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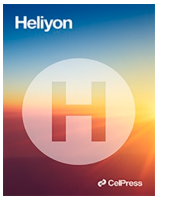

Research article

A study on the methylation patterns of *DIO3* in patients with heart failure and its correlation with key clinical parameters

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ABSTRACT

Objective: This study aimed to analyze the methylation pattern of deoxyribonucleic acid (CpG) sites in the *DIO3*_FA26 promoter region of patients with heart failure (HF) and explore the correlation between differential CpG methylation levels and various clinical parameters.
Methods: Peripheral blood specimens were collected from 20 patients with HF and 20 healthy individuals. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to identify and detect the CpG sites in the *DIO3*_FA26 promoter region. CpG methylation levels were compared between patients with HF and healthy controls and patients with HF with different levels of cardiac function.
Results: The methylation level of *DIO3*_FA26_CpG_17.18 in patients with HF was significantly lower than that in the healthy control group ($P = 0.0002$). Among patients with HF and cardiac function levels of I/II and III/IV, methylation levels of *DIO3*_FA26_CpG_24.25.26.27 ($P = 0.0168$) were significantly lower in those with III/IV cardiac function compared to those with I/II cardiac function.
Conclusion: The methylation level of *DIO3*_FA26_CpG_17.18 is significantly reduced in patients with HF, and that of *DIO3*_FA26_CpG_24.25.26.27 is significantly decreased in patients with III/IV cardiac function. Variations in *DIO3*_FA26 methylation levels influence coagulation, liver and kidney functions, and routine blood indexes, including D-dimer, albumin, calcium, and hemoglobin. This study provides clinical evidence for the involvement of *DIO3*_FA26 methylation in the occurrence and development of HF and proposes novel targets for HF prevention and treatment.

1. Introduction

Heart failure (HF) is a chronic progressive complex clinical syndrome caused by abnormal cardiac structure or function, often presenting with cardiac systolic and/or diastolic dysfunction [1,2]. Clinical manifestations include exertional dyspnea or fatigue, orthopnea, edema, tachycardia, and jugular vein distension [3]. HF is a severe manifestation of terminal-stage cardiovascular disease, with a high incidence, mortality, and treatment costs, posing a substantial public health burden worldwide. As a result of economic

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growth, urbanization, and population aging, HF has become a major public health concern, seriously affecting the well-being of Chinese inhabitants [4].

There are two main types of HF: systolic HF and diastolic HF. Systolic HF occurs when the heart muscle becomes weakened and cannot contract effectively, leading to reduced pumping ability. Diastolic HF, on the other hand, happens when the heart muscle becomes stiff and doesn't relax properly, impairing its ability to fill with blood. Common causes of HF include coronary artery disease, high blood pressure, heart valve problems, heart muscle damage (such as from a heart attack), and certain conditions that affect the heart's rhythm [5–8]. Symptoms of HF can vary but often include shortness of breath, fatigue, swelling in the legs and ankles, and a reduced ability to exercise. The importance of investigating the pathophysiology of heart failure and its relationship with epigenetics has become increasingly recognized. Recent research suggests that epigenetic modifications, which are influenced by environmental factors, may play a crucial role in the development and progression of heart failure [9–13]. Advances in this field have the potential to shed new light on the underlying mechanisms of HF and may ultimately lead to novel therapeutic approaches and preventative strategies.

Selenium (Se), a trace nutrient essential for human health, is prevalent in common foods [14]. Se performs multiple physiological functions, including improving immune function, thyroid function, antioxidation, detoxification, and cell protection and repair [15,16]. The crucial biological functions of Se are achieved through selenoproteins, with 25 selenoproteins being identified to date. The iodothyronine deiodinase (ID) family, an important member of selenoproteins, comprises three subtypes: type I deiodinase (DIO1), type II deiodinase (DIO2), and type III deiodinase (DIO3) [17]. DIO1 is predominantly located in the liver, kidneys, and thyroid; DIO2 is in the pituitary gland, thyroid, and skeletal muscle; *DIO3* is primarily present in the skin and cerebral cortex. The coordinated regulation of thyroid hormones (TH) by these three deiodinases maintains the body's equilibrium [18]. DIO1 and DIO2 primarily convert T4 (thyroxine) into the physiologically more active T3 (triiodothyronine), while DIO3 is involved in converting T3 into the less active rT3 (reverse triiodothyronine). In the heart, increased DIO3 activity reduces the availability of T3, potentially impacting cardiac function. Furthermore, thyroid hormones significantly influence the metabolism of cardiac myocytes, enhancing their energy production and utilization [19,20]. Changes in deiodinase activity can alter how cardiac myocytes respond to thyroid hormones, thereby affecting myocardial contractility and heart rate. Abnormal levels of thyroid hormones (both high and low) are associated with changes in cardiac structure and function. For instance, both hyperthyroidism and hypothyroidism are linked to cardiac pathology and heart failure [21–23].

Epigenetics is the study of heritable changes in gene expression occurring without altering the DNA sequence. Common epigenetic regulatory mechanisms include DNA methylation, histone modification, chromatin remodeling, and non-coding RNA [24]. DNA methylation is the most prevalent and important form, primarily occurring at the cytosine-phosphate-guanine (CpG) sites in DNA sequences. With the action of DNA methyltransferases, a methyl group is added to the 5th carbon atom of cytosine, forming 5-methylcytosine [25]. DNA methylation can regulate gene expression levels, with low methylation increasing gene expression levels and high methylation causing "silencing" of key genes [26]. It plays a crucial role in cell development, genome stability, and disease occurrence [27]. Previous studies have demonstrated that DNA methylation can influence the expression of genes associated with HF, thereby affecting the occurrence and development of HF [28,29]. However, due to various reasons, current research on the methylation levels of HF and the *DIO3* promoter has not reached a consistent conclusion.

In the present study, the methylation status of CpGs in the *DIO3* promoter region was quantitatively assessed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in the peripheral blood of patients with HF. Then, we evaluated the epigenetic modifications in the *DIO3* of patients with HF, elucidated differences in CpG methylation in the *DIO3* promoter region, and further analyzed the roles of *DIO3* promoter methylation in patients with HF. As DNA methylation changes are usually reversible, the CpG sites identified in this research may potentially function as intervention targets, thereby contributing to the promotion of HF prevention and control.

2. Materials and methods

2.1. Study population

The research subjects were selected from the Stroke Center of Ningxia Autonomous Region People's Hospital. Blood samples were collected from 20 patients with HF and 20 healthy individuals undergoing physical examinations. All participants were Han Chinese, underwent relevant examinations, and were diagnosed following the national diagnostic criteria for HF [30–32]. The morning after all research subjects were included in the study, 5 ml of fresh elbow vein blood was collected from each subject while fasting. The blood was anticoagulated with EDTA, genomic DNA was extracted, and the samples were stored at −80 °C for future use. All individuals volunteered to participate and signed informed consent forms.

2.2. DNA extraction, quantification, and quality control

Genomic DNA was extracted from peripheral blood samples using a DNA extraction kit provided by QIAGEN, Germany. The extracted DNA was quantified using a spectrophotometer, and 100 ng of DNA is taken for further analysis. The quality of the extracted DNA was assessed by performing electrophoresis on a 0.8 % agarose gel. The genomic DNA should appear as a band larger than 20 kb with no significant degradation, and the A260/A280 ratio should be between 1.7 and 2.1. The total DNA amount should exceed 2 μg, indicating that the DNA has passed quality control. DNA that meets these criteria was then diluted to a concentration of 75 ng/μL and stored in a 96-well plate at −20 °C for future use.

2.3. Primer design and CpG site selection

We obtained the sequence of the promoter region of the Ref. Seq NM_001362.3 from the NCBI database (https://www.ncbi.nlm.nih.gov/gene/), taking 2000 bp upstream and 1000 bp downstream (−2000 to +1000 bp) of the Transcription Start Site (TSS), totaling 3000 bp. The corresponding physical location on the chromosome is: chr14: 101559351 to 101562350. The primers for methylation detection in *DIO3* promoter region were designed using AGNA software (http://www.epidesigner.com/index.html). The methylation pattern of CpGs was determined based on the target gene sequence, ensuring that the target fragment size for all genes was ranged between 200 and 600 base pairs. The *DIO3*_FA26 segment was 467 bp length contained 38 detectable CpG sites (Fig. 1). Detailed information about the primers were as follows, respectively: Fw: TTTTATTAGGGGTAGTTGTTGTTTGAA and Rv: TTACCC-TAAAAAACACTACAATCCA. These primers were synthesized by Beijing Liuhe Huada Gene Technology Co., Ltd.

2.4. Bisulfite conversion of DNA

First, the DNA sample was freeze-thawed, followed by vortexing and centrifugation. At least 1 μg of the DNA sample was taken, and the total volume was adjusted to 45 μL using triple-distilled water. The bisulfite conversion was then performed on the DNA sample. Following the conversion, the concentration of the bisulfite-converted DNA was measured and recorded, which was reserved for subsequent steps.

2.5. PCR amplification

The 5'-Primer and 3'-(T7) Primer were thoroughly mixed to prepare a Primer Mix with a final concentration of 1 μmol/L. The PCR reaction mixture was prepared according to the following proportions: 10× HotStar Taq buffer (0.50 μL), dNTP mix (25 mmol/L) (0.04 μL), HotStar Taq (5U/μL) (0.09 μL), Primer Mix (2.00 μL), DNA template (1.00 μL), and water (1.37 μL), to achieve a total volume of 5.00 μL. Then, PCR was performed under the following conditions: an initial denaturation at 95 °C for 10 min, followed by 10 cycles of denaturation at 95 °C for 45 s, annealing at 62 °C for 48 s (−0.5 °C/cycle), and extension at 72 °C for 1 min, then followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 57 °C for 48 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 3 min.

2.6. MALDI-TOF MS detection

A 2% agarose gel was prepared. Then, 1 μL of the PCR product was added to 5 μL of 2× Loading Buffer for electrophoresis to assess the amplification results. The SAP reaction mixture was prepared by combining 0.24 μL SAP enzyme, 1.36 μL water, and 4.00 μL PCR product. The mixture was thoroughly mixed, sealed with sealing film, and centrifuged at 4°C for 1 minute at 1000 rpm. PCR was performed using the following program: 37°C for 20 minutes, followed by 85°C for 5 minutes, and then held at 4°C. After in vitro transcription (IVT), RNase digestion, and resin purification, the purified products were loaded onto a Spectro CHIP for detection using MALDI-TOF MS technology. The mass spectrometry platform was provided by CapitalBio Technology in Beijing. The detection data were analyzed, and results were generated using EpiTYPER software.

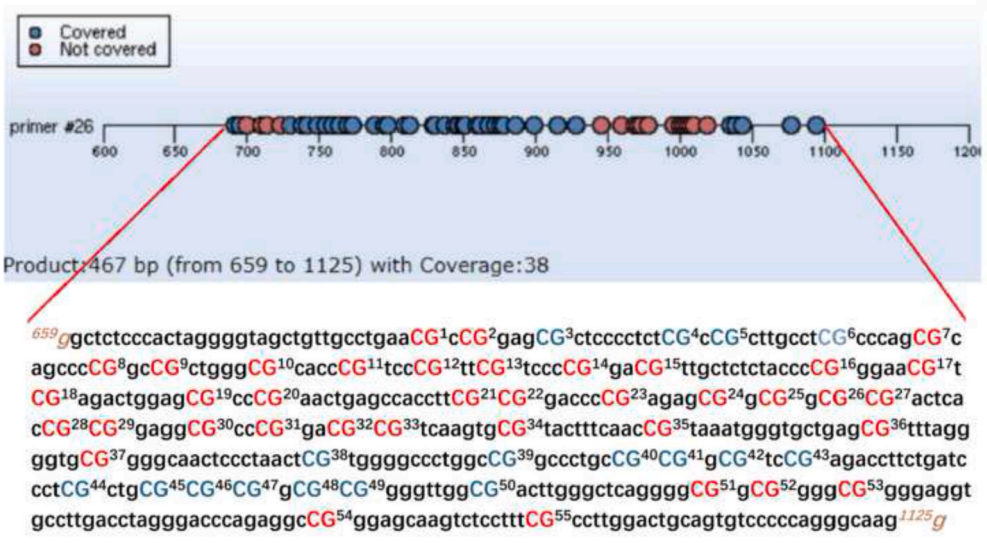

Fig. 1. The detailed information of the DIO3_FA26 segment and 55 CpG sites. (Red CGs were detectable CpGs and blue ones were undetectable due to weak signaling). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.7. Data analysis

Data analysis was conducted using Statistical Package for the Social Sciences (SPSS, version 23.0). The normality or log-normality of all measured data was verified using the Kolmogorov-Smirnov method. Normally distributed quantitative data were presented as Mean ± Standard deviation ($\overline{x} \pm SD$) and compared between groups using *t*-tests; non-normally distributed quantitative data were presented as median and compared using the Mann-Whitney *U* test. Count data were displayed as ratios and proportions (%) and compared using the chi-squared test. For the CpG methylation levels at the 25th, 50th, and 75th percentiles of the HF differential CpG sites, Restricted Cubic Spline regression (RCS) were established for eight kinds of clinical indicators, including blood pressure, blood lipids, coagulation, liver function, kidney function, protein, electrolytes, and complete blood count. Receiver Operating Characteristic (ROC) curves were generated to indentify HF based on individual or combined clinical indicators and the methylation levels of two key CpG sites. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline characteristics and clinical pathological parameters of patients with HF and healthy controls

This study included 20 patients with HF and 20 healthy individuals as research subjects. There were no statistically significant differences in demographic characteristics, including age, gender, lactate dehydrogenase, and other important clinical pathological parameters between the two groups ($P > 0.05$) (Supplementary Table 1).

The comparison results of key biochemical indicators between the HF group and the healthy control group were shown in Fig. 2. The analysis results indicated that Prothrombin time (PT), Prothrombin time ratio (PTR), Activated partial thromboplastin time (APTT), Prothrombin Time - International Normalized Ratio (PT-INR) were significantly elevated in the HF group, with the differences being statistically significant ($Ps < 0.05$).

3.2. Comparison of methylation levels in the selenium protein gene DIO3_FA26 promoter region between the two groups

The analysis results revealed statistically significant differences in the methylation levels of specific CpG sites within the *DIO3*_FA26 promoter region between heart failure patients and healthy controls. CpG sites *DIO3*_FA26_CpG_7 ($P = 0.0047$), *DIO3*_FA26_CpG_17.18 ($P = 0.0002$), *DIO3*_FA26_CpG_23 ($P = 0.0293$), and *DIO3*_FA26_CpG_32.33 ($P = 0.0273$) exhibited statistical significance (Fig. 3).

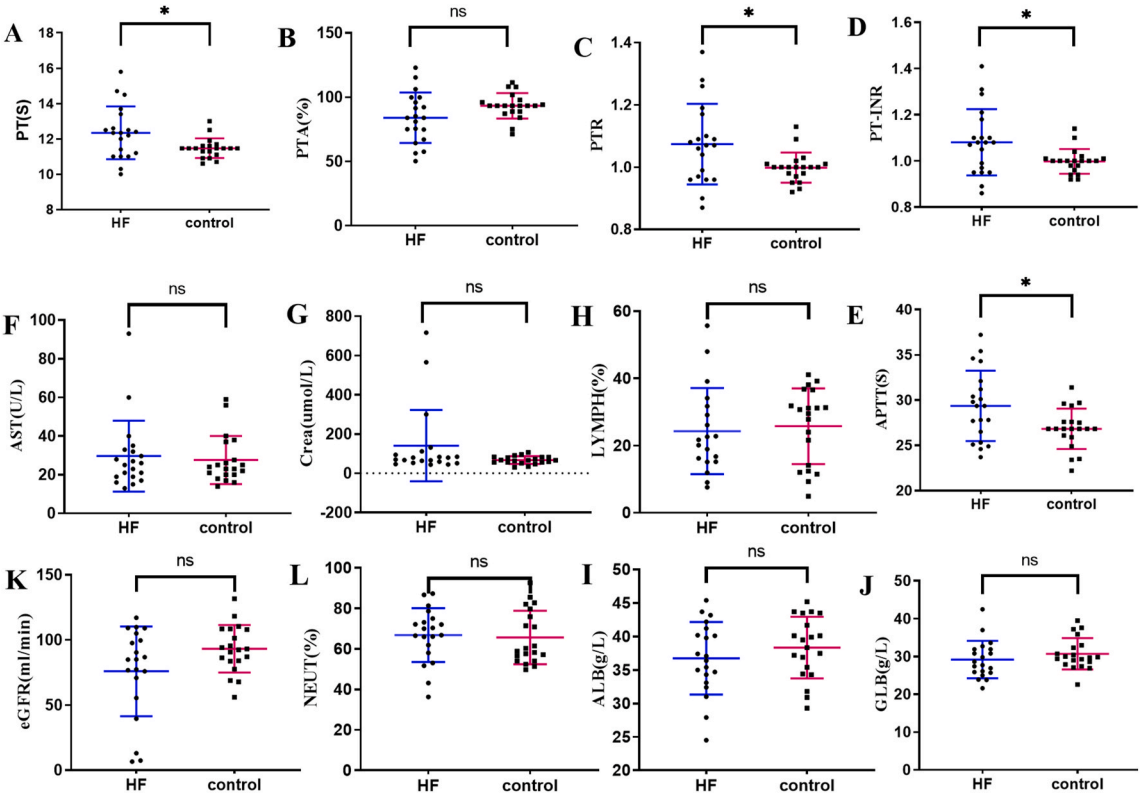

Fig. 2. The comparison of key biochemical indicators between the HF group and the healthy control group.

We selected the *DIO3*_FA26_CpG_17.18 based on its significant differential methylation in HF. This site exhibits the most pronounced alteration and smallest *P*-value among the studied *DIO3* methylation sites. Our study defines this CpGs as pivotal for exploring how *DIO3* methylation changes may contribute to HF mechanisms.

3.3. Comparison of methylation levels in the promoter region of DIO3_FA26 in heart failure patients with different cardiac functions

Further analysis results indicated that compared to HF patients with cardiac function grades I/II, those with grades III/IV exhibited a significant reduction in methylation levels at CpG sites 24.25.26.27 of *DIO3*_FA26 ($P = 0.0168$). Therefore, the current study defines *DIO3*_FA26_CpG_24.25.26.27 as a differential methylation CpG site associated with cardiac function in patients with HF and also proceeds with further comprehensive analysis. (Fig. 4).

3.4. Dose-response relationship between HF related differential methylation CpG sites and blood routine indicators

The results of the restricted cubic spline regression analysis on the methylation levels of *DIO3*_FA26_CpG_17.18 and *DIO3*_FA26_CpG_24.25.26.27 with the blood routine indicators showed a "U-shaped" non-linear association between hemoglobin (HGB) and the methylation level of *DIO3*_FA26_CpG_17.18. As the methylation level of *DIO3*_FA26_CpG_17.18 increases, the OR of HGB first rapidly decreases, then slowly rises before quickly increasing. However, the association between HGB and the methylation level of *DIO3*_FA26_CpG_24.25.26.27 was not statistically significant ($P_{overall} > 0.05$, $P_{non\text{-}linear} > 0.05$) (Fig. 5).

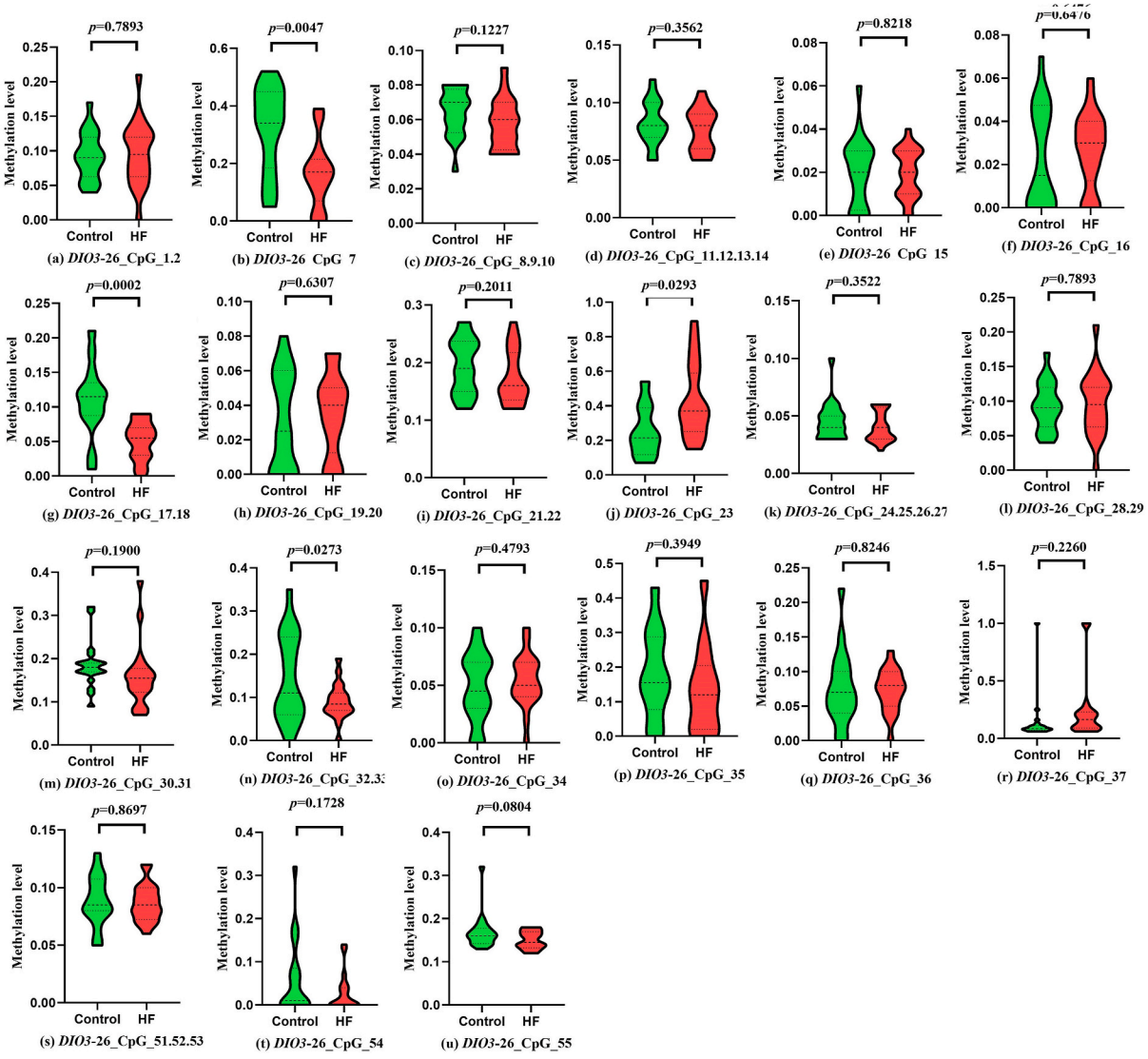

Fig. 3. Comparison of methylation levels in the *DIO3*_FA26 promoter region in HF patients and healthy controls.

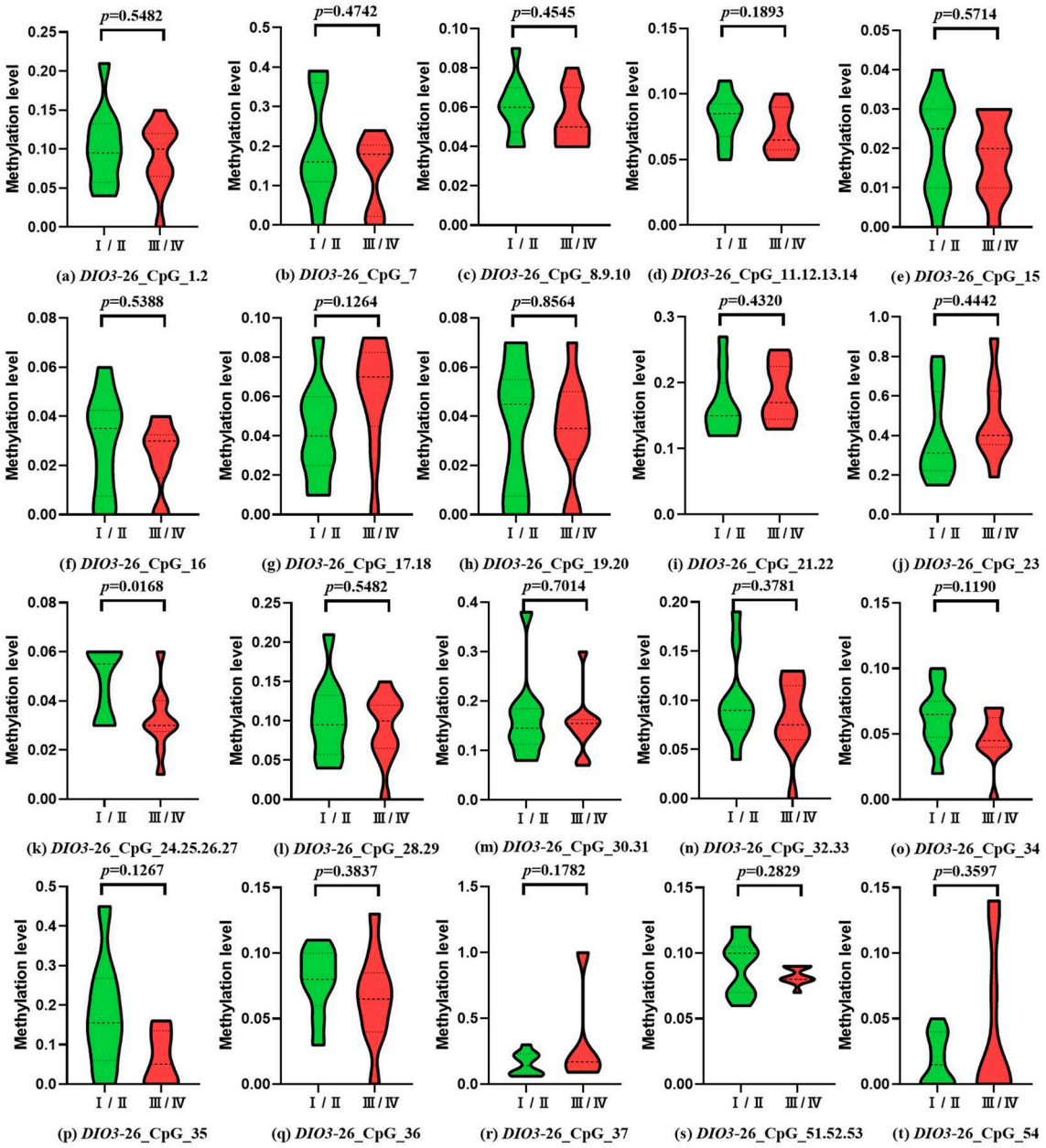

Fig. 4. Comparison of methylation levels in the promoter region of *DIO3*_FA26 in heart failure patients with different cardiac functions.

3.5. Dose-response relationship between methylation levels of two key CpGs and coagulation markers

Using RCS, dose-response curves were fitted for the methylation levels of the *DIO3*_FA26_CpG_17.18 and *DIO3*_FA26_CpG_24.25.26.27 sites concerning coagulation function. The solid lines in the graph represent the odds ratios (OR) values of various coagulation indicators. The analysis results indicated an association between levels of plasma PT, prothrombin activity (PTA), PTR, PT-INR, APTT, and the methylation level of the *DIO3*_FA26_CpG_17.18 (Fig. 6). Particularly, PT, PTA, PTR, and PT-INR exhibited a linear relationship with the methylation level of *DIO3*_FA26_CpG_17.18 and demonstrated a dose-response effect ($P_{overall} < 0.05$, $P_{non\text{-}linear} > 0.05$). There was an inverted U-shaped relationship between the methylation level of *DIO3*_FA26_CpG_17.18 and PT, PTR, and PT-INR, indicating an initial rapid increase followed by a gradual decrease in OR values with increasing methylation levels. Moreover, PTA demonstrated a U-shaped pattern with the methylation level of *DIO3*_FA26_CpG_17.18, showing a rapid decrease followed by a slow increase in OR values with increasing methylation levels. The APTT exhibited an inverted U-shaped non-linear relationship with the methylation level of *DIO3*_FA26_CpG_17.18. However, no dose-response effect was observed ($P_{overall} > 0.05$,

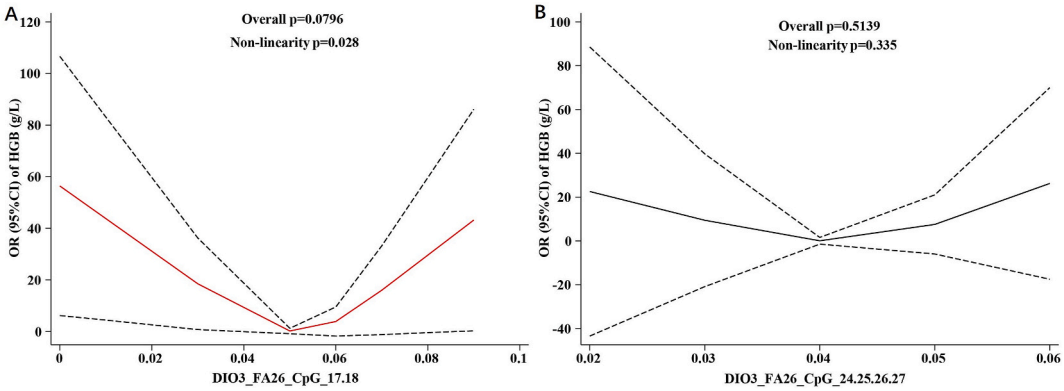

Fig. 5. Dose-response relationship between methylation levels of HF related differential methylation CpG sites and blood routine indicators.

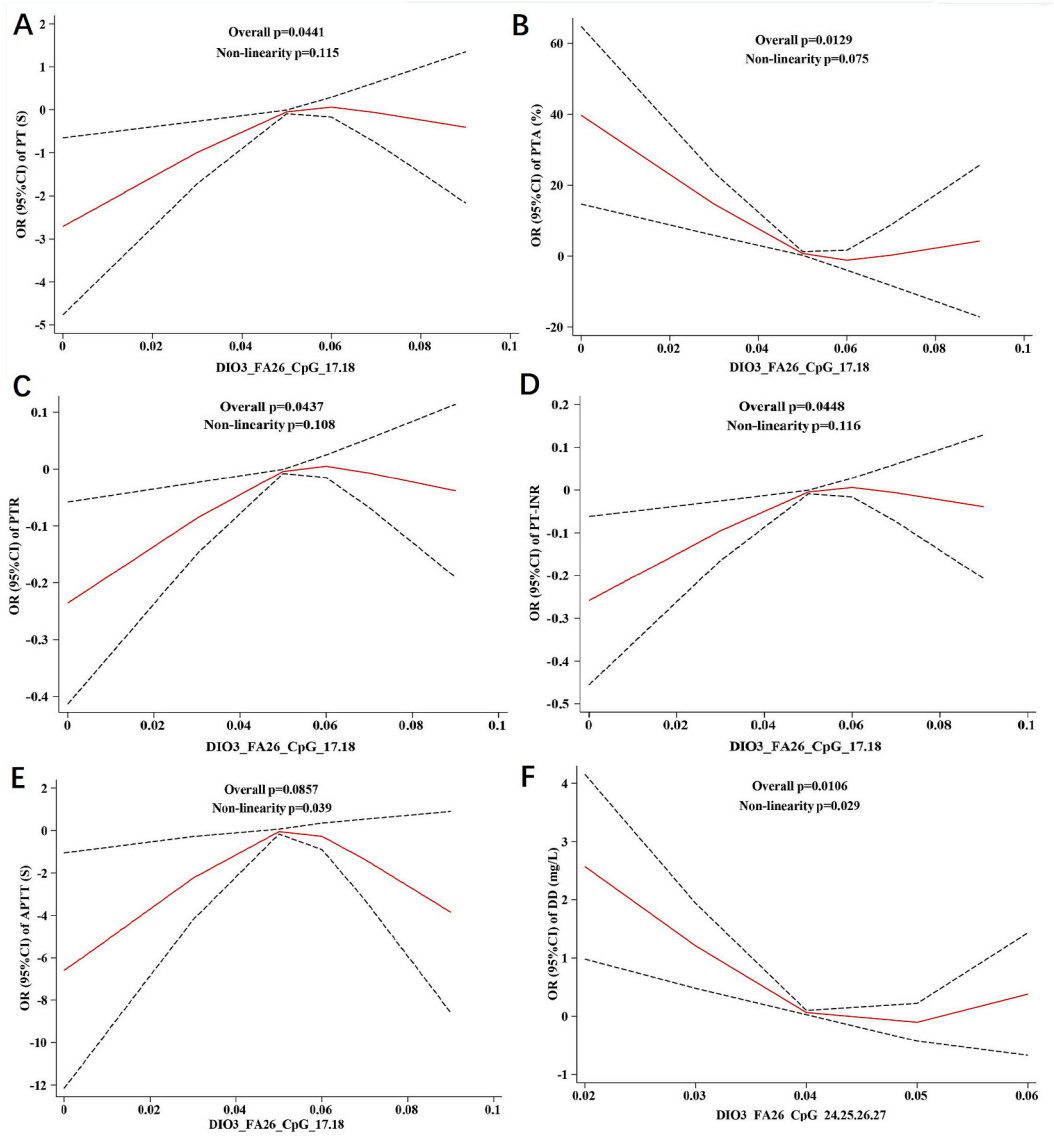

Fig. 6. Dose-response relationship between methylation levels of two key CpGs and coagulation markers.

$P_{non\text{-}linear} < 0.05$), implying a rapid increase followed by a slow decline and then a rapid decrease in OR values with increasing methylation levels. Nonetheless, none of these coagulation indicators showed any association with the methylation level of the *DIO3*_FA26_CpG_24.25.26.27 site ($P_{overall} > 0.05$, $P_{non\text{-}linear} > 0.05$).

Furthermore, a U-shaped non-linear relationship was observed between D-dimer (DD) and the methylation level of the *DIO3*_FA26_CpG_24.25.26.27 site, with a dose-response effect, indicating that as methylation levels at this site increase, DD OR values first decrease rapidly, then decline gradually before slowly increasing (Fig. 6 F). However, DD showed no association with the methylation level of the *DIO3*_FA26_CpG_17.18 site ($P_{overall} > 0.05$, $P_{non\text{-}linear} > 0.05$).

3.6. Dose-response relationship between methylation levels of two key CpGs and liver function

Subsequently, RCS was used to investigate the dose-response relationships between the methylation levels of *DIO3*_FA26_CpG_17.18 and *DIO3*_FA26_CpG_24.25.26.27 and liver function in patients with HF. The findings revealed a "U-shaped" non-linear relationship between albumin (ALB) and the methylation level of the *DIO3*_FA26_CpG_17.18, suggesting a certain dose-response relationship ($P_{overall} > 0.05$, $P_{non\text{-}linear} < 0.05$). As the methylation level of the *DIO3*_FA26_CpG_17.18 increased, the OR of ALB first decreased, then slowly rose before quickly increasing (Fig. 7A). However, the association between ALB and the methylation level of the *DIO3*_FA26_CpG_24.25.26.27 sites was not statistically significant ($P_{overall} > 0.05$, $P_{non\text{-}linear} > 0.05$). There was a linear

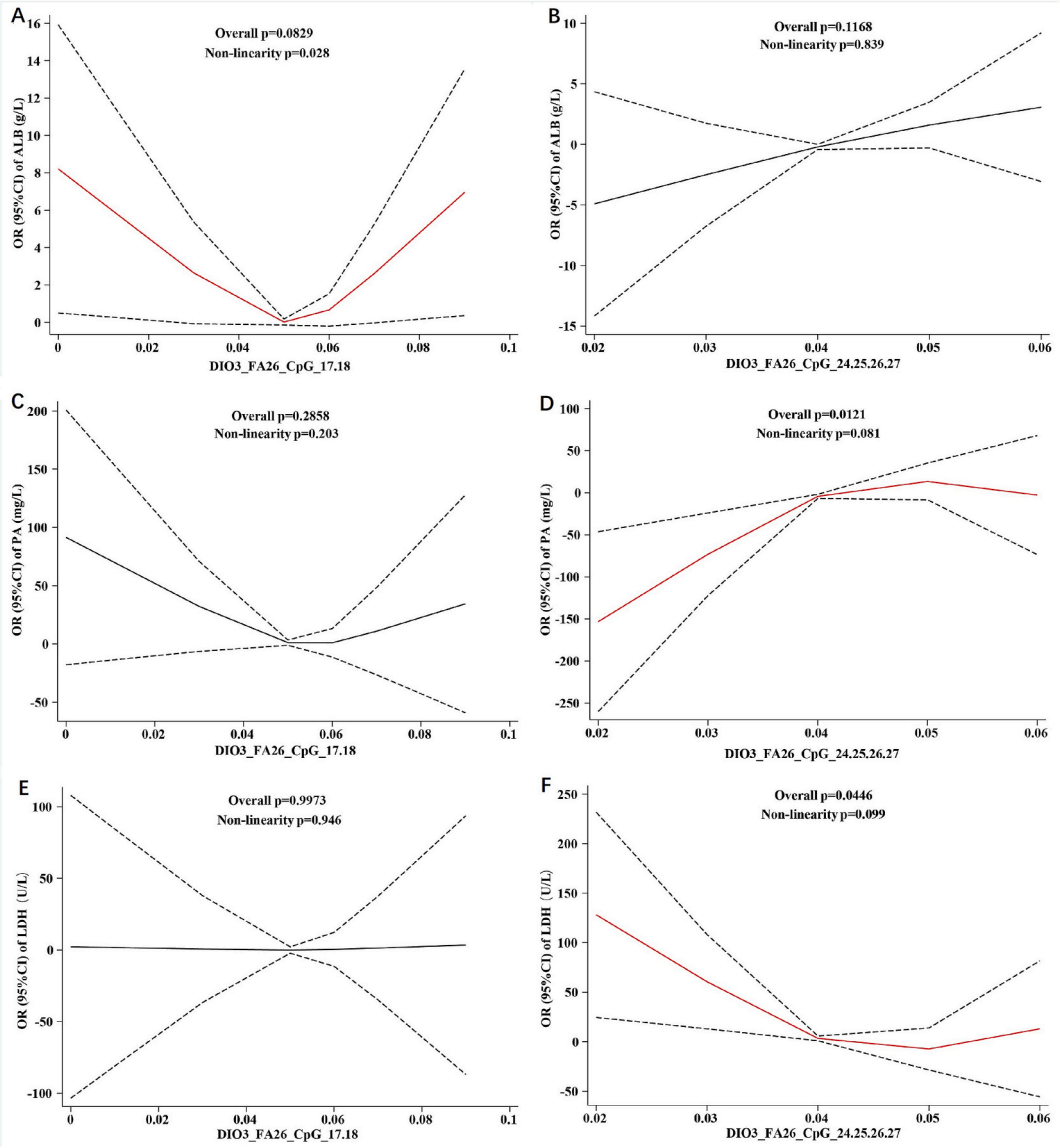

Fig. 7. Dose-response relationship between methylation levels of two key CpGs and liver function.

correlation between prealbumin (PA), lactate dehydrogenase (LDH), and the methylation level of *DIO3*_FA26_CpG_24.25.26.27 ($P_{overall} < 0.05$, $P_{non\text{-}linear} > 0.05$). The relationship between PA and the methylation level of the *DIO3*_FA26_CpG_24.25.26.27 exhibited an "inverse U-shaped" pattern, with the OR of PA first rapidly increasing, then slowly rising before decreasing gradually. The association between LDH and the methylation level of the *DIO3*_FA26_CpG_24.25.26.27 showed a "U-shaped" pattern, with the OR of LDH initially decreasing rapidly, then declining slowly before gradually rising (Fig. 7D, F).

3.7. Dose-response relationship between methylation levels of two key CpGs and kidney function

The findings from RCS analysis on the methylation levels of *DIO3*_FA26_CpG_17.18 and *DIO3*_FA26_CpG_24.25.26.27 with kidney function indicated a "U-shaped" non-linear association between cholinesterase (CHE), calcium (CA), and the methylation level of *DIO3*_FA26_CpG_17.18. As the methylation level of *DIO3*_FA26_CpG_17.18 increases, the OR of CHE and CA first rapidly decrease, then slowly rise before quickly increasing (Fig. 8A, C). However, the associations between CHE, CA, and the methylation level of *DIO3*_FA26_CpG_24.25.26.27 were not statistically significant (*Ps* > 0.05).

3.8. The ROC curve for judging the diagnostic capability

We evaluated the value of clinical indicators and differential CpGs methylation levels in predicting HF using ROC curves and the Area Under the Curve (AUC) analysis. The AUC values of *DIO3*_FA26_CpG_17.18, PT, PTA, PTR, PT-INR, APTT, ALB, CHE, CA, HGB, and their combined predictive model were 0.099, 0.675, 0.323, 0.682, 0.701, 0.420, 0.398, 0.465, 0.351, and 0.940, respectively (Fig. 9).

The predictive values of PT, PTR, PT-INR, and APTT for HF were significant, and the AUC of the combined predictive model was considerably higher than that of individual indicators. For *DIO3*_FA26_CpG_24.25.26.27, DD, LDH, PA, and their combined predictive model, the AUC values were 0.431, 0.366, 0.409, 0.496, and 0.617, respectively, with the combined predictive model AUC significantly higher than the AUC of individual indicators (Fig. 10).

4. Discussion

Recently, there has been an increase in the incidence and mortality of HF annually, particularly affecting younger populations and imposing a significant disease burden on families and society. Numerous studies have indicated that HF is influenced by both environmental and genetic factors. An abnormal expression of certain genes can impact the occurrence and progression of HF [33].

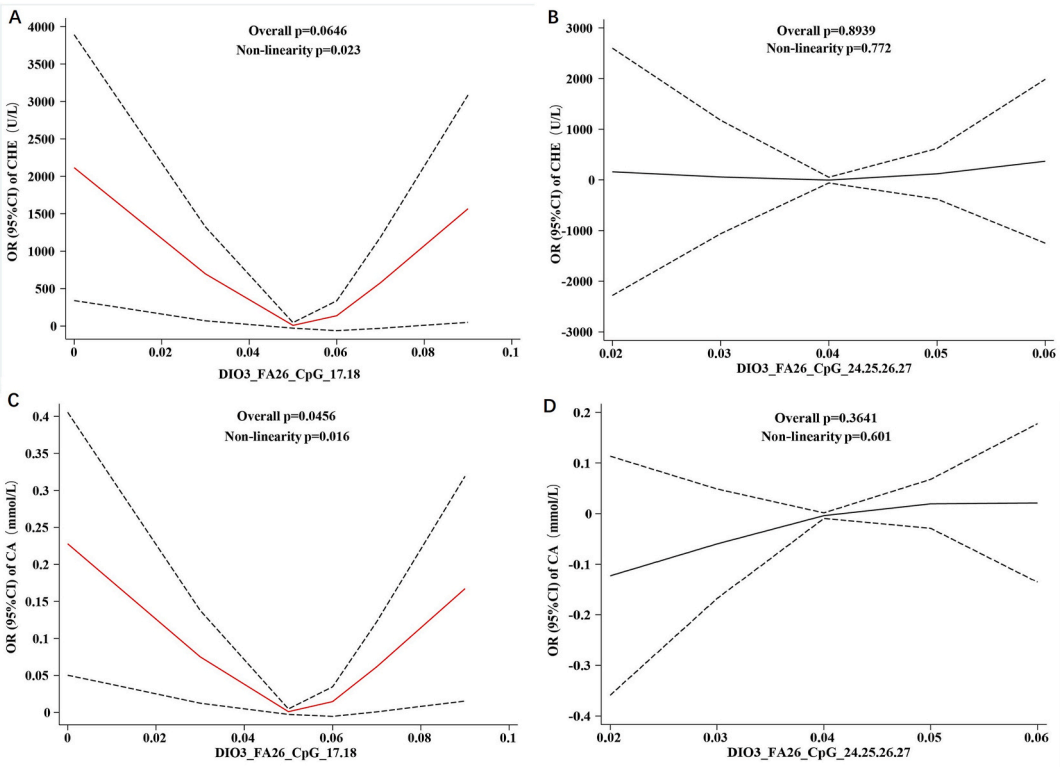

Fig. 8. Dose-response relationship between methylation levels oftwo key CpGs and kidney function.

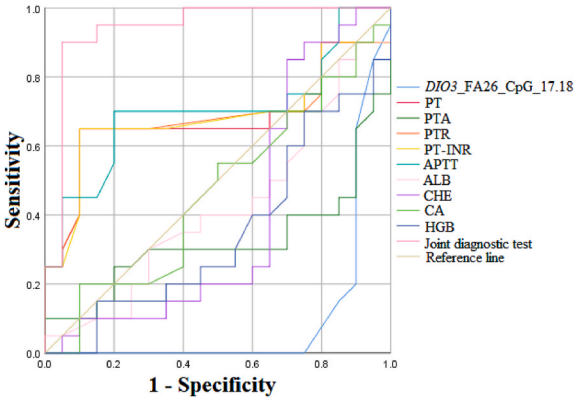

Fig. 9. ROC curves for plasma *DIO3* _ FA 26 _ CpG _ 17.18, PT, PTA, PTR, PT-INR, APTT, ALB, CHE, CA, and HGB for identification of HF.

Epigenetics is the study of the interaction between congenital genetic factors and acquired environmental factors, with DNA methylation being the predominant chemical modification in epigenetics. Many experts have identified DNA methylation sites associated with HF, suggesting that DNA methylation could be involved in the pathogenesis of HF [34,35].

Se is an essential micronutrient for human health, primarily exerting its biological effects in the form of selenoproteins. The correlation between selenoproteins and human health has garnered increased attention recently [36]. Research has shown an association between selenium deficiency and the onset of HF [37]. Selenoproteins can exert antioxidant stress and mitigate a series of effects mediated by reactive oxygen species, thereby alleviating symptoms in patients with HF. Previous studies have established the significant role of selenoproteins in the pathogenesis and treatment of HF [38]. Iodothyronine deiodinase is an important member of the selenoprotein family, with its three members collectively involved in the activation and inactivation of TH, thereby regulating the body's functions [39,40]. Imbalances in TH levels can cause cardiovascular hypertrophy and increased heart rate. A potential association between the ID family and HF has also been reported, although the specific mechanism remained unclear [41]. Therefore, this study aimed to investigate the correlation between *DIO3* methylation levels and HF, providing a theoretical basis for understanding the pathogenesis of HF and offering novel insights and targets for its prevention and treatment.

The methylation levels of various CpG sites of *DIO3*_FA26 in our study were detected using MALDI-TOFMS [42]. The results revealed that, compared to the healthy control group, the methylation level of *DIO3*_FA26_CpG_17.18 in the blood of patients with HF showed the most significant and notable decrease. Among different stages of HF patients, the methylation level of *DIO3*_FA26_CpG_24.25.26.27 was considerably lower in stages III/IV compared to stages I/II. *DIO3* encodes deiodinase, which plays an important role in thyroid hormone metabolism, and the balance of thyroid hormones is crucial for maintaining heart function [24,25]. Changing methylation in the promoter region usually leads to alteration of *DIO3* expression, which may affect thyroid hormone metabolism and subsequently negatively impact heart function. Furthermore, studies have found that the methylation level of the *DIO3* promoter region in myocardial tissue of heart failure patients is significantly higher than that of healthy controls [26,27]. This finding suggests that abnormal methylation of the *DIO3* promoter region may be related to the occurrence and development of HF.

Subsequently, we utilized RCS models to draw curves showing the modifications in methylation levels of different CpG sites and the OR values of various clinical indicators. With regard to coagulation indicators, DD is a specific metabolic product formed by the degradation of cross-linked fibrin by fibrinolytic enzymes [43]. An increased DD expression often indicates secondary fibrinolysis or a hypercoagulable state in the body [44]. Abnormalities in the fibrinolysis-coagulation system causing thrombus formation play a crucial role in the occurrence and progression of HF and other common cardiovascular diseases. Studies have demonstrated that

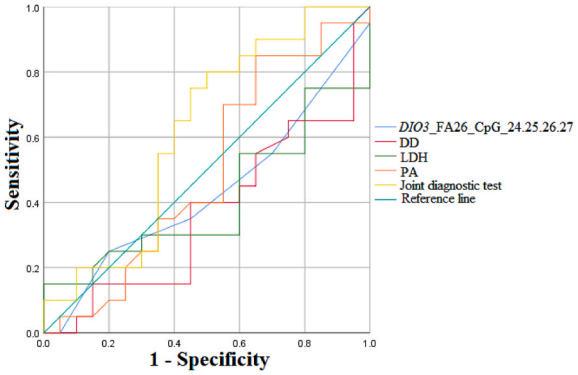

Fig. 10. ROC curves of plasma *DIO3* _ FA 26 _ CpG _ 24.25.26.27, DD, LDH, and PA for identification of HF.

plasma DD can serve as an indicator of cardiovascular diseases, providing an objective quantitative reference for assessing the type, severity, and prognosis of lesions [44,45]. In our study, higher OR values of DD were observed when the methylation levels of the *DIO3*_FA26_CpG_24.25.26.27 sites were lower. Studies reported that patients with heart failure combined with severe pneumonia had significantly elevated D-D levels, which were associated with higher mortality rates [44–46]. In this study, as the methylation level of *DIO3* increased, the risk of elevated D-D decreased, indicating that the methylation level of *DIO3* affects the early process of heart failure. This also further illustrates that the pathogenesis of heart failure is extremely complex.

As for liver function indicators, serum ALB is an abundantly circulating protein produced by hepatocytes in the body. ALB maintains the colloidal osmotic pressure and endothelial stability in the body [47]. It also plays a significant role in anti-thrombotic, antioxidant, and anti-inflammatory processes. ALB levels are influenced by the body's nutritional status; within the normal range, they suggest optimal nutritional status and normal liver and kidney function. Many studies have demonstrated that ALB is an important predictor of the risk of new-onset HF [48] and the clinical prognosis of HF [49]. The lower the level of ALB, the more severe the HF and the higher the risk of death [50]. Our findings were generally consistent with these observations. When the methylation level of *DIO3*_FA26_CpG_17.18 was higher than 0.5, the OR of serum ALB increased. This suggests that when the methylation level at this site exceeds 0.5, ALB may play more important role with the risk of poor prognosis in CHF increases.

In terms of renal function indicators, studies have revealed that high blood CA is a risk factor for cardiovascular disease. An increase in blood CA levels may lead to increased vascular wall calcification, resulting in vascular calcification and consequently cardiovascular disease [50]. In this study, when the methylation level of the *DIO3*_FA26_CpG_17.18 site is low, the OR of blood CA is high.

Regarding blood routine indicators, patients with low HGB have been observed to have a significantly higher incidence of cardiovascular events. Gilbertson et al. [51] have reported that low HGB levels increase the risk of cardiovascular disease mortality. HGB is an indicator reflecting the body's anemia status. Anemia can induce physiological responses in the body, stimulate the sympathetic nervous system, increase heart rate, accelerate blood flow, dilate peripheral blood vessels, reduce systemic vascular resistance, increase cardiac output, and aggravate cardiac preload, leading to cardiac enlargement and myocardial hypertrophy [52]. Left ventricular hypertrophy further causes HF and ischemic heart disease [53]. Our study suggests that during the occurrence and development of heart failure, there may be an interaction between the methylation of *DIO3*_FA26_CpG_17.18 and hemoglobin (HGB). This provides a direction and foundation for future research, and we plan to conduct further in-depth exploration.

The results of the ROC analysis imply that the differential CpG methylation level of the HF-related CpGs in *DIO3*_FA26, combined with clinical indicators, can complement the false positives or negatives of single indicators. Although the joint prediction model has a higher AUC value, the AUC value of *DIO3*_FA26_CpG_17.18 is notably lower, indicating that it does not play a significant role in the joint model. This may be related to the substantive role of methylation itself in the development of HF, or it could be due to the relatively small sample size in this study. In future research, we will conduct further comprehensive validation.

This study has certain limitations: first, it is a small sample, single-center investigation, with all patients coming from the same hospital, which introduces a selection bias; second, this study fails to provide a detailed explanation of the differential methylation level of *DIO3* and its impact on the pathogenesis of HF and different levels of HF. Therefore, reliable evidence for the relationship between gene methylation levels and HF with multi-center, large-sample clinical data is crucial for future studies.

In summary, this study analyzed the methylation levels of *DIO3* promoter region in blood samples from patients with HF and healthy control groups. Based on two important methylation differential CpGs, it was demonstrated that the methylation levels of *DIO3* were lower in patients with HF compared to healthy controls, and lower in patients with HF with class III/IV heart function compared to class I/II heart function. Consequently, we hypothesize that the lower methylation levels of *DIO3* may be linked to the occurrence and progression of HF. Moreover, the methylation levels of the above two key CpG sites showed a dose-response relationship with multiple crucial clinical parameters. We further speculate that the lower methylation levels of *DIO3* may affect the occurrence and progression of HF by influencing changes in important clinical parameters. It can be explained the other way around as well, that is: variations in these parameters after the occurrence and progression of HF may affect the lower methylation levels of *DIO3*. Therefore, the causal relationship between the reduced methylation levels of *DIO3* and HF warrants further validation through large-sample prospective studies. The findings of this study extend the relationship between *DIO3* methylation and HF occurrence and development, offering novel approaches for preventing and treating HF.

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Consent to publish

All authors were involved in reading and revising the draft and approved the final version for publication.

Data availability statement

Data used in the manuscript will be made available upon request.

Ethics declarations

The research design and protocol were approved by the Ethics Committee of Ningxia Autonomous Region People's Hospital (ZDYF-

046; 2021).

CRediT authorship contribution statement

Qi Miao: Writing – original draft, Formal analysis, Data curation. **Min Zhang:** Writing – original draft, Formal analysis, Data curation. **Aoyue He:** Writing – original draft, Investigation, Formal analysis, Data curation. **Chuanyong Qu:** Writing – review & editing, Software, Investigation, Formal analysis, Data curation. **Rongqiang Zhang:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37582.

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