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Epigenetic analysis of glucocorticoid receptor and early childhood stress

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In this study, we extracted DNA from the saliva of 85 children to analyze the methylation of their glucocorticoid receptor gene (NR3C1) promoter region. Sequencing results up to the final stage were obtained for 28 cases that were subsequently divided into two groups based on the presence/absence of methylation to analyze the relationship between factors identified in the questionnaire responses and those found in observational data (which were mainly from early childhood) obtained from the cohort study. Statistically significant differences were observed between the two groups in (1) head circumference at 1 year and 6 months of age and (2) the developmental age for social interaction with adults at 3 years and 6 months of age. In the group with methylation, the head circumference at 1 year 6 months was smaller and the developmental age for social interaction with adults at 3 years 6 months was lower. The methylation group also tended to be smaller in height and weight up to 3 years and 6 months, although no significant difference was observed. In the future, it will be necessary to increase the sensitivity of the analysis method and conduct a study with sufficient number of samples for statistical analysis.

Keywords: cohort study, early childhood stress, glucocorticoid receptor gene, epigenetics

Introduction

The authors of this study participated in the planning of the Japan Science and Technology Agency (JST) cohort study entitled, "Factors Influencing Cognitive and Behavioral Development of Children in Japan" (2004–2008), and have subsequently conducted cohort studies in two prefectures, Mie and Hyogo, with the consent of the participants of the original study.

To build a developmental model using the data on environmental and individual (genetic) factors collected in this study and elucidate the mechanism of developmental changes, it is important to understand how individual and environmental factors interact with each other. In this context, epigenetic mechanisms are cited as one kind of mechanism whereby genetic characteristics, which are individual factors, are influenced by environmental factors. Epigenetic mechanisms have been reported in the onset of developmental disorders such as autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD).1-4) The environment in which the child develops and grows during the fetal stage and early childhood is thought to cause dysregulation in gene expression, such as DNA methylation and histone modification, and contribute to the onset or aggravation of developmental disorders. In addition to genetic factors, the aforementioned epigenetic mechanisms have been shown to be involved in the formation of susceptibility to stress.

The stress of growing up in an unfavorable environment during infanthood is reported to form susceptibility to stress and increase risk for the onset of anxiety, depression, and drug dependence.5,6) One epigenetic mechanism believed to play an important role in psychological susceptibility to stress and in the hypothalamic-pituitary-adrenal axis (HPA) is methylation in the glucocorticoid receptor gene promoter region. Early childhood stresses such as an unfavorable environment promote methylation in the glucocorticoid receptor gene promoter region and suppress the expression of the glucocorticoid receptor. This weakens the physiological effect of glucocorticoids that are activated in response to stress and is thus thought to be involved in susceptibility to stress.7,8)

The objective of this study was to determine the role of epigenetic mechanisms in psychological stress and the HPA axis by analyzing methylation in the glucocorticoid receptor gene promoter region among the Sukusuku Cohort Mie research participants.

Subjects and Methods

Subjects

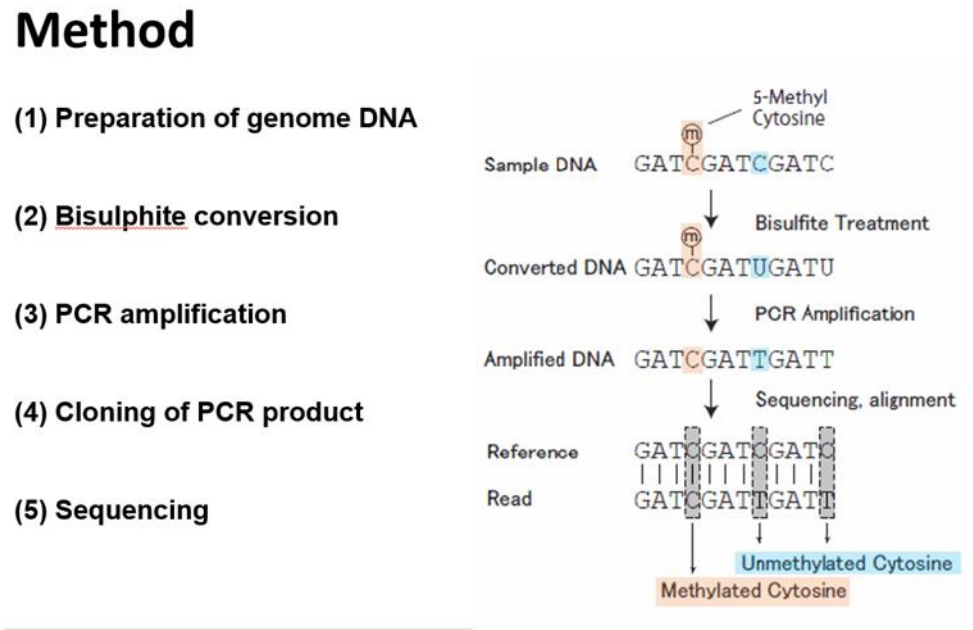
The subjects were children participating in Sukusuku Cohort Mie. We obtained informed consent in writing after providing them a written explanation of the study. We mailed a saliva DNA extraction kit (Saliva DNA Collection and Preservation Devices, NORGEN) to volunteers who consented to participate in the questionnaire survey at 11 years of age. Each volunteer collected

approximately 2ml of saliva using their kit. The saliva samples were collected by mail.

Extraction of genome DNA and bisulfite processing

A Saliva DNA Isolation Kit (Cat# RU45400, NORGEN) was used for the DNA extraction, and the genome DNA was refined according to the product protocol. A MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures) was used on the extracted DNA to perform bisulfite processing according to the kit protocol (See Figure 1 below).

Figure 1
Method of analysis used in the study



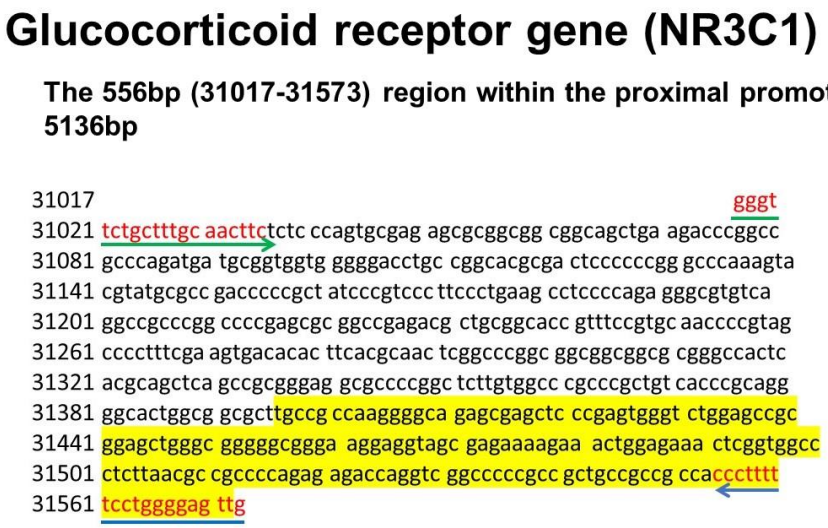
(Note) The right-hand figure shows the methylated and unmethylated cytosines at each stage of the analysis.

PCR amplification and sequencing analysis

The target regions for PCR amplification were the glucocorticoid

receptor gene (NR3C1) exon 1F and the 556bp region containing the promoter (See Figure 2 below). The PCR primer was created using Methyl primer Express software version 1.0 (Applied Biosystems).9) We used TaKaRa EpiTaq HS (for bisulfite-treated DNA) on the PCR to perform the amplification reaction according to the product protocol. The PCR-amplified 556bp fragment was incorporated into T-Vector pMD20 (TaKaRa), and after the host E. coli was cloned in agar medium, the culture was amplified and the plasmid DNA was extracted from it. Sequencing analysis was performed on each extracted clone of DNA using the ABI PRISM Genetic Analyzer (Applied Biosystems).

Figure 2
Sequence of the PCR-amplified region of the glucocorticoid receptor gene (NR3C1)



(Note) The arrows show the sequence of the primers used for the PCR. The yellow highlighted areas show the 13 sites in the CpG structure (numbered 1 to 13) that are reported to be closely correlated with early childhood environmental factors.

Analysis of the relationship between DNA methylation and the

results of the early childhood questionnaire

We examined the relationship between early childhood event factors and methylation in the glucocorticoid receptor gene promoter region based on the observational data obtained from Sukusuku Cohort Mie (which were mainly from early childhood) and the results of the questionnaire survey. We divided the questionnaire results into two groups based on the presence or absence of methylation, with each subject's individual and environmental factors as the quantified data variables. Significance testing was performed between the two groups using a t-test.

Research ethics and conflicts of interest

This study was conducted with the approval of the Mie Central Medical Center Ethics Review Board (approval no. MCERB-202010). The authors have no conflicts of interest to disclose.

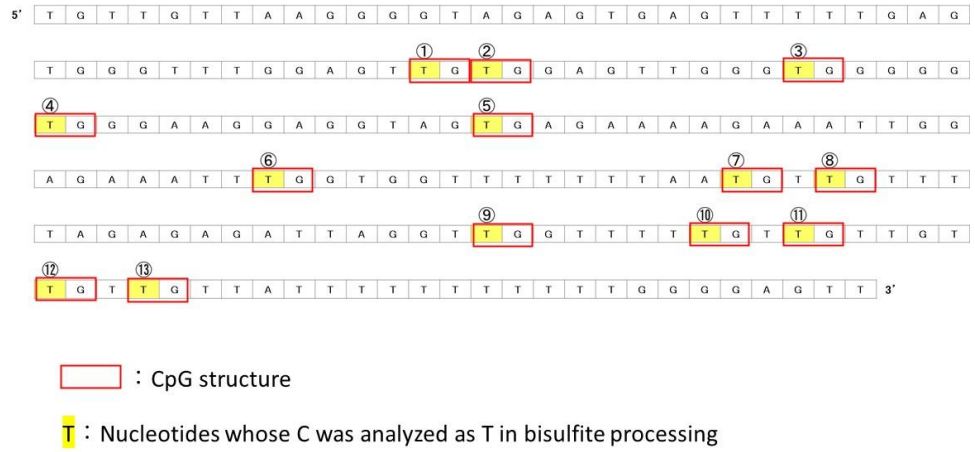
Results

Of the participants in Sukusuku Cohort Mie, there were 85 subjects who consented to participate in a study involving saliva after receiving an explanation about the study. Of those 85 subjects, there were 28 cases where it was possible to extract DNA from the saliva and analyze methylation from the sequence of the target glucocorticoid receptor gene promoter region.

For the region to analyze for methylation in the 556bp fragment that was PCR-amplified and analyzed, we chose the 177bp region from the 3' end, which contains 13 CpG structure sites

and is reported to have a close correlation with early childhood factors (10,11) (See Figure 3).

Figure 3
CpG structure sites analyzed in the glucocorticoid receptor gene promoter region



(Note) This figure shows the region in the amplified and analyzed 556bp fragment where the nucleic acid sequence was analyzed. It corresponds to the 177bp region from the 3' end, which contains 13 CpG structure sites (#1 to 13) and has been reported to have a close correlation with early childhood factors.

Of the 28 cases that could be analyzed up to the final-stage of sequencing, there were 11 in which the CpG structure had undergone methylation, almost all of which were CpG site number 2, 5, 6, 7, or 8 (See Table 1).

We divided the 28 cases into two groups based on the presence or absence of methylation, and performed significance testing of factors in the observational data obtained from the Sukusuku Cohort Mie study (which were mainly from early childhood) and the results of the questionnaire survey.

In terms of physical factors such as height and weight, the group in which methylation was observed temporarily had a smaller

Table 1
Summary of samples in which methylation was observed

Sample	Site No. of Met+	Sample	Site No. of Met+
1	② ⑤⑥⑦⑧	7	② ⑤⑥⑦⑧
2	② ⑤⑥⑦⑧	8[b]	② ⑤⑥⑦⑧
3	② ⑤⑥⑦⑧	9	② ⑤⑥⑦⑧
4	② ⑤⑥⑦⑧	10	⑧⑬
5	② ⑤⑥⑦⑧	11	② ⑥⑦⑧
6[a]	② ⑤⑥⑦⑧		

(Note) This table shows the 11 samples in which methylation was observed and the sites of their CpG structure confirmed to have methylated cytosines (notated as #1 to 13), as well as samples with circumstances worthy of special mention.

[a] Single-mother household, moved house to a faraway location, [b] One of the twin (#7&#8) had a history of wrist-cutting in middle school

head circumference (at the 5% significance level) in the data taken at 1 year and 6 months of age, but no difference was observed thereafter. The group in which methylation was observed had smaller bodies until they were 3 years and 6 months old, and this trend tended to reverse after 4th grade, but this phenomenon was not found to be statistically significant.

No clear difference was found between the two groups in terms of factors relating to taking iron medication, drinking, smoking, regular visits by the mother to the hospital for phototherapy or other treatment, or conditions during pregnancy and delivery.

In the observational data on development, the developmental age for social interaction with adults was temporarily significantly lower (at the 5% significance level) at 3

years and 6 months of age.

Although no statistically significant difference was observed in the home environment, in the group with methylation, there were two noteworthy cases – the case of a child who belonged to a single-mother household that had moved far away from the family, and the case of a child (who had an identical twin) who had a history of wrist-cutting in middle school. The twins showed a similar pattern of methylation.

Discussion

From the present analysis, we were not able to confirm a relationship between methylation in the glucocorticoid receptor gene (NR3C1) exon 1F and promoter region, factors in the observational data (which were mainly from early childhood), and the results of the questionnaire survey. The inability to perform sufficient data analysis owing to the low efficiency of proceeding from collection of saliva samples to DNA analysis of methylation was a major challenge. Of the 13 CpG structure sites reported to have a close correlation with early childhood environmental factors, sites 2, 5, 6, 7, and 8 were often seen to be methylated, which is consistent with previous reports.

While we obtained several statistically significant analytical results, the small sample size made it difficult to perform a sufficiently reliable statistical analysis. However, the fact that (1) the group with methylation tended to be smaller in height and weight up to 3 years and 6 months, and (2) the developmental age for social interaction with adults at 3 years and 6 months was

significantly lower did suggest a relationship with previous reports that difficult experiences in early childhood are closely correlated with the promotion of methylation. Furthermore, it is interesting that the group in which methylation was observed included a case where the child belonged to a single-mother household that moved far away, as a result of which stress due to the home environment was suspected to be a factor, as well as a case in which the child attempted self-harm by cutting their wrist after the samples were collected. The wrist-cutting case was done by one child out of two identical twins, and it would not be a contradiction if either environmental factors or individual factors affected methylation, since the twins did not display a difference in their methylation patterns and grew up in the same home environment.

Unfortunately, we were unable to obtain a statistical analytical result in this study that could prove a relationship between the conditions of these cases and methylation in the glucocorticoid receptor gene promoter region. To elucidate such a relationship would require performing a statistical analysis using a highly sensitive analytical method and a larger sample size.

Conclusion

We analyzed methylation in the glucocorticoid receptor gene (NR3C1) promoter region using DNA from saliva provided by participants in Sukusuku Cohort Mie. Methylation was observed in 11 of the 28 cases that we were able to analyze, but we were not able to confirm a relationship between methylation and the observational data obtained from the cohort study (which were

mainly from early childhood) and the results of the questionnaire survey. However, the group in which methylation was observed included a case that suggested the presence of stress in the home environment, which could possibly have some significance. In the future, it would be beneficial to perform a statistical analysis using a higher-sensitivity analytical method and a larger sample size to obtain more conclusive results.

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