

Short Communication

Methylome analysis of thyroid ectopy shows no disease-specific DNA methylation signature

Satoshi Narumi^{1, 2}, Keiko Matsubara¹, Tomohiro Ishii², and Tomonobu Hasegawa²

¹Department of Molecular Endocrinology, National Research Institute for Child Health and Development, Tokyo, Japan

²Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan

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Introduction

Congenital hypothyroidism is the most frequent congenital endocrine disorder, affecting about 1 in 3,000 newborns worldwide. The most common form of permanent and severe congenital hypothyroidism is thyroid dysgenesis (TD), in which athyreosis, thyroid ectopy, and thyroid hypoplasia are included. Among the three forms, thyroid ectopy is the most common. The etiology of TD remains largely unknown (1). A nationwide survey in France revealed that only 2.7% of patients with TD (athyreosis or thyroid ectopy in this study) had a family history of TD in their first-degree relatives (2), indicating that single gene disorders are not the major cause of TD. It is also noteworthy that the onset of TD (athyreosis or thyroid ectopy in this study) has been discordant between 12

out of 13 monozygotic twin pairs (3), suggesting that not only single gene disorders, but also the intrauterine environment are not the major causes. In 2004, based on these epidemiological and clinical observations, De Felice and Di Lauro postulated a DNA methylation defect as a possible mechanism for TD (4), although this hypothesis has never been tested in a structured manner. In this study, we performed a genome-wide DNA methylation analysis (*i.e.*, methylome analysis), using peripheral blood DNA samples from 10 thyroid ectopy patients, to test the hypothesis linking TD and a DNA methylation defect.

Materials and Methods

Subjects

This study was approved by the ethics committees of National Center for Child Health and Development and Keio University School of Medicine. Written informed consent for molecular analysis was obtained from the study subjects and/or their parents. We enrolled 10 Japanese patients with congenital hypothyroidism due to thyroid ectopy. For all 10 subjects, thyroid ectopy was diagnosed by thyroid scintigraphy with ¹²³I. For comparison, we used peripheral blood genomic DNA samples derived from 16 healthy children.

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Corresponding author: Satoshi Narumi, M.D., Ph.D., Department of Molecular Endocrinology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
E-mail: narumi-s@ncchd.go.jp

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DNA methylation analysis

From each subject, genomic DNA was extracted from peripheral blood using the Genra Puregene Blood kit (QIAGEN, Hilden, Germany). Using the EZ DNA methylation kit (Zymo Research, Irvine, CA, USA), 500 ng of DNA was treated with sodium bisulfite to convert cytosine to uracil, while 5-methyl cytosine remained unreactive to the treatment. Bisulfite-treated DNA was hybridized to an Infinium MethylationEPIC BeadChip Kit microarray (Illumina, San Diego, CA, USA) that was scanned with the iScan system (Illumina). The methylation levels (β values) were calculated using GenomeStudio software (V2011.1). Probes on sex chromosomes, non-CpG probes, and probes with missing value(s) or with a detection P value above 0.01 in one or more of the samples were removed from the analysis. In total, 761,047 probes (87.9% of the total probes) were subject to clustering analysis with R3.3.3 (<https://www.r-statistics.com/>). We excluded samples with aberrantly low signal intensities (mean < 2000) or with fewer than 90% of the CpG loci detected.

For targeted analysis of the three thyroid-specific transcription factors [*PAX8* (NM_003466), *NKX2-1* (NM_003317), and *FOXE1* (NM_004473)], β values derived from probes located in the three genes were analyzed. We defined “promoter” probes and “gene body” probes as ones located between +0 to -1,000 and between +1 to transcription termination sites, respectively. Volcano plots were used to display probes with differential methylation levels.

Results

In this study, 10 patients (3 boys and 7 girls) with thyroid ectopy were enrolled. All 10 patients were confirmed to have permanent congenital hypothyroidism by reevaluation of the thyroid function test with the cessation of therapy. The medians (IQRs) of the serum TSH levels and FT₄ levels at reevaluation were 56.4 mU/L (33.4–69.0) and 0.30 ng/dL (0.20–0.58), respectively.

We determined the genome-wide DNA methylation signature for the 10 patients and 16 healthy control children using the Infinium MethylationEPIC BeadChip Kit. Of the samples, 9 patients and 15 healthy controls passed the quality control criteria. In total, 761,047 probes, derived from the 24 subjects (9 patients and 15 controls), were evaluated in the subsequent analyses. To characterize the disease-specific DNA methylation signature, we conducted an unsupervised hierarchical clustering analysis of the genome-wide data (Fig. 1A). The analysis showed no characteristic clustering of the data derived from the patients with thyroid ectopy. This indicated that there are no genome-wide differences in the DNA methylation signatures between the patient group and the control group.

Considering the possibility that the defective DNA methylation of gene(s) that have indispensable roles in thyroid gland development can cause thyroid ectopy without affecting the genome-wide methylation signature, we performed a focused analysis on three thyroid-specific transcription factors, namely, *PAX8*, *NKX2-1*, and *FOXE1*. We compared the β values (*i.e.*, methylation levels) for the “promoter” probes and “gene body” probes between the patient group and the control group. However, again, no probes with hypermethylation/hypomethylation were detected in the patient group, compared to the control group (Fig. 1B).

Discussion

The possible roles for DNA methylation in the thyroid gland have been investigated in thyroid cancer research. Kondo *et al.* showed that *NKX2-1* was hypermethylated and downregulated in 6 out of 10 surgical specimens of undifferentiated thyroid carcinoma (5). For TD, Abu-Khudir *et al.* compared the methylation signatures of resected thyroid tissues (three from patients with thyroid ectopy and three from patients with a hyperfunctioning thyroid nodule), but found no significant differences (6). In our study, a larger

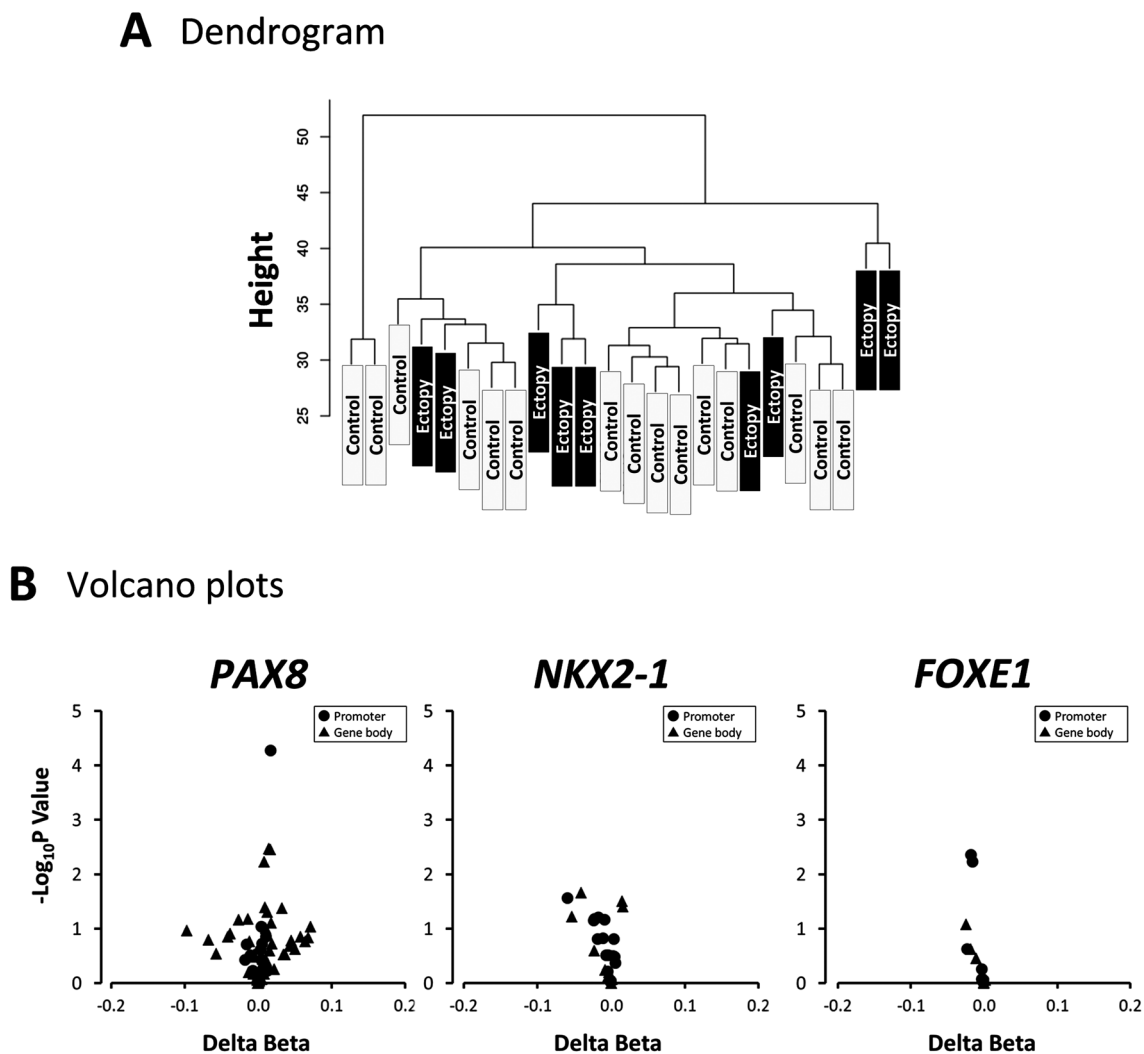


Fig. 1. DNA methylation analysis of the thyroid ectopy group and the control group. (A) The dendrogram for the unsupervised hierarchical clustering analysis showed that the samples were clustered irrespective of the group, indicating that there were no group differences at the whole-genome level. (B) Focused DNA methylation analysis of the three thyroid-specific transcription factors (*PAX8*, *NKX2-1*, and *FOXE1*). Volcano plots showed that there were no probes that fulfilled both a high effect size (low or high Delta Beta value) and a low *P* value.

number of patients (N=10) and controls (N=16) were used to examine the possible association between DNA methylation signatures and TD, but again, the results were negative. At present, the major cause of TD is unknown. It could be due to complex genetic and/or environmental interactions or due to a stochastic mechanism, as previously suggested (1, 4).

We analyzed peripheral blood DNA. This

is a limitation of this study, as the methylation signatures could vary depending on the types of tissues (7). However, there are at least two well-established examples of endocrine disorders due to defective methylation, namely, (i) transient neonatal diabetes mellitus and (ii) pseudohypoparathyroidism type IB, in which disease-associated DNA methylation defects can be reliably detected in peripheral blood DNA.

Therefore, we suppose that tissue specificity is not a major obstacle. Other limitations include the relatively small number of subjects, and the restricted thyroid morphology type (thyroid ectopy).

We presume two approaches would be feasible and worth considering in the future. One is a genome-wide association study, in which genetic variations with relatively weak effects can be detected. The other is *in vitro* recapitulation of thyroid development with the use of iPS cells derived from TD patients. This cell-based approach could possibly allow for the elucidation of non-genetic mechanism(s).

In conclusion, we performed a comprehensive DNA methylation analysis of 10 patients with thyroid ectopy, but could not find any significant differences in the DNA methylation signatures. The major mechanism responsible for TD remains unclear at present.

Conflict of Interest: The authors have nothing to disclose.

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