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Effects of different iodine levels on the DNA methylation of intrinsic apoptosis-associated genes and analysis of gene–environment interactions in patients with autoimmune thyroiditis

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Abstract

Iodine is an essential nutrient that may change the occurrence of autoimmune thyroiditis (AIT). Apoptosis and DNA methylation participate in the pathogenesis and destructive mechanism of AIT. We detected the methylation and the expression of mRNA of intrinsic apoptosis-associated genes (*YWHAG*, *ING4*, *BRSK2* and *GJA1*) to identify the potential interactions between the levels of methylation in these genes and different levels of iodine. 176 adult patients with AIT in Shandong Province, China, were included. The MethylTarget™ assay was used to verify the levels of methylation. We used PCR to detect the mRNA levels of the candidate genes. Interactions between methylation levels of the candidate genes and iodine levels were evaluated with multiplicative and addictive interaction models and GMDR. In the AIT group, *YWHAG_1* and six CpG sites and *BRSK2_1* and eight CpG sites were hypermethylated, whereas *ING4_1* and one CpG site were hypomethylated. A negative correlation was found between methylation levels of *YWHAG* and mRNA expression. The combination of iodine fortification, *YWHAG_1* hypermethylation and *BRSK2_1* hypermethylation was significantly associated with elevated AIT risk. A four-locus model (*YWHAG_1* × *ING4_1* × *BRSK2_1* × iodine level) was found to be the best model of the gene–environment interactions. We identified abnormal changes in the methylation status of *YWHAG*, *ING4* and *BRSK2* in patients with AIT in different iodine levels. Iodine fortification not only affected the methylation levels of *YWHAG* and *BRSK2* but also interacted with the methylation levels of these genes and may ultimately increase the risk of AIT.

Key words: **Iodine: Autoimmune thyroiditis: DNA methylation: Apoptosis: Gene–environment interaction**

Iodine, a reactant in the synthesis of thyroid hormones, is an essential nutrient absorbed mainly through drinking water and food to meet the needs of the human. Autoimmune thyroiditis (AIT) is a common thyroid disease, and the characteristic of AIT is the infiltration of lymphocytes and positivity for thyroid antibodies in the thyroid. Studies have confirmed that iodine as a nutritional factor intake could change the occurrence of AIT[(1)].

The association of genetic susceptibility factors and environmental factors could cause the development of AIT. Among environmental factors, iodine nutrition is an identified risk factor. On the one hand, the occurrence of AIT could be induced by iodine excess (IE). Excessive iodine consumption is a risk factor for the development of thyroid autoimmunity[(2)]. On the other hand, iodine supplementation in the areas of iodine deficiency increases the prevalence of AIT, and a cohort study has indicated

Abbreviations: AIT, autoimmune thyroiditis; HT, Hashimoto's thyroiditis; IA, iodine adequate; IE, iodine excess; IF, iodine fortification; TgAb, thyroglobulin antibodies; TPOAb, thyroid peroxidase antibodies; UIC, urinary iodine concentration.

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that the prevalence of AIT elevated after iodine fortification (IF) in iodine-deficient areas[3]. For the genetic factors, the role of epigenetics in thyroid diseases has attracted increasing attention. DNA methylation is the most widely studied epigenetic mechanism[4]. Studies found that aberrant DNA methylation patterns in the genes such as *ICAM-1* and *PTPN22* have been associated with AIT[5,6], and DNA methylation variations may affect the development and prognosis of AIT[7,8]. Therefore, whether iodine nutrition, as an environmental factor, can affect the methylation of certain genes contributing to AIT pathogenesis must be studied.

Apoptosis is a biological process in which cells actively destroy themselves. Genetic programmes can control apoptosis, which leads to the destruction of DNA and changes in cell morphology[9]. Increasing evidence suggests that apoptosis plays an important role in AIT[10,11]. Apoptosis can affect the homeostasis of thyroid cells and participate in the destructive mechanism of AIT. The percentage of apoptotic thyrocytes increased in Hashimoto's thyroiditis (HT), and apoptotic cells were mainly distributed in disrupted follicles and on the periphery of infiltrating lymphoid cells[12]. Studies have found intrinsic apoptotic signalling pathway is involved in the pathogenesis of AIT[11]. Among the intrinsic apoptotic signalling pathways, the fate of thyrocytes was determined by the activity of the anti-apoptotic and apoptotic genes. For instance, in HT, the *BCL2* gene is elevated in lymphocytes but decreased in thyrocytes, and this suggests that the relationship between *BCL2* family expression and thyrocyte destruction is considered to be a key factor in regulating thyrocyte survival[13]. However, the relationship between intrinsic apoptosis-associated gene methylation and AIT risk has not been reported.

Therefore, in this study, we investigated the effects of different levels of iodine on intrinsic apoptosis-associated gene methylation in AIT. We also investigated the relationships between intrinsic apoptosis-associated gene methylation in the whole-blood DNA and the environmental factor of iodine level, as well as their addictive and multiplicative interactions in AIT.

Materials and methods

Survey areas and participants

Based on the recent data[14], participants from three survey areas with different levels of iodine (water iodine content and the use of iodised salt) were chosen to conduct an epidemiological study in Shandong province. The details were as follows: 1. IF, with water iodine content ≤ 10 μg/l and > 90 % coverage rates of qualified iodised salt, including Dongtan and Qianlv villages. These areas were iodine-deficient areas until the implementation of universal salt iodisation in 1995; 2. iodine adequate (IA), with 40 μg/l < water iodine content < 100 μg/l and non-iodised salt supplied, including Liuxiangzhuang and Dongding villages; and 3. IE, with water iodine content ≥ 100 μg/l and non-iodised salt supplied, including Xieyuanji village. In total, 176 pairs of AIT cases and controls were selected (IF: 89 pairs, IA: 40 pairs and IE: 47 pairs). For the AIT group, the inclusion criteria were 1. serum thyroid peroxidase antibodies (TPOAb), thyroglobulin antibodies (TgAb) or double antibody positivity[15]; 2. Thyroid ultrasound examination has the following conditions: goitre or echo heterogenicity or multiple hypoechoic areas; 3. absence of hyperthyroidism and subclinical hyperthyroidism. For the control group, the inclusion criteria were 1. normal healthy participants matched to the case group based on sex, age, place of residence and BMI; 2. no family or personal history of autoimmune diseases or other thyroid diseases, no chronic or acute diseases, no use of long-term thyroid drugs or hormones and no pregnancy; 3. no goitre; negative TgAb and TPOAb; normal findings for other thyroid function indicators and no thyroid ultrasound abnormalities. This study was consistent with the Declaration of Helsinki and was approved by the Harbin Medical University Ethics Review Committee (No. hrbmuecdc20200320). Participants in the study all signed informed consent forms.

Laboratory testing

Venous blood was collected from the participants after an 8-hour fast. The levels of FT_3, FT_4, TSH, TGAb and TPOAb were measured using chemiluminescence immunoassays (Siemens Inc.). The reference ranges were as follows: 11·5 pmol/l < FT_4 < 22·7 pmol/l; 3·1 pmol/l < FT_3 < 6·8 pmol/l; 0·27 mIU/l < TSH < 4·20 mIU/l. Isolated positive TPOAb was defined as having TPOAb ≥ 34 μg/ml and TGAb < 115 μg/ml.; Isolated positive TGAb was defined as having TGAb ≥115 μg/ml and TPOAb < 34 μg/ml; TPOAb(+)&TGAb(+) were defined as TPOAb ≥ 34 μg/ml and TGAb ≥ 115 μg/ml. The water iodine content and urinary iodine concentrations (UIC) were determined with the As^{3+}-Ce^{4+} catalytic spectrophotometry method[16]. Certified reference materials (GBW09108, GBW9109 and GBW9110) from the NRLIDD in China were used to control measurement quality, and the target values were 69·5 ± 9·0 μg/l, 134 ± 10 μg/l and 239 ± 15 μg/l. The intra-assay CV was 2·7, 1·4 and 2·3 %, and the inter-assay CV was 2·3, 2·5 and 2·4 %.

Determination of candidate genes and DNA methylation measurements

In our previous study[17], an Illumina Methylation 850K BeadChip was used to detect genome-wide DNA methylation levels in whole blood samples from the case and control groups. A total of 853 307 CpG sites were detected, and a total of 312 differential methylation sites in 257 differentially methylated genes were screened according to methylation differences and P values. To further investigate the key biological processes associated with these distinct genes, we assessed the significant key biological processes associated with AIT according to the GO databases, including intrinsic apoptosis. The genes associated with apoptosis are shown in Supplementary Table 1. *YWHAG*, *ING4*, *BRSK2* and *GJA1* genes were enriched in the intrinsic apoptosis process and were closely associated with AIT. DNA was extracted by the TIANGEN Extraction Kit (TIANGEN). DNA methylation was evaluated with MethylTarget performed by Genesky Biotechnologies Inc.. Table 1 lists the primers of selected genes. Genomic DNA was transformed into bisulphite by EZ DNA methylation kit (Zymo). According to the guidelines, all

Table 1. Primer sequences in MethylTarget™ assay

Gene	Target	Chr	Start	End	Length	Primer F	Primer R
YWHAG	YWHAG_1	7	75957936	75957786	151	GTTATTTATTTTGTATTGGGGYGTTTTT	TTTTTCTACCAATAAACTTCTAACACCT
ING4	ING4_1	12	6 762 584	6 762 375	210	TAGGTTTTTGTTTTTGTTGTAGATTTG	AAACCATAAACAAAACCATACCATCT
BRSK2	BRSK2_1	11	1 474 808	1 475 079	272	GAGTGAGGGTTGGATTTGGTT	TAACTATTCAAAAATAACACRCTCTACTAAC
	BRSK2_2	11	1 457 129	1 457 371	243	GGGTAGGGGTTTTYGGTTTTG	CACCCACCTTCATCAACAC
GJA1	GJA1_1	6	122391199	122391354	156	ATAGGAAAGAGGATTATAGTATAGGTTTGTAG	AAAACCACTCTCTCATAAAACTTACA

the samples were amplified, barcoded and sequenced (Illumina).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from whole blood with RNAiso Plus (Takara). NanoDrop 2000C (Thermo Fisher) was used to determine the RNA concentrations. The value of OD 260/280 between 1·8 and 2·0 was thought to be sufficient quality. Each sample was performed with a QuantStudio™5 real-time PCR (Thermo Fisher). The amplification reaction system was as follows: 5·0 μl SYBR Green (Roche), 1·0 μl cDNA, 0·5 μl upstream and downstream primer and 3·0 μl ddH_2O. The reaction steps were as follows: 1. hold stage, 95°C 10 min; 2. PCR stage (40 cycles): 95°C 15 s and 60°C 1 min; 3. melt curve stage: 95°C 15 s, 60°C 1 min and 95°C 15 s. Specific primers were as follows: *YWHAG*-F: 5′-CGG CAA TGA GAA GAA GAT TGA G-3′; *YWHAG*-R: 5′-GCT GCA ATT CTT GAT CAG GTA G-3′; *ING4*-F: 5′-AGT TGG CCA CTG AGT ATA TGA G-3′; *ING4*-R: 5′-TGT GTT TGT CCA CCA TCT CAT A-3′; *BRSK2*-F: 5′-TTC CAC ATG CCG CAC TTT ATC-3′; *BRSK2*-R: 5′-GTG TTT CTG AAT GTG CTC TAG C-3′; *β*-actin-F: 5′-CCT TCC TGG GCA TGG AGT CCT G-3′; *β*-actin-R: 5′-GGA GCA ATG ATC TTG ATC TTC-3′. The $2^{-\Delta\Delta Ct}$ method was used to analyse the levels of mRNA of the target genes.

Statistical analysis

In this study, we used SPSS 23·0 for statistical analysis. Normally distributed data are characterised by the mean ± standard deviation (SD) and were analysed with the Student's *t* test and one-way ANOVA. Non-normally distributed data are described with median and 25th and 75th percentiles and were analysed with the Kruskal–Wallis H test and Mann–Whitney U test. The chi-square (χ^2) test was used to compare the rates of the subjects with different thyroid antibody groups. The correlations between relative mRNA expression and the methylation levels of candidate genes were analysed with Pearson correlation analysis. Interactions between different levels of iodine and methylation levels of candidate genes on AIT were evaluated with multiplicative and addictive interaction models. The methylation levels in target regions were transformed into a bicategorical variable of hypomethylation and hypermethylation, the different levels of iodine were divided into IF, IA and IE and two risk factors (methylation levels and different levels of iodine) and their interaction terms were included in a logistic regression model[18]. GMDR is an alternative linear or logistic regression to non-parametric and gene-free models for detecting and characterising non-linear interactions between environmental attributes and discrete genetics[19]. We used this method to analyse the high-dimensional interactions among three candidate genes (*YWHAG*, *ING4* and *BRSK2*) and different levels of iodine on AIT. Age, sex, BMI, smoking, drinking, thyroid function, UIC and family history of thyroid disease were chosen as covariates. Among the candidate models, the most suitable models were those with a sign test $P < 0{\cdot}05$, and the highest cross-validation consistency, training balanced accuracy and testing balanced accuracy[20].

Results

Demographic characteristics and DNA methylation levels in participants

The distribution of basic demographic characteristics and thyroid function is shown in Table 2. According to different levels of iodine, a total of 352 participants were divided into three groups. No significant differences were found between AIT and control groups in terms of sex, age, UIC and BMI. In IF and IE, the levels of TSH in the AIT groups were significantly higher than that in the control groups (both $P < 0{\cdot}050$). The prevalence of i-TGAb(+) and TPOAb(+) and TGAb(+) are significantly different among the different levels of iodine (both $P < 0{\cdot}050$). In Table 3, we detected the methylation levels of five target regions in four genes. The AIT group exhibited higher methylation in the target regions of *YWHAG_1* and *BRSK2_1* than the control group (both $P < 0{\cdot}010$). *BRSK2_2* methylation level was not detected in the control group; for DNA methylation in the target regions of *ING4_1* and *GJA1_1*, no significant differences were found between the AIT and control groups ($P > 0{\cdot}050$). To find the details of the methylation status in these genes, all CpG sites on the targets were calculated. In Fig. 1, there are a total of thirty-two CpG sites in five target regions in four genes that were analysed. For *YWHAG_1*, six CpG sites exhibited higher methylation. There are eight hypermethylation CpG sites in *BRSK2_1* and one hypomethylation CpG site in *ING4_1* in AIT patients compared with controls (all $P < 0{\cdot}050$).

DNA methylation status of participants with different levels of iodine

In Table 4, based on the different levels of iodine, a stratified analysis was performed. In IF, compared with the healthy controls, the AIT patients exhibited higher methylation of *YWHAG_1* and *BRSK2_1* (both $P < 0{\cdot}050$), but lower methylation of *ING4_1* ($P = 0{\cdot}049$). In IA and IE, we observed no significant differences in DNA methylation in the target regions for all candidate genes (all $P > 0{\cdot}050$). As shown in Table 5, in IF, there were six hypermethylation CpG sites in *YWHAG_1* and nine hypermethylation CpG sites in *BRSK2_1*, one CpG site exhibited lower methylation in *ING4_1* in patients with AIT than in controls (all $P < 0{\cdot}05$). In IA, six CpG sites exhibited higher methylation in *BRSK2_1* in patients with AIT than in controls (all $P < 0{\cdot}050$). In IE, no CpG site in the four genes exhibit a difference between AIT and the control group (all $P > 0{\cdot}050$).

Correlations among intrinsic apoptosis-associated genes' DNA methylation levels, age, UIC, thyroid function and thyroid antibodies

As shown in Fig. 2, the associations between the methylation levels of target regions and age were analysed with Pearson correlation. The associations between the UIC, thyroid function and the methylation levels in target regions in patients with AIT were analysed with Spearman's rank correlation. Positive correlations were found between the methylation levels of *YWHAG_1* and *BRSK2_1* and age (both $P < 0{\cdot}001$). The levels of TSH were positively correlated with the DNA methylation levels of *YWHAG_1* and *BRSK2_1* (both $P < 0{\cdot}050$), but for UIC and FT_4, the correlation was negative (all $P < 0{\cdot}050$) in patients with AIT. Positive correlations were found between the methylation level of *GJA1_1* and FT_4 ($P = 0{\cdot}021$). No correlations were observed between FT_3 and target regions' methylation. In Supplementary Table 2, we analysed the relationship between *YWHAG*, *ING4* and *BRSK2* gene methylation and serum TPOAb and TGAb levels. Among different thyroid antibody groups, the methylation levels of *BRSK2_1* are significantly different ($P = 0{\cdot}039$). TPOAb and TGAb were divided into three groups according to different levels of titre, and we further analysed the relationship between thyroid antibodies' titre and DNA methylation levels. Among three different titres of TPOAb, we observed a significant difference in DNA methylation of the *ING4* gene ($P = 0{\cdot}045$) and *BRSK2* gene ($P = 0{\cdot}033$).

Combined and interactive effects between methylation levels in target regions and different levels of iodine in autoimmune thyroiditis

As shown in Tables 6 and 7, we performed a cross-over analysis to find the combined effect between different levels of iodine and methylation levels in AIT. Combinations of IF, *YWHAG_1* hypermethylation (OR = 2·63, 95 % CI: 1·22, 5·69, $P = 0{\cdot}014$) and *BRSK2_1* hypermethylation (OR = 4·48, 95 % CI: 2·22, 10·59, $P < 0{\cdot}001$) were significantly associated with elevated AIT risk, as compared with that in the reference group. Meanwhile, the interactive effects were analysed. We observed no significant multiplicative effects between methylation levels in target regions and IF in AIT. Moreover, we did not find any combined and multiplicative effects between IE and methylation levels in target regions in AIT. Because no difference was found between the methylation levels of the *GJA1* gene between the AIT patients and the controls at different iodine levels, we did not analyse the interaction between the iodine level and *GJA1*.

GMDR analysis of gene–environment interactions

Table 8 presents the GMDR gene–environment interaction model. The four-locus model (*YWHAG_1* × *ING4_1* × *BRSK2_1* × iodine level) was the best model, with the highest training balanced accuracy (0·661) and testing balanced accuracy (0·606). This model also had the greatest cross-validation consistency (10/10; sign test $P = 0{\cdot}010$). Therefore, this was the best model for assessing the interactions between different levels of iodine exposure and candidate gene DNA methylation. Figure 3 demonstrate the specific distribution of score in the best gene–environment interaction model. In different grids of Fig. 3, the scores for AIT groups are varied, suggesting the patterns of risk differed across multi-locus dimensions.

Relationships between DNA methylation and mRNA expression of YWHAG, ING4 *and* BRSK2 *genes*

As shown in Fig. 4, the mRNA expression levels of the *YWHAG* and *BRSK2* genes in AIT patients were significantly lower than in controls (0·599 ± 0·215 *v.* 1·004 ± 0·003; 0·604 ± 0·248 *v.*

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Table 2. The demographic data in case and control groups with different levels of iodine

	IF				IA				IE			
	Case		Control		Case		Control		Case		Control	
n	89		89		40		40		47		47	
Gender												
Female	81		81		35		35		37		37	
Male	8		8		5		5		10		10	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age	45	8	45	8	44	10	44	10	43	11	43	11
BMI (kg/m^2)	24·4	3·2	24·3	2·9	24·0	3·3	24·0	3·4	25·6	3·6	25·5	3·3
	P_{50}	P_{25}–P_{75}	P_{50}	P_{25}–P_{75}	P_{50}	P_{25}–P_{75}	P_{50}	P_{25}–P_{75}	P_{50}	P_{25}–P_{75}	P_{50}	P_{25}–P_{75}
Water iodine content (μg/l)	2·6	1·9–2·8			71·4	18·2–98·2			325·0	300·3–375·2		
UIC (μg/l)	224·6	149·7–319·5	211·7	134·1–229·8	258·2	152·9–406·4	229·2	116·9–339·8	451·8	250·4–583·8	363·8	214·3–508·1
TSH (μIU/ml)	2·6	1·8–4·9*	2·1	1·5–2·7	2·5	1·6–4·2	2·4	1·8–3·1	3·1	1·9–4·1*	1·9	1·4–2·6
FT_3 (pmol/l)	5·2	4·7–5·6	5·3	4·8–5·6	5·1	4·8–5·4	5·2	5·0–5·6	5·2	4·8–5·4	5·1	4·6–5·4
FT_4 (pmol/l)	15·1	13·6–16·7	15·7	14·0–16·8	15·1	13·2–16·4	16·1	14·0–17·0	16·6	15·3–18·5	16·3	15·1–17·4
	n	%			*n*	%			*n*	%		
i-TGAb(+)	26	29·2†			12	30·0†			4	8·5		
i-TPOAb(+)	30	33·7			10	25·0			19	40·4		
TPOAb(+)&TGAb(+)	21	23·6			17	42·5‡			19	40·4‡		

IF, iodine fortification; IA, iodine adequate; IE, iodine excess; UIC, urinary iodine concentrations; TSH, thyroid stimulating hormone; FT_3, free triiodothyronine; FT_4, free thyroxine; i-TPOAb(+), isolated positive TPOAb; i-TGAb(+), isolated positive TGAb; TPOAb(+)&TGAb(+), double positive TPOAb and TGAb.

* Significant differences between case and control groups.

† Significant differences compare to IE.

‡ Significant differences compare to IF. $P < 0·05$.

Table 3. DNA methylation levels of regions between cases and controls (mean ± SD)

Targets	Chr	Case (*n* 176)		Control(*n* 176)		Meth. Diff	*P* value
		Mean	SD	Mean	SD		
YWHAG_1	7	0·601	0·040	0·587	0·049	0·014	0·003*
ING4_1	12	0·972	0·003	0·973	0·003	–0·001	0·083
BRSK2_1	11	0·415	0·148	0·371	0·152	0·044	0·005*
BRSK2_2	11	0·456	0·148	NA		NA	NA
GJA1_1	6	0·390	0·087	0·383	0·084	0·007	0·459

Chr, Chromosome; Meth. Diff = The methylation level of case-The methylation level of control; NA, not applicable.
* $P < 0·01$.

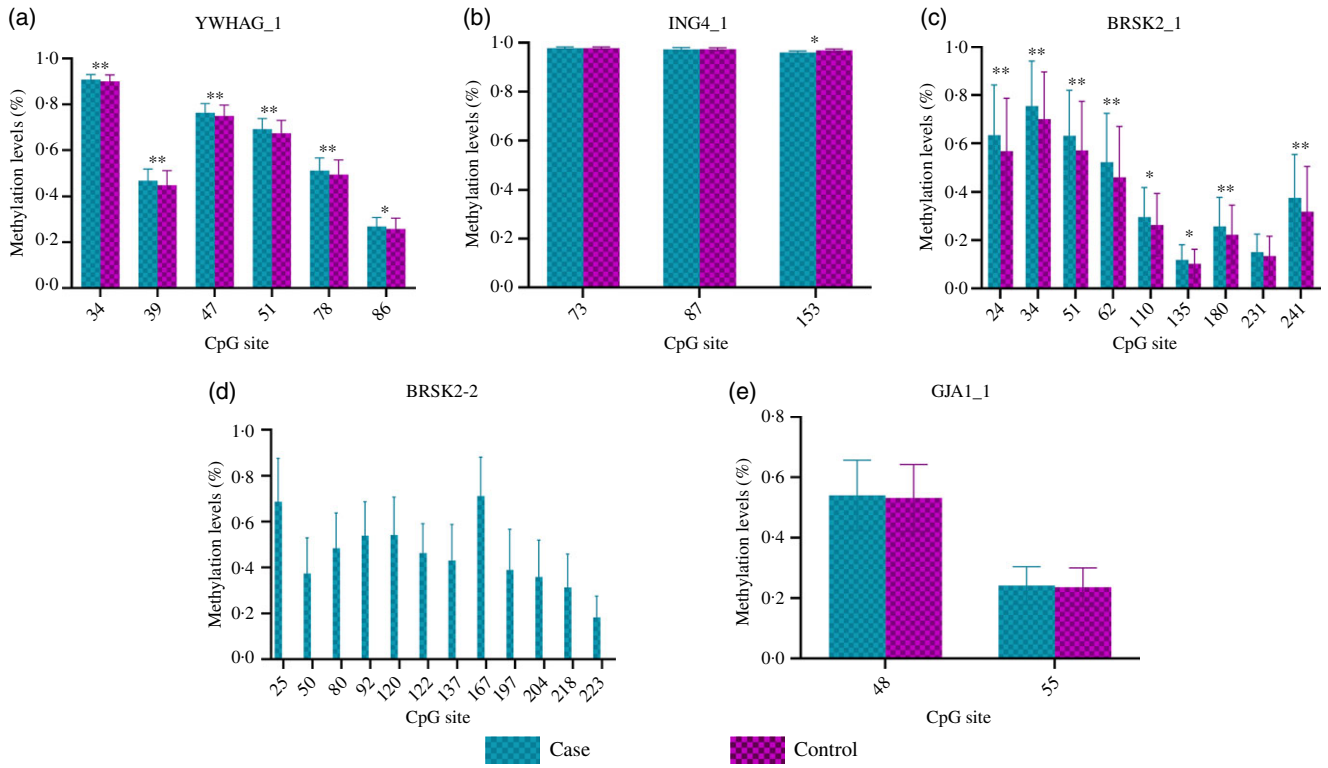

Fig. 1. DNA methylation levels of CpG sites between cases and controls. (a) *YWHAG_1*; (b) *ING4_1*; (c) *BRSK2_1*; (d) *BRSK2_2*; (e) *GJA1_1*; The case group compared with the control group, * $P < 0·05$, ** $P < 0·001$.

Table 4. DNA methylation levels of targets between cases and controls with different levels of iodine

Target	Chr	IF (Case : Control = 89:89)		IA (Case : Control = 40:40)		IE (Case :Control = 47:47)	
		Meth. Diff	*P* value	Meth. Diff	*P* value	Meth. Diff	*P* value
YWHAG_1	7	0·016	0·011*	0·005	0·632	0·017	0·090
ING4_1	12	–0·001	0·049*	0·001	0·558	–0·001	0·254
BRSK2_1	11	0·070	0·001*	0·064	0·054	–0·021	0·529
BRSK2_2	11	NA	NA	NA	NA	NA	NA
GJA1_1	6	0·021	0·105	–0·001	0·629	–0·008	0·657

IF, iodine fortification; IA, iodine adequate; IE, iodine excessive; Chr, Chromosome; Meth. Diff = The methylation level of case-The methylation level of control; NA, not applicable.
* $P < 0·05$.

1·007 ± 0·004, both $P < 0·001$), but that for *ING4* were higher (1·483 ± 0·568 *v.* 1·006 ± 0·003, $P < 0·001$) than those in healthy controls. A negative correlation was observed between DNA methylation levels and expression of the *YWHAG* gene ($r = -0·111$, $P = 0·038$). In supplementary Table 3, the mRNA expression of the *YWHAG* and *BRSK2* genes in AIT patients was lower than controls in different levels of iodine, but for *ING4* was higher (all $P < 0·001$).

Table 5. DNA methylation levels of CpG sites between cases and controls with different levels of iodine

					IF (Case: Control = 89:89)		IA (Case: Control = 40:40)		IE (Case: Control = 47:47)	
Gene	Target_site	Chr	Genome Position	Type	Meth. Diff	*P* value	Meth. Diff	*P* value	Meth. Diff	*P* value
YWHAG_1	34	7	75957903	CG	0·008	0·021*	0·002	0·678	0·009	0·091
YWHAG_1	39	7	75957898	CG	0·02	0·015*	0·002	0·833	0·021	0·066
YWHAG_1	47	7	75957890	CG	0·016	0·011*	0·007	0·427	0·017	0·079
YWHAG_1	51	7	75957886	CG	0·02	0·006**	0·007	0·531	0·019	0·095
YWHAG_1	78	7	75957859	CG	0·018	0·029*	0·008	0·543	0·023	0·08
YWHAG_1	86	7	75957851	CG	0·014	0·025*	0·001	0·917	0·011	0·244
ING4_1	73	12	6 762 512	CG	0·001	0·562	0·002	0·106	–0·001	0·065
ING4_1	87	12	6 762 498	CG	–0·001	0·722	–0·002	0·442	–0·001	0·861
ING4_1	153	12	6 762 432	CG	–0·003	0·003**	0·001	0·656	0·001	0·653
RSK2_1	24	11	1 474 831	CG	0·112	< 0·001**	0·069	0·154	–0·027	0·579
BRSK2_1	34	11	1 474 841	CG	0·1	< 0·001**	0·043	0·312	–0·021	0·617
BRSK2_1	51	11	1 474 858	CG	0·094	0·001**	0·078	0·079	–0·021	0·634
BRSK2_1	62	11	1 474 869	CG	0·095	0·001**	0·096	0·038*	–0·032	0·483
BRSK2_1	110	11	1 474 917	CG	0·047	0·011**	0·062	0·021*	–0·019	0·499
BRSK2_1	135	11	1 474 942	CG	0·02	0·024*	0·04	0·005**	–0·011	0·343
BRSK2_1	180	11	1 474 987	CG	0·054	0·001**	0·056	0·031*	–0·021	0·448
BRSK2_1	231	11	1 475 038	CG	0·024	0·036*	0·037	0·037*	–0·017	0·289
BRSK2_1	241	11	1 475 048	CG	0·082	0·002**	0·091	0·025*	–0·017	0·66
GJA1_1	48	6	122391246	CG	0·026	0·131	–0·009	0·569	–0·011	0·622
GJA1_1	55	6	122391253	CG	0·016	0·092	–0·008	0·535	–0·003	0·755

IF, iodine fortification; IA, iodine adequate; IE, iodine excess; Chr, Chromosome; Meth. Diff = The methylation level of case-The methylation level of control.
* $P < 0·05$, ** $P < 0·01$.

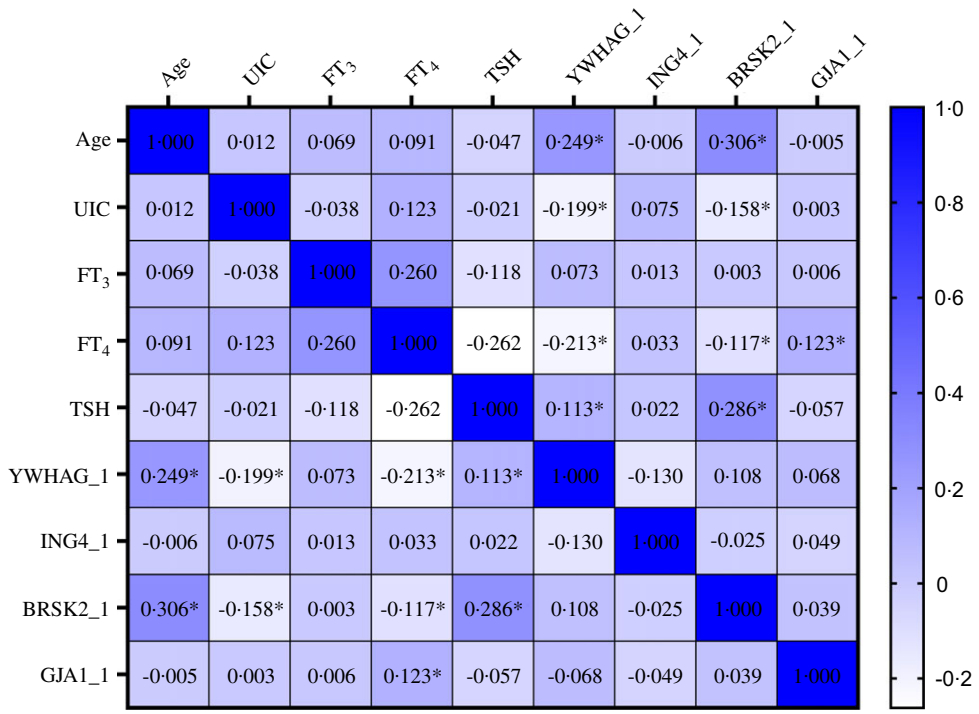

Fig. 2. Heat map of correlation between DNA methylation status of target region, age, UIC and thyroid function in AIT patients. *$P < 0·05$.

Discussion

In this study, firstly, we revealed the status of DNA methylation of apoptosis-associated genes in AIT patients and analysed the relationship between mRNA expression and DNA methylation. Secondly, we investigated whether iodine nutrition could affect DNA methylation and plays a potential role in the development of AIT. Our study not only demonstrated that iodine as a nutrient could induce epigenetic changes in the body and affect thyroid health but also screened out potential candidate genes and CpG sites that may be affected by iodine. Our research provides a theoretical basis for future nutrition research.

Table 6. Combined and interactive effects between methylation levels in the target region and IF in AIT

	Additive effects*					Interactive effects*		
	IA		IF					
Methylation levels in the target region	OR_{eg}	95 % CI	OR_{eg}	95 % CI	*P*-value	OR_i	95 %CI	*P*-value
YWHAG_1								
Hypomethylation	1		1·36	0·63,2·96		0·93	0·35,2·51	0·891
Hypermethylation	2·07	0·91,4·74	2·63	1·22,5·69	0·014			
ING4_1								
Hypomethylation	1		0·68	0·32,1·43		2·19	0·76,6·35	0·148
Hypermethylation	0·55	0·26,1·33	0·81	0·38,1·73	0·588			
BRSK2_1								
Hypomethylation	1		2·33	1·08,5·06		0·81	0·30,2·17	0·674
Hypermethylation	2·56	1·14,5·80	4·84	2·22,10·59	< 0·001			

IA, iodine adequate; IF, iodine fortification; OR_{eg}: OR genetic&environment, additive effects of methylation and IF; OR_i: OR interaction, multiplicative effects of methylation and IF.
* Adjustment: age, gender, BMI, smoking, drinking thyroid function and family history of thyroid disease.

Table 7. Combined and interactive effects between methylation levels in target region and IE in AIT

	Additive effects					Interactive effects*		
	IA		IE					
Methylation levels in target region	OR_{eg}	95 % CI	OR_{eg}	95 % CI	*P*-value	OR_i	95 %CI	*P*-value
YWHAG_1								
Hypomethylation	1		1·22	0·51,2·94		0·69	0·21,2·28	0·54
Hypermethylation	1·20	0·52,2·80	1·01	0·43,2·42	0·974			
ING4_1								
Hypomethylation	1		0·92	0·39,2·14		1·19	0·36,3·98	0·773
Hypermethylation	0·55	0·26,1·33	0·60	0·26,1·40	0·235			
BRSK2_1								
Hypomethylation	1		1·48	0·65,3·38		0·44	0·13,1·47	0·183
Hypermethylation	1·35	0·56,3·67	0·89	0·38,2·04	0·774			

IA, iodine adequate; IE, iodine excessive OR_{eg}: OR genetic&environment, additive effects of methylation and IE; OR_i: OR interaction, multiplicative effects of methylation and IE.
* Adjustment: age, gender, BMI, smoking, drinking thyroid function and family history of thyroid disease.

Table 8. GMDR analysis for the best gene–environment interaction models

Model	Training Bal. Acc.	Testing Bal. Acc.	Sign test	*P*	CV Consistency
Gene–environment interactions*					
BRSK2_1	0·598	0·530	7	0·172	5/10
YWHAG_1 × BRSK2_1	0·609	0·575	9	0·031	7/10
YWHAG_1 × BRSK2_1 × ING4_1	0·637	0·582	8	0·055	6/10
YWHAG_1 × BRSK2_1 × ING4_1 × iodine levels	0·661	0·606	9	0·010	10/10

Training Bal. Acc. Training balanced accuracy; Testing Bal. Acc. Testing balanced accuracy, CV consistency cross-validation consistency.
* Adjustment: age, gender, BMI, smoking, drinking thyroid function and family history of thyroid disease.

YWHAG (14–3–3γ) regulates diverse cellular processes, including apoptosis and cell proliferation[21]. Two interaction points exist between the *YWHAG* gene and the apoptotic machinery. Signals arriving from growth factor receptors activate kinases (e.g., *PI3K* and *Akt*), which phosphorylate several intrinsic proapoptotic factors (e.g., *Bad* and *FOXO1*), and consequently promote the binding of *YWHAG* proteins and inhibit intrinsic apoptosis. In contrast, pro-death signals activate the kinase JNK, among others, which phosphorylates *YWHAG* isoforms and induces the release of intrinsic proapoptotic client proteins (e.g., *Bax* and *Bim*), thereby triggering intrinsic apoptosis[22,23]. In our study, the *YWHAG* gene was hypermethylated, and the expression of mRNA was diminished in patients with AIT. We speculate that in AIT patients, the high methylation levels of the target region and CpG sites of the *YWHAG* gene led to the lower expression, thus affecting the combination of *YWHAG* and the intrinsic proapoptotic factors, and ultimately intrinsic apoptosis. Nevertheless, in the future, more studies are needed to confirm this process.

Some research has shown that *ING4* plays important roles in many biological processes, including DNA damage, cell proliferation and apoptosis[24]. It is a well-defined tumour suppressor in cancers such as lung cancer, breast cancer and glioma[25,26]. Researchers treated thyroid cancer cells with recombinant *ING4* protein and the results indicate that the rate of intrinsic apoptosis in thyroid cancer cells increased significantly, while the migration ability of thyroid cancer cells was inhibited[27]. *ING4* is also thought to enhance the intrinsic

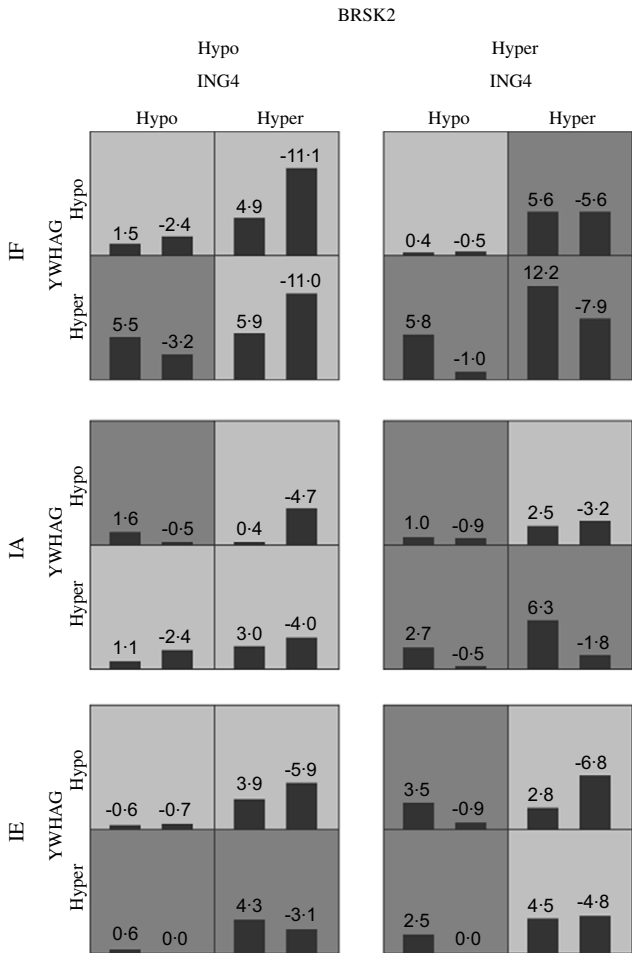

Fig. 3. The best adjusted GMDR model for gene–environment interaction. Hyper indicates hypermethylation; Hypo indicates hypomethylation; IF, iodine fortification; IA, iodine adequate; IE, iodine excessive; The adjusted covariates included age, gender, BMI, smoking, drinking thyroid function and family history of thyroid disease. The best model is composed of iodine levels, *YWHAG_1*, *ING4_1* and *BRSK2_1*. In each cell, the left bar represents a positive score, and the right bar represents a negative score. High-risk cells are indicated by dark shading. The pattern of high-risk and low-risk cells differs across each of the different multilocus dimensions, presenting evidence of epistasis.

apoptosis of human lung adenocarcinoma cells by activating the mitochondrial apoptotic pathway[28]. To our knowledge, little research explores the association between *ING4* gene methylation and AIT. Even so, the results of our study are consistent with results on the role of *ING4* in thyroid cancer cells and the pathogenesis of other tumours. Our results showed that the methylation level of CpG site 153 in *ING4* significantly differed between cases and controls. According to the biological role of *ING4* in intrinsic apoptosis, our results suggest that the *ING4* gene may affect AIT by intrinsic apoptosis pathway; however, the related details must be further studied.

BRSK2 is a serine/threonine kinase in the *AMPK* family[29]. Endoplasmic reticulum stress can regulate the levels of *BRSK2* protein and then participates in intrinsic apoptosis induced by endoplasmic reticulum stress[30]. Under the conditions of AIT, the function of the endoplasmic reticulum in sustaining proteostasis is perturbed, thus, leading to endoplasmic reticulum stress and intrinsic apoptosis. When *BRSK2* is knocked down, the expression of CHOP and caspase-3 increases, thus, eventually enhancing endoplasmic reticulum stress-mediated intrinsic apoptosis in cells. These findings suggest that the effects of *BRSK2* on intrinsic apoptosis under endoplasmic reticulum stress may be mediated through caspase-3. Therefore, this study is the first to investigate the correlation between *BRSK2* methylation and AIT. The results showed that DNA methylation levels of the target region *BRSK2_1* and eight CpG sites in AIT patients were significantly higher than those in controls, while the mRNA expression levels were significantly lower than those in controls. We hypothesised that *BRSK2* hypermethylation might silence *BRSK2* gene expression, thereby affecting intrinsic apoptosis induced by endoplasmic reticulum stress in AIT. However, all these hypotheses must be further studied.

We illustrate the effects of the methylation status of *YWHAG*, *ING4* and *BRSK2* on various signalling pathways related to apoptosis in Fig. 5. Signals from growth factor receptors activate *PKA* and *Akt*. *PKA* phosphorylates *BRSK2*. On the one hand, the phosphorylated *BRSK2* can be combined with *YWHAG* and fixed in the cytoplasm[31]. On the other hand, both endoplasmic reticulum and *BRSK2* hypermethylation can significantly down-regulate the expression of *BRSK2*, followed by up-regulation of CHOP and ultimately leading to the up-regulation of Caspase 3 and apoptosis[30]. PI3K-Akt can phosphorylate *Bad* protein. When *YWHAG* is normally expressed, it will be combined with the phosphorylated *Bad* and inactivate the Bad. In our study, *YWHAG* is hypermethylated, which leads to a decrease in its expression level and its ability to combine with Bad. After that, uncombined *Bad* will play a proapoptotic role on the mitochondrial membrane[32]. Meanwhile, the low expression of *YWHAG* could not be effectively combined with the phosphorylated *FOXO1*, so *FOXO1* could continue to play a proapoptotic role in the following process[33]. The death signal activates *JNK* and then breaks up the combination of phosphorylated *BAX* and *YWHAG*. After the separation, BAX is dephosphorylated, and the *YWHAG* is phosphorylated. The dephosphorylated BAX promotes cytochrome C release at the mitochondrial membrane and promotes apoptosis[34,35]. *JNK* can also activate *p53*. When *ING4* is hypomethylated, the over-expression of *ING4* will combine with *p53* and cause apoptosis. The overexpression of *ING4* can also promote the expression of *Bax* and inhibit the expression of *BCL2*, resulting in intrinsic apoptosis[36]. The above mechanism will be verified by relevant molecular biology experiments in our follow-up work.

Above, we analysed the relationship between methylation of our candidate genes and AIT and elaborated on the related mechanism. Next, we will discuss the effects of different iodine levels on the DNA methylation of candidate genes. In this study, the iodine levels mainly refer to the water iodine content and the use of iodised salt in the population because these are the main source of iodine nutrition for Chinese residents, dietary iodine and biomarker iodine were not discussed in this study. We found that according to recommendations from WHO/UNICEF/IGN, the iodine nutritional status of IF and IA were both adequate, but the difference in DNA methylation levels of the candidate genes (target and site) between the AIT cases and the control mainly occurred with the status of IF but not IA. Many studies have

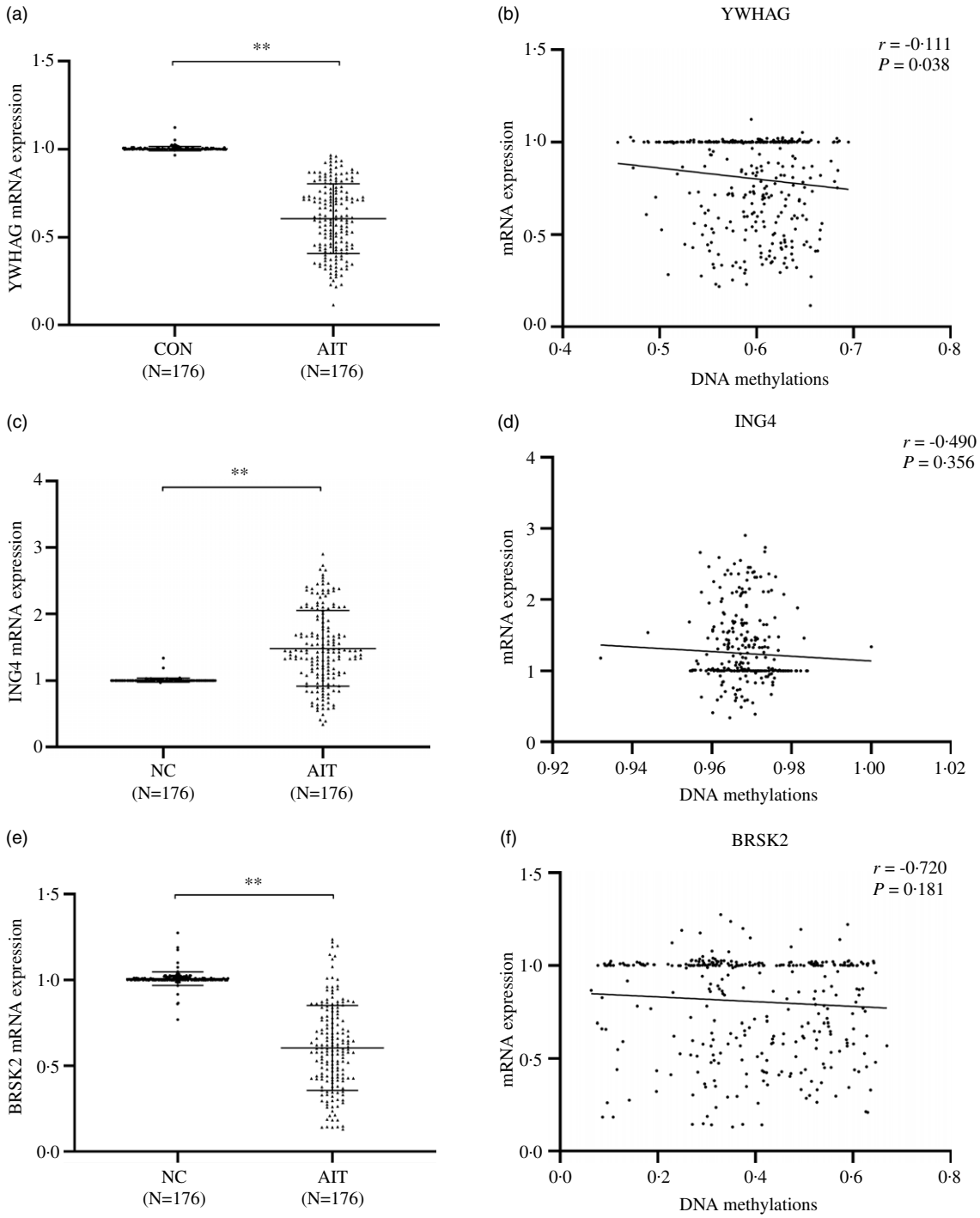

Fig. 4. Correlation analysis between DNA methylation and relative mRNA expression of *YWHAG*, *ING4* and *BRSK2* genes. (a) *YWHAG* mRNA expression; (b) Correlation between DNA methylation and relative mRNA expression of *YWHAG_1*; (c) *ING4* mRNA expression; (d) Correlation between DNA methylation and relative mRNA expression of *ING4_1*; (e) *BRSK2* mRNA expression; (f) Correlation between DNA methylation and relative mRNA expression of *BRSK2_1*.

shown that after IF[37,38], the status of thyroid autoimmune among some residents could change. Our results indicated that IF after iodine deficiency is more likely to alter the methylation levels of *YWHAG*, *BRSK2* and *ING4*. At the same time, the universal salt iodisation could be further fine-tuned and start with small doses of iodine in the future to reduce the impact of IF after iodine deficiency on epigenetics. Patients in IE were exposed to high water iodine content for a long time, and according to recommendations from WHO/UNICEF/IGN, their iodine nutritional status was IE. But there were no differences in the methylation levels of candidate genes between AIT cases and controls. Our results indicated that the effect of IE on these candidate genes is relatively weak. In addition, we also explored the effects of iodine nutrition on the DNA methylation levels of

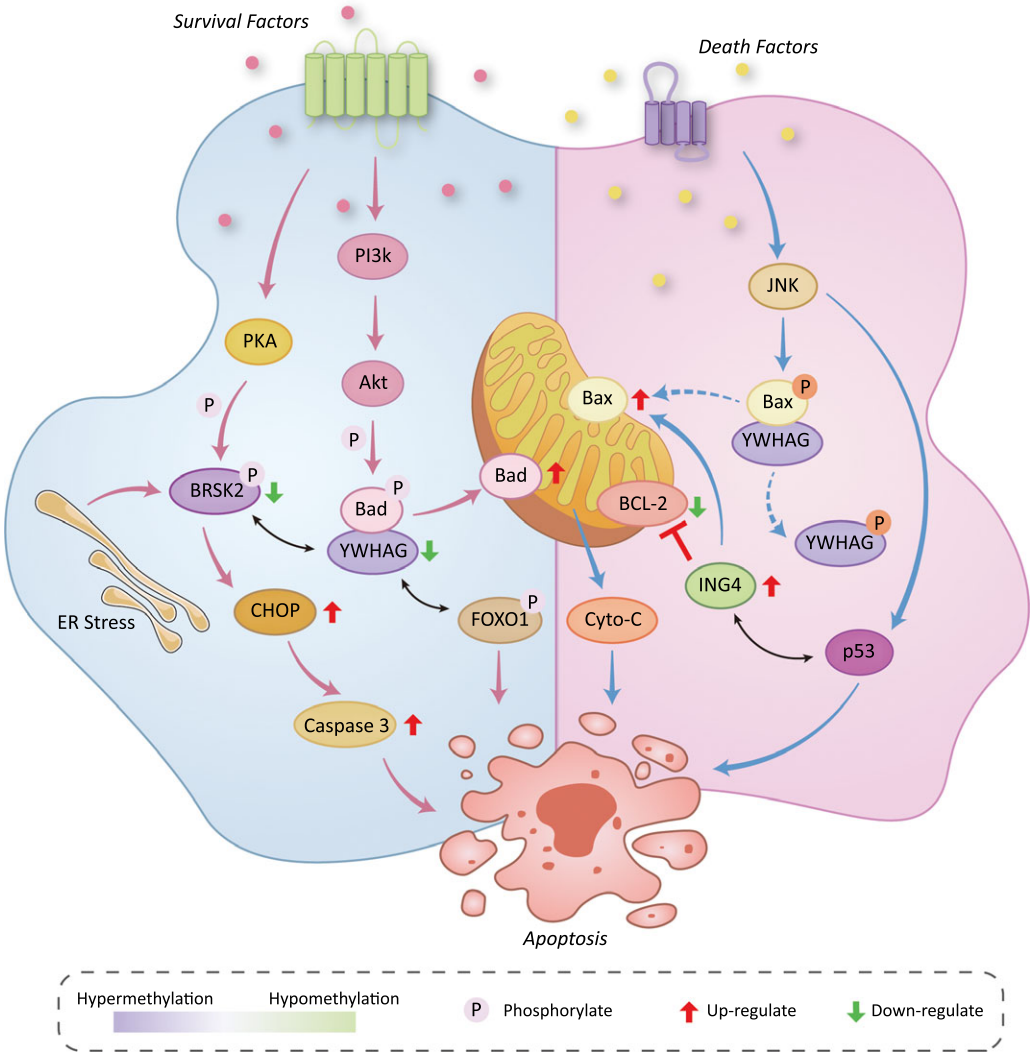

Fig. 5. Effects of the methylation status of *YWHAG*, *ING4* and *BRSK2* on various signaling pathways related to apoptosis.

target regions through UIC and found a negative correlation between DNA methylation levels of *YWHAG*, *BRSK2* and UIC, and this further indicates that the methylation levels of *YWHAG* and *BRSK2* are more likely to be high when the iodine nutritional status is deficient. Over recent years, there has been growing evidence showing that the effects of nutrition on health could be mediated by DNA methylation[39]. On the one hand, nutrients can change the level of global DNA methylation. For example, folic acid and vitamin B_{12} could significantly increase the levels of global DNA methylation, whereas selenium, bioflavonoids and green tee polyphenols could significantly decrease the levels of global DNA methylation[40,41]. On the other hand, many studies have found that some specific methylation sites are closely related to the nutritional status of the body; for example, the use of folic acid in the perimenopausal period can significantly increase methylation levels in the promoter region of the *IGF2* gene[42]. There are few studies on iodine nutrition and DNA methylation. One study in the elderly showed that iodine could not modulate the global DNA methylation profile of leukocytes[43], and another study in animals showed that IE did not change the global methylation levels in lymphocytes[44]. Our previous studies have shown that different iodine levels could affect the methylation patterns of *DAPK1* and *ITGA6*[45,46]. This study further confirms that iodine status could affect the methylation levels of intrinsic apoptosis genes.

Based on the above findings, we further investigated whether combined and interactive effects might exist among different iodine levels and the DNA methylation levels of these genes, to explore the potential interaction between iodine nutrition and epigenetics. In this study, IF combined with *YWHAG_1* hypermethylation and *BRSK2_1* hypermethylation significantly increased the risk of AIT. These findings suggest that IF not only influences the methylation pattern of intrinsic apoptosis-associated genes but also interacts with the methylation levels of these genes and may ultimately increase the risk of AIT. In the occurrence or development of AIT, this IF–methylation interaction model may play an important role. We analysed the single interactions between each gene and different levels of iodine, but the four genes are all associated with intrinsic apoptosis regulation, and the result of one gene–iodine level interaction

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could be influenced by other genes. So, we structured gene–environment interaction models by the GMDR method. We found a four-order high–dimensional interaction among *YWHAG*, *ING4* and *BRSK2* methylation and iodine levels, which strongly contributed to the risk of AIT. These epidemiological findings suggest that the methylation of the intrinsic apoptosis gene is associated with AIT.

Studies have shown that there is a positive relationship between the prevalence of thyroid antibodies and age[47]. Methylation has also been shown to be associated with ageing processes. However, few studies have assessed the relationship between intrinsic apoptosis-associated gene methylation and age in patients with AIT. In this study, we explored whether changes in the methylation of intrinsic apoptosis-associated genes might be correlated with age. Older age was associated with increased methylation levels of *YWHAG* and *BRSK2*. Thus, in older AIT patients, the changes in methylation in these two genes deserve more attention. In addition, we discussed the relationship between intrinsic apoptosis-associated gene methylation levels and thyroid function. Some studies have found that interindividual variations in FT_4, FT_3 and TSH are influenced by genetic factors[48]. Animal studies have shown that thyroid hormones affect DNA methylation, such as T_3, which leads to a significant reduction in DNA methylation[49]. An Australian cohort study has described six CpG sites associated with increased levels of FT_3 and two CpG sites associated with increased levels of TSH[50]. There are few studies on the association between the levels of DNA methylation in intrinsic apoptosis-associated genes and thyroid function in AIT patients. A positive correlation was found between the methylation level of *YWHAG_1* and *BRSK2_1* and TSH and a negative correlation with FT_4. We hypothesised that the methylation levels of intrinsic apoptosis-associated genes might affect thyroid function and predict changes in thyroid function in AIT. However, these possibilities require further verification.

There are some following strengths and limitations in our study. First, we not only analysed levels of DNA methylation in intrinsic apoptosis-associated genes in different levels of iodine, which have been rarely studied in the past but also comprehensively analysed gene–environment interactions and proposed the calculational model for assessing the interactions between iodine exposure and DNA methylation. Second, the intrinsic apoptosis-associated genes in our study were novel differentially methylated genes, which have rarely been reported before. However, the present study also had several limitations. First, this study was a case–control study, and we could not get the past methylation data of the participants, especially those who have experienced iodine supplementation. Second, the relationship between intrinsic apoptosis gene methylation and increased risk of AIT was based on the results of population epidemiological studies, but we did not study the molecular functional mechanism in depth because of the difficulty in obtaining thyroid tissues from patients with AIT. Thus, more experiments in animals, such as the NOD. H2 ^{h4} model and more molecular mechanism research are needed to extend the results of this study in the future.

In conclusion, our study showed that changes in methylation levels and mRNA expression in the *YWHAG*, *ING4* and *BRSK2* genes are associated with AIT. IF not only affects the methylation levels of *YWHAG* and *BRSK2* but also interacts with the methylation levels of these genes and may ultimately increase the risk of AIT. A four-order high–dimensional interaction among *YWHAG*, *ING4* and *BRSK2* methylation and iodine levels appear to contribute to the risk of AIT strongly. These findings suggest a mechanism in which intrinsic apoptosis-associated gene methylation plays a critical role in AIT.

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The original data generated during the current study are available in the SequenceRead Archive (SRA) repository, the Accession ID is PRJNA876118. https://www.ncbi.nlm.nih.gov/bioproject/PRJNA876118.

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The contribution of each author is as follows: H. S. designed the study; H. S., Z. Z., M. J., B. L., Y. H., L. L., B. R., J. L., F. L., J. L., Y. C., S. W. conducted the research. Z. Z. analysed the data. Z. Z. drafted the manuscript. All authors revised the report and approved the final version before submission.

The authors declare that no conflict of interest that could be perceived as prejudicing the impartiality of this study.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114523001216

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