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Associations of single nucleotide polymorphisms in precursor-microRNA (miR)-125a and the expression of mature miR-125a with the development and prognosis of autoimmune thyroid diseases

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Summary

It is important to search the biomarker to predict the development and prognosis of autoimmune thyroid diseases (AITDs) such as Hashimoto's disease (HD) and Graves' disease (GD). MicroRNA (miR) bind directly to the 3′ untranslated region of specific target mRNAs to suppress the expression of proteins, promote the degradation of target mRNAs and regulate immune response. miR-125a is known to be a negative regulator of regulated upon activation normal T cell expressed and secreted (RANTES), interleukin (IL)-6 and transforming growth factor (TGF)-β; however, its association with AITDs remains unknown. To clarify the association between AITDs and miR-125a, we genotyped the rs12976445 C/T, rs10404453 A/G and rs12975333 G/T polymorphisms in the *MIR125A* gene, which encodes miR-125a, using direct sequencing and polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) methods in 155 patients with GD, 151 patients with HD and 118 healthy volunteers. We also examined the expression of miR-125a in peripheral blood mononuclear cells (PBMCs) from 55 patients with GD, 79 patients with HD and 38 healthy volunteers using quantitative real-time PCR methods. We determined that the CC genotype and C allele of the rs12976445 C/T polymorphism were significantly more frequent in patients with HD compared with control subjects ($P < 0{\cdot}05$) and in intractable GD compared with GD in remission ($P < 0{\cdot}05$). The expression of miR-125a was correlated negatively with age ($P = 0{\cdot}0010$) and down-regulated in patients with GD compared with control subjects ($P = 0.0249$). In conclusion, miR-125a expression in PBMCs and the rs12976445 C/T polymorphism were associated with AITD development and prognosis.

Keywords: autoimmune thyroid disease, intractability, miR-125a, polymorphism, severity

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Introduction

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

MicroRNAs (miR) are endogenously encoded single-stranded RNAs approximately 22 nucleotides in length, and they have been known to play essential roles in a variety of pathological conditions [5]. miR suppress post-transcriptional gene expression by binding specifically to their target messenger RNAs (mRNAs) and inducing either translational repression or mRNA degradation [6]. miR regulate various biological processes such as cell proliferation, differentiation, metabolism, apoptosis, development, inflammation and immunity [5–15]. Recently, strong associations between miR and the immune system have been reported, and it has been suggested that miR also have

regulatory roles in the immune response [8,10,13]. miR are initially transcribed as long primary transcripts (pri-miRNA), and they are processed into a ~65 nt hairpin-shaped precursor miR (pre-miRNA) [16]. The pri-miRNA processing is a critical step in miRNA biogenesis because it regulates the expression level of mature miRNA [17,18]. Pre-miRNA are subsequently exported to the cytoplasm and cleaved to generate a 18~25 nt mature miRNA [13].

The *MiR125A* gene, which encodes miR-125a, is located on chromosome 19q13.41 in a gene cluster containing *MIR99B* and *MIR7E*. miR-125a is known to be a negative regulator of Kruppel-like factor 13 (KLF13) and the tumour necrosis factor α-induced protein 3 (TNFAIP3), inhibits the production of regulated on activation normal T cell expressed and secreted (RANTES) and promotes the nuclear factor kappa B (NF-κB) pathway [12,15]. It has been reported that miR-125a is down-regulated in systemic lupus erythematosus (SLE) [15,19], breast cancer [20], gastric cancer [21], ovarian cancer [22] and verrucous carcinoma [14], although the roles of miR-125a in AITD still remain unclear. Therefore, we performed a quantification of miR-125a expression in peripheral blood mononuclear cells (PBMCs).

A G/T single-nucleotide polymorphism, named rs12975333, has been identified in the *MIR125A* gene, and its T allele blocks the pri- to pre-miR-125a processing step [17]. There are also two polymorphisms in this gene, rs10404453 A/G and rs12976445 C/T, which may be correlated with the expression of mature miR-125a in patients with AITDs. In this study, we genotyped these three *MIR125A* SNPs in patients with AITD.

Materials and methods

Subjects for genotyping

We screened the study polymorphisms in 155 patients with GD, 151 patients with HD and 118 healthy volunteers. Among GD patients, 60 GD patients had been treated with methimazole for at least 5 years and were still positive for anti-thyrotrophin receptor antibody (TRAb) (intractable GD), 45 GD patients had maintained a euthyroid state and were negative for TRAb for more than 2 years without medication (GD in remission), and 50 patients who could not be categorized to intractable GD or GD in remission groups at the time of analysis. All patients with GD had clinical histories of positive TRAb and thyrotoxicosis. Among HD patients, 59 HD patients had developed moderate to severe hypothyroidism before 50 years of age and been treated with thyroxine (severe HD), 41 untreated euthyroid HD patients were aged more than 50 years (mild HD), and 51 patients who could not be categorized to severe HD or mild HD groups at the time of analysis. All patients with HD were positive for anti-thyroid microsomal antibody (McAb) and/or anti-thyroglobulin antibody (TgAb) and all patients with mild HD had a palpable diffuse goitre. All healthy volunteers were euthyroid and negative for thyroid-specific autoantibodies (control subjects). All patients and control subjects were Japanese and unrelated. Written informed consent was obtained from all patients and controls and the study protocol was approved by the Ethics Committee of Osaka University. Clinical characteristics of the subjects selected for genotyping are given in Table 1.

Table 1. Clinical characteristics of the subjects for genotyping.

		GD		HD	
	Controls	Intractable	In remission	Severe	Mild
n (female/male)	118 (78/40)	60 (50/10)	45 (39/6)	59 (47/12)	41 (35/6)
Age of onset (years) (range)	37·0 ± 14·6‡ (21~66)	34·5 ± 14·7 (11~71)	33·4 ± 14·3 (10~67)	37·1 ± 11·1 (10~49)	62·2 ± 10·9‡ (50~92)
Goitre size (cm)	n.d.	4·8 ± 1·3	4·2 ± 0·7	4·1 ± 1·0	4·5 ± 1·1
Free T4 (ng/dl)	n.d.	1·2 ± 0·3	1·2 ± 0·1	1·3 ± 0·3	1·2 ± 0·2
Free T3 (pg/dl)	n.d.	2·7 ± 0·5	2·7 ± 0·4	2·6 ± 0·6	2·8 ± 0·4
TSH (μU/ml)	n.d.	1·8 ± 1·4	1·9 ± 1·3	4·8 ± 16·9	2·7 ± 1·6
TRAb (IU/l) (range)	<2·0	6·1 ± 11 (2~71)*	<2·0	<2·0	<2·0
TgAb ($2^n \times 100$)	Negative	3·6 ± 3·0	2·9 ± 3·2	6·8 ± 3·4**	3·6 ± 4·4
McAb ($2^n \times 100$)	Negative	5·1 ± 2·3	5·4 ± 2·1	5·0 ± 3·2	3·3 ± 3·0
Current treatment	None	Methimazole or PTU	None	L-thyroxine	None
Duration of treatment (years)	None	12·1 ± 6·7	3·0 ± 1·2§	10·0 ± 8·5	None
Current dose of anti-thyroid drug (mg/day) (range)†	None	12·4 ± 11·5 (2·5~50)	None	None	None
Current dose of L-thyroxine drug (μg/day) (range)	None	None	None	88·7 ± 39·0 (25~250)	None

Data are means ± standard deviations. *$P < 0{\cdot}01$ [*versus* Graves' disease (GD) in remission]. **$P < 0{\cdot}01$ [*versus* mild Hashimoto's disease (HD)]. †Doses were expressed as comparable doses of methimazole [50 mg of propylthiouracil (PTU) was converted to 5 mg of methimazole]. ‡Age at the time of sampling. §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.

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

	Primer pairs	PCR condition	Restriction enzymes
PCR–RFLP			
rs12976445 C/T	5′-TTTTGGTCTTTCTGTCTCTGG -3′ 5′-TGGAGGAAGGGTATGAGGAGT-3′	96°C for 5 min (94°C for 30 s, 58·4°C for 30 s, 72°C for 30 s) × 35 cycles 72°C for 7 min	BaeGI
Direct sequence			
rs10404453 A/G	5′-CTGACTCCCTCTTATTCTGG-3′ 5′-TAGAGACTGGCAACATGG-3′	96°C for 5 min (94°C for 30 s, 54·9°C for 30 s, 72°C for 30 s) × 35 cycles 72°C for 7 min	
Direct sequence			
rs12975333 G/T	5′-GAATGTCTCTGTGCCTATCTCC-3′ 5′-TCAGGCCAGCAATTCCC-3′	96°C for 5 min (94°C for 30 s, 58·5°C for 30 s, 72°C for 30 s) × 35 cycles 72°C for 7 min	

PCR–RFLP = polymerase chain reaction–restriction fragment length polymorphism.

Genotyping of polymorphisms

Genotyping was performed using the PCR–restriction fragment length polymorphism (RFLP) method and direct sequencing. Briefly, target sequences of DNA were amplified using PCR. The PCR products were then digested with BaeGI (New England Biolabs, Ipswich, MA, USA). DNA fragment length polymorphisms were visualized by ultraviolet transillumination after electrophoretic separation on 10% polyacrylamide gels. The PCR primers used for *MIR125A* sequencing are presented in Table 2.

miRNA extraction from PBMCs

We collected blood samples in ethylenediamine tetraacetic acid (EDTA)-treated tubes and isolated PBMCs from each AITD patient and healthy volunteer with Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). PBMCs were washed once in sterile phosphate-buffered saline (PBS) and preserved in RNAlater® solution (Ambion, Austin, TX, USA) at –80°C until required. Total RNA was isolated from preserved PBMCs with the mirVana™ PARIS™ Kit (Ambion), in accordance with the manufacturer's protocol.

Quantitative RT–PCR

Reverse transcription (TR) of miR-125a and U6 was performed using a TaqMan® MicroRNA RT kit and target-specific stem loop primers provided in TaqMan® MicroRNA assays (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol. Quantitative RT–PCR was performed using TaqMan® MicroRNA assays (Applied Biosystems), in accordance with the manufacturer's protocol. The following TaqMan MicroRNA assays were used: hsa-miR-125a and RNU6B. Detection and quantification were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Expression levels of miRNA were normalized to U6. All reactions were performed in triplicate. The relative expression levels of each miRNA were calculated using the ΔΔCt method.

Subjects for quantification of miR expression

We examined the expression level of miRNAs in PBMCs from 55 patients with GD, 79 patients with HD and 38 healthy volunteers. Among these, 15 patients had intractable GD, 17 patients had GD in remission, 22 patients who could not be categorized to intractable GD or GD in remission groups at the time of analysis, 32 patients had severe HD and 19 had mild HD, and 28 patients who could not be categorized to severe HD or mild HD groups at the time of analysis. The clinical characteristics of the subjects used for miR quantification are given in Table 3.

Statistical analysis

We used Student's *t*-test for two-group comparisons to analyse the significance of differences in the expression level of miRNAs. For multiple group comparisons, parametric comparisons used analysis of variance (ANOVA). If ANOVA was significant, significances of patients with GD or HD compared with control subjects were evaluated by Dunnett's test. A χ^2 test was used to evaluate differences in genotype frequencies and alleles among the subject groups. Pearson's correlation coefficient was used to analyse the associations between the expression of miR125a and age. Data were analysed using JMP9 software (SAS Institute, Inc., Tokyo, Japan). Probability values of less than 0·05 were considered significant.

Results

MIR125A rs12976445 C/T polymorphism

Initially, we performed direct sequencing for three SNPs (rs12976445, rs10404453, rs12976333) in a randomly

Table 3. Clinical characteristics of the subjects for quantification of microRNA (miR)-125a expression.

		GD		HD	
	Controls	Intractable	In remission	Severe	Mild
n (female/male)	38 (24/14)	15 (14/1)	17 (15/2)	32 (25/7)	19 (18/1)
Age of onset (years) (range)	–	34·7 ± 14·6 (16~64)	28·3 ± 8·7 (15~46)	39·0 ± 9·8 (23~49)	–
Age of sampling (years) (range)	46·6 ± 12·7 (21~66)	53·6 ± 11·0 (38~83)	52·6 ± 15·4 (26~87)	53·0 ± 15·5 (26~83)	62·2 ± 9·7 (50~79)
Goitre size (cm)	n.d	4·6 ± 1·1	4·4 ± 0·5	4·2 ± 1·2	4·7 ± 1·3
Free T4 (ng/dl)	1·1 ± 0·2	1·1 ± 0·2	1·3 ± 0·2	1·2 ± 0·2	1·2 ± 0·4
Free T3 (pg/dl)	2·8 ± 0·2	2·6 ± 1·1	2·8 ± 0·3	2·8 ± 0·8	2·8 ± 0·3
TSH (μU/ml)	2·1 ± 1·3	1·9 ± 1·3	2·1 ± 1·7	4·8 ± 4·9	2·7 ± 2·1
TRAb (IU/l) (range)	<2·0	11·6 ± 27·8 (2~99)	<2·0	<2·0	<2·0
TgAb ($2^n \times 100$)	Negative	5·0 ± 2·6	6·0 ± 2·8	9·0 ± 2·6*	2·15 ± 2·4
McAb ($2^n \times 100$)	Negative	7·0 ± 3·8	7·3 ± 3·0	9·5 ± 4·2	6·3 ± 2·9
Current treatment	None	Methimazole or PTU	None	L-thyroxine	None
Current dose of anti-thyroid drug (mg/day) (range)	None	37·5 ± 59·8 (5~200)	None	None	None
Current dose of L-thyroxine drug (μg/day) (range)	None	None	None	79·6 ± 32·1(25~150)	None

Data are means ± standard deviations. *$P < 0·01$ [*versus* mild Hashimoto's disease (HD)]. Doses were expressed as comparable doses of methimazole [50 mg of propylthiouracil (PTU) was converted to 5 mg of methimazole]. T4 = thyroxine; T3 = triiodothyronine; TSH = thyrotrophin; TRAb = anti-thyrotrophin receptor antibody; McAb = anti-thyroid microsomal antibody; n.d. = not determined; TgAb = anti-thyroglobulin antibody.

selected group of 16 patients with HD and determined that rs12976445 was the most suitable polymorphism for further examination (Table 4). We found a significant difference in the genotype frequency between control subjects and patients with HD ($P = 0·037$) for this polymorphism. The C allele and CC genotype of this polymorphism were significantly more frequent in patients with HD compared with control subjects ($P = 0·049$ and $P = 0·036$, respectively) (Table 5). Furthermore, the C allele and CC genotype were significantly more frequent in patients with intractable GD

Table 4. Genotype frequencies of the MIR125A polymorphisms (preliminary investigation).

		Severe Hashimoto's disease (HD)	Mild HD
rs12976445	CC	6 (75·0%)	7 (87·5%)
C/T	CT	2 (25·0%)	1 (12·5%)
	TT	0 (0%)	0 (0%)
rs10404453	GG	8 (100%)	8 (100%)
G/A	GA	0 (0%)	0 (0%)
	AA	0 (0%)	0 (0%)
rs12975333	GG	8 (100%)	8 (100%)
G/T	GT	0 (0%)	0 (0%)
	TT	0 (0%)	0 (0%)

Table 5. Genotype and allele frequencies of the miR-125a polymorphism in patients with Graves' disease (GD), Hashimoto's disease (HD) and in control subjects.

		Control	All patients with GD		All patients with HD	
miR-125a	CC	87 (73·73%)	122 (78·71%)	n.s.	127 (84·11%)	0·037
rs12976445	CT	31 (26·27%)	31 (20%)		24 (15·89%)	
	TT	0 (0%)	2 (1·29%)		0 (0%)	
	CC + CT	118 (100%)	153 (98·71%)	n.s.	151 (100%)	n.s.
	TT	0 (0%)	2 (1·29%)		0 (0%)	
	CC	87 (73·73%)	122 (78·71%)	n.s.	127 (84·11%)	0·036
	CT + TT	31 (26·27%)	33 (21·29%)		24 (15·89%)	
	C allele	205 (86·86%)	275 (88·71%)	n.s.	278 (92·05%)	0·049
	T allele	31 (13·14%)	35 (11·29%)		24 (7·95%)	

n.s. = not significant.

Table 6. Genotype and allele frequencies of the miR-125a polymorphism in patients with Graves' disease (GD), Hashimoto's disease (HD).

		GD			HD		
		Intractable	In remission		Severe	Mild	
miR-125a	CC	51 (85%)	30 (66·67%)	n.s.	48 (81·36%)	36 (87·80%)	n.s.
rs12976445	CT	8 (13·33%)	14 (31·11%)		11 (18·64%)	5 (12·20%)	
	TT	1 (1·67%)	1 (2·22%)		0 (0%)	0 (0%)	
	CC + CT	59 (98·33%)	44 (97·78%)	n.s.	59 (100%)	41 (100%)	n.s.
	TT	1 (1·67%)	1 (2·22%)		0 (0%)	0 (0%)	
	CC	51 (85%)	30 (66·67%)	0·027	48 (81·36%)	36 (87·80%)	n.s.
	CT + TT	9 (15%)	15 (33·33%)		11 (18·64%)	5 (12·20%)	
	C allele	110 (91·67%)	74 (82·22%)	0·040	107 (90·68%)	77 (93·90%)	n.s.
	T allele	10 (8·33%)	16 (17·78%)		11 (9·32%)	5 (6·10%)	

n.s. = not significant.

compared with those with GD in remission ($P = 0·040$ and $P = 0·027$, respectively) (Table 6).

Expression of miR-125a in PBMCs and clinical characteristics

The expression of miR-125a in control subject PBMCs was correlated negatively with age (Fig. 1, $r = -0·52$, $P = 0·0010$). However, in GD and HD patients, the expression of miR125a was not correlated with any clinical characteristics.

PBMC miR-125a expression

The expression of miR-125a in GD patient PBMCs was decreased significantly compared with control subjects ($P = 0·0249$, Fig. 2a). We found no significant differences in miR-125a expression in PBMCs among the patients with different prognoses of AITDs (Fig. 2b,c).

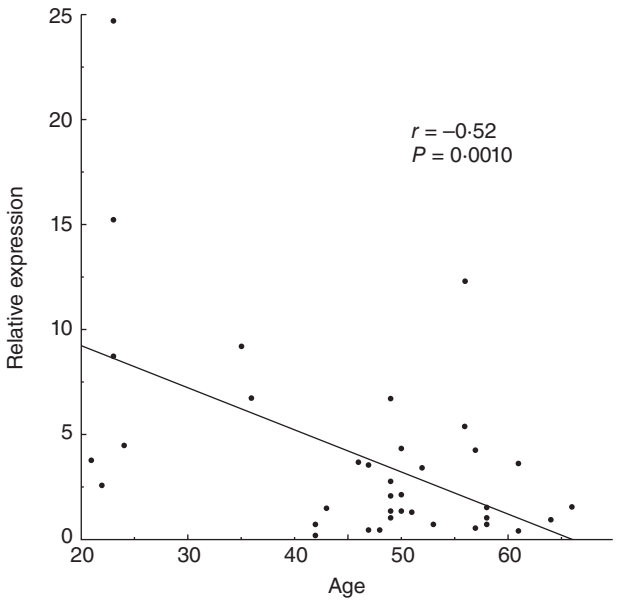

Fig. 1. The correlation between microRNA (miR)-125a expression in peripheral blood mononuclear cells (PBMCs) and age. Pearson's correlation coefficient was used to analyse the associations between the PBMC miR125a expression level and age at sampling in control subjects.

PBMC miR-125a expression stratified by genotype

PBMC miR-125a expression did not differ significantly among patients with each genotype of the *MIR125A* rs12976445C/T polymorphism (Fig. 3).

Discussion

Initially, our data indicated that miR-125a expression decreased with age (Fig. 1). Therefore, prior to the analysis of miR-125a expression, we confirmed that our subject groups were age- and sex-matched (Table 3). To our knowledge, this is the first report demonstrating the correlation between miR expression and age.

We determined in this study that the *MIR125A* rs12976445 C/T polymorphism was associated with the pathogenesis and prognosis of AITDs. Our data indicate that the C allele and CC genotype are correlated with HD development and GD intractability (Tables 5 and 6). A previous study reported that C allele carriers (CC + CT genotypes) correlated with low miR-125a expression compared with the TT genotype in a Polish population [23]. However, in the present study, the association between genotype and miR-125a expression level was not observed in control subject PBMCs (Fig. 3). We suggest that this polymorphism may be associated with the development and prognosis of AITDs through a role in pri-miRNA processing, because this polymorphism is known to be related to pri-miR processing rather than through variations in miR-125a expression levels [17].

Our data also demonstrated that miR-125a expression in PBMCs was decreased significantly in patients with GD (Fig. 2a). This indicates that miR-125a expression in PBMCs may suppress GD development. It has been demonstrated that miR-125a acts as a negative regulator of RANTES, interleukin (IL)-6 and transforming growth

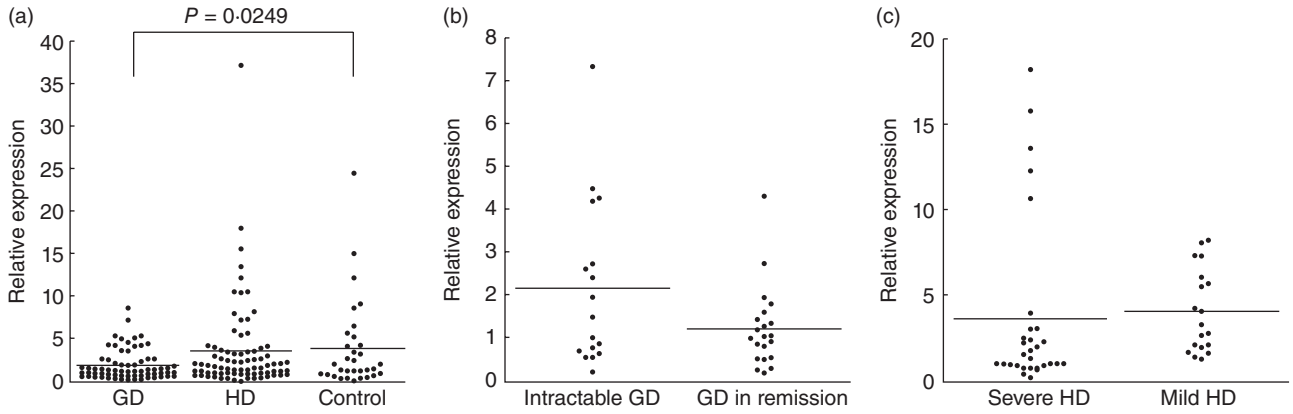

Fig. 2. Peripheral blood mononuclear cell (PBMCs) microRNA (miR)-125a expression in study groups. The PBMC miR-125a expression level in patients with autoimmune thyroid diseases (AITDs) and control subjects (a), with different prognoses of Graves' disease (GD) (b) and Hashimoto's disease (HD) (c). A Student's *t*-test was used to compare the expression level of miR-125a between two groups and Dunnett's test was used between three groups.

factor (TGF)-β [9,15]. Additionally, we have reported previously that the high genetic producibilities of IL-6 and TGF-β, which promote the differentiation of T helper type 17 (Th17) cells, were associated with the GD development [24,25]. Therefore we speculate that, in GD patients, the down-regulation of miR-125a may indirectly promote the differentiation of Th17 cells and cause GD development. This is supported by our previous study, where we demonstrated that Th17 cells were increased in GD patients [26]. Conversely, in some patients with severe HD, a higher miR-125a expression was observed (Fig. 2b). In these subjects, high miR-125a expression may play some role to cause a severe condition in HD through some factors such as Th1 cells, which are predominant in severe HD [26]. However, Yamada *et al.* reported that serum miRNA levels (miR-16, miR-22, miR-375 and miR-451) were associated with the development of AITD [27]. Therefore, further studies are necessary to clarify the association between circulating miRNA levels and the development and prognosis of AITD.

In conclusion, miR-125a expression in PBMCs was down-regulated in patients with GD. The rs12976445 C/T polymorphism was associated with HD development and GD intractability.

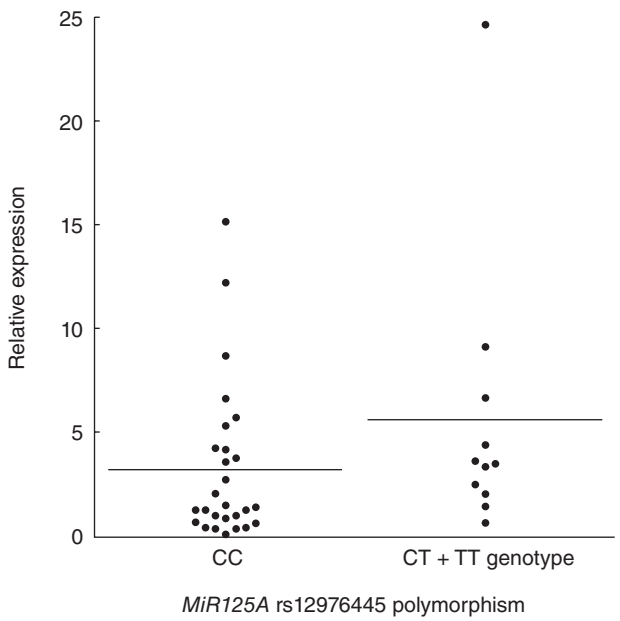

Fig. 3. The expression of microRNA (miRNA) in patients stratified by genotype. miR-125a expression in peripheral blood mononuclear cells (PBMCs) from control subjects with each genotype of *MIR125A* rs12976445 C/T polymorphism. A Student's *t*-test was used to compare the expression levels of miR-125a among each group.

Disclosure

The authors declare that they have no conflicts of interest.

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