

# Association of polymorphisms in *DNMT1*, *DNMT3A*, *DNMT3B*, *MTHFR* and *MTRR* genes with global DNA methylation levels and prognosis of autoimmune thyroid disease

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## Introduction

Autoimmune thyroid diseases (AITDs), such as Graves' disease (GD) and Hashimoto's disease (HD), are typical autoimmune diseases [1,2]. The severity of HD and the intractability (that is, inducibility to remission) of GD varies among patients. Some patients with HD develop hypothyroidism earlier in life, while some maintain a euthyroid state even up to old age. Some patients with GD achieve remission through medical treatment, whereas others do not [3,4]. However, the intractability of GD and the severity of HD are very difficult to predict at diagnosis.

DNA methylation occurs at cytosine residues in cytosine–phosphate–guanosine (CpG) dinucleotides and involves methylation of the fifth carbon of the pyrimidine ring leading to the formation of 5-methylcytosine (5-mC). The majority of CpG sites (70–80%) in human DNA are methylated and many of the non-methylated sites are found in so-called CpG islands, which are sites of transcription

## Summary

To clarify the association between factors regulating DNA methylation and the prognosis of autoimmune thyroid diseases (AITDs), we genotyped single nucleotide polymorphisms in genes encoding DNA methyltransferase 1 (DNMT1), DNMT3A, DNMT3B, methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR), which are enzymes essential for DNA methylation. Subjects for this study included 125 patients with Hashimoto's disease (HD), including 48 patients with severe HD and 49 patients with mild HD; 176 patients with Graves' disease (GD), including 79 patients with intractable GD and 47 patients with GD in remission; and 83 healthy volunteers (control subjects). The *DNMT1* +32204GG genotype was more frequent in patients with intractable GD than in patients with GD in remission. Genomic DNA showed significantly lower levels of global methylation in individuals with the *DNMT1* +32204GG genotype than in those with the AA genotype. The *MTRR* +66AA genotype was observed to be more frequent in patients with severe HD than in those with mild HD. The *DNMT1* +14395A/G, *DNMT3B* –579G/T, *MTHFR* +677C/T and +1298A/C polymorphisms were not correlated with the development or prognosis of AITD. Our study indicates that the *DNMT1* +32204GG genotype correlates with DNA hypomethylation and with the intractability of GD, and that the *MTRR* +66AA genotype may correlate with the severity of HD.

**Keywords:** DNMT1, intractability, methylation, single nucleotide polymorphism, severity

initiation [5]. Several studies have reported a strong correlation between DNA methylation and gene expression [6]. In addition, DNA methylation is one of the epigenetic processes regulating several biological events, including embryonic development, transcriptional regulation, X-chromosome inactivation, genomic imprinting and chromatin modification [7]. Altered DNA methylation patterns have been associated with tumorigenic events and development of autoimmune diseases [8].

DNA methylation is established and maintained by DNA methyltransferases (DNMTs). In humans, three enzymes, DNMT1, DNMT3A and DNMT3B, are known to have DNMT activity. DNMT3A and DNMT3B are responsible for *de-novo* methylation and modification of unmethylated DNA, whereas DNMT1 is required to maintain DNA methylation [9,10]. Previous studies have shown that mRNA levels of DNMT1 and DNMT3A are reduced in patients with atopic dermatitis [11], and that DNMT1 mRNA levels were also decreased in patients with systemic lupus erythematosus

(SLE) [12]. There are several noteworthy polymorphisms in the genes encoding enzymes.

It has been reported that the A-allele of the *DNMT1* +14395A/G polymorphism (rs16999593) is present more frequently in patients with infiltrating ductal breast carcinoma than among controls [13]. The *DNMT1* +32204A/G (rs2228612) polymorphism is a non-synonymous substitution in which the frequency of the minor allele is 5% in the Japanese population, according to the National Center for Biotechnology Information (NCBI)-SNP (<http://www.ncbi.nlm.nih.gov/snp/>) and Japanese (J)-SNP databases (<http://snp.ims.u-tokyo.ac.jp/>). The A-allele of the *DNMT3A* -448A/G (rs1550157) polymorphism showed significantly higher promoter activity (>twofold) compared to the G-allele [14]. Carriers of the T-allele of the *DNMT3B* -283T/C polymorphism (rs6087990) showed significantly lower promoter activity compared to carriers of the C-allele [15]. However, unambiguous genotyping of the *DNMT3B* -283T/C polymorphism by restriction fragment length polymorphism (RFLP) analysis is complex.

Therefore, we examined The *DNMT3B* -579G/T polymorphism (rs1569686), which is in linkage disequilibrium (LD) with the -283T/C polymorphism. The two common haplotypes formed by these SNPs, -283T/-579G and -283C/-579T, account for 98% of the chromosome [15].

Methylenetetrahydrofolate reductase (MTHFR), which is involved in the supply of the methylation group, is an enzyme necessary for the folate metabolic pathway (Fig. 1) and is considered to result in hypermethylation of genomic DNA [16,17]. The *MTHFR* +677C/T polymorphism (rs1801133) results in an alanine (C)-to-valine (T) substitution and renders the enzyme less active [18,19]. The *MTHFR* +1298A/C polymorphism (rs1801131) results in a glutamic acid (A)-to-alanine (C) substitution and the CC genotype of the SNP results in a significant decrease of MTHFR activity [20].

Methionine synthase reductase (MTRR) plays a crucial role in maintaining the active state of methionine synthase

(MTR), which is associated with an increase in the DNA methylation level [21,22]. Because the minor allele frequency of a functional polymorphism in the *MTR* gene (rs1805087) [23] was less than 5% in the Japanese population, we focused on another polymorphism in the *MTRR* gene as an alternative candidate. The most common polymorphism in the *MTRR* gene is the +66A/G polymorphism (rs1801394), which results in an isoleucine (A)-to-methionine (G) substitution at position 22; its minor allele frequency in the Japanese population is 30%. The *MTRR* +66AA genotype may induce hypomethylation of DNA by the regulation of homocysteine levels [21,24].

In this study, to elucidate the association of *DNMT1*, *DNMT3A*, *DNMT3B*, *MTHFR* and *MTRR* polymorphisms with the prognosis of AITDs and DNA methylation levels, we genotyped these polymorphisms and investigated global methylation levels of DNA.

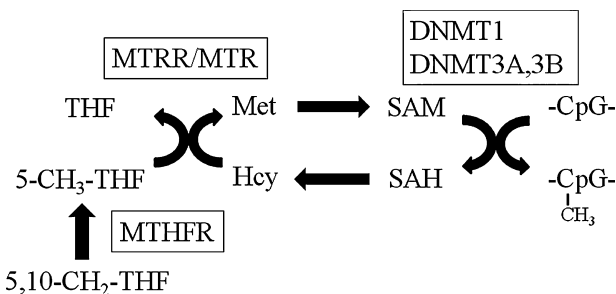
## Materials and methods

### Subjects

We screened each polymorphism among 125 patients (17 men and 108 women) with HD, 176 patients (30 men and 146 women) with GD, and 83 healthy volunteers (control subjects; 29 men and 54 women). Patients with HD were positive for anti-thyroid microsomal antibody (McAb) or anti-thyroglobulin antibody (TgAb), and showed hypothyroidism or euthyroidism with palpable diffuse goitre. Patients with GD had a clinical history of thyrotoxicosis with a positive test for anti-thyrotrophin receptor antibody (TRAb). Healthy volunteers were euthyroid and negative for thyroid autoantibodies.

Forty-eight of these patients (seven men and 41 women) with HD developed moderate to severe hypothyroidism before 50 years of age, and were treated on a daily basis (subgroup with severe HD). Forty-nine untreated euthyroid HD patients (six men and 43 women) were more than 50 years of age (subgroup with mild HD). Seventy-nine euthyroid patients (15 men and 64 women) with GD had been treated with methimazole for at least 5 years and were still positive for TRAb (subgroup with intractable GD). Forty-seven patients (seven men and 40 women) with GD in remission had maintained a euthyroid state and were negative for TRAb for more than 2 years without medication (subgroup with GD in remission).

All patients and control subjects were Japanese and were unrelated to each other. All patients were followed-up closely for more than 5 years as out-patients at our thyroid clinic. Genomic DNA was isolated from ethylenediamine tetraacetic acid (EDTA)-treated peripheral blood mononuclear cells with a commercially available kit (Dr. GenTLE™, Takara Bio Inc., Shiga, Japan). Written informed consent was obtained from all patients and controls, and the study protocol was approved by the Ethics Committee of Osaka University.



**Fig. 1.** Summary of folate metabolic pathway. MTHFR: 5,10-methylenetetrahydrofolate reductase; MTR: methionine synthase; MTRR: methionine synthase reductase; THF: tetrahydrofolate; Met: methionine; 5,10-CH<sub>2</sub>-THF: 5,10-methylene-THF; 5-CH<sub>3</sub>-THF: 5-methyltetrahydrofolate; SAM: S-adenosylmethionine; Hcy: homocysteine; SAH: S-adenosylhomocysteine.

**Table 1.** Clinical characteristics of the patients with autoimmune thyroid disease at the time of sampling.

	Controls	Graves' disease Past clinical history of thyrotoxicosis with elevated TRAb		Hashimoto's disease Diffuse goitre and positive TgAb and/or McAb	
		Intractable	In remission	Severe	Mild
<i>n</i> (female/male)	83 (54/29)	50 (42/8)	47 (40/7)	48 (41/7)	49 (43/6)
Age of onset (years) (range)	40.2 ± 15.9 <sup>‡</sup> (19–76)	34.0 ± 13.5 (11–66)	32.6 ± 13.3 (10–66)	37.7 ± 10.5 (10–49)	59.0 ± 10.3 <sup>‡</sup> (50–92)
Goitre size (cm)	n.d.	5.0 ± 1.4*	4.2 ± 0.6	4.2 ± 1.0**	4.7 ± 1.3
Free T4 (ng/dl)	n.d.	1.2 ± 0.3	1.2 ± 0.2	1.4 ± 0.3	1.2 ± 0.2
Free T3 (pg/ml)	n.d.	2.7 ± 0.6	2.6 ± 0.3	2.6 ± 0.6	2.8 ± 0.4
TSH (μU/ml)	n.d.	1.6 ± 1.2	1.8 ± 1.2	1.8 ± 1.3	2.8 ± 1.7
TRAb (IU/l) (range)	<1.0	7.9 ± 13.2 (1.1–71.0)	<1.0	<1.0	<1.0
TgAb (2 <sup>n</sup> × 100)	Negative	2.8 ± 2.9	2.3 ± 2.7	7.1 ± 3.4**	1.5 ± 2.2
McAb (2 <sup>n</sup> × 100)	Negative	4.3 ± 2.7	5.3 ± 2.1	5.4 ± 3.1	3.9 ± 3.1
Current treatment	None	Methimazole or PTU	None	L-thyroxine	None
Duration of treatment (years)	None	11.7 ± 7.3	3.2 ± 1.3 <sup>§</sup>	10.9 ± 8.5	None
Current dose of anti-thyroid drug (mg/day) <sup>†</sup> (range)	None	18.2 ± 32.2 (2.5–200)	None	None	None
Current dose of L-thyroxine (μg/day) (range)	None	None	None	91.1 ± 36.8 (50–250)	None

Data are expressed as mean ± standard deviation. \**P* < 0.01 (*versus* Graves' disease in remission); \*\**P* < 0.01 (*versus* mild Hashimoto's disease).  
<sup>†</sup>Dose were expressed as the comparable dose of methimazole (50 mg of PTU was converted to 5 mg of methimazole). <sup>‡</sup>Age at the time of sampling.  
<sup>§</sup>Duration of treatment with anti-thyroid drug before remission. T4: thyroxine; T3: triiodothyronine; TSH: thyrotrophin; TRAb: anti-thyrotrophin receptor antibody; McAb: anti-thyroid microsomal antibody; n.d.: not determined; TgAb: anti-thyroglobulin antibody; PTU: propylthiouracil.

Clinical characteristics of the examined subjects are given in Table 1.

### Genotyping of polymorphisms

We used RFLP analysis for genotyping the *DNMT1* +32204A/G, *DNMT1* +14395A/G, *DNMT3B* –579G/T, *MTHFR* +677C/T and *MTHFR* +1298A/C polymorphisms. Target sequences of each gene were amplified using polymerase chain reaction (PCR), and the PCR product was digested by the addition of restriction enzyme. The

sequences of forward and reverse primers, the PCR conditions and restriction enzymes used are summarized in Table 2. *TaqMan* SNP genotyping assays (Applied Biosystems, Tokyo, Japan) were used to genotype *DNMT3A* –448A/G and *MTRR* +66A/G polymorphisms.

### Global methylation levels of DNA in peripheral whole blood cells

The global methylation levels of genomic DNA isolated from the whole blood were determined by a commercially

**Table 2.** The primers, polymerase chain reaction (PCR) conditions and restriction enzymes used in this study.

	Primer pairs	PCR conditions	Restriction enzymes
<i>DNMT1</i>	+32204A/G 5'-AGAACCTGAAAAAGTAAATCCACCG-3' 5'-CATGTGATTCACCCGCTTCAG-3'	96°C for 5 min (94°C for 30 s, 62°C for 30 s, 72°C for 30 s) × 35 cycles 72°C for 7 min	MspI
	+14395A/G 5'-TGTTTCCTTGTCTCTGACAC-3' 5'-TTGGTTCCCGTTTCTAGAC-3'	94°C for 5 min (94°C for 30 s, 62°C for 30 s, 72°C for 30 s) × 30 cycles 72°C for 5 min	BstEII
<i>DNMT3B</i>	–579G/T 5'-GAGGTCTCATTATGCCTAGG-3' 5'-GGGAGCTCACCTTCTAGAAA-3'	96°C for 5 min (96°C for 30 s, 56°C for 30 s, 72°C for 30 s) × 30 cycles 72°C for 5 min	PvuII
<i>MTHFR</i>	+677C/T 5'-TGAAGGAGAAGGTGTCTGCGG-3' 5'-AGGACGGTGCGGTGAGAGTG-3'	94°C for 5 min (96°C for 30 s, 66°C for 30 s, 72°C for 30 s) × 30 cycles 72°C for 5 min	HinfI
	+1298G/T 5'-CTTGGGGGAGCTGAAGGACTACTAC-3' 5'-CACTTTGTGACCATTCCGGTTTG-3'	96°C for 5 min (96°C for 30 s, 61°C for 30 s, 72°C for 30 s) × 32 cycles 72°C for 5 min	MboII

**Table 3.** Genotype and allele frequencies of polymorphisms genotyped in this study in patients with autoimmune thyroid diseases and control subjects.

		Control	All patients with AITD		All patients with Graves' disease		All patients with Hashimoto's disease	
<i>DNMT1</i>	AA	30 (45.5%)	128 (46.5%)	n.s.	83 (48.3%)	n.s.	45 (43.7%)	n.s.
rs2228612	AG	26 (39.4%)	118 (42.9%)		70 (40.7%)		48 (46.6%)	
+32204A/G	GG	10 (15.1%)	29 (10.6%)		19 (11.0%)		10 (9.7%)	
	A allele	86 (65.1%)	374 (68.0%)	n.s.	236 (68.6%)	n.s.	138 (67.0%)	n.s.
	G allele	46 (34.9%)	176 (32.0%)		108 (31.4%)		68 (33.0%)	
<i>DNMT1</i>	AA	42 (61.8%)	175 (64.0%)	n.s.	101 (66.5%)	n.s.	74 (66.1%)	n.s.
rs16999593	AG	24 (35.2%)	77 (31.5%)		44 (29.0%)		33 (29.4%)	
+14395A/G	GG	2 (3.0%)	12 (4.5%)		7 (4.5%)		5 (4.5%)	
	A allele	108 (79.4%)	427 (80.9%)	n.s.	246 (80.9%)	n.s.	181 (80.8%)	n.s.
	G allele	28 (20.6%)	101 (19.1%)		58 (19.1%)		43 (19.2%)	
<i>DNMT3A</i>	GG	36 (54.6%)	163 (56.8%)	n.s.	98 (57.0%)	n.s.	65 (56.5%)	n.s.
rs1550157	GA	25 (37.9%)	111 (38.7%)		68 (39.5%)		43 (37.4%)	
-448A/G	AA	5 (7.5%)	13 (4.5%)		6 (3.5%)		7 (6.1%)	
	G allele	97 (73.5%)	437 (76.1%)	n.s.	264 (76.7%)	n.s.	173 (72.1%)	n.s.
	A allele	35 (26.5%)	137 (23.9%)		80 (23.3%)		57 (27.9%)	
<i>DNMT3B</i>	TT	68 (82.9%)	216 (82.1%)	n.s.	115 (81.0%)	n.s.	101 (83.5%)	n.s.
rs1569686	TG	13 (15.9%)	44 (16.7%)		25 (17.6%)		19 (15.7%)	
-579 G/T	GG	1 (1.2%)	3 (1.2%)		2 (1.4%)		1 (0.8%)	
	T allele	131 (91.0%)	476 (90.5%)	n.s.	255 (89.8%)	n.s.	221 (91.3%)	n.s.
	G allele	13 (9.0%)	50 (9.5%)		29 (10.2%)		21 (8.7%)	
<i>MTHFR</i>	CC	36 (42.9%)	99 (35.5%)	n.s.	54 (33.8%)	n.s.	45 (37.8%)	n.s.
rs1801133	CT	35 (41.7%)	142 (50.9%)		79 (49.4%)		63 (52.9%)	
+677C/T	TT	13 (15.4%)	38 (13.6%)		27 (16.8%)		11 (9.3%)	
	C allele	106 (63.9%)	340 (60.9%)	n.s.	187 (56.7%)	n.s.	153 (64.3%)	n.s.
	T allele	60 (36.1%)	218 (39.1%)		143 (43.3%)		85 (35.7%)	
<i>MTHFR</i>	AA	37 (57.8%)	170 (62.7%)	n.s.	95 (60.5%)	n.s.	75 (65.8%)	n.s.
rs1801131	AC	26 (40.6%)	96 (35.4%)		60 (38.2%)		36 (31.6%)	
+1298A/C	CC	1 (1.6%)	5 (1.9%)		2 (1.3%)		3 (2.6%)	
	A allele	84 (76.4%)	436 (80.4%)	n.s.	250 (79.6%)	n.s.	186 (81.6%)	n.s.
	C allele	26 (23.6%)	106 (19.6%)		64 (20.4%)		42 (18.4%)	
<i>MTRR</i>	AA	37 (52.9%)	141 (49.1%)	n.s.	80 (51.6%)	n.s.	61 (46.2%)	n.s.
rs1801394	AG	27 (38.6%)	117 (40.8%)		58 (37.4%)		59 (44.7%)	
+66A/G	GG	6 (8.5%)	29 (10.1%)		17 (11.0%)		12 (9.1%)	
	A allele	101 (72.1%)	399 (70.0%)	n.s.	218 (70.3%)	n.s.	181 (68.6%)	n.s.
	G allele	39 (27.9%)	175 (30.0%)		92 (29.7%)		83 (31.4%)	

AITD: autoimmune thyroid diseases; DNMT: DNA methyltransferase; MTRR: methionine synthase reductase; MTHFR: 5,10-methylenetetrahydrofolate reductase; n.s.: not significant.

available kit (MethylFlash™ Methylated DNA Quantification Kit; Epigentek, New York, USA). We examined 13 patients with GD in remission (three men and 10 women;  $42.4 \pm 15.5$ ), 12 patients with mild HD (two men and 10 women;  $60.8 \pm 9.6$ ) and 28 healthy volunteers (10 men and 18 women;  $38.2 \pm 12.3$ ), because these subjects did not receive any medical treatment. The proportion of 5-methylcytosine in the whole DNA was expressed as a percentage of methylation.

### Statistical analysis

We used the  $\chi^2$  and Fisher's exact tests to evaluate the significance of differences between the genotype and allele frequencies among the subject's groups. Tukey's honestly significant difference test was used for multiple comparisons

to analyse the differences between global DNA methylation levels among each genotype. Data were analysed with JMP8 software (SAS Institute Inc., Tokyo, Japan). Probability values of less than 0.05 were considered significant.

## Results

### *DNMT1* polymorphisms

Although there was no significant difference in genotype and allele frequencies of the *DNMT1* +32204A/G polymorphisms between healthy controls and patients with AITD (Table 3), the G allele and GG genotype of this polymorphism was significantly more frequent in patients with intractable GD than in those with GD in remission ( $P = 0.033$  and  $P = 0.005$ , respectively, Table 4).

**Table 4.** Genotype and allele frequencies of polymorphisms genotyped in this study in patients with Graves' disease and Hashimoto's disease.

		Graves' disease				Hashimoto's disease		
		Intractable	In remission	P value	Odds ratio (95% CI)	Severe	Mild	P value
<i>DNMT1</i>	AA	35 (44.3%)	25 (53.2%)	0.009*		18 (40.0%)	17 (44.7%)	n.s.
rs2228612	AG	29 (36.7%)	21 (44.7%)			22 (48.9%)	16 (42.1%)	
+32204A/G	GG	15 (19.0%)	1 (2.1%)			5 (11.1%)	5 (13.2%)	
	A Allele	99 (62.7%)	71 (75.5%)	0.033*	1.8	58 (64.4%)	50 (65.8%)	n.s.
	G Allele	59 (37.3%)	23 (24.5%)		(1.0–3.3)	32 (35.6%)	26 (34.2%)	
	AA+AG	64 (81.0%)	46 (97.9%)	0.005**	10.8	40 (88.9%)	33 (86.8%)	n.s.
	GG	15 (19.0%)	1 (2.1%)		(1.4–84.5)	5 (11.1%)	5 (13.2%)	
<i>DNMT1</i>	AA	46 (62.2%)	25 (67.6%)	n.s.		29 (60.4%)	28 (68.3%)	n.s.
rs16999593	AG	24 (32.4%)	11 (29.7%)			18 (37.5%)	10 (24.4%)	
+14395A/G	GG	4 (5.4%)	1 (2.7%)			1 (2.7%)	3 (7.3%)	
	A Allele	116 (78.4%)	61 (82.4%)	n.s.		76 (79.2%)	66 (80.5%)	n.s.
	G Allele	32 (21.6%)	13 (17.6%)			20 (20.8%)	16 (19.5%)	
<i>DNMT3A</i>	GG	38 (50.7%)	25 (56.8%)	n.s.		23 (60.5%)	22 (44.9%)	n.s.
rs1550157	GA	34 (45.3%)	17 (38.6%)			12 (31.6%)	23 (46.9%)	
–448A/G	AA	3 (4.0%)	2 (4.6%)			3 (7.9%)	4 (8.2%)	
	G Allele	110 (73.3%)	67 (76.1%)	n.s.		58 (76.3%)	67 (68.4%)	n.s.
	A Allele	40 (26.7%)	21 (23.9%)			18 (23.7%)	31 (31.6%)	
<i>DNMT3B</i>	TT	51 (79.7%)	30(76.9%)	n.s.		39 (86.7%)	36 (78.3%)	n.s.
rs1569686	TG	13 (20.3%)	8 (20.5%)			5 (11.1%)	10 (21.7%)	
–579 G/T	GG	0 (0.0%)	1 (2.6%)			1 (2.2%)	0 (0.0%)	
	T Allele	91 (87.5%)	65 (87.8%)	n.s.		85 (92.4%)	84 (89.4%)	n.s.
	G Allele	13 (12.5%)	9 (12.2%)			7 (7.6%)	10 (10.6%)	
<i>MTHFR</i>	CC	27 (36.5%)	13 (27.7%)	n.s.		15 (40.5%)	25 (45.5%)	n.s.
rs1801133	CT	36 (48.6%)	25 (53.2%)			18 (48.7%)	28 (50.9%)	
+677C/T	TT	11 (14.9%)	9 (19.1%)			4 (10.8%)	2 (3.6%)	
	C Allele	90 (60.8%)	45 (59.2%)	n.s.		58 (61.7%)	78 (71.0%)	n.s.
	T Allele	58 (39.2%)	31 (40.8%)			36 (38.3%)	32 (29.0%)	
<i>MTHFR</i>	AA	43 (55.1%)	28 (62.2%)	n.s.		26 (57.8%)	29 (63.0%)	n.s.
rs1801131	AC	34 (43.6%)	17 (37.8%)			18 (40.0%)	15 (32.6%)	
+1298A/C	CC	1 (1.3%)	0 (0.0%)			1 (2.2%)	2 (4.4%)	
	A Allele	120 (76.9%)	73 (75.7%)	n.s.		48 (80.0%)	73 (79.3%)	n.s.
	C Allele	36 (23.1%)	17 (24.3%)			12 (20.0%)	19 (20.7%)	
<i>MTRR</i>	AA	34 (50.0%)	21 (53.9%)	n.s.		26 (57.8%)	22 (37.9%)	n.s.
rs1801394	AG	28 (41.2%)	16 (41.0%)			16 (35.5%)	30 (51.7%)	
+66A/G	GG	6 (8.8%)	2 (5.1%)			3 (6.7%)	6 (10.4%)	
	A Allele	96 (70.6%)	58 (74.4%)	n.s.		68 (75.6%)	74 (63.8%)	n.s.
	G Allele	40 (29.4%)	20 (25.6%)			22 (24.4%)	42 (36.2%)	
	AA	34 (50.0%)	21 (53.8%)	n.s.		26 (57.8%)	22 (37.9%)	0.045*
	AG+GG	34 (50.0%)	18 (46.2%)			19 (42.2%)	36 (62.1%)	

\*Value calculated by the  $\chi^2$  test, \*\*Value calculated by Fisher's exact test. DNMT: DNA methyltransferase; MTRR: methionine synthase reductase; MTHFR: 5,10-methylenetetrahydrofolate reductase; n.s.: not significant.

The genotype and allele frequencies of the *DNMT1* +14395A/G polymorphisms showed no difference between healthy controls and patients with AITD (Table 3), and showed no evidence of being involved in the prognosis of AITD (Table 4).

#### *DNMT3A* and *DNMT3B* polymorphisms

Genotype and allele frequencies of the *DNMT3A* –448A/G and *DNMT3B* –579G/T polymorphisms showed no significant differences between healthy controls and patients with

AITD (Table 3), and were not involved in the prognosis of AITD (Table 4).

#### *MTHFR* polymorphisms

Genotype and allele frequencies of the *MTHFR* +677C/T and +1298A/C polymorphisms showed no significant differences between healthy controls and patients with AITD (Table 3); these genotype and allele frequencies did not influence prognosis of AITD (Table 4).

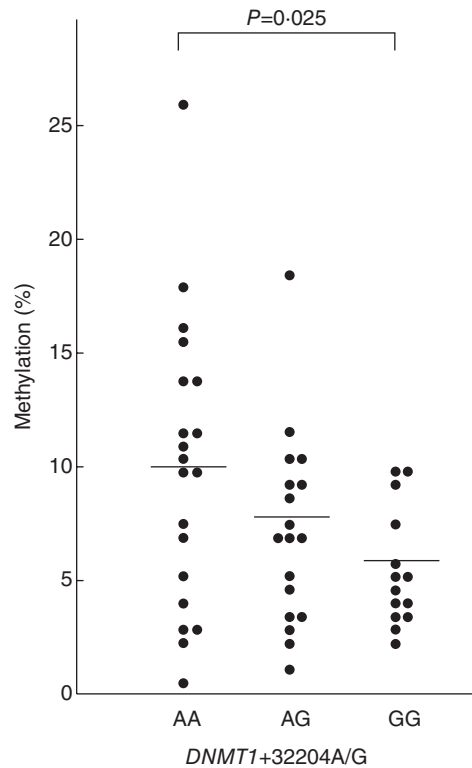


Fig. 2. Methylation levels in individuals with each genotype of *DNMT1* +32204 A/G polymorphism.

### *MTRR* polymorphism

The *MTRR* +66AA genotype was observed to be more frequent in patients with severe HD than in patients with mild HD ( $P = 0.045$ , Table 4), although we found no significant differences in genotype or allele frequencies of this polymorphism between patients with AITD and healthy control subjects (Table 3).

### DNA methylation levels and polymorphisms in methylation-related genes

DNA methylation levels in individuals with *DNMT1* +32204GG genotype were significantly lower than those in carriers of the AA genotype ( $P = 0.025$ , Fig. 2). We were unable to obtain any evidence of a significant relationship between each of the polymorphisms and DNA methylation levels (data not shown).

Conversely, *MTRR* +66AG polymorphisms were not associated with DNA methylation levels (Fig. 3).

### Clinical characteristics and polymorphisms in methylation-related genes

No association was observed between each allele or genotype of the examined polymorphisms and the levels of TRAb, McAb or TgAb, or goitre size (data not shown).

### Discussion

In the present study, we observed that the *DNMT1* +32204GG genotype was correlated with lower levels of global DNA methylation (Fig. 2). Because this polymorphism is located in the region of the *DNMT1* gene encoding the nuclear localization sequence (NLS)-containing domain (NlsD) of the enzyme, which plays an important role in binding to CpG sites [25], we anticipate that the substitution of Ile (+32204A allele) to Val (+32204G allele) may transform the structure of the NlsD and decrease the DNA-binding ability of the enzyme; therefore, DNA methylation levels may be lower in individuals with the +32204GG genotype. To the best of our knowledge, this is the first report demonstrating that a functional polymorphism in the *DNMT1* gene is related to methylation levels of DNA. In addition, The *DNMT1* +32204GG genotype was observed to be more frequent in patients with intractable GD than in those with GD in remission (Table 3). This indicates that the +32204GG genotype, which is related to a lower level of global DNA methylation, is associated with the intractability of GD.

In addition, we have reported previously that genetic programming for production of higher interleukin (IL)-1 $\beta$  and transforming growth factor (TGF)- $\beta$  levels are associated with intractability of GD [26,27]. It is well known that demethylation of the CpG site in the promoter regions enhance gene expression [28,29]. Therefore, lowered global DNA methylation ability in individuals with the +32204GG genotype may decrease the methylation levels of *IL1B* and *TGFB*

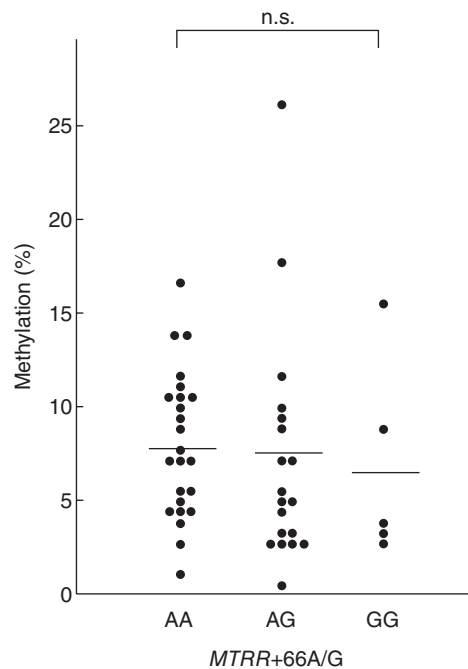


Fig. 3. Methylation levels in individuals with each genotype of methionine synthase reductase (*MTRR*) +66A/G polymorphism.

promoter regions and enhance the production of IL-1 $\beta$  and TGF- $\beta$ . This may be a potential candidate mechanism to impede induction remission in GD patients with *DNMT1* +32204GG genotype.

In addition, we clarified that the *MTRR* +66AA genotype was more frequent in patients with severe HD than in those with mild HD. Previous reports showed that homocysteine levels were increased in plasma obtained from individuals with the *MTRR* +66AA genotype [21]. There are two possible mechanisms that may explain how the *MTRR* +66AA genotype is related to the severity of HD. One possibility is that increased homocysteine levels may cause an insufficient supply of methyl groups and result in a decrease in global DNA methylation [24]. In this study, however, global methylation levels of DNA were not associated with the genotypes of the *MTRR* +66A/G polymorphism (Fig. 3). Another possibility is that an increase in homocysteine levels may increase caspase-3 activity and, thus, apoptosis in thyroid epithelial cells in HD patients with the *MTRR* +66AA genotype, as it has been reported that homocysteine induces caspase-3 activity and apoptosis in various cells [30–33]. Previous reports also suggest that caspase-3 is highly expressed in thyroid epithelial cells in HD patients [34].

In conclusion, the *DNMT1* +32204GG genotype is associated with hypomethylation of DNA and is related to the intractability of GD, while the *MTRR* +66A/G polymorphism is associated with the severity of HD.

## Declarations of interest

No potential conflicts of interest were disclosed.

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