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Methylation analysis of the glucocorticoid receptor using next-generation sequencing

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Introduction

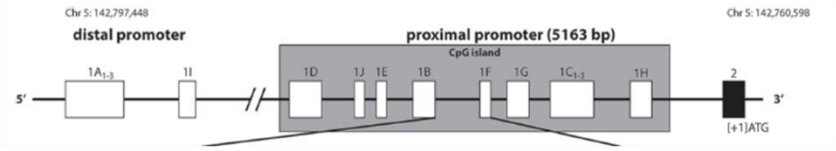
We participated in the Japan Science and Technology Agency (JST) cohort study “Elucidating Factors Influencing Cognitive and Behavioral Development in Children in Japan” (2004–2008). Following its completion, another study with the same cohort has continued with the consent of research collaborators, as part of our ongoing basic research initiatives.

To construct a developmental model and elucidate the mechanisms underlying developmental change using the environmental and individual (genetic) factor data collected in this study, it is essential to examine the interactions between individual and environmental factors. Among these, epigenetic mechanisms represent one pathway through which genetic traits, as individual factors, are influenced by environmental conditions. Epigenetic

processes have been implicated in the pathogenesis of developmental disorders such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD). It is suggested that the prenatal and early postnatal environment induces dysregulation of gene expression, including alterations in DNA methylation and histone modification, thereby contributing to the onset and severity of these disorders. In the development of stress vulnerability, not only genetic factors but also epigenetic mechanisms, as described above, have been shown to play a role. Exposure to stress associated with adverse early-life environments has reportedly contributed to the formation of stress vulnerability and to the increased risk of anxiety, depression, and substance dependence. One epigenetic mechanism considered to be particularly important to psychological stress vulnerability and regulation of the hypothalamic–pituitary–adrenal (HPA) axis is

Figure 1. Glucocorticoid receptor gene (NR3C1)

The regions highlighted in yellow indicate areas containing 13 CpG sites that previous studies have reported to be strongly associated with early-life environmental factors.



556bp (31017–31573)

31017 gggt
31021 tctgctttgc aacttctctc ccagtgcgag agcgcggcgg cggcagctga agacccggcc
31081 gcccagatga tgcggtggtg ggggacctgc cggcacgcga ctccccccgg gcccaaagta
31141 cgtatgcgcc gacccccgct atcccgtccc ttccctgaag cctccccaga gggcgtgtca
31201 ggccgcccgg ccccgagcgc ggccgagacg ctgcggcacc gtttccgtgc aaccccgtag
31261 ccccctttcga agtgacacac ttcacgcaac tcggcccggc ggcggcggcg cgggccactc
31321 acgcagctca gccgcgggag gcgccccggc tcttgtggcc cgcccgctgt cacccgcagg
31381 ggcactggcg gcgcttgccg ccaaggggca gagcgagctc ccgagtgggt ctggagccgc
31441 ggagctgggc gggggcggga aggaggtagc gagaaaagaa actggagaaa ctcggtggcc
31501 ctcttaacgc cgccccagag agaccaggtc ggcccccgcc gctgccgccg ccacccctttt
31561 tcctggggag ttg

methylation of the promoter region of the glucocorticoid receptor gene. Early-life stress, such as that arising from unfavorable caregiving environments, is thought to promote methylation in this promoter region, leading to reduced expression of the glucocorticoid receptor. This reduction may attenuate the physiological effects of glucocorticoids during stress and is considered to be associated with increased stress vulnerability.

To date, DNA has been extracted from saliva samples of 85 consenting participants in the "Sukusuku Cohort in Mie," and methylation analysis of the promoter region of the glucocorticoid receptor gene (NR3C1) has been conducted (Figures 1 and 2). However, final sequencing results using the Sanger method were obtained for only 28 cases, representing approximately one-third of the participants (Figure 3), which limited the ability to adequately examine the association between early-life environmental factors and methylation of the glucocorticoid receptor gene promoter region. This low analytical efficiency is considered to reflect the limitations of conventional methods based

Figure 2. Sites of highly frequent methylation closely correlates with GR expression:①～⑬

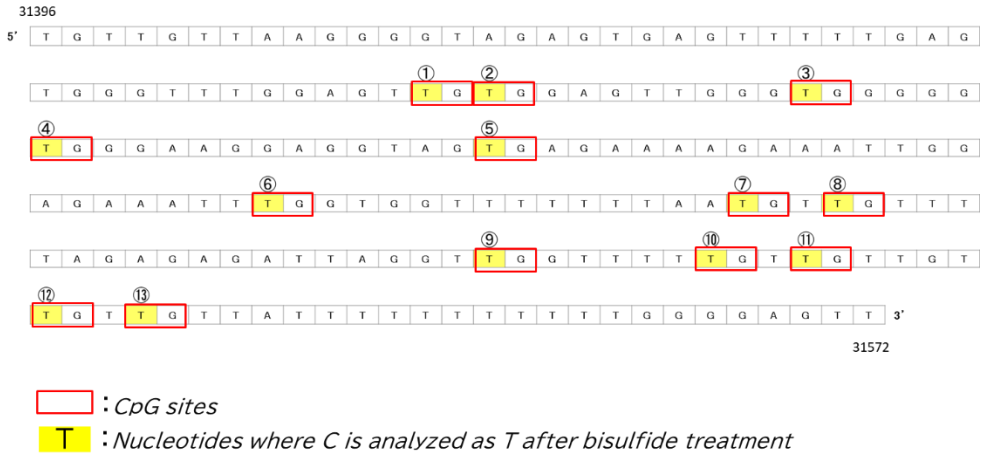
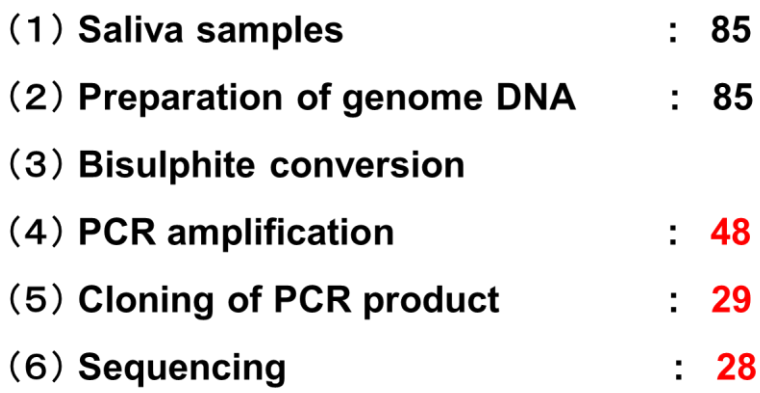


Figure 3. Issues related to analytical efficiency



on capillary sequencing.

In this study, methylation analysis of the promoter region of the glucocorticoid receptor gene (NR3C1) was conducted using stored saliva samples from 85 participants, with the expectation that the introduction of a next-generation sequencer (NGS) in our department would improve analytical sensitivity and accuracy. Therefore, we established a method for methylation analysis of the NR3C1 promoter region by designing primers for the target region and optimizing analytical conditions using next-generation sequencing. We report our findings herein.

Subjects and Methods

Participants: Four volunteers from our research department who provided informed consent were included in this study. Approximately 2 mL of saliva was collected from each participant using a saliva DNA collection and preservation device (Saliva DNA Collection and Preservation Devices, NORGEN), consistent with the procedures used in the cohort study.

Extraction of genomic DNA and bisulfite treatment: For DNA extraction, the Saliva DNA Isolation Kit (NORGEN) was used, and genomic DNA was purified in accordance with the manufacturer's protocol. The extracted DNA was subsequently subjected to bisulfite treatment using the EpiJET Bisulfite Conversion Kit (Thermo Scientific), in accordance with the manufacturer's protocol.

PCR amplification: The PCR amplification target comprised a 300 bp region (Figure 4) within a 556 bp segment (Figure 1) encompassing exon 1F and the promoter region of the glucocorticoid receptor gene (NR3C1). PCR primers were designed using Methyl Primer Express software version 1.0 (Applied Biosystems). Amplification was performed using Platinum™ PCR SuperMix High Fidelity (Invitrogen™) in accordance with the

Figure 4. Primer Pairs aligned with GRNR3C1

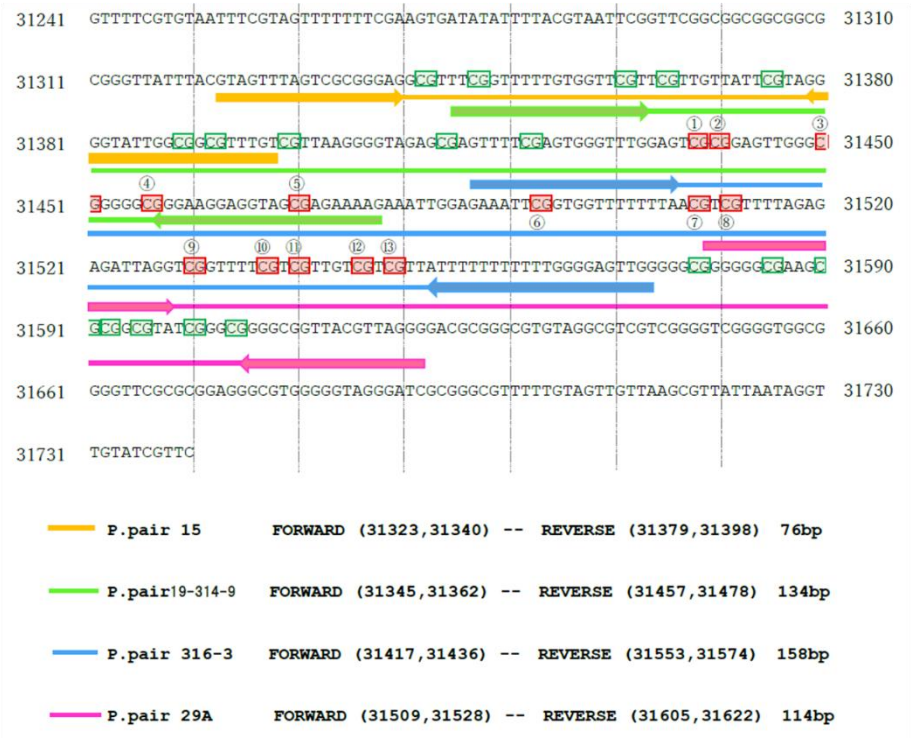


Table 1. Results of hotspot analysis: Sample 1

Position	Ref	Variant	Allele Call	Frequency	Possible Polyploidy Allele	Quality	Type	Allele Source
31438	C	T	Homozygous	99.2	0	3892.96	SNP	Hotspot
31440	C	T	Homozygous	99	0	3860.28	SNP	Hotspot
31450	C	T	Homozygous	99	0	3855.81	SNP	Hotspot
31456	C	T	Homozygous	99.7	0	3931.8	SNP	Hotspot
31470	C	T	Heterozygous	70.1	0	1783.85	SNP	Hotspot
31493	C	T	Homozygous	99.5	0	3924.89	SNP	Hotspot
31508	C	T	Homozygous	99	0	3875.01	SNP	Hotspot
31511	C	T	Heterozygous	89.5	0	3021.37	SNP	Hotspot
31530	C	T	Homozygous	99.5	0	3937.64	SNP	Hotspot
31537	C	T	Homozygous	99.2	0	3724.79	SNP	Hotspot
31540	C	T	Homozygous	99	0	3841.02	SNP	Hotspot
31546	C	T	Homozygous	98.5	0	3806.76	SNP	Hotspot
31549	C	T	Homozygous	99.8	0	3964.7	SNP	Hotspot

manufacturer's protocol. To accommodate the fusion method used in next-generation sequencing, each PCR amplicon was designed to be 100–200 nucleotides in length. Multiple amplicons were combined to cover the 264 bp methylation region (31341–31605), and PCR conditions were optimized to evaluate amplification efficiency. Ultimately, four primer sets demonstrating high amplification efficiency and compatibility with next-generation sequencing were identified, enabling coverage of the entire 264 bp target region.

NGS methylation analysis: Libraries prepared from PCR amplicons for each sample were subjected to template preparation using the Ion Chef system (Thermo Fisher Scientific). The resulting templated semiconductor chips were sequenced using the Ion GeneStudio S5 system (Thermo Fisher Scientific). The analysis results for the samples included in this study are presented in Tables 1–5.

Research ethics and COI: This study was conducted with

approval from the Ethics Review Committee of Mie Central Medical Center (Approval Number: MCERB-202010). The authors have no conflicts of interest to declare in relation to this study.

Results

In the glucocorticoid receptor gene (NR3C1), 13 CpG sites

Table 2. Results of hotspot analysis: Sample 2

Position	Ref	Variant	Allele Call	Frequency	Possible Polyploidy Allele	Quality	Type	Allele Source
31438	C	T	Homozygous	99.2	0	3903.14	SNP	Hotspot
31440	C	T	Homozygous	98.5	0	3809.79	SNP	Hotspot
31450	C	T	Homozygous	98.5	0	3799.09	SNP	Hotspot
31456	C	T	Homozygous	100	0	3950.2	SNP	Hotspot
31470	C	T	Heterozygous	65.5	0	1574.42	SNP	Hotspot
31493	C	T	Homozygous	99.8	0	3967.04	SNP	Hotspot
31508	C	T	Homozygous	99.8	0	3965.77	SNP	Hotspot
31511	C	T	Heterozygous	81.8	0	2488.2	SNP	Hotspot
31530	C	T	Homozygous	100	0	4002.89	SNP	Hotspot
31537	C	T	Homozygous	99.5	0	3723.13	SNP	Hotspot
31540	C	T	Homozygous	99.2	0	3870.58	SNP	Hotspot
31546	C	T	Homozygous	99	0	3822.94	SNP	Hotspot
31549	C	T	Homozygous	99.2	0	3876.39	SNP	Hotspot

Table 3. Results of hotspot analysis: Sample 3

Position	Ref	Variant	Allele Call	Frequency	Possible Polyploidy Allele	Quality	Type	Allele Source
31438	C	T	Homozygous	99.8	0	3966.22	SNP	Hotspot
31440	C	T	Homozygous	99	0	3869.38	SNP	Hotspot
31450	C	T	Homozygous	99	0	3863.63	SNP	Hotspot
31456	C	T	Homozygous	99.5	0	3906.19	SNP	Hotspot
31470	C	T	Heterozygous	69.8	0	1792.61	SNP	Hotspot
31493	C	T	Homozygous	99.8	0	3966.81	SNP	Hotspot
31508	C	T	Homozygous	98.8	0	3847.44	SNP	Hotspot
31511	C	T	Heterozygous	83.2	0	2586.65	SNP	Hotspot
31530	C	T	Homozygous	99.7	0	3956.48	SNP	Hotspot
31537	C	T	Homozygous	99.7	0	3880.49	SNP	Hotspot
31540	C	T	Homozygous	99.5	0	3921.72	SNP	Hotspot
31546	C	T	Homozygous	98.5	0	3814.65	SNP	Hotspot
31549	C	T	Homozygous	99.2	0	3893.26	SNP	Hotspot

have been identified as candidate methylation sites within exon 1F and the promoter region. The corresponding base positions are ①31438, ②31440, ③31450, ④31456, ⑤31470, ⑥31493, ⑦31508, ⑧31511, ⑨31530, ⑩31537, ⑪31540, ⑫31546, and ⑬31549 (Tables 1–5).

In the analysis tables, positions where the “Allele Call” is indicated as “Heterozygous” are sites where methylation was

Table 4. Results of hotspot analysis: Sample 4

Position	Ref	Variant	Allele Call	Frequency	Possible Polyploidy Allele	Quality	Type	Allele Source
31438	C	T	Homozygous	99.5	0	3935.34	SNP	Hotspot
31440	C	T	Homozygous	99	0	3871.82	SNP	Hotspot
31450	C	T	Homozygous	99.7	0	3913.26	SNP	Hotspot
31456	C	T	Homozygous	99	0	3864.22	SNP	Hotspot
31470	C	T	Heterozygous	76.5	0	2182.71	SNP	Hotspot
31493	C	T	Homozygous	100	0	4003.23	SNP	Hotspot
31508	C	T	Homozygous	99.2	0	3904.42	SNP	Hotspot
31511	C	T	Heterozygous	83.2	0	2591.61	SNP	Hotspot
31530	C	T	Homozygous	99.5	0	3923.98	SNP	Hotspot
31537	C	T	Homozygous	99	0	3638.69	SNP	Hotspot
31540	C	T	Homozygous	99.2	0	3901.69	SNP	Hotspot
31546	C	T	Homozygous	99.5	0	3925.87	SNP	Hotspot
31549	C	T	Homozygous	99.5	0	3914.11	SNP	Hotspot

Table 5. Results of hotspot analysis: Sample 5

Position	Ref	Variant	Allele Call	Frequency	Possible Polyploidy Allele	Quality	Type	Allele Source
31438	C	T	Homozygous	99.2	0	3879.26	SNP	Hotspot
31440	C	T	Homozygous	99.8	0	3933.91	SNP	Hotspot
31450	C	T	Homozygous	98.8	0	3845.83	SNP	Hotspot
31456	C	T	Homozygous	100	0	3982.89	SNP	Hotspot
31470	C	T	Heterozygous	67.8	0	1685.2	SNP	Hotspot
31493	C	T	Homozygous	99.5	0	3934.85	SNP	Hotspot
31508	C	T	Homozygous	98.8	0	3846.38	SNP	Hotspot
31511	C	T	Homozygous	93	0	3290.27	SNP	Hotspot
31530	C	T	Homozygous	100	0	3980.55	SNP	Hotspot
31537	C	T	Homozygous	99.2	0	3721.33	SNP	Hotspot
31540	C	T	Homozygous	100	0	3990.01	SNP	Hotspot
31546	C	T	Homozygous	99.5	0	3846.2	SNP	Hotspot
31549	C	T	Homozygous	99.5	0	3913.77	SNP	Hotspot

observed. Methylation at position ⑤31470 was observed in all samples, whereas methylation at position ⑧31511 was detected in all samples except sample 5. Samples 1 and 2 were derived from DNA extracted from the same individual and were analyzed to assess the reproducibility and accuracy of the analytical system.

Discussion

Next-generation sequencing is characterized by its ability to simultaneously determine base sequences at numerous loci using short DNA fragments as templates. In this analysis, data from thousands of reads were integrated into a consensus sequence using computational methods. Methylation sites could be readily and automatically identified as SNP-like (single nucleotide polymorphism-like) variations. Because the accuracy of each sample's analysis was evaluated based on the proportion of data derived from thousands of reads, it is considered substantially higher than that achieved with conventional sequencing methods. In terms of sensitivity, even when the concentration of PCR-amplified DNA fragments used for library preparation is low, template semiconductor chips can be generated using the Ion Chef system, enabling a substantially higher number of analyses compared to conventional methods. At present, this analytical system is being applied to reanalyze methylation in 85 cryopreserved samples (saliva and genomic DNA).

Conclusion

Previously, methylation analysis of the promoter region of the glucocorticoid receptor gene (NR3C1) was conducted using

salivary DNA obtained from collaborators in the “Sukusuku Cohort in Mie” through conventional methods. However, analytical efficiency was low, with successful analysis achieved in only 28 of 85 samples, and final determinations were based on sequencing results from approximately 10 clones, resulting in limitations in sensitivity and accuracy. In the present study, the availability of next-generation sequencing enabled investigation of analytical conditions using five volunteer samples. The results demonstrated high sensitivity and accuracy, yielding informative findings. Further improvements in reliability are anticipated through reanalysis of preserved samples using this approach.