) and SG (n=44, r=0.927) ; (C) hsa-miR-223-5p and hsa-miR-30b-5p in CG-boys (n=21, r=0.302) and SG-boys (n=29, r=0.938) ; (D) hsa-miR-223-5p and hsa-miR-30b-5p in CG-girls (n=23, r=0.374) and SG-girls (n=15, r=0.917) . FC indicates a fold change expression to 100 pmol of an external control, cel-39-3p.

has not yet been established. In this study, we investigated whether miRNAs, which play a central role in physiological and psychological stress response pathways, can be used to diagnose child abuse. Normally, miRNAs are purified from the peripheral blood, but we have purified them as a noninvasive method from the saliva 40),41). Studies using saliva miRNA for diagnosis have already been reported, and this is speculated as the optimal method for collection from children 42),43). In the present study, we compared the expression levels of saliva-derived miRNAs in abused children with those in non-abused children and assessed their effectiveness as biomarkers for detecting child abuse. Additionally, as the miRNA expression differs depending on the gender, we examined the expression levels of child-abuse related miRNA within each gender 44)-46).

First, we used the miRNA array to screen for miRNAs those expression levels are upregulated by child abuse (Fig 1). As a result, we determined the expression levels of six miRNAs (hsa-miR-103-3a, hsa-miR-1229-3p, hsa-miR-181a-5p, hsa-miR-19b-3p, hsa-miR-223-5p, and hsa-miR-30b-5p) were significantly increased in SG than those of CG. Then, we calculated the expression levels of those six miRNAs in other 88 children with quantitative RT-PCR. In SG, three miRNAs (hsa-miR-181a-5p, -223-5p and -30b-5p) were significantly expressed higher than those of CG. Moreover, no difference was observed in the expression levels within gender. Although the hsa-miR-103-3a was the most upregulated among the six miRNAs, there were no significant difference in the expression levels between CG and SG, and the miRNA was especially upregulated in SG-boys (Table 1).

Three miRNAs (hsa-miR-181a-5p, -223-5p, and -30b-5p) have been reported as biomarkers, and their functions have been revealed in several studies 47)-51). However, these miRNAs have not been reported to be associated with abusive stress. The enhancement of their expression levels with stress in our findings may indicate a new function for hsa-miR-181a-5p, -223-5p, and -30b-5p.

Next, we investigated whether these three miRNAs could be effective biomarkers for the detection and distribution of abused children using ROC curve analysis. The miRNA hsa-miR-223-5p has the highest AUC score among the three miRNAs (Figs 2A–C). These results indicated that hsa-miR-223-5p has high diagnostic accuracy as a biomarker for detecting child abuse; however, the sensitivity and specificity of this diagnostic method were insufficient. Therefore, the diagnosis of child abuse can be misleading when tested alone with hsa-miR-223-5p. It is necessary to test not only hsa-miR-223-5p but also hsa-miR-181a-5p or hsa-miR-30b-5p to improve the diagnostic accuracy of detecting child abuse. We suggest that it is important to select either one of the miRNA that does not depend on the expression level of hsa-miR-223-5p to achieve high diagnostic accuracy. Thus, the expression level of hsa-miR-30b-5p was not significantly correlated with that of hsa-miR-223-5p in CG (Fig 3B–D). Additionally, hsa-miR-30b-5p showed moderate scores, sensitivity, and specificity (Fig 2A to C). Thus, it was speculated that hsa-miR-30b-5p can be considered a useful second biomarker for detecting child abuse.

From the easily noticeable changes in salivary miRNAs, it was speculated that chronic stress associated with child abuse that has a significant impact on the mental and

physical growth and development of children could be detected as soon as possible.

To detect the children who have been abused using the expression levels of miRNA in the early stage of child abuse may lead to save these children.

It is necessary to apply this suggestion to the identification of that age group of children who cannot complain of abuse by repeating the verification by cohort and for whom additional samples cannot be collected for repeat verification.

Conclusions

In summary, we speculate that hsa-miR-223-5p and hsa-miR-30b-5p are saliva-derived biomarkers that can diagnose child abuse. These microRNAs are expected to lead to early detection of child abuse and provide a basis for preventive action. Furthermore, it is necessary to investigate whether the three miRNAs (hsa-miR-223-5p, hsa-miR-181a-5p, and hsa-miR-30b-5p) could be used as biomarkers for child abuse- and neglect-induced borderline personality disorder, such as ADHD, and PTSD. However, since these results were investigated in Japanese children, it will be necessary to scrutinize it all over the world in the future.

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Author's contributions

T.Oz.: Conceptualization, Methodology, Formal analysis, Data curation, and Writing of original draft. T. Oh.: Conceptualization, Methodology, Formal analysis, Investigation, and Data curation. H.M.: Formal analysis, Data curation, and Supervision. K.S.: Conceptualization, Formal analysis, Data curation, and Writing of original draft. T.Oz. and T.Oh. have contributed equally to this article. All authors have read and agree to the published version of the manuscript.

Competing interests

The authors declare no conflict of interest.

References

1) UNICEF. Hidden in plain sight: a statistical analysis of violence against children. 2014;https://data.unicef.org/wp-content/uploads/2015/12/VR-full-report_Final-LR-3_2_15_189.pdf
2) Prados, J., Stenz, L., Courtet, P., et al.; Borderline personality disorder and childhood maltreatment: a genome-wide methylation analysis. Genes Brain Behav., 14, 177-188, 2015.
3) Endo, T., Sugiyama, T., Someya, T.; Attention-deficit/hyperactivity disorder and dissociative disorder among abused children. Psychiatry Clin Neurosci., 60, 434-438, 2006.
4) Mehta, D., Klengel, T., Conneely, K.N., et al.; Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder. Proc Natl Acad Sci U S A, 110, 8302-8307, 2013.
5) Insana, S.P., Banihashemi, L., Herringa, R.J., Kolko, D.J., Germain, A.; Childhood maltreatment is associated with altered frontolimbic neurobiological activity during wakefulness in adulthood. Dev Psychopathol, 28, 551-564, 2016.
6) Li, L., Pereira, S.M.P., Power, C.; Childhood maltreatment and biomarkers for cardiometabolic disease in mid-adulthood in a prospective British birth cohort: associations and potential explanations. BMJ Open, 23, 9 (3), e024079, 2019.

7) Soares, A.L.G., Hammerton, G., Howe, L.D., Rich-Edwards, J., Halligan, S., Fraser, A.; Sex differences in the association between childhood maltreatment and cardiovascular disease in the UK Biobank. Heart, 106（17）, 1310-1316, 2020.
8) Hovdestad, W.E., Shields, M., Shaw, A., Tonmyr, L.; Childhood maltreatment as a risk factor for cancer: findings from a population-based survey of Canadian adults. BMC Cancer, 29, 20（1）, 70, 2020.
9) Essabar, L., Khalqallah, A., Benjelloun Dakhama, B.S.; Child sexual abuse: report of 311 cases with review of literature. Pan Afr Med J, Jan 19;20:47, 2015.
10) Levitskiy, D., Confair, A., Wagner, K.E., et al.; Longitudinal stability of salivary microRNA biomarkers in children and adolescents with autism spectrum disorder.Res Autism Spectr Disord, 85, 101788, 2021.
11) Kim, MY., Shin, H., Moon, H.W., Park, Y.H., Park, J., Lee, J.Y.; Urinary exosomal microRNA profiling in intermediate-risk prostate cancer.Sci Rep, 1, 11（1）, 7355, 2021.
12) Salahandish, R., Ghaffarinejad, A., Omidinia, E., Zargartalebi, H., Majidzadeh-A, K., Naghib, S.M., Sanati-Nezhad, A.; Label-free ultrasensitive detection of breast cancer miRNA-21 biomarker employing electrochemical nano-genosensor based on sandwiched AgNPs in PANI and N-doped graphene. Biosens Bioelectron, 30（120）, 129-136, 2018.
13) Cao, Y., Green, K., Quattlebaum, S., et al.; Methylated genomic loci encoding microRNA as a biomarker panel in tissue and saliva for head and neck squamous cell carcinoma. Clin Epigenetics, 10, 43, 2018.
14) Kutwin, P., Borkowska, E.M., Bogucka, P., Jablonowski, Z.; Expression profile of microRNAs（106b-3p, 130b-3, 145-3p, 199a-5p）in urine and serum samples from patients with the diagnosis of bladder cancer. Pol Merkur Lekarski, 18, 49（290）, 103-107, 2021.
15) Gao, Y., Wang, J., Zhou, Y., Sheng, S., Qian, S.Y., Huo, X.; Evaluation of Serum CEA, CA19-9, CA72-4, CA125 and Ferritin as Diagnostic Markers and Factors of Clinical Parameters for Colorectal Cancer. Sci Rep, 9, 8（1）, 2732, 2018.
16) Dochez ,V., Caillon, H., Vaucel, E., Dimet, J., Winer, N., Ducarme, G.; Biomarkers and algorithms for diagnosis of ovarian cancer: CA125, HE4, RMI and ROMA, a review. J Ovarian Res, 27, 12（1）, 28, 2019.
17) Miyakawa, M., Matsui, T., Kishikawa, H., Murayama, R., Uchiyama, I., Itoh, T., Yoshidaet, T.; Salivary chromogranin A as a measure of stress response to noise. Noise Health, 8, 108-13, 2006.
18) Van Andel, H.W.H, Jansen, L.M., Grietens, H., Knorth, E.J., Van der Gaag, R.J.; Salivary cortisol: a possible biomarker in evaluating stress and effects of interventions in young foster children? Eur Child Adolesc Psychiatry, 23, 3-12, 2014.
19) Linnemann, A., Kappert, M.B., Fischer, S., Doerr, J.M., Strahler, J., Nater, U.M.; The effects of music listening on pain and stress in the daily life of patients with fibromyalgia syndrome. Front Hum Neurosci, 9, 434, 2015.
20) Chennaoui, M., Bougard, C., Drogou, C., Langrume, C., Miller, C., Gomez-Merino, D., Vergnoux-et, F.; Stress Biomarkers, Mood States, and Sleep during a Major Competition: "Success" and "Failure" Athlete's Profile of High-Level Swimmers. Front Physiol, 14, 7, 94, 2016.
21) Kaneko, S., Liu, L., Kakamu, T., Minami-Hori, M., Morita, E.; Salivary chromogranin A levels correlate with disease severity but do not reflect anxiety or personality of adult patients with atopic dermatitis. J Dermatol, 44（8）, 920-926, 2017.
22) Nandam, L.S., Brazel, M., Zhou, M., Jhaveri, D.J.; Cortisol and Major Depressive Disorder-Translating Findings From Humans to Animal Models and Back. Front Psychiatry, 22, 10, 974, 2020.
23) Benesova, S., Kubista, M.; Valihrach L.Small RNA-Sequencing: Approaches and Considerations for miRNA Analysis. Diagnostics（Basel）, 27, 11（6）, 964, 2021.
24) Chorley, B.N., Atabakhsh, E., Doran, G., et al.; Methodological considerations for measuring biofluid-based microRNA biomarkers. Crit Rev Toxicol, 51（3）, 264-282, 2021.
25) Sassen, S., Miska, E.A., Caldas, C.; MicroRNA: implications for cancer. Virchows Arch, 452, 1-10, 2008.
26) O'Brien, J., Hayder, H., Zayed, Y., Peng, C.; Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Front Endocrinol（Lausanne）, 3, 9, 402, 2018.
27) Asakura, K., Kadota, T., Matsuzaki, J., et al.; A miRNA-based diagnostic model predicts resectable lung cancer in humans with high accuracy. Commun Biol, 19, 3（1）, 134, 2020.
28) Wu, H.Z.Y., Thalamuthu, A., Cheng, L., Fowler, C., Masters, C.L., Sachdev, P., Mather, K.A.; Differential blood miRNA expression in brain amyloid imaging-defined Alzheimer's disease and controls. Alzheimer's Research & Therapy, 12（1）, 59, 2020.
29) Liu, X., Chen, J., Guan, T., Hui, Yao., Zhang, W., Guan, Z., Wang, Y.; miRNAs and target genes in the blood as biomarkers for the early diagnosis of Parkinson's disease. BMC Systems Biology, 13, 10, 2019.
30) Dobrowolny, G., Martone, J., Lepore, E., et al.; A

longitudinal study defined circulating microRNAs as reliable biomarkers for disease prognosis and progression in ALS human patients. Cell Death Discovery, 7, 4, 2021.

31) Jacobsen, D.P., Eriksen, M.B., Rajalingam, D., Nymoen, I., Nielsen, M.B., Einarsen, S., Gjerstad, J.; Exposure to workplace bullying, microRNAs and pain; evidence of a moderating effect of miR-30c rs928508 and miR-223 rs3848900. Stress, 23 (1), 77-86, 2020.

32) Linnstaedt, S.D., Rueckeis, C.A., Riker, K.D., et al.; MicroRNA-19b predicts widespread pain and posttraumatic stress symptom risk in a sex-dependent manner following trauma exposure. Pain, 161 (1), 47-60, 2020.

33) Prasad, S.R., Pai, A., Shyamala, K., Yaji, A.; Expression of Salivary miRNA 21 in Oral Submucous Fibrosis (OSMF) : An Observational Study. Microrna, 9 (4), 295-302, 2020.

34) Kondratov, K., Kurapeev, D., Popov, M., Sidorova, M., Minasian, S., Galagudza, M., Kostareva, A., Fedorov, A.; Heparinase treatment of heparin-contaminated plasma from coronary artery bypass grafting patients enables reliable quantification of microRNAs. Biomol Detect Quantif, 8, 9-14, 2016.

35) Fischer, J.E., Bachmann, L.M., Jaeschke, R.; A readers' guide to the interpretation of diagnostic test properties: clinical example of sepsis; Intensive Care Med, 29,1043-1051,2003.

36) Lippard, E.T.C., Nemeroff, C.B.; The Devastating Clinical Consequences of Child Abuse and Neglect: Increased Disease Vulnerability and Poor Treatment Response in Mood Disorders. Am J Psychiatry, 1, 177 (1), 20-36, 2020.

37) Kaufman, J., Torbey, S.; Child maltreatment and psychosis. Neurobiol Dis, 131, 104378, 2019.

38) Kimber, M., Gonzalez, A., MacMillan, H.L.; Recognizing and Responding to Child Maltreatment: Strategies to Apply When Delivering Family-Based Treatment for Eating Disorders. Front Psychiatry, 10, 11, 678, 2020.

39) Bosquet, E.M., Englund, M.M., Egeland, B.; Maternal Childhood Maltreatment History and Child Mental Health: Mechanisms in Intergenerational Effects. J Clin Child Adolesc Psychol, 47, 1-62, 2018.

40) Kichukova, T.M., Popov, N.T., Ivanov, I.S., Vachev, T.I.; Profiling of Circulating Serum MicroRNAs in Children with Autism Spectrum Disorder using Stem-loop qRT-PCR Assay. Folia Med (Plovdiv), 59, 43-52, 2017.

41) He, K., Guo, C., He, L., Shi, Y.; MiRNAs of peripheral blood as the biomarker of schizophrenia. Hereditas, 155, 9, 2017.

42) Humeau, M., Vignolle-Vidoni, A., Sicard, F., Martins, F., Bournet, B., Buscail, L., Torrisani, J., Cordelier, P.; Salivary MicroRNA in Pancreatic Cancer Patients. PLoS One, 10, e0130996, 2015.

43) Wu, L., Zheng, K., Yan, C., et al.; Genome-wide study of salivary microRNAs as potential noninvasive biomarkers for detection of nasopharyngeal carcinoma. BMC Cancer, 19, 843, 2019.

44) Mavrikaki,M., Pantano,L., Potter,D., Rogers-Grazado,M.A., Anastasiadou,E., Slack,F.J., Amr,S.S., Ressler, K.J., Daskalakis N.P., Chartoff,E.; Sex-Dependent Changes in miRNA Expression in the Bed Nucleus of the Stria Terminalis Following Stress. Front Mol Neurosci, Oct 4, 12:236, 2019.

45) Piscopo,P., Bellenghi,M., Manzini,V., Crestini,A., Pontecorvi,G., Corbo, M., Ortona,E., Carè,A., Confaloni,A ; A Sex Perspective in Neurodegenerative Diseases: microRNAs as Possible Peripheral Biomarkers. International Journal of Molecular Sciences, 23,22 (9) ,4423, 2021.

46) Huen,K., Lizarraga,D., Kogut,K., Eskenazi,B., Holland,N. Age-Related Differences in miRNA Expression in Mexican-American Newborns and Children; Int J Environ Res Public Health, Feb 13,16 (4) ,524, 2019.

47) Ansari, A., Maffioletti, E., Milanesi, E., et al.; miR-146a and miR-181a are involved in the progression of mild cognitive impairment to Alzheimer's disease. Neurobiol Aging, 82, 102-109, 2019.

48) Zhang,Q., Ma,X.F., Dong,M.Z., Tan,J., Zhang,J., Zhuang,L.K., Liu,S.S., Xin,Y.N.; MiR-30b-5p regulates the lipid metabolism by targeting PPARGC1A in Huh-7 cell line. Lipids Health Dis, 16,19 (1) ,76, 2020.

49) Woo, C.C., Liu, W., Lin, X.Y., Dorajoo, R., Lee, K.W., Richards, A.M., Lee, C.N., Wongsurawat, T., Nookaew, I., Sorokin, V.; The interaction between *30b-5p* miRNA and *MBNL1* mRNA is involved in vascular smooth muscle cell differentiation in patients with coronary atherosclerosis. Int J Mol Sci, 21, 11, 2019.

50) Brennan, S., Keon, M.,Liu, B., Su, Z., Saksena, N.K.; Panoramic Visualization of Circulating MicroRNAs Across Neurodegenerative Diseases in Humans. Mol Neurobiol, 56, 7380-7407, 2019.

51) Dietz, C., Müller, M., Reinhold, A.K., et al.; What is normal trauma healing and what is complex regional pain syndrome I? An analysis of clinical and experimental biomarkers. Pain, 160, 2278-2289, 2019.