British Journal of Nutrition (2023), 129, 206–217 doi:10.1017/S0007114522000721

The whole blood DNA methylation patterns of extrinsic apoptotic signalling pathway-related genes in autoimmune thyroiditis among areas with different iodine levels

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(Submitted 14 December 2021 – Final revision received 5 February 2022 – Accepted 28 February 2022 – First published online 9 March 2022)

Abstract

Autoimmune thyroiditis (AIT) has a complex aetiology and the susceptibility to it is determined by a combination of genetic and environmental factors, although these are not yet fully understood. The present research aimed to explore the DNA methylation patterns in whole blood of extrinsic apoptotic signalling pathway-related genes in AIT among areas with different iodine levels. We selected the iodine-fortification areas, iodine-adequate areas and water-based iodine-excess areas from Shandong Province of China as survey sites. Totally, 176 AIT cases and 176 controls were included. MethylTarget™ and QT-PCR technology were used to detect candidate genes' DNA methylation levels and mRNA expression levels, respectively. We found that death associated protein kinase 1 (DAPK1) DNA methylation levels in AIT cases (especially in female) were significantly higher than controls ($t = 2{\cdot}7715$, $P = 0{\cdot}0059$; $t = 2{\cdot}4638$, $P = 0{\cdot}0143$ in female). There were differences in DAPK1 ($t = 2{\cdot}5384$, $P = 0{\cdot}0121$), TNF superfamily member 8 ($t = 2{\cdot}1667$, $P = 0{\cdot}0334$) and TNF-α-induced protein 8 (TNFAIP8) ($t = 2{\cdot}5672$, $P = 0{\cdot}0121$) genes methylation between cases and controls with different water iodine levels. The mRNA expression of DAPK1 ($t = 4{\cdot}329$, $P < 0{\cdot}001$) and TNFAIP8 ($t = 3{\cdot}775$, $P < 0{\cdot}001$) in the cases was increased. We identified the differences in the DNA methylation status of the extrinsic apoptotic signalling pathway-related genes between AIT and controls and in different iodine levels areas. The results were verified at the mRNA level. The environmental iodine may affect DNA methylation to some extent.

Key words: **Autoimmune thyroiditis: DNA methylation: Apoptotic: Iodine**

Autoimmune thyroiditis (AIT) is the most common organ-specific autoimmune disease mediated by T lymphocytes[1] which has a complex aetiology, and the susceptibility to it is determined by a combination of genetic and environmental factors, although these are not yet fully understood[2]. In recent years, increasing evidence has suggested that apoptosis plays a significant role in the development of autoimmune thyroid disease[3–6]. Apoptosis can be triggered by the extrinsic or the intrinsic death pathways[7]. A study had shown that the serum concentration of soluble Fas (sFas) in AIT patients was significantly decreased compared with healthy controls[8]. The level of sFasL in children with hypothyroidism was higher than controls[9]. The serum concentration level of CD30, a member of TNF receptor superfamily, had been found increased in patients with Hashimoto thyroiditis (HT)[10]. TNF-α was elevated in serum of patients with chronic lymphocytic thyroiditis[11].

Abbreviations: AIT, autoimmune thyroiditis; DAPK1, death associated protein kinase 1; HOXA5, homeobox A5; HT, Hashimoto thyroiditis; IAA, iodine-adequate areas; IEA, iodine-excess areas; IFA, iodine-fortification areas; PF4, platelet factor 4; SIC, serum iodine concentration; TNFAIP8, TNF-α-induced protein 8; TNFSF8, TNF superfamily member 8; TSH, thyroid-stimulating hormone; UIC, urinary iodine concentration.

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Platelet factor 4 (PF4) is involved in the extrinsic apoptosis pathway mediated by caspase[12]. The level of circulating PF4 was proved to decrease in subclinically hypothyroid AIT patients[13]. The abnormal expression of these molecules in the serum of AIT patients suggests that extrinsic apoptotic pathway-related molecules may be involved in the pathogenesis of AIT and play an important role.

In recent years, increasing evidence has revealed the roles of epigenetic in autoimmune thyroid disease[14–17]. DNA methylation is an essential form of epigenetic modification. Several studies have revealed altered methylation patterns of cells and tissues of AIT patients. Patients with HT showed higher methylation levels at the -426 CpG site of IL6 gene than controls[18]. Sally M Shalaby *et al.* found the differences in the frequency of ICAM-1 methylation status between Graves' disease or HT and healthy individuals[19]. Liu *et al.* identified aberrant DNA methylation and hydroxymethylation of the ICAM1 gene promoter in the thyrocytes of AIT patients[20]. However, the available data are still limited. Moreover, it is believed that iodine excess, a nutritional factor in the environment, will lead to AIT[21]. Therefore, to explore the DNA methylation patterns of extrinsic apoptotic signalling pathway-related genes in AIT among areas with different iodine levels, we assessed DNA methylation levels using DNA extracted from 352 whole blood samples from populations in different water iodine levels areas in Shandong Province, China.

Materials and methods

Survey areas and participants

According to the drinking water iodine survey of China, iodine-fortification areas (IFA) (median water iodine < 10 μg/l, with coverage rates of qualified iodised salt > 90 %, Qianlv and Dongtan Villages), iodine-adequate areas (IAA) (median water iodine: 40–100 μg/l, Liuxiangzhuang and Dongding Villages) and iodine-excess areas (IEA) (median water iodine > 300 μg/l, Jieyuanji Village) from Shandong Province were selected. The sample size included in this study was calculated with reference to our previous study and the expected sample for each area was at least 384[22]. In total, 1225 adults (409 from IFA; 392 from IAA; 424 from IEA) were included to screen AIT. Finally, a total of 176 AIT cases and 176 healthy controls were enrolled in our study, including 153 females and 23 males, respectively. Healthy controls were matched to patients with AIT by age, sex, BMI and places of residence for 1:1. Among these subjects, eighty-nine cases and eighty-nine controls were from IFA, forty cases and forty controls were from IAA, forty-seven cases and forty-seven controls were from IEA. The diagnostic criteria for AIT were thyroid peroxidase antibody > 60 U/ml and/or thyroglobulin antibody > 60 U/ml, and patients with thyroid-stimulating hormone (TSH) < 0·27 μ/ml were excluded[23].

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethical Review Board of Harbin Medical University (No. hrbmuecdc20200320). Written informed consents were obtained from all subjects.

Urinary sample collection and urinary iodine detection

Urine samples were collected on site from all subjects and stored in tightly sealed tubes at 4°C. Urinary iodine concentration (UIC) was measured by As^{3+}–Ce^{4+} catalytic spectrophotometry (WS/T 107·1–2006) according to the China Health Standard Method of Determination of Iodine in Urine[24].

Blood sample collection, serum iodine and thyroid function detection

Non-anticoagulated venous blood was collected in the morning after overnight fasting, and serum was separated after centrifugation at 3000 ***g*** and stored at −80°C. Serum iodine concentration (SIC) was measured by the inductively coupled plasma-MS system (ICP-MS, PerkinElmer NexION 350)[22]. The levels of free triiodothyronine (FT_3), free thyroxine (FT_4), TSH, thyroid peroxidase antibody and thyroglobulin antibody were determined using the chemiluminescent immunoassay (Siemens Healthcare Diagnostics Inc.). The reference values were as follows: 52–109 μg/l for SIC; 3·1–6·8 pmol/l for FT_3; 11·5–22·7 pmol/l for FT_4; 0·27–4·2 μ/ml for TSH and 0–60 U/ml for both thyroid peroxidase antibody and thyroglobulin antibody.

Detection of DNA methylation

Anticoagulative blood samples were stored at −80°C for DNA extraction. Genomic DNA was extracted from whole blood using TIANGEN Gel Extraction Kit (TIANGEN) according to the manufacturer. Quantitative detection of DNA methylation levels using MethylTarget™ (Genesky Biotechnologies Inc., Shanghai, 201315), a multiple targeted CpG methylation analysis method based on next-generation sequencing technology. Samples were treated with bisulphite using the EZ DNA Methylation-Gold Kit (ZYMO) following the manufacturer's protocols. Bisulphite treatment could transform cytosine, which was not methylated, to uracil. The optimised multiplex PCR primer panel was used to perform the multiplex PCR amplification (HotStart Taq polymerase; TaKaRa) using the transformed sample genome as a template. After PCR amplification of target CpG regions and methylation library construction, high-throughput sequencing was carried out on the Illumina Hiseq (Illumina) to get FastQ data. The primer sequences used for PCR were designed by primer 3 (http://primer3.ut.ee/) and present in Table 1. The methylation level of each CpG site was calculated as the percentage of the methylated cytosines over total tested cytosines. The average methylation level was calculated using methylation levels of all measured CpG sites within the gene. In this study, the proportion of samples with an average amplicon sequencing depth > 1000× was 99·18 %. For samples with an average amplicon sequencing depth > 1000×, the proportion of fragments sequencing depth > 20× was 95·70 %.

Detection of mRNA expression

The total RNA was isolated from a 250 μl whole blood sample using 750 μl Trizol reagent according to manufacture's protocols. RNA concentration was measured by the NanoDrop 2000 spectrophotometer (NanoDrop Technologies). The ratio of the

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absorbance value at 260–280 nm (A260/280 = 1·8–2·0) was used to determine the purity of RNA. PrimeScript™ RT reagent kit with gDNA Eraser (TAKARA BIO INC.) was used for the reverse transcription strictly following the instructions. The death associated protein kinase 1 (DAPK1) and TNF-α-induced protein 8 (TNFAIP8) genes mRNA expression levels were measured in the QuantStudio 5 quantitative Real-Time PCR system (Applied Biosystems) using the SYBR Green kit (Roche Diagnostics GmbH). The amplification reaction system consisted of 10·0 μl, including cDNA 1·0 μl, SYBR 5·0 μl, upstream primer 0·5 μl, downstream primer 0·5 μl and enzyme-free water 3·0 μl. In order to ensure the accuracy of experimental results, each sample was run in triplicate. The PCR reaction was carried out according to the following conditions: hold stage: step 1, 95°C 10 min; PCR stage (forty cycles): step 1, 95°C 15 s, step 2, 60°C 1 min; melt curve stage: step 1, 95°C 15 s, step 2, 60°C 1 min, step 3, 95°C 15 s. β-actin was used as the internal reference[19,25,26], and gene mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences of DAPK1, TNFAIP8 and β-actin are shown in Table 1.

Table 1. Primer sequences

Method	Gene	Target	Primer forward	Primer reverse	Product length
Detection of DNA methylation	DAPK1	DAPK1_1	TGGGGYGAGTGGGTGTGTG	CRAAACAACCTCTCRACTCCTTACC	226
	TNFAIP8	TNFAIP8_1	GATTGAAGGTTTAGATTTTATTTTATTTTGG	TTTTCTACRAACCTTAAATCTACCCTAC	242
	TNFSF8	TNFSF8_1	TTATTTAGAAGTTTATAGTTTAGTAGGGAGGTAAA	ACTACACTACCCTAAAACTACTTAACCTAATACC	260
	HOXA5	HOXA5_1	TTTTYGGAGTTAAAGTGGTYGGAGTT	AACCACAAATCAAACACACATATCAA	236
		HOXA5_2	TTGATATGTGTGTTTGATTTGTGGTT	CACCCAACTCCCCTATTAATACAC	227
		HOXA5_3	TATTAATAGGGGAGTTGGGTGGAG	CCCCCAATCCTCTACATCCTC	283
		HOXA5_4	GGGGGAGGTGGTGATTTG	CAATTAACTAAATAACTTTCCCCCTTC	173
		HOXA5_5	GGGGGAAAGTTATTTAGTTAATTGGAG	AACTAATAACACRAAAACCATATTCACAC	239
		HOXA5_6	GATTTTTTTTTYGTGTGAATATGGTTTT	CCAAACCCCAAACAACCTC	193
	PF4	PF4_1	GGTAGAGTTTTTAGTAGGATTTTAGTGTTTAGTG	AAAACCCAAATCTTCCCAATACTATC	189
Detection of mRNA expression	DAPK1	–	5′-CTGGATGAGGAAGACTCCTTTG-3′	5′-AGTTGGATAATGAGCCCAGAAG-3′	100
	TNFAIP8	–	5′-CCATCGCCACCACCTTAAT-3′	5′-GATCTTCTCTGCCTCCTTCTTG-3′	104
	β-actin	–	5′-CCTTCCTGGGCATGGAGTCCTG-3′	5′-GGAGCAATGATCTTGATCTTC-3′	202

Statistical analysis

Data processing and statistical analysis were performed using Excel 2019 and IBM SPSS Statistics 20.0. GraphPad Prism 9.0 was used to make figures. We used mean values and standard deviations to describe normally distributed data, while non-normally distributed data were presented as median and 25th and 75th percentiles. In comparing the case and control groups, variables distributed normally were assessed with a group *t* test, and variables with non-normally distribution were assessed with a Mann–Whitney *U* test. One-way ANOVA was used to compare normally distributed data among the groups; further pairwise comparison was performed by Student Newman Keuls (SNK)-*q* test. The Kruskal–Wallis *H* test was used to compare non-normally distributed data among the groups. Pearson or Spearman correlation analysis was used to explore the correlation between variables. Two-sided *P* values of less than 0·05 were regarded as statistically significant.

Results

Demographic characteristics

Demographic characteristics, UIC and SIC of AIT patients and controls are presented in Table 2. There is a more than six-fold sex difference among the study population. There were no statistically significant differences in sex, age, BMI, UIC and SIC between the AIT patients and controls.

Selection of candidate genes and DNA methylation analysis

According to the previous Illumina 850k DNA methylation chip analysis results of our research group, which was performed on ten AIT patients and ten matched controls[27], we found the methylation differences of several apoptotic function-related genes between AIT cases and healthy controls. In combination with searching related literature, we finally chose the following five extrinsic apoptotic signalling pathway-related genes as the

Table 2. Demographic characteristics and iodine nutrition of AIT patients and controls (Mean values and standard deviations; median values and percentiles)

			Sex		Age (years)		BMI (kg/m^2)		UIC (μg/l)		SIC (μg/l)	
Area	Group	*n*	Male	Female	Mean	SD	Mean	SD	Median	P_{25}-P_{75}	Median	P_{25}-P_{75}
IFA	Case	89	8	81	45	8	24·47	3·28	224·60	149·65–319·50	73·62	63·35–86·83
	Control	89	8	81	45	8	24·34	2·99	211·70	134·05–299·80	76·57	68·92–85·28
IAA	Case	40	5	35	44	10	24·07	3·32	258·25	152·92–406·40	70·50	64·21–84·01
	Control	40	5	35	44	10	24·01	3·48	229·90	116·90–339·80	75·52	62·82–83·62
IEA	Case	47	10	37	43	11	25·63	3·69	451·75	250·42–583·80	79·96	70·00–96·37
	Control	47	10	37	43	11	25·54	3·34	363·80	214·30–508·10	83·05	70·10–93·70
Overall	Case	176	23	153	44	9	24·69	3·44	259·60	157·10–439·25	74·82	64·23–86·98
	Control	176	23	153	44	9	24·59	3·24	230·90	144·90–363·80	77·89	68·08–86·41

IFA, iodine-fortification areas; IAA, iodine-adequate areas; IEA, iodine-excess areas; UIC, urinary iodine concentration; SIC, serum iodine concentration.
Compared with the control group, there was no significant difference in the case group ($P > 0·05$).

candidate genes: DAPK1, TNFAIP8, TNF superfamily member 8 (TNFSF8), homeobox A5 (HOXA5) and PF4. Then we enlarged the sample size on the basis of 10:10 to verify the methylation differences of the five candidate genes in 176 AIT cases and 176 matched controls using the MethylTarget™ technology.

We detected the DNA methylation levels of twenty-six CpG sites of the DAPK1 gene, of which the DNA methylation levels of 15 CpG sites in the AIT group were statistically higher than controls (all $P < 0·05$; Fig. 1(a)). A total of eleven CpG sites were detected in the TNFAIP8 gene, one site in TNFSF8, seventy-six sites from six targets in HOXA5 and ten sites in PF4. However, the DNA methylation levels of these CpG sites in AIT patients and controls were not significantly different (all $P > 0·05$; Fig. 1(b)–(j)). In order to further understand the overall methylation status of the candidate genes, the average of all the CpG sites methylation levels on the target was calculated as the target methylation level. There was an association between DAPK1 (DAPK1_1) DNA methylation and AIT; the DNA methylation levels of DAPK1_1 in the cases were significantly higher than controls ($t = 2·7715$, $P = 0·0059$; Table 3). However, the DNA methylation levels of TNFAIP8 (TNFAIP8_1), TNFSF8 (TNFSF8_1), HOXA5 (HOXA5_1, HOXA5_2, HOXA5_3, HOXA5_4, HOXA5_5, HOXA5_6) and PF4 (PF4_1) in patients with AIT and healthy controls were not significant different (all $P > 0·05$; Table 3).

Stratified (areas and sex) analysis of candidate genes DNA methylation

We performed a stratified analysis based on the different water iodine areas. In IFA, the DNA methylation levels of twelve CpG sites (all $P < 0·05$; Fig. 2(a)) and the target ($t = 2·5384$, $P = 0·0121$; Table 4) of DAPK1 in the AIT group were significantly higher than that in the controls. In IAA, the DNA methylation levels of 1 CpG site ($P < 0·05$; Fig. 2(b)) and the target ($t = 2·1667$, $P = 0·0334$; Table 4) of TNFSF8 in the AIT group were significantly higher than that in the controls. In IEA, the DNA methylation levels of eight CpG sites (all $P < 0·05$; Fig. 2(c)) and the target ($t = 2·5672$, $P = 0·0121$; Table 4) of TNFAIP8 in the AIT group were significantly higher than that in the controls. However, neither the CpG sites nor the targets of HOXA5 and PF4 DNA methylation levels were significantly different between cases and controls in the three areas.

In the stratified analysis of sex, the DNA methylation levels of ten CpG sites (all $P < 0·05$; Fig. 3) and the target ($t = 2·4638$, $P = 0·0143$; Table 5) of DAPK1 in female cases were significantly higher than those in female controls. However, no difference was found between the female cases and controls in the other four candidate genes' DNA methylation levels (including CpG sites and targets). Besides, there was no significant difference in the five candidate genes' DNA methylation levels (including CpG sites and targets) between the male cases and controls.

Differences in DNA methylation of candidate genes among cases in three areas

A comparison was conducted among the cases in the three areas with different water iodine levels. The DNA methylation levels of eight CpG sites (all $P < 0·05$; Table 6) and the target ($F = 4·969$, $P = 0·008$; Table 7) of DAPK1 among cases in the three areas were statistically significant. Further pairwise comparisons brought out significant differences between IFA and IEA, and the DNA methylation levels of DAPK1 in IFA were higher than in IEA (SNK-q test $P < 0·05$). The DNA methylation levels of DAPK1 (including CpG sites and target) of cases in IAA were higher than that in IEA while lower than that in IFA, but the differences were not statistically significant. Besides, there was no significant difference in DNA methylation levels of the other four candidate genes (including CpG sites and targets) among the three case groups with different water iodine levels.

Correlation between the candidate genes DNA methylation levels and iodine nutrition levels, age and thyroid function

The results of correlation analysis are depicted in Tables 8 and 9. No correlation was found between the DNA methylation levels of candidate genes and UIC, SIC, TSH and FT_3 levels of AIT patients, while the DAPK1_1 and HOXA5_5 DNA methylation levels were positively associated with the age of AIT patients. Besides, the DNA methylation levels of HOXA5_1, HOXA5_3, HOXA5_5 and HOXA5_6 were positively correlated with the FT_4 level of AIT cases. Thus, we further performed association analysis of the DNA methylation levels of HOXA5_1, HOXA5_3, HOXA5_5 and HOXA5_6 and the FT_4 levels of AIT cases adjusting by age. After adjusting age, the DNA

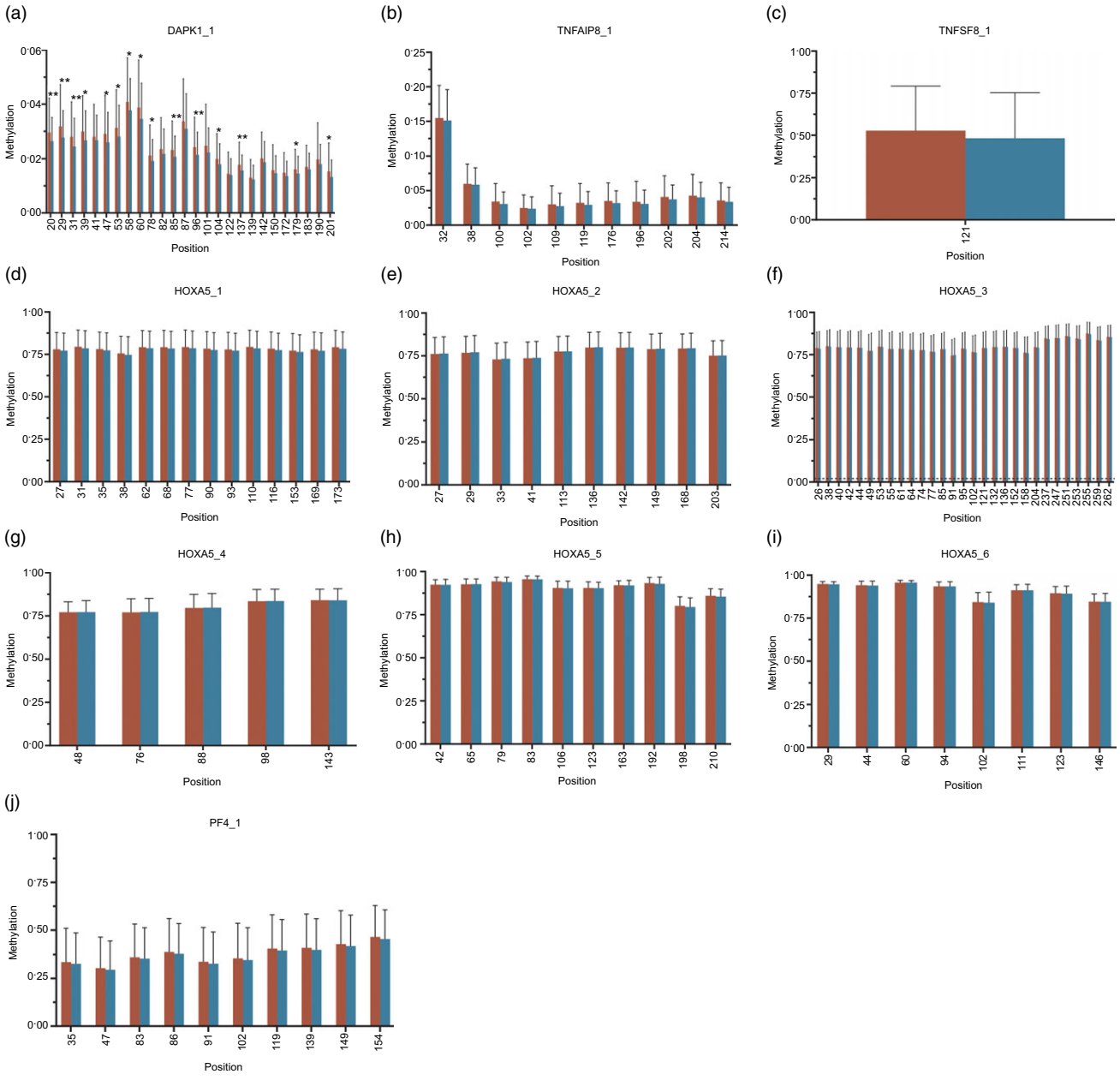

Fig. 1. Result of candidate genes CpG sites DNA methylation. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, *t* test. ▬, case; ▬, control.

methylation levels of HOXA5_1 (r_s 0·152, $p = 0{\cdot}045$), HOXA5_3 (r_s 0·169, $P = 0{\cdot}026$) and HOXA5_5 (r_s 0·204, $P = 0{\cdot}007$) were still positively correlated with the FT_4 levels of AIT cases. No correlation was found between the DNA methylation levels of candidate genes and UIC, SIC, FT_3 and FT_4 levels of controls. The DAPK1_1, TNFAIP8_1, HOXA5_1, HOXA5_2, HOXA5_3 and HOXA5_4 DNA methylation levels were positively associated with the age of controls, while the PF4_1 DNA methylation levels were negatively associated with the age of controls. Besides, the DNA methylation levels of TNFAIP8_1 were positively correlated with the TSH levels of controls. After adjusting age, the DNA methylation levels of TNFAIP8_1 (r_s 0·162, $p = 0{\cdot}032$) were still positively correlated with the TSH levels of controls.

Death associated protein kinase 1 and TNF-α-induced protein 8 genes mRNA expression

To verify the DNA methylation results, we selected the DAPK1 gene, which has a significant difference between cases and controls, and the TNFAIP8 gene, which has a significant difference between cases and controls in IEA, to detect their mRNA expression. Compared with the control group, the mRNA expression levels of DAPK1 ($t = 4{\cdot}329$, $P < 0{\cdot}001$) and TNFAIP8 ($t = 3{\cdot}775$, $P < 0{\cdot}001$) in the whole blood of AIT were significantly higher (Fig. 4). We used Pearson correlation analysis to analyse the correlation between DNA methylation levels and mRNA expression levels of DAPK1 and TNFAIP8 genes. No significant correlation was found between them (Fig. 5). We performed a stratified

Table 3. Differences of DNA methylation levels (%) of candidate genes between cases and controls (Mean values and standard deviations)

		Case		Control			
Gene	Target	Mean	SD	Mean	SD	GroupDiff (%)	*P*
DAPK1	DAPK1_1	2·39*	0·85	2·17	0·64	0·22	0·0059
TNFAIP8	TNFAIP8_1	4·77	2·67	4·51	2·02	0·26	0·3089
TNFSF8	TNFSF8_1	52·75	26·46	48·22	27·06	4·53	0·1165
HOXA5	HOXA5_1	78·38	9·83	77·61	10·09	0·77	0·4720
	HOXA5_2	77·01	9·00	77·20	9·16	–0·20	0·8410
	HOXA5_3	80·05	8·57	79·85	9·29	0·20	0·8335
	HOXA5_4	80·29	6·76	80·37	7·04	–0·08	0·9166
	HOXA5_5	90·73	2·99	90·48	3·08	0·25	0·4393
	HOXA5_6	91·00	2·82	90·87	3·18	0·13	0·6884
PF4	PF4_1	37·86	17·21	36·94	15·80	0·92	0·6027

GroupDiff = The methylation level of case – The methylation level of control.
* AIT group compared with control group, $P < 0·05$, *t* test.

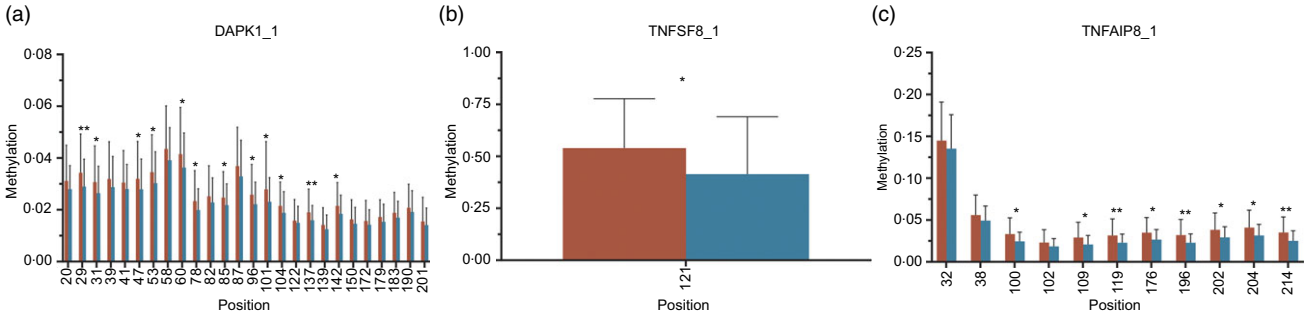

Fig. 2. (a) Result of DAPK1_1 CpG sites DNA methylation in iodine-fortification areas (IFA). (b) Result of TNFSF8_1 CpG site DNA methylation in iodine-adequate areas (IAA). (c) Result of TNFAIP8_1 CpG sites DNA methylation in iodine-excess areas (IEA). —, case; —, control.

Table 4. Differences of DNA methylation levels (%) of candidate genes between cases and controls in three areas

		IFA (case: control = 89:89)		IAA (case: control = 40:40)		IEA (case: control = 47:47)	
Gene	Target	GroupDiff (%)	*P*	GroupDiff (%)	*P*	GroupDiff (%)	*P*
DAPK1	DAPK1_1	0·30*	0·0121	0·21	0·1717	0·08	0·5680
TNFAIP8	TNFAIP8_1	0·23	0·5565	–0·36	0·5189	0·84*	0·0121
TNFSF8	TNFSF8_1	1·81	0·6615	12·49*	0·0334	2·77	0·6146
HOXA5	HOXA5_1	1·55	0·3091	–0·11	0·9592	–0·02	0·9929
	HOXA5_2	0·57	0·6755	–0·78	0·7030	–1·20	0·5394
	HOXA5_3	1·14	0·3786	–0·44	0·8399	–1·00	0·5907
	HOXA5_4	0·19	0·8549	–0·47	0·7588	–0·29	0·8482
	HOXA5_5	0·48	0·3024	–0·19	0·7802	0·19	0·7619
	HOXA5_6	0·42	0·3480	–0·42	0·5414	0·04	0·9492
PF4	PF4_1	3·12	0·1817	–4·73	0·2230	1·58	0·6647

IFA, iodine-fortification areas; IAA, iodine-adequate areas; IEA, iodine-excess areas.
GroupDiff = The methylation level of case – The methylation level of control.
* AIT group compared with control group in each area, $P < 0·05$, *t* test.

analysis based on the different water iodine areas (Fig. 6) and sexes (Fig. 7). In IFA, the mRNA expression levels of TNFAIP8 ($t = 2·890$, $P = 0·005$; Fig. 6(b)) in the AIT group were significantly higher than that in the controls. In IEA, the mRNA expression levels of DAPK1 ($t = 4·208$, $P < 0·001$; Fig. 6(a)) and TNFAIP8 ($t = 2·248$, $P = 0·030$; Fig. 6(b)) of AIT were significantly higher than controls. The mRNA expression levels of DAPK1 ($t = 4·018$, $P < 0·001$; Fig. 7(a)) and TNFAIP8 ($t = 3·184$, $P = 0·002$; Fig. 7(b)) in female cases were significantly higher than those in female controls. However, these significant differences were not found in male.

Discussion

AIT is the leading cause of hypothyroidism, affecting about 10 % of the population in China. Although the aetiology of AIT has not been fully elucidated, it is related to the interaction among genetic, environmental and epigenetic factors. As one of the epigenetic mechanisms, DNA methylation is a hot topic in exploring the pathogenesis of many diseases, including AIT. In our research, we selected five extrinsic apoptotic signalling pathway-related genes (DAPK1, TNFAIP8, TNFSF8, HOXA5, PF4) as candidate genes to explore the relationship between these

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Table 5. Differences of DNA methylation levels (%) of candidate genes between cases and controls in different sexes

		Male (case: control = 23:23)		Female (case: control = 153:153)	
Gene	Target	GroupDiff (%)	*P*	GroupDiff (%)	*P*
DAPK1	DAPK1_1	0·33	0·1995	0·21*	0·0143
TNFAIP8	TNFAIP8_1	0·28	0·5625	0·26	0·3507
TNFSF8	TNFSF8_1	–0·95	0·9109	5·31	0·0852
HOXA5	HOXA5_1	–0·14	0·9628	0·86	0·4477
	HOXA5_2	–0·08	0·9766	–0·25	0·8078
	HOXA5_3	0·78	0·7682	0·10	0·9229
	HOXA5_4	–0·70	0·7365	–0·02	0·9823
	HOXA5_5	–0·31	0·7178	0·32	0·3633
	HOXA5_6	–0·55	0·5850	0·22	0·5212
PF4	PF4_1	–3·78	0·4888	1·62	0·3860

GroupDiff = The methylation level of case – The methylation level of control.
* AIT group compared with control group in female, $P < 0·05$, *t* test.

Table 6. Differences of DNA methylation levels (%) of candidate genes CpG sites among cases in three areas (Mean values and standard deviations)

				IFA case		IAA case		IEA case		
Gene	Target	Position	Type	Mean	SD	Mean	SD	Mean	SD	*P*
DAPK1	DAPK1_1	31	CG	3·11*	1·36	2·75	1·15	2·42	0·99	0·009
		41	CG	3·08*	1·20	2·78	1·13	2·50	0·93	0·018
		47	CG	3·23*	1·42	2·78	1·28	2·54	1·32	0·017
		53	CG	3·46*	1·45	3·03	1·48	2·64	1·01	0·004
		78	CG	2·36*	1·16	2·00	1·04	1·87	0·95	0·030
		87	CG	3·73*	1·46	3·15	1·47	3·05	1·61	0·024
		101	CG	2·79*	1·85	2·31	1·15	2·08	0·82	0·025
		183	CG	1·90*	0·77	1·58	0·69	1·50	0·83	0·008

IFA, iodine-fortification areas; IAA, iodine-adequate areas; IEA, iodine-excess areas.
* IFA case group compared with IEA case group, $P < 0·05$, SNK-*q* test (ANOVA analysis).

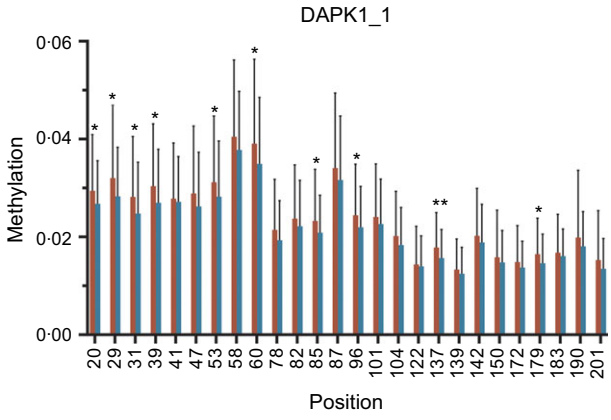

Fig. 3. Result of DAPK1_1 DNA methylation in female. *$P < 0·05$, **$P < 0·01$, *t* test. ▬, case; ▬, control.

genes and AIT from the perspective of epigenetics, so as to provide a basis for the diagnosis and treatment of the disease from a molecular point of view.

DAPK1 is an important serine/threonine kinase that is involved in multiple cellular processes including extrinsic apoptosis pathway[28–30]. The present study identified that the DAPK1 gene DNA methylation in AIT patients was significantly higher than controls. A previous study also showed that the methylation rate of DAPK gene in the HT patients combined with thyroid nodules group (5 %) was higher than controls (3·33 %), but the difference was not statistically significant[31]. The reason may be due to the small sample size of HT and controls in their study. DNA methylation plays a vital role in gene transcriptional regulation. Therefore, to verify the role of the DAPK1 gene DNA methylation in AIT, we detected mRNA expression levels in the whole blood. The results showed that the mRNA expression levels of DAPK1 in the AIT group were significantly higher than that in the controls. But we did not find a significant correlation between DNA methylation levels and mRNA expression levels. Initially, it was thought that the only function of DNA methylation was to silence gene expression. But with the emergence of updated research, the location of DNA methylation in the genome determines the effect of methylation on gene expression[32,33]. For example, methylation of CpG islands in the promoter region was observed in suppressed genes. On the contrary, methylation in the gene bodies showed a positive correlation with gene expression[32]. Most of the methylation sites of the DAPK1 gene detected in this study were located in the downstream region of the transcriptional starting site, which may be the reason why the DNA methylation level and mRNA expression level of DAPK1 in the AIT group were higher than those in controls at the same time. The research conducted by Xu Zhang *et al.* showed that the DNA methylation levels and mRNA expression levels of IL12B gene in ankylosing spondylitis

Table 7. Differences of DNA methylation levels (%) of candidate genes among cases in three areas (Mean values and standard deviations)

		IFA case		IAA case		IEA case		
Gene	Target	Mean	SD	Mean	SD	Mean	SD	*P*
DAPK1	DAPK1_1	2·58*	0·90	2·30	0·79	2·12	0·70	0·008
TNFAIP8	TNFAIP8_1	5·02	3·07	4·49	2·53	4·54	1·87	0·461
TNFSF8	TNFSF8_1	56·37	25·56	55·31	22·45	49·56	26·70	0·331
HOXA5	HOXA5_1	78·55	9·97	76·36	9·71	79·77	9·60	0·266
	HOXA5_2	77·63	8·47	74·80	9·67	77·73	9·29	0·210
	HOXA5_3	80·72	8·03	77·64	9·29	80·85	8·73	0·127
	HOXA5_4	80·38	6·44	78·85	6·88	81·37	7·14	0·218
	HOXA5_5	90·81	3·01	90·17	3·12	91·05	2·86	0·377
	HOXA5_6	91·13	2·74	90·40	3·04	91·26	2·77	0·303
PF4	PF4_1	37·41	16·63	36·19	16·49	40·14	18·95	0·535

IFA, iodine-fortification areas; IAA, iodine-adequate areas; IEA, iodine-excess areas.
* IFA case group compared with IEA case group, $P < 0·05$, SNK-*q* test (ANOVA analysis).

Table 8. Correlation between DNA methylation levels of candidate genes and UIC, SIC, age, FT_3, FT_4 and TSH in AIT patients

		UIC (μg/l)		SIC (μg/l)		Age (years)		FT_3 (pmol/l)		FT_4 (pmol/l)		TSH (μIU/ml)	
Gene	Target	r_s	*P*	r_s	*P*	*r*	*P*	r_s	*P*	r_s	*P*	r_s	*P*
DAPK1	DAPK1_1	–0·139	0·069	0·064	0·410	0·372*	< 0·001	0·030	0·689	0·044	0·559	0·017	0·818
TNFAIP8	TNFAIP8_1	–0·065	0·395	0·109	0·157	0·128	0·091	–0·062	0·415	–0·025	0·740	0·115	0·128
TNFSF8	TNFSF8_1	–0·023	0·766	–0·007	0·927	0·104	0·177	0·060	0·440	0·001	0·991	0·114	0·142
HOXA5	HOXA5_1	–0·011	0·888	0·108	0·160	0·123	0·104	–0·030	0·690	0·163*	0·030	0·027	0·724
	HOXA5_2	0·011	0·889	0·086	0·265	0·141	0·062	–0·064	0·398	0·127	0·095	0·086	0·257
	HOXA5_3	0·007	0·930	0·128	0·098	0·124	0·104	–0·055	0·474	0·174*	0·021	0·072	0·346
	HOXA5_4	0·035	0·647	0·088	0·255	0·096	0·204	–0·062	0·415	0·136	0·073	0·094	0·214
	HOXA5_5	0·023	0·767	0·098	0·211	0·205*	0·007	–0·047	0·543	0·210*	0·006	0·076	0·325
	HOXA5_6	0·023	0·765	0·092	0·231	0·116	0·125	–0·046	0·548	0·150*	0·047	0·064	0·399
PF4	PF4_1	0·058	0·445	0·000	0·999	0·059	0·438	0·070	0·353	0·057	0·452	0·052	0·491

UIC, urinary iodine concentration; SIC, serum iodine concentration; FT_3, free triiodothyronine; FT_4, free thyroxine; TSH, thyroid-stimulating hormone; r_s, Spearman correlation coefficient; *r*, Pearson correlation coefficient.
* $P < 0·05$, correlation analysis.

Table 9. Correlation between DNA methylation levels of candidate genes and UIC, SIC, age, FT_3, FT_4 and TSH in controls

		UIC (μg/l)		SIC (μg/l)		Age (years)		FT_3 (pmol/l)		FT_4 (pmol/l)		TSH (μIU/ml)	
Gene	Target	r_s	*P*	r_s	*P*	*r*	*P*	r_s	*P*	r_s	*P*	r_s	*P*
DAPK1	DAPK1_1	–0·024	0·757	–0·013	0·864	0·375*	< 0·001	0·049	0·521	–0·042	0·587	0·129	0·091
TNFAIP8	TNFAIP8_1	–0·096	0·220	–0·058	0·453	0·159*	0·039	0·031	0·686	–0·100	0·196	0·173*	0·024
TNFSF8	TNFSF8_1	0·107	0·166	–0·017	0·821	0·115	0·134	0·067	0·385	–0·098	0·201	0·051	0·509
HOXA5	HOXA5_1	0·128	0·098	–0·023	0·768	0·215*	0·005	–0·013	0·864	0·059	0·440	–0·114	0·138
	HOXA5_2	0·095	0·223	0·015	0·844	0·205*	0·007	0·040	0·606	0·051	0·511	–0·127	0·098
	HOXA5_3	0·092	0·232	–0·010	0·895	0·191*	0·012	0·046	0·550	0·039	0·608	–0·140	0·068
	HOXA5_4	0·121	0·116	–0·046	0·551	0·169*	0·027	0·036	0·642	–0·005	0·952	–0·125	0·102
	HOXA5_5	0·112	0·148	–0·064	0·412	0·117	0·128	0·066	0·390	0·034	0·661	–0·081	0·290
	HOXA5_6	0·139	0·071	–0·050	0·515	0·096	0·209	0·063	0·410	0·068	0·378	–0·109	0·154
PF4	PF4_1	0·060	0·436	0·099	0·195	–0·168*	0·027	0·034	0·651	0·026	0·735	–0·032	0·679

UIC, urinary iodine concentration; SIC, serum iodine concentration; FT_3, free triiodothyronine; FT_4, free thyroxine; TSH, thyroid-stimulating hormone; r_s, Spearman correlation coefficient; *r*, Pearson correlation coefficient.
* $P < 0·05$, correlation analysis.

were both higher than those in the controls group, which was consistent with us[26]. In summary, we speculate that the DAPK1 gene DNA methylation can regulate the mRNA expression, which may participate in AIT by affecting apoptosis. Nevertheless, further studies are needed to confirm the underlying mechanism of this process.

TNFAIP8 is the first TNF-α-induced protein 8-like (TNFAIP8/TIPE) family member protein[34]. The expression of TNFAIP8 mRNA could be activated by TNF-α and up-regulation of TNFAIP8 mRNA, thus inhibited cell apoptosis stimulated by TNF-α[35]. The present research on TNFAIP8 and autoimmune diseases focuses primarily on rheumatoid arthritis[36]. There

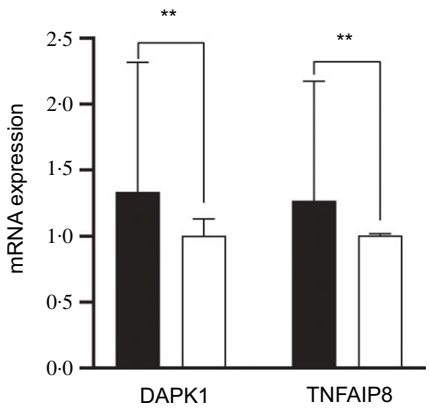

Fig. 4. The mRNA expression levels of DAPK1 and TNFAIP8 genes in the whole blood of cases and controls. $^{**}P < 0{\cdot}01$, *t* test. ▬, case; ▭, control.

are no reports on the role of the TNFAIP8 gene in AIT. Therefore, in our study, the association between TNFAIP8 and AIT was investigated for the first time, but we did not find the relationship between the DNA methylation levels of TNFAIP8 and AIT. We also detected the mRNA expression levels of TNFAIP8. The result showed that the mRNA expression levels of this gene in AIT patients were significantly higher than that in controls. But there was no significant correlation between the DNA methylation levels and the mRNA expression levels of the gene. The increased expression of mRNA indicates that the gene may play a role in the occurrence of AIT by participating in apoptosis. It is necessary to further explore the role of TNFAIP8 DNA methylation in AIT and the relationship between the DNA methylation levels and the levels of mRNA expression of this gene.

The TNFSF8 gene, as known as CD30L, belongs to the TNF ligand family. TNFSF8 has significant homology with TNF-α, TNF-β, CD40L and FasL and involves cell differentiation, apoptosis and immune response[37]. The soluble form of CD30L (sCD30L) has the ability to induce apoptosis of CD30+ cells, thus promoting the persistence of active inflammation[38]. Studies on DNA methylation of TNFSF8 are limited. A previous study showed that CD30/CD30L plays a role in thyroid autoimmune diseases[39]. Therefore, we explored the association between the TNFSF8 gene DNA methylation and AIT. However, a significant difference in DNA methylation levels of the TNFSF8 gene between AIT and controls was not found.

The expression of HOXA5 can induce extrinsic apoptosis through an apoptotic mechanism mediated by caspases 2 and 8[40–42]. Methylation of this gene may result in the loss of its expression and, since the encoded protein up-regulates the tumour suppressor p53, this protein may play an essential role in tumourigenesis[43]. However, the relationship between HOXA5 and AIT is unclear. In this study, we investigated the association between HOXA5 and AIT, but the results showed no statistically significant differences in the HOXA5 DNA methylation levels between AIT and control.

PF4 inhibits tumour growth and metastasis by inhibiting tumour-induced angiogenesis and apoptosis in many solid and non-solid tumours[44,45]. A study has confirmed that PF4 plays a role through caspase-mediated exogenous apoptosis pathway[12]. It was found that the levels of circulating PF4 in sub-clinical hypothyroidism AIT patients were lower than that in AIT patients with normal thyroid function[13]. In our study, PF4 was selected as a candidate gene to compare the differences in DNA methylation levels between the AIT group and the healthy controls group. But there was no significant difference in DNA methylation levels between the two groups.

As a nutritional factor in the environment, iodine plays a crucial role in the pathogenesis of AIT. Therefore, we performed a stratified analysis according to different water iodine levels and compared the differences of DNA methylation levels of candidate genes between cases and controls in IFA, IAA and IEA. The results showed a significant difference in DNA methylation level of DAPK1 between IFA cases and controls, TNFSF8 between IAA cases and controls and TNFAIP8 between IEA cases and controls. We verified the methylation differential genes on the mRNA expression levels. The results showed a significant difference in mRNA expression levels of DAPK1 between IEA cases and controls, and TNFAIP8 between IFA and IEA cases and controls. These results indicated that iodine in the environment may affect DNA methylation status, but this still needs to be confirmed by more in-depth studies. To further explore the effects of iodine in the environment and human iodine nutrition on the DNA methylation of candidate genes, we compared the differences of DNA methylation levels of the candidate genes among cases in different water iodine levels areas and analysed the correlation between UIC, SIC and DNA methylation levels of candidate genes. The DNA methylation levels of DAPK1 in IEA cases were significantly lower than that in IFA. But no obvious correlation between UIC or SIC and methylation levels was found. This suggests that iodine nutrition may affect the levels of gene DNA methylation. Still, more in-depth studies are needed to explore the mechanism of iodine and DNA methylation on AIT.

There are significant sex differences in the prevalence of AIT[46], so we conducted a stratified analysis according to sex. We found that the DNA methylation levels of DAPK1 in female AIT patients were significantly higher than that in controls. In addition, the results of mRNA expression detection also showed a significant difference between female cases and controls. This suggests that the change of DNA methylation status of DAPK1 is related to the occurrence of AIT in women. There was no significant difference in the methylation levels of the candidate genes between male cases and controls, which may due to the insufficient sample size of male cases for analysis because of the low prevalence of AIT in men. There is a certain correlation between DNA methylation levels and age, and a study had shown that the levels of methylation were positively correlated with age[47]. Our results showed that the DNA methylation levels of DAPK1, HOXA5 and PF4 were correlated with age, which is consistent with the above study. This suggests that age has a specific effect on DNA methylation, which may lead to an increase in the prevalence of the disease with age. We analysed the correlation between thyroid functions (FT_3, FT_4, TSH) and the DNA methylation levels of candidate genes of AIT patients for the first time. After age adjustment, we found that the DNA methylation levels of some target regions of HOXA5 (HOXA5_1, HOXA5_3, HOXA5_5) were positively correlated with the FT_4 levels of AIT,

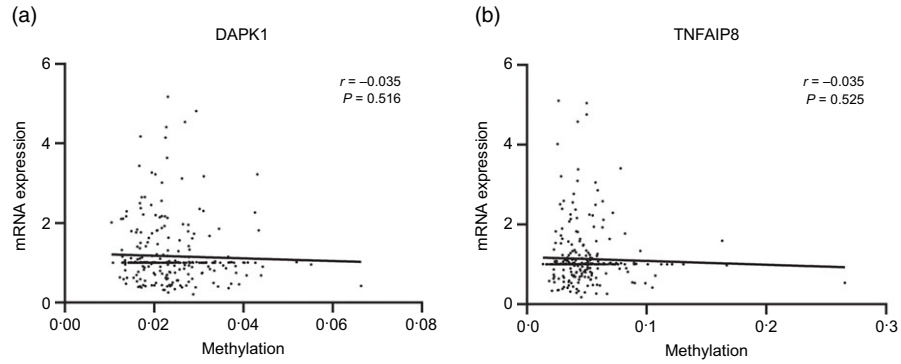

Fig. 5. Correlation analysis between DNA methylation levels of DAPK1 and TNFAIP8 genes and mRNA expression levels.

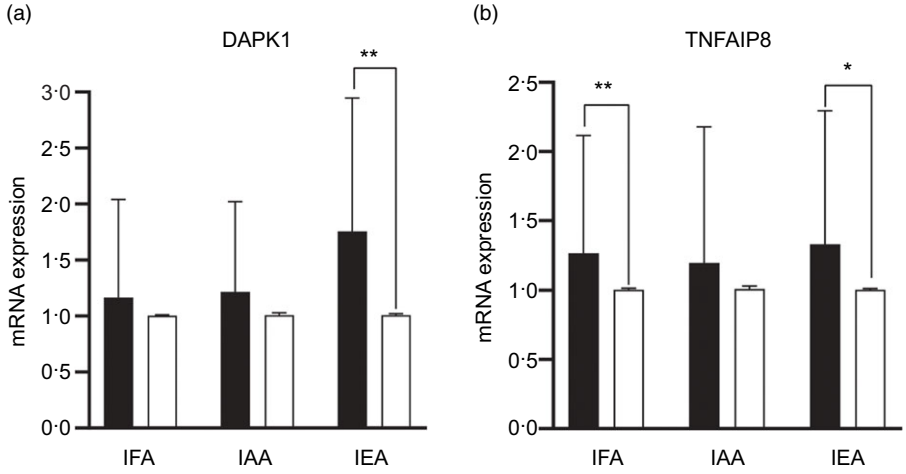

Fig. 6. The mRNA expression levels of DAPK1 and TNFAIP8 genes in the whole blood of different areas between cases and controls. IFA, iodine-fortification areas; IAA, iodine-adequate areas; IEA, iodine-excess areas; $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, *t* test. ▬, case; ▭, control.

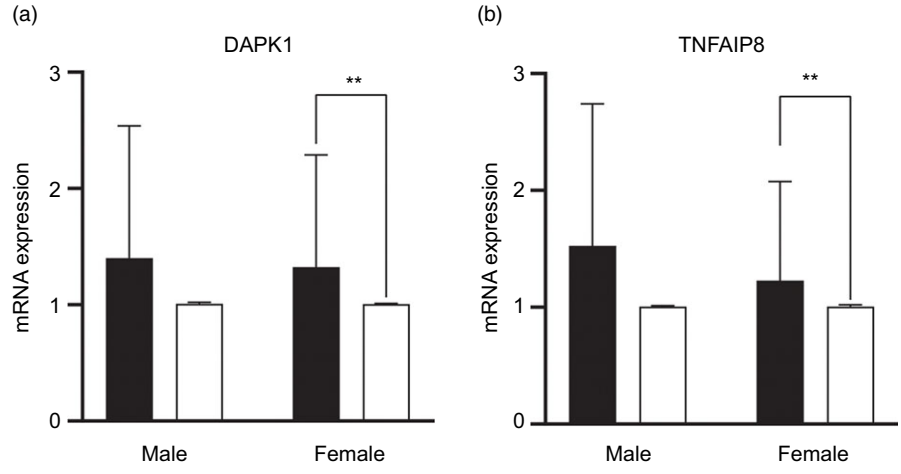

Fig. 7. The mRNA expression levels of DAPK1 and TNFAIP8 genes in the whole blood of different sexes between cases and controls. $^{**}P < 0{\cdot}01$, *t* test. ▬, case; ▭, control.

and the DNA methylation levels of TNFAIP8_1 were positively correlated with the TSH levels of controls.

In conclusion, we identified the differences in the DNA methylation status of the extrinsic apoptotic signalling pathway-related genes between AIT patients and healthy controls. And these results were verified at the mRNA level. This is the first study to show an association between AIT and the DNA methylation status of the extrinsic apoptotic signalling pathway-related genes. The results

of our research improved our knowledge of the role of the extrinsic apoptotic signalling pathway in the development of AIT and filled the gap in correlative research on the extrinsic apoptotic signalling pathway-related genes DNA methylation in AIT. However, the present study also had limitations. In our research, we only explored the association between the extrinsic apoptotic signalling pathway-related genes DNA methylation and AIT but did not study the molecular functional mechanism. Because it is difficult to obtain thyroid tissues from AIT patients, our study only detected the DNA methylation levels in whole blood. Although blood methylation has been suggested as a potential biomarker in several diseases and environmental conditions, it mostly indicates the epigenetic regulation in blood cells, which may be biased by cell composition, and does not reflect the methylation or pathological status of other tissues. In future research, it is necessary to conduct a more in-depth study of the molecular mechanism combined with the results of this study to provide a new basis for the pathogenesis of AIT.

Acknowledgements

We are grateful for the assistance provided by the Institute for Prevention and Treatment of Endemic Disease of Shandong Province for collecting epidemiological data and samples, and contributions and support from all participants.

This study was supported by the National Natural Science Foundation of China (grant no. 82073490).

H. S. and M. Q. designed this research. H. S., L. L., M. Q., S. W., H. W., B. R. and Y. C. conducted the research. M. Q. analysed the data and wrote the paper. H. S. had primary responsibility for the final content. All authors read and approved the final manuscript for submission.

The authors declare that they have no conflict of interest.

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British Journal of Nutrition