Original Article

DNA Methylation of KLRC1 and KLRC3 in Autoimmune Thyroiditis: Perspective of Different Water Iodine Exposure*

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Abstract

Objective This study aimed to identify differentially methylated genes (DMGs) associated with natural killer cells in patients with autoimmune thyroiditis (AIT), focusing on the influence of varying water iodine exposure levels.

Methods Participants were divided into categories based on median water iodine (MWI) concentrations: iodine-fortified areas (IFA, MWI < 10 μg/L), iodine-adequate areas (IAA, 40 ≤ MWI ≤ 100 μg/L), and iodine-excessive areas (IEA, MWI > 300 μg/L). A total of 176 matched AIT cases and controls were recruited and divided into 89, 40, and 47 pairs for IFA, IAA, and IEA, respectively. DMGs were identified using 850K BeadChip analysis for 10/10 paired samples. Validation of DNA methylation and mRNA expression levels of the DMGs was conducted using MethylTarget™ and QRT-PCR for 176/176 paired samples.

Results *KLRC1*, *KLRC3*, and *SH2D1B* were identified as significant DMGs. Validation revealed that *KLRC1* was hypomethylated and highly expressed, whereas *KLRC3* was hypermethylated and highly expressed in individuals with AIT. Furthermore, *KLRC1* was hypomethylated and highly expressed in both IFA and IEA.

Conclusion The DNA methylation status of *KLRC1* and *KLRC3* may play crucial roles in AIT pathogenesis. Additionally, DNA methylation of *KLRC1* seems to be influenced by different iodine concentrations in water.

Key words: Autoimmune thyroiditis; DNA methylation; KLRC1; KLRC3; SH2D1B; Iodine

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INTRODUCTION

Autoimmune thyroiditis (AIT), also known as Hashimoto's thyroiditis (HT) or chronic lymphoid thyroiditis, is the most prevalent organ-specific autoimmune disease. It is characterized by the presence of thyroid peroxidase antibody (TPOAb) and/or thyroglobulin antibody (TgAb) in the serum, extensive lymphocyte infiltration, and damage to the follicular cell structure of the thyroid gland[1,2]. AIT is a leading cause of acquired hypothyroidism and goiter and predominantly affects middle-aged women[1]. Recent epidemiological studies indicated an increasing incidence of AIT worldwide, particularly in regions with uneven iodine distribution[3,4].

Iodine is a micronutrient essential for thyroid hormone synthesis and significantly influences the pathogenesis of thyroid diseases. Research has indicated that iodine deficiency can result in endemic goiters and cognitive deficits[5,6], whereas an iodine surplus may impair thyroid function, causing goiter, hyperthyroidism, and hypothyroidism[7,8]. The chronic consumption of high iodine levels and extended iodine fortification following prolonged deficiency are associated with an increased risk of AIT[9,10].

From the genomic perspective, AIT susceptibility genes are primarily classified into immune-associated and thyroid-specific categories[11]. Natural Killer (NK) cells, a subset of lymphocytes crucial to the innate immune system, are involved in the pathogenesis of common autoimmune diseases, including juvenile rheumatoid arthritis, Type I Diabetes Mellitus, and autoimmune thyroid disease[12-14]. Wenzel et al. reported reduced NK cell activity in peripheral blood lymphocytes of patients with HT[15]. This reduction in NK cell function may precede the development of thyroid-specific autoantibodies and ensuing lymphocyte migration and infiltration into the thyroid gland, corresponding to the clinical phenotype of AIT[16]. Thus, we suggest that genes related to NK cells contribute to AIT susceptibility. Increasing evidence has shown that epigenetic alterations, especially DNA methylation, are significant in autoimmune thyroid disease pathogenesis[17,18]. Previous DNA methylation studies on thyroid pathology have mainly focused on Graves' disease and thyroid carcinomas[19,20], leaving AIT comparatively unexamined. DNA methylation is affected by nutrient levels, environmental factors, sex, and age[21-24]. Tingting et al. observed abnormal DNA methylation and hydroxymethylation at the *ICAM1* gene promoter in the thyroid cells of patients with AIT, linking this epigenetic alteration to *ICAM1* gene expression[25]. Therefore, we hypothesized that DNA methylation, influenced by environmental and genetic factors, plays a role in AIT pathogenesis.

In conclusion, AIT results from both genetic and environmental factors. Given the limited research on differentially methylated genes (DMGs) associated with AIT, our primary goal was to identify DMGs associated with NK cell activity in patients with AIT. Considering the role of waterborne iodine as an environmental factor in AIT pathogenesis, our secondary objective was to examine the DNA methylation status of NK cells in patients with AIT living in areas with different iodine levels.

MATERIALS AND METHODS

Survey Regions

Following the Health Industry Standard issued by the People's Republic of China, iodine zones were identified based on median water iodine concentrations (MWI): iodine-fortified areas (IFA, MWI < 10 μg/L, with iodized salt qualification rates > 90%), iodine-adequate areas (IAA, 40 ≤ MWI ≤ 100 μg/L, with non-iodized salt supplement), and iodine-excessive areas (IEA, MWI > 300 μg/L, with non-iodized salt supplement)[26,27]. Cluster sampling was used to select the villages of Dongtan and Qianlv as IFA, Dongding and Liuxiangzhuang as IAA, and Xieyuan as IEA.

Sequencing and Survey Cohort

To form the sequencing cohort, ten pairs of patients with AIT and controls were enrolled at the Affiliated Hospital of the Shanxi Institute of Endemic Diseases in November 2018. The diagnostic criteria included: 1) presence of serum TPOAb and TgAb or positivity for both antibodies concomitant with hypothyroidism or subclinical hypothyroidism; 2) thyroid ultrasound revealing goiter, echo heterogeneity, or multiple hypoechoic areas. The inclusion criteria were: 1) healthy individuals corresponding to the case group in sex, age, residence, and Body Mass Index (BMI); 2) no history of autoimmune or other thyroid diseases, no chronic or acute conditions, no prolonged thyroid medication or hormone treatment, and no pregnancy; 3) no goiter, negative for TgAb and TPOAb, normal thyroid function test results, or unremarkable thyroid ultrasound findings. The data for the 10 pairs are presented in Supplementary

Table S1 (available in www.besjournal.com).

To assemble the verification cohort, the study enlisted 1,225 local participants, comprising 852 females and 373 males. This group included 409, 392, and 424 individuals from the IFA, IAA, and 424 from IEA. Participants were required to be 18 years or older and have lived in the survey areas for at least five years. The exclusion criteria were pregnant or lactating women, individuals on medication or undergoing treatments that might affect thyroid function, and those who had consumed iodine-enriched foods in the last 72 h. Clinical characteristics have been outlined in a previous study[28]. For detailed analysis, AIT cases and controls were matched by age, sex, BMI, and residence. The inclusion and exclusion criteria for the AIT cases and controls were consistent with those of the sequenced population. In total, 176 matched pairs were established, including 89, 40, and 47 pairs from IFA, IAA, and IEA, respectively. Informed consent was obtained from all the participants, and the study protocol was approved by the Ethics Review Committee of Harbin Medical University (hrbmuecdc20200320).

Sample Procurement and Laboratory Analyses

Water samples were collected from the specified survey areas, with each aliquot containing at least 15 mL and stored at 4 °C. Water iodine concentrations (WIC) was determined using As^{3-}-Ce^{4+} catalytic spectrophotometry, following the protocols of the National Reference Laboratory for Iodine Deficiency Disorders and the Chinese Center for Disease Control and Prevention. The Chinese National Reference Laboratory for Iodine Deficiency Disorders provides internal quality control for iodine in water. Urinary specimens were collected from participants between 08:00 and 11:00 in sterilized, labeled polyethylene containers and kept at 4 °C. Urinary iodine concentrations (UIC) were measured using As^{3-}-Ce^{4+} catalytic spectrophotometry according to the China Ministry of Health directive (WS/T 107.1-2016)[29]. Venous blood was drawn from the subjects after an 8-hour fast. Serum iodine concentrations (SIC) were assessed using an inductively coupled plasma mass spectrometry system (PerkinElmer NexION 350; Shelton, CT, USA) according to the standard (WS/T 783-2021)[30]. Thyroid function parameters, free triiodothyronine (FT_3), free thyroxine (FT_4), thyroid-stimulating hormone (TSH), TPOAb, and TgAb were quantified using a chemiluminescence immunoassay (Siemens Healthcare Diagnostics Inc.). The normative reference ranges for thyroid function were set as follows: FT_3, 3.1–6.8 pmol/L; FT_4, 11.5–22.7 pmol/L; TSH, 0.27–4.2 μIU/mL; TPOAb, 0–60 U/mL; and TgAb, 0–60 U/mL.

850K Beadchip

The Illumina Methylation EPIC 850K Beadchip was used for genomic screening of whole blood samples from 10/10 matched pairs. Genomic DNA was extracted using a TIANGEN Extraction Kit (TIANGEN, Beijing, China), and their purity and concentration were assessed using a Nanodrop 2000 spectrophotometer. A total of 500 ng of DNA from each sample was bisulfite-converted using an EZ DNA Methylation Kit (Zymo Research, USA) and hybridized onto an Illumina Infinium Human Methylation 850K BeadChip (Illumina Inc., CA, USA), according to the manufacturer's instructions.

A total of 853,307 CpG sites were examined, with 257 sites showing differential methylation across 139 DMGs, based on the criteria for methylation variance and *P* values. Functional enrichment and signaling pathway analyses were conducted using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases. Following these analyses, DMGs related to NK cells were further examined, and the results are listed in Supplementary Table S2 (available in www.besjournal.com). *KLRC1*, *KLRC3*, and *SH2D1B* were identified as key genes associated with AIT.

MethylTarget™

MethylTarget™ (Genesky Corporation, Shanghai, China) was used to verify the methylation differences of candidate genes, using whole blood samples from 176/176 matched pairs. The primer details are provided in Supplementary Table S3 (available in www.besjournal.com).

Genomic DNA was extracted using a TIANGEN Extraction Kit (TIANGEN, Beijing, China) and converted into bisulfite using an EZ DNA Methylation Kit (Zymo, Irvine, CA, USA). Amplification, barcoding, and sequencing of samples were conducted on a MiSeq platform (Illumina, Inc., San Diego, CA, USA), strictly following the manufacturer's protocols.

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

In this study, the mRNA expression levels of KLRC3 and KLRC1 were quantified using QRT-PCR in 176/176 matched pairs. Total RNA was extracted from whole blood using RNAiso Plus (Takara, Dalian, China) following the manufacturer's instructions. The quantity of isolated RNA was measured with a

NanoDrop2000 spectrophotometer (NanoDrop Technologies, USA), considering an optical density value between 1.8 and 2.0 at 260/280 as indicative of adequate sample quality. Reverse transcription was conducted using the PrimeScript™ RT Reagent Kit (Takara, Japan) on a gradient PCR machine (ABI, USA). Transcription levels were measured using a QuantStudio5 Real-Time PCR System (Applied Biosystems). The specific PCR primer sequences are listed in Supplementary Table S4 (available in www.besjournal.com).

Statistical Analysis

The data were organized and processed using Microsoft Excel 2019, and subsequent statistical analyses were performed using SPSS software version 22.0. Graphical displays were created using GraphPad Prism Version 5.0 (GraphPad Software Inc., CA, USA). For datasets following a normal distribution, values are expressed as means ± standard deviations and analyzed using *t*-tests or one-way analysis of variance, with further pairwise comparisons conducted using the Least Significant Difference test. For datasets that were not normally distributed, values were presented as medians with interquartile ranges [25th–75th percentiles] and analyzed using the Mann–Whitney U test or Kruskal-Wallis H test. The Chi-square test was used to compare rates between distinct groups. Correlations between the variables were evaluated using Spearman's rank or Pearson's correlation analysis. All statistical tests were two-sided, with a *P*-value < 0.050 deemed statistically significant.

RESULTS

Demographic Characteristics

After applying strict matching criteria for age, sex, and BMI, a cohort of 176 paired participants was established for further analyses. These pairs were divided into three groups: 89 pairs in the IFA, 40 pairs in the IAA, and 47 pairs in the IEA, as detailed in Table 1. Within this cohort, TSH levels in the AIT groups were significantly higher than those in the respective control groups, with $P < 0.001$ indicating statistical significance. Specifically, in the IFA and IEA groups, TSH concentrations in the AIT groups were significantly higher than those in the control groups ($P < 0.050$ for both). Differences in the prevalence of TgAb+, and TPOAb+ and TgAb+ were notable across the different water iodine regions, with $P < 0.05$ for

Table 1. Demographic characteristics of AIT and control groups

Characteristics	IFA (89:89)		IAA (40:40)		IEA (47:47)		All pairs (176:176)	
	Case	Control	Case	Control	Case	Control	Case	Control
Sex (male/female)	8/81	8/81	5/35	5/35	10/37	10/37	23/153	23/153
Age (years)	45 ± 8	45 ± 8	44 ± 10	44 ± 10	43 ± 11	43 ± 11	44 ± 9	44 ± 9
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	24.69 ± 3.44	24.59 ± 3.24
UIC (μg/L)	224.6 (149.6–319.5)	211.7 (134.0–299.8)	258.2 (152.9–406.4)	229.9 (116.9–339.8)	451.7 (250.4–583.8)	363.8 (214.3–508.1)	259.60 (157.10–439.25)	230.90 (144.90–363.80)
SIC (μg/L)	73.6 (63.3–86.8)	76.5 (68.9–85.2)	70.5 (64.2–84.0)	75.5 (62.8–83.6)	79.9 (70.0–96.3)	83.0 (70.1–93.7)	74.82 (64.23–86.98)	77.89 (68.08–86.41)
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)	5.2 (4.8–5.5)	5.2 (4.9–5.6)
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)	15.4 (13.9–17.6)	16.0 (14.4–17.1)
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)	2.8 (1.7–4.4)*	2.1 (1.5–2.8)
TGAb (+), *n* (%)	26 (29.2)†	–	12 (30)†	–	4 (8.5)	–	42 (23.9)	–
TPOAb (+), *n* (%)	30 (33.7)	–	10 (25.0)	–	19 (40.4)	–	59 (33.5)	–
TGAb (+) & TPOAb (+), *n* (%)	21 (23.6)	–	17 (42.5)‡	–	19 (40.4)‡	–	57 (32.4)	–

Note. Data are expressed as means ± standard deviations or medians withinterquartile ranges (25th–75th percentiles) or number (%). IFA, iodine-fortification area; IAA, iodine-adequate area; IEA, iodine-excess area; UIC, urinary iodine concentration; SIC, serum iodine concentration; FT_3, free triiodothyronine; FT_4, free thyroxine; TSH, thyroid-stimulating hormone; TPOAb (+), thyroid peroxidase antibody positive; TGAb (+), thyroglobulin antibody positive. *Significant differences compared with control groups; †Significant differences compared with IEA; ‡Significant differences compared with IFA. $P < 0.05$.

both. The prevalence of TgAb+ was significantly higher in the IFA and IAA groups than in IEA ($P < 0.05$ for both). Similarly, the prevalence of TPOAb+ and TgAb+ was significantly higher in the IAA and IEA groups than in IFA ($P < 0.05$ for both).

DNA Methylation Levels in Candidate Genes in the Context of 850K Array

Table 2 presents the methylation status of the selected candidate genes, specifically *KLRC1*, *KLRC3*, and *SH2D1B*, as identified using the 850K Beadchip array. These genes were classified as DMGs with *P* values less than 0.05 and a group differential greater than 0.1 and were significantly involved in the NK cell-mediated immune regulation pathway. These genes were hypomethylated in the AIT cohort compared with the control group, with all instances reaching statistical significance (all $P < 0.05$).

DNA Methylation Levels of Candidate Genes and CpG Sites Between the AIT and Control Groups in the MethylTarget™

As outlined in Table 3, we identified three candidate genes–*KLRC1*, *KLRC3*, and *SH2D1B*–encompassing 15 CpG sites. Specifically, KLRC1 was associated with seven CpG sites, KLRC3 with seven

Table 2. DNA methylation levels of candidate genes between AIT and control groups in the 850K

Probe	Gene	Chr	Position	Feature	Case	Control	Group. diff	*P*
cg23810434	*KLRC1*	12	10603937	5'UTR	0.689 ± 0.097	0.789 ± 0.061	−0.101	0.010*
cg04531182	*KLRC3*	12	10563981	TSS1500	0.272 ± 0.180	0.499 ± 0.299	−0.228	0.046*
cg01062020	*SH2D1B*	1	162382848	TSS1500	0.213 ± 0.125	0.405 ± 0.230	−0.193	0.026*

Note. Chr, chromosome; 5'UTR, in the range of 5'UTR sequence; TSS1500, in the range of 200 bp–1,500 bp upstream of the transcription start site; Group. diff, methylation level of case - the methylation level of control; *$P < 0.05$.

Table 3. DNA methylation levels of candidate genes and CpG sites between AIT and control groups in the MethylTarget™

Gene	Site	Case	Control	Group. diff	*P*
KLRC1	—	0.892 ± 0.026	0.907 ± 0.019	−0.015	9.37×10^{-10}**
	28	0.893 ± 0.029	0.904 ± 0.022	−0.012	4.71×10^{-5}**
	97	0.740 ± 0.052	0.749 ± 0.051	−0.010	0.085
	138	0.935 ± 0.036	0.942 ± 0.037	−0.008	0.059
	142	0.890 ± 0.048	0.919 ± 0.031	−0.030	8.02×10^{-11}**
	144	0.892 ± 0.045	0.922 ± 0.031	−0.031	2.44×10^{-12}**
	154	0.949 ± 0.019	0.958 ± 0.015	−0.010	1.48×10^{-6}**
	182	0.947 ± 0.018	0.956 ± 0.016	−0.009	4.71×10^{-5}**
KLRC3	—	0.658 ± 0.208	0.611 ± 0.202	0.047	0.033*
	65	0.621 ± 0.233	0.573 ± 0.227	0.049	0.049*
	99	0.545 ± 0.283	0.478 ± 0.280	0.067	0.027*
	122	0.581 ± 0.261	0.515 ± 0.256	0.067	0.017*
	133	0.558 ± 0.280	0.492 ± 0.275	0.067	0.026*
	162	0.675 ± 0.204	0.627 ± 0.199	0.048	0.027*
	174	0.802 ± 0.118	0.780 ± 0.116	0.022	0.080
	178	0.823 ± 0.099	0.812 ± 0.088	0.011	0.283
SH2D1B	—	0.147 ± 0.154	0.175 ± 0.191	−0.028	0.132
	81	0.147 ± 0.154	0.175 ± 0.191	−0.028	0.132

Note. Group.diff, the methylation level of case - the methylation level of control; *$P < 0.05$, **$P < 0.001$.

CpG sites, and SH2D1B with one CpG site. Comparative analysis indicated significant hypomethylation in *KLRC1* within the AIT cohort compared with the control group (t = −6.314, P < 0.001), whereas *KLRC3* was hypermethylated in the AIT cohort (t = 2.143, P = 0.033). The methylation status of SH2D1B did not significantly differ between the AIT and control groups.

At the individual CpG site level, five CpG sites in *KLRC1* in the AIT cohort were significantly hypomethylated compared with those in the control group (t = −4.127, −6.749, −7.309, −4.905, −4.875; all P < 0.001). Similarly, five CpG sites in *KLRC3* in the AIT cohort showed significant hypermethylation compared with the control group (t = 1.974, 2.220, 2.400, 2.243, and 2.223; all P < 0.050). No statistically significant difference was found in the methylation status of the CpG site within *SH2D1B* between the two groups.

Associations between DNA Methylation Levels of Candidate Genes and Age, Iodine Nutrition Status, and Thyroid Function among Patients With AIT

Figure 1 and Supplementary Table S5 (available in www.besjournal.com) show a significant inverse relationship between the methylation levels of the *KLRC1* gene and SIC in patients with AIT (r = −0.223, P = 0.004). Additionally, there was a negative correlation between the methylation levels of *KLRC3* and age in the AIT cohort (r = −0.235, P = 0.002). However, no significant associations were observed between the DNA methylation levels of the candidate genes and UIC, FT_3, FT_4, or TSH levels.

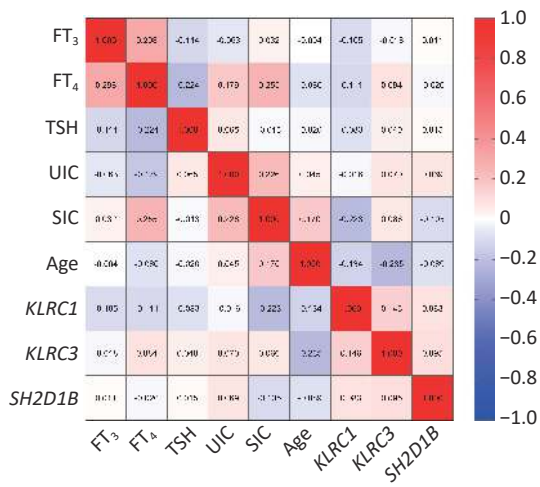

Figure 1. Correlation between DNA methylation levels of the candidate genes and age, iodine nutrition levels, and thyroid function in patients with autoimmune thyroiditis (AIT).

Sex, Age, SIC, and UIC Stratified Analysis of DNA Methylation Levels in AIT and Control Cohorts

Stratified analyses were performed following correlation analyses and insights from existing research on the factors influencing DNA methylation in the pathogenesis of AIT.

Supplementary Table S6 (available in www.besjournal.com) shows that in the male subgroup, *KLRC1* exhibited significantly lower methylation levels in the AIT cohort than in the control group (t = −2.744, P = 0.010). In the female subgroup, pronounced hypomethylation of *KLRC1* was observed in the AIT cohort compared with the controls (t = −5.701, P < 0.001). Regarding CpG sites associated with candidate genes, the male subgroup showed two CpG sites within *KLRC1* in the AIT cohort with decreased methylation compared with that in the control group (both P < 0.050). In the female subgroup, significant differences in methylation were found for five CpG sites in *KLRC1* and four CpG sites in *KLRC3* between the AIT and control cohorts (all P < 0.001 for *KLRC1* and all P < 0.050 for *KLRC3*).

Age-stratified analyses revealed significant findings, as detailed in Supplementary Table S7 (available in www.besjournal.com). In individuals aged ≤ 29 years, a single CpG site within *KLRC1* in the AIT cohort showed decreased methylation compared with that in the control group (P = 0.008). For the age group of 30–39 years, *KLRC1* and its five associated CpG sites in the AIT cohort were significantly hypomethylated relative to those in the control group (all P < 0.050). In the 40–49 years age group, the *KLRC1* gene and three of its CpG sites in the AIT cohort exhibited lower methylation levels than those in the control group (all P < 0.050), and the *SH2D1B* gene in the AIT cohort also showed hypomethylation (P = 0.019). In the subgroup aged > 50 years, three CpG sites within *KLRC1* in the AIT cohort had reduced methylation levels compared with those in the control group (all P < 0.050), and *SH2D1B* also demonstrated lower methylation in the AIT cohort (P = 0.017).

Following the analyses presented in Supplementary Table S8 (available in www.besjournal.com), within the subgroup having SIC between 50–109.9 μg/L, the *KLRC1* gene and its five associated CpG sites in the AIT cohort were found to have significantly lower methylation levels compared with the control group (all P < 0.050).

Additionally, the *SH2D1B* gene in the AIT cohort showed markedly reduced methylation levels compared with those in the control group (P = 0.001).

As indicated in Supplementary Table S9 (available in www.besjournal.com), for the subgroup with UIC less than 100 μg/L, three CpG sites associated with the *KLRC1* gene in the AIT cohort exhibited lower methylation (all $P < 0.050$). In the subgroup with UIC between 100–199 μg/L, two CpG sites associated with the *KLRC1* gene in the AIT cohort showed lower methylation (all $P < 0.050$). In the subgroup with UIC between 200–299 μg/L, four CpG sites associated with the KLRC1 gene showed lower methylation, three CpG sites of the *KLRC3* gene showed higher methylation, and the *SH2D1B* gene showed lower methylation in the AIT cohort (all $P < 0.050$). In participants with UIC greater than 300 μg/L, both the *KLRC1* gene and its five associated CpG sites in the AIT cohort exhibited lower methylation, and the *SH2D1B* gene also showed reduced methylation levels (all $P < 0.050$).

DNA Methylation Levels of Candidate Genes and CpG Sites between AIT and Control Groups in Different Water Iodine Areas

In an analysis presented in Table 4, significant hypomethylation of the *KLRC1* gene in the AIT cohort compared with the control group was observed across various iodine-rich water environments, including IFA, IAA, and IEA (t = −2.758, −5.010, and −4.232, respectively; all $P < 0.050$). Specifically, in IFA, four CpG sites associated with *KLRC1* in the AIT cohort showed hypomethylation (t = −2.519, −2.618, −3.285, and −1.979; all $P < 0.050$). In IAA, five CpG sites in KLRC1 exhibited hypomethylation in the AIT cohort (t = −2.557, −5.484, −5.991, −4.693, and −3.514; all $P < 0.050$), with similar findings in IEA (t = −2.737, −5.034, −4.690, −4.817, and −4.086; all $P < 0.050$). No statistically significant differences were observed in the methylation levels of *KLRC3* and *SH2D1B* or their CpG sites between the AIT and control groups.

Further investigation into DNA methylation

Table 4. DNA methylation levels of candidate genes and CpG sites between AIT and control groups in different water iodine areas

Gene	Sites	IFA (89:89)		IAA (40:40)		IEA (47:47)	
		Group.diff	*P*	Group.diff	*P*	Group.diff	*P*
KLRC1	—	−0.010	0.006**	−0.021	4.00×10^{-6}**	−0.021	6.73×10^{-5}**
	28	−0.010	0.013*	−0.011	0.059	−0.016	0.008*
	97	−0.006	0.461	−0.010	0.362	−0.017	0.139
	138	−0.008	0.162	−0.010	0.013*	−0.006	0.605
	142	−0.017	0.010*	−0.045	7.93×10^{-7}**	−0.040	4.35×10^{-6}**
	144	−0.020	0.001*	−0.046	8.96×10^{-8}**	−0.037	1.32×10^{-5}**
	154	−0.002	0.489	−0.015	1.25×10^{-5}**	−0.018	8.73×10^{-6}**
	182	−0.006	0.049*	−0.011	7.70×10^{-3}**	−0.013	1.06×10^{-3}**
KLRC3	—	0.037	0.250	0.054	0.265	0.060	0.127
	65	0.036	0.311	0.053	0.339	0.069	0.122
	99	0.050	0.250	0.076	0.253	0.090	0.098
	122	0.051	0.208	0.067	0.276	0.096	0.054
	133	0.053	0.220	0.076	0.249	0.085	0.116
	162	0.040	0.205	0.051	0.280	0.060	0.118
	174	0.021	0.240	0.034	0.217	0.013	0.582
	178	0.005	0.718	0.023	0.289	0.010	0.586
SH2D1B	—	−0.021	0.412	−0.058	0.126	−0.016	0.675
	1	−0.021	0.412	−0.058	0.126	−0.016	0.675

Note. IFA, iodine-fortification areas; IAA, iodine-adequate areas; IEA, iodine-excess areas; Group.diff, the methylation level of case - the methylation level of control; *$P < 0.05$, **$P < 0.001$.

differences among patients with AIT across these iodine-rich areas, as detailed in Supplementary Table S10 (available in www.besjournal.com). We confirmed the demographic consistency in age, sex, and BMI among the case cohorts. No significant differences in methylation levels were found for *KLRC1*, *KLRC3*, or *SH2D1B* among cases. Nonetheless, significant differential methylation was observed at the three CpG sites of *KLRC1* across these areas (F = 3.903, 4.175, and 6.301; all $P < 0.050$). Pairwise comparisons further highlighted the increased DNA methylation levels in patients with AIT in the IFA compared with those in the IEA, which was statistically significant (all $P < 0.050$).

mRNA Expression Levels of KLRC1 and KLRC3 Gene

Due to the observed differential methylation in *KLRC1* and *KLRC3* genes between AIT and control groups, as reported in the context of the MethylTarget™ assay (referenced in Table 3), these genes were selected for validation analysis of their mRNA expression levels using QRT-PCR. Figure 2A shows that the transcriptional activity of both *KLRC1* (1.356 ± 0.939 *vs.* 1.007 ± 0.016, t = −4.926, P < 0.001) and *KLRC3* (1.281 ± 0.893 *vs.* 1.009 ± 0.015, t = −3.849, P < 0.001) was significantly higher in the AIT cohort compared with the control group. Figure 2B shows a significant increase in *KLRC1* mRNA expression in patients with AIT over controls within IFA (1.319 ± 0.955 *vs.* 1.006 ± 0.019, t = −3.077, P = 0.002) and IEA (1.299 ± 0.917 *vs.* 1.005 ± 0.008, t = −2.196, P = 0.031). Similarly, Figure 2C indicates a rise in *KLRC3* mRNA expression levels in the AIT cohort versus the control group in IFA (1.260 ± 0.811 *vs*. 1.011 ± 0.016, t = −2.043, P = 0.043) and IEA (1.454 ± 0.999 *vs.* 1.009 ± 0.014, t = −3.048, P = 0.003). Further analysis revealed no significant variation in the mRNA expression levels of *KLRC1* and *KLRC3* across the iodine areas (F = 1.609, P = 0.203; F = 1.270, P = 0.284).

Correlation Analysis of DNA Methylation Levels and mRNA Expression Levels of KLRC1 and KLRC3 Genes

As shown in Figure 3, correlative analyses showed that DNA methylation levels in *KLRC1* and its four associated CpG sites were inversely related to mRNA expression (all $P < 0.050$). However, no linear correlation was observed between the DNA methylation status of *KLRC3* and its seven CpG sites or mRNA expression levels. In addition, there were no linear associations between DNA methylation and mRNA expression levels of *KLRC1* and *KLRC3* when analyzed with varying water iodine concentrations (all $P > 0.050$).

DISCUSSION

The present study identified NK cell-associated genes, specifically *KLRC1*, *KLRC3*, and *SH2D1B*, as DMGs. Further investigation in a larger cohort demonstrated that *KLRC1* was hypomethylated and exhibited increased transcription, whereas *KLRC3* showed hypermethylation and elevated expression levels in individuals with AIT. Environmentally, KLRC1 was found to be hypomethylated and transcriptionally active, particularly in IFA and IEA.

KLRC1 (NKG2A), an inhibitory receptor predominantly expressed in NK and T cells, plays a crucial role in modulating NK cell exhaustion and inhibiting cytotoxicity[31]. Our findings indicate that *KLRC1* was hypomethylated and showed increased mRNA expression in the AIT cohort. Additionally, an

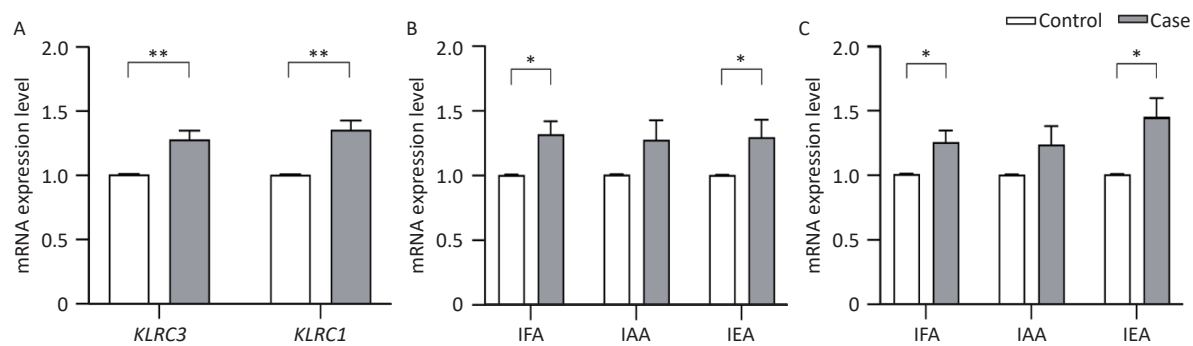

Figure 2. mRNA expression levels of *KLRC1* and *KLRC3* gene. (A) mRNA expression levels of KLRC1 and *KLRC3* in cases and controls; (B) mRNA expression levels of *KLRC1* in cases and controls from different water-iodine areas; (C) mRNA expression levels of *KLRC3* in cases and controls from different water-iodine areas. IFA, iodine-fortified areas; IAA, iodine-adequate areas; IEA, iodine-excessive areas. $^{*}P < 0.05$; $^{**}P < 0.01$.

inverse relationship was observed between the DNA methylation status and mRNA expression levels of *KLRC1*, aligning with the principle that promoter hypomethylation is associated with increased gene expression (as observed with *KLRC1* promoter methylation changes in Table 2)[32]. Previous studies have also noted increased expression of *KLRC1* in patients with HT[33], suggesting that the methylation status of *KLRC1* may regulate NK cell function and contribute to AIT pathogenesis. However, further research is required to confirm this hypothesis.

KLRC3 (NKG2E) forms a complex with CD94 in NK cells and acts as an activating receptor that modulates the immune response[34]. Our data showed that *KLRC3* was hypermethylated and had higher transcriptional activity in the AIT cohort. This finding may seem counterintuitive to traditional views as emerging evidence suggests that promoter hypermethylation sometimes coincides with increased transcriptional activity[35]. The CpG sites examined near KLRC3 were primarily in the distal promoter region (TSS1500), which typically has a reduced effect on gene expression[36]. The complex role of DNA methylation in immune-mediated diseases[37] indicates that the relationship between *KLRC3* methylation and AIT pathogenesis requires further investigation.

SH2D1B (EAT-2) functions as an inhibitory component in NK cell operations and is involved in signal transduction modulation through signaling lymphocyte activation molecule family receptors, affecting both the innate and adaptive immune systems[38]. Previous studies have shown that SH2D1B increases the secretion of pro-inflammatory cytokines and chemokines, thereby increasing the regulatory and effector functions of human immune cells[39]. Research using animal models has identified SH2D1B as a negative regulator of NK cell activity, with these regulatory changes potentially leading to autoreactivity and autoimmunity[40]. Although identified as a DMG in the 850K array, our analysis did not reveal significant differences in *SH2D1B* methylation between the AIT and control groups.

Correlation and stratification analyses were performed to explore the influence of potential confounding factors on the methylation of candidate genes. These findings suggest that the methylation status of certain genes, particularly *KLRC1*, is associated with variables such as age, sex, SIC, and WIC. Autoimmune diseases generally show a female predilection, especially pronounced in thyroid disorders such as HT and Graves' disease[41], which is consistent with the observations of this study. A notable number of CpG sites near the candidate genes displayed differences in methylation, primarily in individuals older than 29 years. It has been established that AIT is more common in females and increases with age[1], particularly affecting age

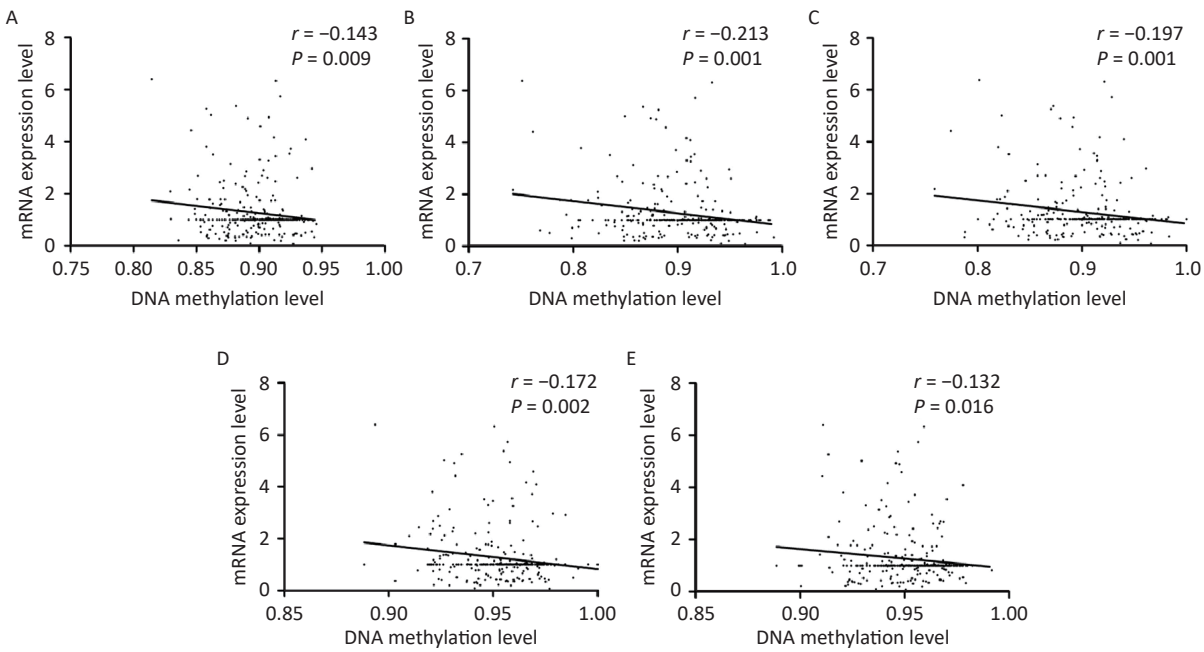

Figure 3. Scatter plots for mRNA expression levels and DNA methylation levels of *KLRC1*. (A) *KLRC1*, (B) *KLRC1*_142, (C) *KLRC1*_144, (D) *KLRC1*_154, and (E) *KLRC1*_182.

groups beyond 45–50 years[42].This finding is supported by our data. Iodine is a crucial component in the synthesis of thyroid hormones, which is a complex process that occurs in thyroid follicular cells. This study identified a significant association between serum and water iodine concentrations and DNA methylation status of genes implicated in AIT.

Our investigation was centered on the distinct methylation patterns observed between AIT cases and controls in different iodine environments. Our findings revealed that in IEA, *KLRC1* was hypomethylated and showed increased expression in the AIT cohort. These findings support the hypothesis that excessive iodine intake increases the risk of developing AIT. For instance, a five-year prospective study indicated an increasing cumulative incidence of AIT with varying iodine intake levels: 0.2% in mildly deficient, 1.0% in more than adequate, and 1.3% in excessive iodine intake scenarios[9]. Additionally, our group observed a higher incidence rate of AIT among lactating women in iodine-excess regions than among those in optimal iodine areas[7]. In areas where groundwater consumption is prevalent and groundwater iodine levels are high, iodine nutritional status largely depends on groundwater iodine concentration[43]. IEA, which lacks iodized salt provision, relies heavily on waterborne iodine, leading to a significantly higher UIC in IEA than in IFA and IAA[28]. This suggests that excessive waterborne iodine exposure in the IEA influences *KLRC1* methylation and mRNA transcription, contributing to the development of AIT.

In IFA, *KLRC1* exhibited hypomethylation and increased transcription in the AIT cohort. The exacerbation of thyroid autoimmunity following iodine supplementation after prolonged deficiency has been well documented. A previous study highlighted a potential link between increased iodine consumption and AIT onset, suggesting that excess iodine may trigger thyroid autoimmunity, leading to hypothyroidism[10]. Moreover, a longitudinal study by Zois et al. involving 302 children over seven years recorded a threefold increase in AIT prevalence post-iodization[44].

In areas with lower iodine levels, the iodine nutritional index primarily depends on the iodine content in salt and dietary iodine intake, especially with the implementation of the USI[43]. In our study, the IFA was supplied with an iodized salt, making it the primary source of salt-derived iodine. Our previous research indicated that the UIC in IFA (228.4 μg/L) was similar to that in IAA (243.9 μg/L)[28], suggesting comparable iodine nutritional levels despite the different iodine sources. We observed hypomethylation of the *KLRC1* gene in the AIT cohort in IAA, where iodized salt was not supplied, and iodine intake mainly came from water. This suggests that diet in IFA or waterborne in IAA may influence KLRC1 methylation irrespective of the iodine source, contributing to AIT pathogenesis. To reduce the disease risk associated with iodine nutrition, different strategies may be considered: reducing the iodized salt supply in IFA to lower iodine nutritional levels and potentially decrease AIT risk and in IAA, focusing on environmental health impacts, such as water treatment methods to reduce drinking water iodine levels.

A significant challenge in this study involved comparing DNA methylation profiles between the AIT and control groups from different iodine environments, particularly when controlling for confounding variables. Although the water iodine levels in the IFA, IAA, and IEA regions were very different, the differences in iodine nutritional levels (urinary iodine level and serum iodine) among the populations of the three regions were not obvious because China adopted different salt iodization policies (iodized salt in the IFA and non-iodized salt in the IAA and IEA regions). Urinary and serum iodine levels in the human body are more reflective of the iodine nutritional status, which may lead to a smaller difference in DNA methylation levels among the three regions. Moreover, owing to the broad scope of our research and ethical considerations, we used whole blood samples instead of specific thyroid tissues. Whole blood comprises various cell types, which limits our ability to obtain detailed biological insights specific to certain tissues or organs. This limitation may obscure subtle biological differences and disease mechanisms pertinent to specific tissue types, highlighting the complexity of dissecting the molecular underpinnings of diseases, such as AIT, within the constraints of available sample types.

CONCLUSION

This study provides cross-sectional empirical evidence to clarify the relationship between genomic methylation patterns and AIT in populations exposed to varying waterborne iodine concentrations. The DNA methylation status of *KLRC1* and *KLRC3* is closely associated with AIT pathogenesis. Our findings indicate that changes in KLRC1 DNA methylation in patients with AIT may vary significantly between IFA and IEA.

CONFLICT OF INTEREST

No competing financial interests exist.

AUTHORS CONTRIBUTION

Yao Chen: Writing-original draft preparation and formal analysis. Jinjin Liu: Writing-reviewing and editing. Mengying Qu, Bingxuan Ren, Huaiyong Wu, Li Zhang, and Zheng Zhou: Investigation and validation. Lixiang Liu: Project administration. Hongmei Shen: Supervision and funding acquisition.

DATA AVAILABILITY

The data described in the manuscript, codebook, and analytic code will not be made available.

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