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Blood-based *HYAL2* methylation as a potential marker for the preclinical detection of coronary heart disease and stroke

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Abstract

Background Coronary heart disease (CHD) and stroke have become the leading cause of premature mortality and morbidity worldwide. Therefore, sensitive and accurate biomarkers for early detection of CHD and stroke are urgently needed for effective prevention and treatment. We aim to investigate the association between blood-based *HYAL2* methylation and the risk of CHD and stroke in Chinese population.

Methods In a prospective nested case–control study comprising 171 CHD cases, 139 stroke cases, who developed the diseases after recruitment and 356 controls who remained healthy during the 2.5 years of follow-up time, the methylation level of *HYAL2* in the peripheral blood was quantified using mass spectrometry, and the association was calculated by logistic regression adjusted for covariant.

Results Significant association between *HYAL2* methylation in the peripheral blood and increased risk of preclinical CHD and stroke were identified [odds ratios (ORs) per − 10% methylation: 1.35–1.64, $p \le 0.045$ for HYAL2_CpG_1, HYAL2_CpG_2 and HYAL2_CpG_3 in CHD; ORs per − 10% methylation: 0.76–1.64, $p \le 0.033$ for HYAL2_CpG_2 and HYAL2_CpG_4 in stroke]. The association in CHD was further enhanced by female gender, younger age (< 70 years old), without the history of hypertension and cancer. The combination of four *HYAL2* methylation sites showed an effective discrimination of CHD and stroke cases without hypertension from controls [area under curve (AUC) = 0.78 and 0.75, respectively].

Conclusions This study presents a strong association of altered *HYAL2* methylation in peripheral blood with preclinical CHD and stroke, providing a novel biomarker for risk assessment and early detection of cardiovascular diseases.

Keywords DNA methylation, *HYAL2*, Coronary heart disease, Stroke, Early detection

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Introduction

Cardiovascular diseases (CVDs) are chronic noncommunicable diseases that impose a substantial economic and health burden worldwide. From 2017 to 2020, the prevalence of CVDs in adults aged ≥ 20 years old was 48.6% overall and increased with age [1]. Coronary heart disease (CHD) and stroke account for a large proportion of CVDs. Atherosclerosis is a pathological condition characterized by fibrous proliferation, chronic inflammation, lipid accumulation, which often occurs at the asymptomatic stage of diseases [2]. As the illnesses progresses, it eventually contributes to ischemic diseases, such as CHD and stroke [3, 4]. A considerable number of new or recurrent CHD and stroke cases each year are underdiagnosed due to silent infractions that do not meet the clinical diagnosis threshold [1, 5]. Although coronary CT angiography (CCTA) is currently the gold standard method for confirming the diagnosis of atherosclerosis, it is unable to recognize sub-millimeter-sized which likely represent very early atherosclerosis [6]. Cardiac troponins (cTn), the preferred noninvasive markers of myocardial injury, have been widely used in blood tests to diagnose symptomatic patients. However, cTn lack specificity for the detection of CVDs, as its level may increase in other pathological conditions, such as sepsis, chronic obstructive pulmonary disease, diabetes mellitus and renal failure [7, 8]. Besides, the high level of cTn cannot explain the specific mechanisms underlying CVD pathogenesis. Moreover, due to the heterogeneity of stroke etiology and the variability in laboratory measurement of these biomarkers, the use of biomarkers for diagnosing stroke has been limited [9]. Therefore, novel and sensitive biomarkers are critically needed to be identified. Genome-wide association studies (GWAS) have been conducted to predict heritable components of disease risks and phenotypes by polygenic risk scores (PRSs) [10]. A study showed that the ability of genome-wide PRS for predicting lipid traits and atherosclerosis among East Asians was not competent in the general population (AUCs = 0.63–0.67) [11].

DNA methylation is recognized as a fundamental and widespread epigenetic mechanism encompassing inheritable alterations in gene structure and function without changes in the DNA sequence. It plays a crucial role in regulating various cellular processes, including embryonic development, transcription, chromatin structure, X-chromosome inactivation, genome imprinting and chromosome stability. Recent studies have demonstrated the correlation between DNA methylation and cardiovascular disorders, as well as the correlation between candidate gene expression and CHD [12]. Using a mouse model of acute myocardial infarction (AMI), Luo et al. [12, 13] revealed genes participating in AMI process through DNA methylation regulation. Additionally, the biological link between differentially methylated position and stroke has been established in rat models [14], indicating DNA methylation would be a biomarker for the diagnosis in cardiovascular diseases.

Hyaluronoglucosaminidase 2 (*HYAL2*), a member of the hyaluronidase family, is a key regulator of hyaluronan (HA) metabolism. *HYAL2* cleaves high molecular weight HA (HMW-HA), resulting in the generation of intermediate size fragments of 20 kDa. Low molecular weight HA (LMW-HA) promotes inflammation and angiogenesis by stimulating the expression of cytokines, chemokines and growth factors in a TLR2/TLR4-dependent manner, which subsequently contribute to CVDs and tumor formation [15, 16]. Previous studies have shown that *HYAL2* methylation is associated with different types of cancer, including breast cancer [17], thyroid cancer [18] and pancreatic cancer [19]. However, there is currently a lack of studies on the association between *HYAL2* methylation and CVDs, especially CHD and stroke. Here, we examined *HYAL2* methylation level in peripheral blood in a prospective nested case–control study with a total of 666 subjects, aiming to explore *HYAL2* methylation differences between CHD cases, stroke cases and controls.

Methods

Population of prospective nested case–control study

Subjects were free from CHD and stroke at the time of enrollment in a prospective cohort from Jurong City, Jiangsu Province, from October of 2015. And a total of 11,151 individuals aged ≥ 18 years were recruited. Written informed consents have been obtained from all participants. Questionnaires were used to collect demographic data including age, gender, status of smoking, alcohol consumption frequency, history of diabetes, hypertension and cancer. Anthropometric measurements including height, weight, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were taken simultaneously. In addition, blood samples of all participants were collected upon enrollment, and the levels of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and glucose (GLU) were measured.

Till the July of 2018, a total of 171 subjects who developed CHD and 139 subjects who developed stroke within 2.5 years after recruitment were included in this study as CHD and stroke cases, respectively. The incidence of CHD and stroke was ascertained through coronary angiography and head computed tomography, respectively, by local hospitals, centers for disease control and community health service centers. A total of 356 gender and age-matched subjects who were defined as CHD-free and stroke-free according to the healthy check reports were

randomly selected as controls. The demographic and clinical characteristics of participants in this prospective nested case–control study are shown in Table 1.

Sample collection, DNA extraction and bisulfite conversion

The peripheral blood samples were collected in ethylene diamine tetraacetic acid (EDTA) tubes and stored at −80 °C until usage. DNA extraction kit (TANTICA, Nanjing, China) was used to isolate genomic DNA from peripheral whole blood. Genomic DNA was bisulfite converted by the DNA methylation kit (TANTICA, Nanjing, China) according to the standard protocol. Non-methylated cytosine (C) at CpG sites was converted to uracil (U), while methylated cytosines remained unchanged after the bisulfite conversion. The samples from all subjects were treated and analyzed in parallel through all the progress.

MALDI-TOF mass spectrometry

The level of *HYAL2* methylation was determined by Agena matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry as described by Yang et al. [20]. The bisulfite-converted DNA was amplified by bisulfite-specific primers. The amplified PCR products were incubated with shrimp alkaline phosphatase (SAP) and then treated with T7 transcriptase along with RNase according to the manufacturer's instructions of Agena EpiTyper Assay. The products were cleaned by resin and further dispensed to a 384 SpectroCHIP by a Nanodispenser. The MassARRAY system was

Table 1 Baseline characteristics of coronary heart disease cases, stroke cases, and controls

Characteristics	Controls (N = 356) N (%)	CHD cases (N = 171) N (%)	Stroke cases (N = 139) N (%)	p^a-value	p^b-value
Gender					
Male	180 (50.6)	74 (43.3)	81 (58.3)		
Female	176 (49.4)	97 (56.7)	58 (41.7)	0.117	0.123
Smoking					
Yes	80 (22.5)	36 (21.0)	35 (25.2)		
No	276 (77.5)	135 (79.0)	104 (74.8)	0.713	0.521
Drinking					
Yes	114 (32.0)	47 (27.5)	47 (33.8)		
No	242 (68.0)	123 (71.9)	92 (66.2)		
Unknown	0 (0.0)	1 (0.6)	0 (0.0)	0.309	0.702
Hypertension					
Yes	232 (65.2)	121 (70.8)	96 (69.1)		
No	124 (34.8)	50 (29.2)	43 (30.9)	0.201	0.410
Diabetes					
Yes	53 (14.9)	20 (11.7)	27 (19.4)		
No	303 (85.1)	151 (88.3)	112 (80.6)	0.321	0.218
Characteristics	**Controls (N = 356) Median (IQR)**	**CHD cases (N = 171) Median (IQR)**	**Stroke cases (N = 139) Median (IQR)**	**p^a-value**	**p^b-value**
Age	66.59 (60.27–71.42)	62.83 (54.25–69.58)	67.67 (62.17–74.00)	**0.001**	0.059
BMI, kg/m^2	25.21 (22.91–27.38)	25.44 (22.95–27.95)	24.88 (22.60–27.03)	0.417	0.240
SBP, mmHg	144.67 (130.42–159.33)	142.00 (129.00–159.00)	144.67 (133.67–162.00)	0.662	0.491
DBP, mmHg	81.33 (75.00–88.00)	81.33 (74.00–89.67)	80.33 (72.67–89.67)	0.951	0.366
TG, mmol/L	1.35 (0.95–1.90)	1.39 (1.02–1.89)	1.32 (0.95–1.90)	0.397	0.989
TC, mmol/L	4.93 (4.40–5.49)	4.85 (4.20–5.53)	5.17 (4.48–5.85)	0.361	**0.022**
HDL-C, mmol/L	1.42 (1.20–1.78)	1.41 (1.18–1.74)	1.50 (1.22–1.80)	0.365	0.231
LDL-C, mmol/L	2.74 (2.32–3.26)	2.66 (2.19–3.34)	2.97 (2.44–3.49)	0.370	**0.018**
GLU, mmol/L	5.73 (5.17–6.66)	5.62 (5.18–6.23)	5.88 (5.39–7.25)	0.205	0.090

BMI body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *TG*: triglyceride, *TC*: total cholesterol, *HDL-C*: high-density lipoprotein cholesterol, *LDL-C*: low-density lipoprotein cholesterol, *GLU*: glucose. Signifcant *p*-values are in bold

[a] Represents CHD cases versus controls

[b] Represents stroke cases versus controls

used to read the chips, and data were visualized with EpiTyper v1.2 software. The most effective discrimination between fully methylated and non-methylated template DNA can be achieved in reactions above [21]. To investigate the association between *HYAL2* methylation and the risk of CHD and stroke, the same amplicon reported by Yang et al. was used in this study [22]. The *HYAL2* amplicon (349 bp, chr3: 50,360,508–50,360,856) covers 4 CpG sites, and HYAL2_CpG_4 refers to cg27091787 as previously reported (Figure S1) [22]. There are no single-nucleotide polymorphisms (SNPs) located at the primer regions or overlapped with any of the CpG sites in the amplicon. Cases and controls were processed and analyzed in parallel for each batch of MassARRAY analysis.

Statistical analysis

The data were analyzed by IBM SPSS Statistics 26.0 and GraphPad Prism software version 8.0. Quantitative variables with abnormal distribution were expressed as median [interquartile range (IQR)], and the Mann–Whitney test was employed to assess the difference between groups. Chi-square(χ^2) test was used for comparing qualitative variables. Methylation differences of *HYAL2* between CHD cases, stroke cases and controls were accessed by logistic regression adjusted for covariates including age, gender, smoking, drinking, hypertension and diabetes and estimated by odds ratios (ORs) and 95% confidence intervals (CIs). Cardiovascular-related factors were categorized according to commonly used criteria [23], including TC (< 5.0 mmol/L vs. ≥ 5.0 mmol/L), TG (< 1.7 mmol/L vs. ≥ 1.7 mmol/L), LDL-C (< 3.0 mmol/L vs. ≥ 3.0 mmol/L), HDL-C (< 1.0 mmol/L vs. ≥ 1.0 mmol/L), and BMI (< 24 kg/m^2 vs. ≥ 24 kg/m^2). Forest plots were used to show the methylation difference among subjects overall, as well as stratified by hypertension, TC level, LDL-C level and cancer history. The discriminatory power of *HYAL2* methylation levels was evaluated by the receiver operator characteristic (ROC) curve and calculated by the area under the curve (AUC) with 95% CI. All the statistical tests were two-sided, and *p*-values less than 0.05 were considered statistically significant.

Results

Altered *HYAL2* methylation in peripheral blood is associated with CHD and stroke

In this prospective nested case–control study with 171 CHD cases, 139 stroke cases and 356 gender-matched controls, we quantitatively measured methylation levels at 4 CpG loci in the *HYAL2* amplicon [22] by Agena MALDI-TOF spectrometry. Baseline characteristics of the cases and controls are shown in Table 1.

Logistic regression adjusted for age, gender, smoking, drinking, hypertension and diabetes revealed that three out of four CpG sites of *HYAL2* showed significantly lower methylation in CHD cases compared to controls [for HYAL2_CpG_1, OR per −10% methylation (95% CI) = 1.35 (1.01–1.81), $p = 0.045$; for HYAL2_CpG_2, OR per −10% methylation (95% CI) = 1.64 (1.25–2.15), $p = 3.46 \times 10^{-4}$; for HYAL2_CpG_3, OR per −10% methylation (95% CI) = 1.41 (1.01–1.99), $p = 0.042$, Fig. 1, Supplementary Table 1]. Comparing stroke cases and controls, two CpG sites showed significant methylation differences [for HYAL2_CpG_2, OR per −10% methylation (95% CI) = 0.76 (0.59–0.98), $p = 0.033$; for HYAL2_CpG_4, OR per −10% methylation (95% CI) = 1.64 (1.25–2.15), $p = 3.98 \times 10^{-4}$, Fig. 1, Supplementary Table 1].

The methylation difference of *HYAL2* in CHD and stroke stratified by gender and age

Gender-incongruent individuals highlighted the influence of genetic makeup on differences in DNA methylation, and several articles have shown gender disparities in cardiovascular epigenetics [24–26]. Interestingly, we found that gender-specific methylation differences varied across different CVDs. As indicated by our results, female and male individuals have completely different methylation patterns in CHD cases. In females, three CpG sites show lower methylation levels in CHD cases than in controls [for HYAL2_CpG_1, OR per −10% methylation (95% CI) = 1.97 (1.26–3.06), $p = 0.003$; for HYAL2_CpG_2, OR per −10% methylation (95% CI) = 1.86 (1.28–2.71), $p = 0.001$; for HYAL2_CpG_3, OR per −10% methylation (95% CI) = 2.11 (1.29–3.45), p 0.003, Table 2]. In contrast, none of four CpG sites of *HYAL2* show methylation differences between male CHD cases and controls. In the case of stroke, only HYAL2_CpG_4 showed difference in both female and male stroke cases compared to controls [for females, OR per −10% methylation (95% CI) = 1.69 (1.06–2.69), $p = 0.027$; for males, OR per −10% methylation (95% CI) = 1.60 (1.14–2.23), $p = 0.006$, Table 2]. Gender-stratified methylation logistic regression analyses adjusted for age, smoking, drinking, hypertension and diabetes.

Previous studies have reported an increase in DNA methylation variability with advancing age [27, 28]. To mitigate the impact of age, we next investigated the methylation difference stratified by the age of 70 years old via logistic regression analyses adjusted for age, gender, smoking, drinking, hypertension and diabetes, according to our previous established age grouping [29]. In the individuals younger than 70 years old, two CpG loci showed lower methylation levels of *HYAL2* in CHD cases than in controls [for HYAL2_CpG_2, OR per −10% methylation

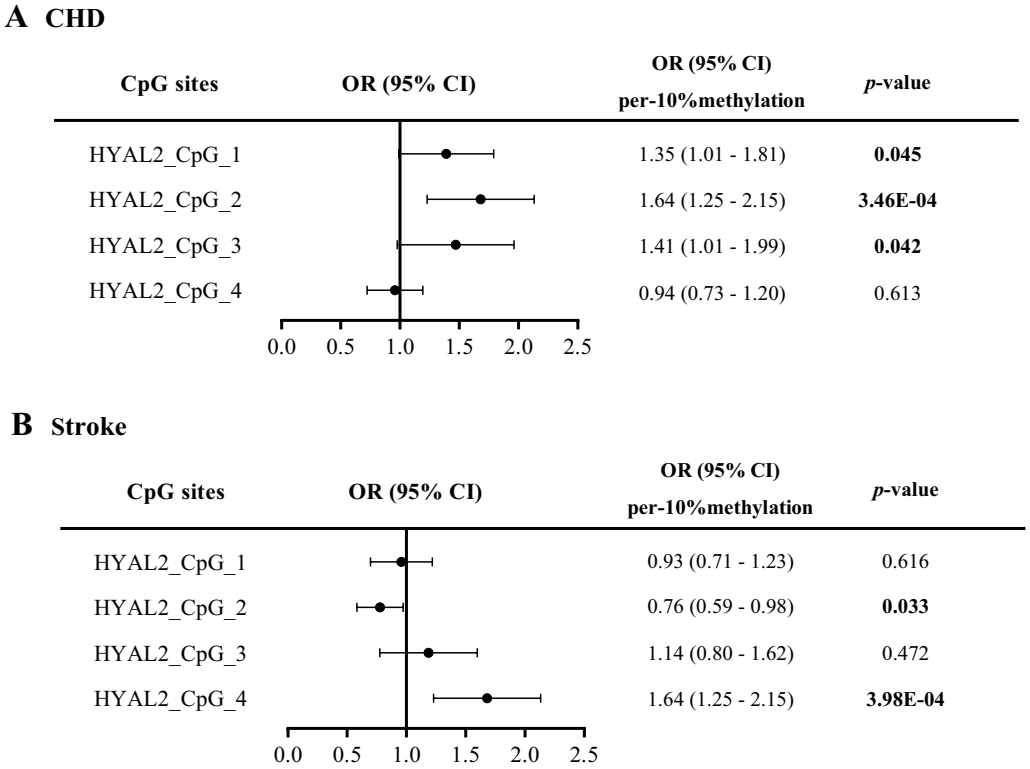

Fig. 1 Forest plots of *HYAL2* methylation difference between CHD cases, stroke cases and controls. **A** Methylation difference of *HYAL2* between CHD cases and controls. **B** Methylation difference of *HYAL2* between stroke cases and controls. The *p*-values were calculated by logistic regression adjusted for age, gender, smoking, drinking, hypertension and diabetes

Table 2 Gender-stratified methylation difference of *HYAL2* between CHD cases, stroke cases, and controls

CpG sites	Controls (N = 176)	CHD cases (N = 97)	Stroke cases (N = 58)	CHD cases vs. controls		Stroke cases vs. controls	
	Median (IQR)	Median (IQR)	Median (IQR)	OR (95% CI) per − 10% methylation	*p*-value[a]	OR (95% CI) per − 10% methylation	*p*-value[a]
Females							
HYAL2_CpG_1	0.33 (0.30–0.37)	0.31 (0.28–0.36)	0.35 (0.31–0.38)	1.97 (1.26–3.06)	**0.003**	0.82 (0.51–1.32)	0.411
HYAL2_CpG_2	0.25 (0.21–0.31)	0.22 (0.18–0.28)	0.28 (0.23–0.33)	1.86 (1.28–2.71)	**0.001**	0.72 (0.49–1.06)	0.096
HYAL2_CpG_3	0.38 (0.34–0.41)	0.35 (0.32–0.39)	0.37 (0.34–0.40)	2.11 (1.29–3.45)	**0.003**	1.08 (0.59–1.97)	0.803
HYAL2_CpG_4	0.55 (0.51–0.59)	0.54 (0.49–0.58)	0.52 (0.48–0.58)	1.25 (0.89–1.77)	0.204	1.69 (1.06–2.69)	**0.027**
CpG sites	**Controls (N = 180)**	**CHD cases (N = 74)**	**Stroke cases (N = 81)**	**CHD cases vs. controls**		**Stroke cases vs. controls**	
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**OR (95% CI) per − 10% methylation**	***p*-value[a]**	**OR (95% CI) per − 10% methylation**	***p*-value[a]**
Males							
HYAL2_CpG_1	0.32 (0.29–0.37)	0.33 (0.29–0.36)	0.32 (0.29–0.36)	1.00 (0.69–1.45)	0.982	1.00 (0.71–1.41)	0.981
HYAL2_CpG_2	0.25 (0.19–0.31)	0.24 (0.20–0.27)	0.27 (0.21–0.32)	1.46 (0.97–2.19)	0.071	0.79 (0.56–1.11)	0.174
HYAL2_CpG_3	0.36 (0.32–0.40)	0.36 (0.33–0.39)	0.36 (0.32–0.40)	0.95 (0.58–1.56)	0.837	1.18 (0.76–1.84)	0.458
HYAL2_CpG_4	0.54 (0.48–0.59)	0.56 (0.51–0.60)	0.52 (0.45–0.57)	0.70 (0.50–1.00)	0.051	1.60 (1.14–2.23)	**0.006**

[a] Logistic regression adjusted for age, smoking, drinking, hypertension and diabetes. Signifcant *p*-values are in bold

(95% CI) = 1.69 (1.22–2.34), $p=0.002$; for HYAL2_CpG_3, OR per −10% methylation (95% CI) = 1.71 (1.11–2.64), $p=0.016$, Table 3]. In subjects older than 70 years old, the methylation difference between CHD cases and controls was not significant. As for stroke, there were two CpG sites showing significant methylation differences between cases and controls in the group younger than 70 years old [for HYAL2_CpG_2, OR per −10% methylation (95% CI) = 0.60 (0.43–0.84), $p=0.003$; for HYAL2_CpG_4, OR per −10% methylation (95% CI) = 1.53 (1.06–2.23), $p=0.024$, Table 3]. The methylation pattern observed in stroke cases aged ≥ 70 years old was similar as those in individuals aged < 70 years, and only HYAL2_CpG_4 exhibited a lower methylation level compared to controls [OR per −10% methylation (95% CI) = 1.70 (1.10–2.61), $p=0.016$, Table 3].

The influence of different clinical characteristics on the methylation levels of *HYAL2* in CHD and stroke

CVDs are widely recognized to be closely associated with various clinical factors. In comparison with numerous indicators of clinical characteristics in Supplementary Table 2, we found significant differences in *HYAL2* methylation levels in the study subjects when stratified by the status of hypertension, TC and LDL-C levels while there was no or weak correlation between *HYAL2* methylation and lifestyles (smoking and drinking) as well as body obesity indexes (TG, HDL-C, BMI and diabetes). We next investigated *HYAL2* methylation difference between CHD cases, stroke cases and controls stratified by different clinical characteristics with logistic regression model adjusted for age, gender, smoking, drinking, hypertension and diabetes.

When stratified by hypertension, in the case of CHD, the methylation difference of *HYAL2* mainly manifested in subjects without hypertension, with two out of four CpG sites presented significance between CHD cases and controls [for HYAL2_CpG_1, OR per −10% methylation (95% CI) = 2.10 (1.14–3.87), $p=0.018$; for HYAL2_CpG_3, OR per −10% methylation (95% CI) = 2.58 (1.24–—5.37), $p=0.012$, Fig. 2A], whereas only HYAL2_CpG_2 presented methylation difference in subjects with hypertension [OR per −10% methylation (95% CI) = 1.60 (1.15–2.23), $p=0.005$, Supplementary Fig. 2A]. In the analysis of stroke cases, only HYAL2_CpG_4 displayed significant hypomethylation compared to controls [for

Table 3 Age-stratified methylation difference of *HYAL2* between CHD cases, stroke cases and controls

CpG sites	Controls (N = 247)	CHD cases (N = 131)	Stroke cases (N = 88)	CHD cases vs. controls		Stroke cases vs. controls	
	Median (IQR)	Median (IQR)	Median (IQR)	OR (95% CI) per −10% methylation	*p-value*[a]	OR (95% CI) per −10% methylation	*p-value*[a]
Age < 70 years							
HYAL2_CpG_1	0.33 (0.29–0.37)	0.32 (0.28–0.36)	0.34 (0.31–0.37)	1.38 (0.97–1.95)	0.072	0.82 (0.58–1.17)	0.277
HYAL2_CpG_2	0.25 (0.20–0.29)	0.22 (0.19–0.27)	0.28 (0.23–0.33)	1.69 (1.22–2.34)	**0.002**	0.60 (0.43–0.84)	**0.003**
HYAL2_CpG_3	0.37 (0.34–0.40)	0.36 (0.32–0.39)	0.37 (0.34–0.40)	1.71 (1.11–2.64)	**0.016**	0.92 (0.54–1.55)	0.742
HYAL2_CpG_4	0.54 (0.50–0.59)	0.55 (0.50–0.58)	0.51 (0.47–0.56)	0.90 (0.66–1.22)	0.479	1.53 (1.06–2.23)	**0.024**
CpG sites	**Controls (N = 109)**	**CHD cases (N = 40)**	**Stroke cases (N = 51)**	**CHD cases vs. controls**		**Stroke cases vs. controls**	
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**OR (95% CI) per −10% methylation**	***p-value***[a]	**OR (95% CI) per −10% methylation**	***p-value***[a]
Age ≥ 70 years							
HYAL2_CpG_1	0.32 (0.29–0.38)	0.32 (0.29–0.34)	0.32 (0.28–0.37)	1.20 (0.67–2.16)	0.544	1.10 (0.69–1.76)	0.686
HYAL2_CpG_2	0.26 (0.19–0.32)	0.23 (0.19–0.28)	0.25 (0.20–0.32)	1.30 (0.78–2.17)	0.314	1.02 (0.68–1.54)	0.908
HYAL2_CpG_3	0.36 (0.32–0.41)	0.35 (0.32–0.40)	0.36 (0.32–0.40)	0.94 (0.54–1.64)	0.814	1.31 (0.78–2.19)	0.304
HYAL2_CpG_4	0.54 (0.49–0.62)	0.56 (0.51–0.61)	0.52 (0.45–0.57)	0.92 (0.60–1.43)	0.721	1.70 (1.10–2.61)	**0.016**

[a] Logistic regression adjusted for age, gender, smoking, drinking, hypertension and diabetes. Signifcant *p*-values are in bold

(See figure on next page.)

Fig. 2 Forest plots of *HYAL2* methylation difference between CHD cases, stroke cases and controls stratified by different clinical characteristics. **A** Methylation difference of *HYAL2* in subjects without hypertension. **B** Methylation difference of *HYAL2* in subjects with TC ≥ 5.0 mmol/L. **C** Methylation difference of *HYAL2* in subjects with LDL-C ≥ 3.0 mmol/L. **D** Methylation difference of *HYAL2* in subjects with cancer history. The *p*-values were calculated by logistic regression models, **A** adjusted for age, gender, smoking, drinking and diabetes; **B**–**D** adjusted for age, gender, smoking, drinking, hypertension and diabetes

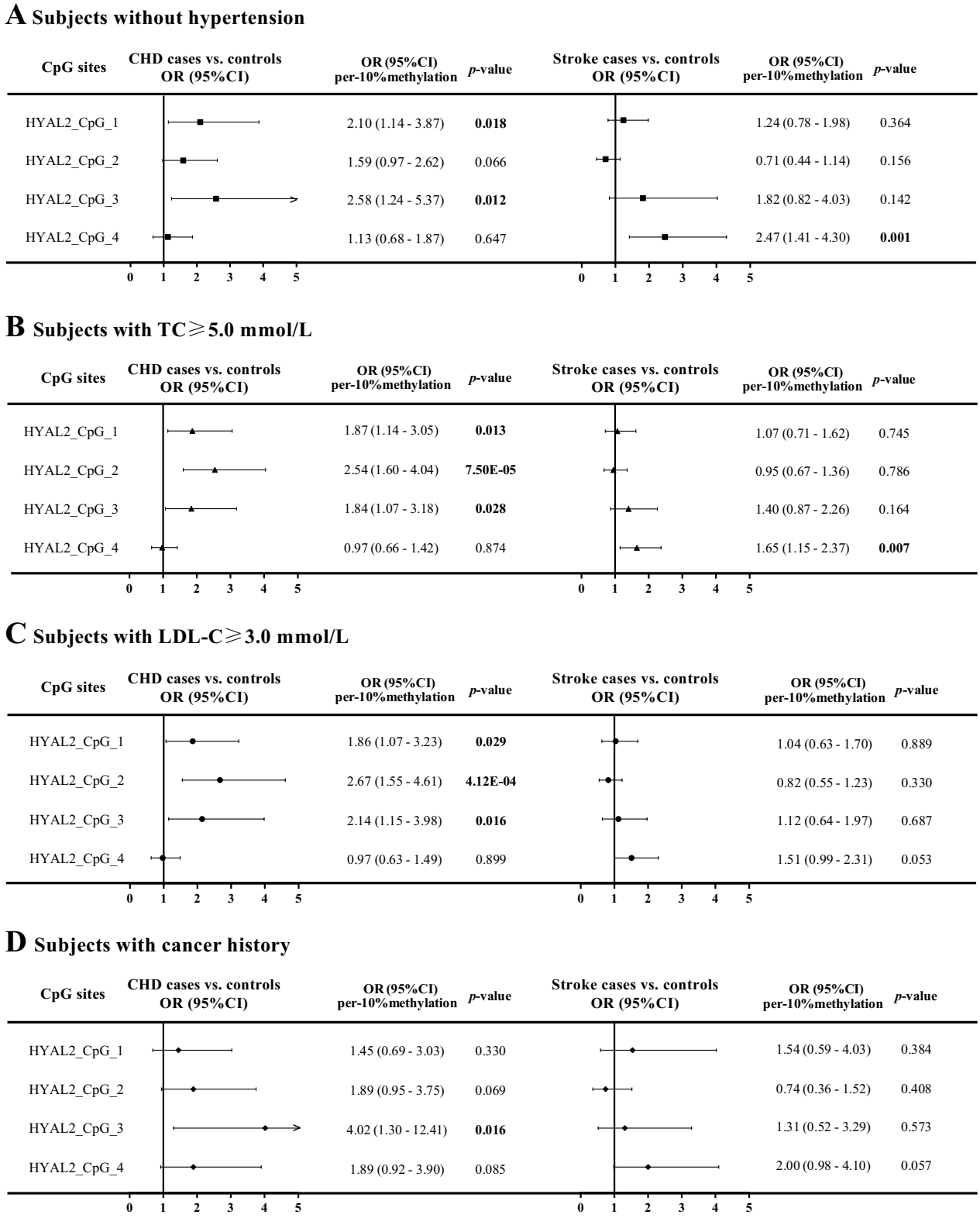

Fig. 2 (See legend on previous page.)

subjects without hypertension, OR per −10% methylation (95% CI) = 2.47 (1.41–4.30), $p=0.001$; for subjects with hypertension, OR per −10% methylation (95% CI) = 1.38 (1.01–1.88), $p=0.042$, Fig. 2A, Supplementary Fig. 2A].

As the underlying pathology of CVDs, lipids and lipoprotein particles play a crucial role in the development of atherosclerosis [30]. For subjects with TC ≥ 5.0 mmol/L, three CpG sites exhibited significant lower methylation levels in CHD cases compared to the controls, among which HYAL2_CpG_2 showed the most significant difference [for HYAL2_CpG_1, OR per −10% methylation (95% CI) = 1.87 (1.14–3.05), $p=0.013$; for HYAL2_CpG_2, OR per −10% methylation (95% CI)= 2.54 (1.60–4.04), $p=7.50\times10^{-5}$; for HYAL2_CpG_3, OR per −10% methylation (95% CI)= 1.84 (1.07–3.18), $p=0.028$, Fig. 2B]. In contrast, no significant association between CHD cases and controls was observed in subjects with TC < 5.0 mmol/L (Supplementary Fig. 2B). As for stroke, methylation changes of HYAL2_CpG_4 were observed in both groups [for subjects with TC ≥ 5.0 mmol/L, OR per −10% methylation (95% CI) = 1.65 (1.15–2.37), $p=0.007$; for subjects with TC < 5.0 mmol/L, OR per −10% methylation (95% CI) = 1.68 (1.08–2.62), $p=0.020$, Fig. 2B, Supplementary Fig. 2B]. Interestingly, the methylation level at HYAL2_CpG_2 was significantly increased in stroke cases compared to controls in the subjects with TC < 5.0 mmol/L [OR per −10% methylation (95% CI) = 0.60 (0.40–0.89), $p=0.012$, Supplementary Fig. 2B].

In addition to TC levels, it has been demonstrated that LDL-C levels are associated with the risk of CHD and stroke. HYAL2_CpG_2 became the most pronounced in CHD cases compared to controls with LDL-C ≥ 3.0 mmol/L [OR per −10% methylation (95% CI) = 2.67 (1.55–4.61), $p=4.12\times10^{-4}$, Fig. 2C] and other two CpG loci exhibited methylation differences [for HYAL2_CpG_1, OR per −10% methylation (95% CI) = 1.86 (1.07–3.23), $p=0.029$; for HYAL2_CpG_3, OR per −10% methylation (95% CI) = 2.14 (1.15–3.98), $p=0.016$, Fig. 2C]. In subjects with LDL-C < 3.0 mmol/L, only HYAL2_CpG_2 had significant methylation difference in CHD versus controls analysis [OR per −10% methylation (95% CI) = 1.39 (1.00–1.92), $p=0.050$, Supplementary Fig. 2C]. There was no significant association found in stroke cases with LDL-C ≥ 3.0 mmol/L (Fig. 2C), but a significant methylation difference at HYAL2_CpG_4 was observed between stroke cases and controls in the analysis of subjects with LDL-C < 3.0 mmol/L [OR per −10% methylation (95% CI) = 1.89 (1.30–2.77), $p=0.001$, Supplementary Fig. 2C].

Arterial thrombotic events are serious and potentially life-threatening adverse events associated with various anticancer treatments [31]. The association between blood-based *HYAL2* methylation and breast, thyroid, pancreatic cancer, colon and head and neck cancers has been reported [17–19, 32, 33]. Altered *HYAL2* methylation was also found in the tissue of around ten cancer types according to the human disease methylation database (http://bio-bigdata.hrbmu.edu.cn/diseasemeth/). Meanwhile, only the cancer history in general is available in our study, but no detailed information about cancer types. We therefore stratified the subjects with cancer history. Compare to controls with cancer history, the methylation levels of *HYAL2* in CHD cases with cancer history were significantly reduced at HYAL2_CpG_3 [OR per −10% methylation (95% CI)= 4.02 (1.30–12.41), $p=0.016$, Fig. 2D]. In non-cancer subjects when comparing CHD and controls, HYAL2_CpG_2 retained its differential methylation but lower ORs [OR per −10% methylation (95% CI)= 1.61 (1.19–2.18), $p=0.002$, Supplementary Fig. 2D], whereas HYAL2_CpG_3 did not [HYAL2_CpG_3, OR per −10% methylation (95% CI)= 1.18 (0.81–1.73), $p=0.393$, Supplementary Fig. 2D]. As for stroke, no methylation difference was observed in subjects with cancer history (Fig. 2D).

The methylation difference of *HYAL2* stratified by onset times of CHD and stroke

Since the onset time of CHD and stroke may influence *HYAL2* methylation levels, we next performed further logistic regression analyses comparing controls and stratified cases by time of onset adjusted for age, gender, smoking, drinking, hypertension and diabetes. A total of 171 subjects who developed CHD and 139 subjects who developed stroke within 2.5 years were included in this study. There was only one CpG site presented significantly altered methylation level between CHD cases with time of onset ≤ 1.5 years and controls [HYAL2_CpG_2, OR per −10% methylation (95% CI)= 1.69 (1.17–2.44), $p=0.006$, Table 4]. Compared to controls, the methylation levels of three CpG sites (HYAL2_CpG_1, HYAL2_CpG_2 and HYAL2_CpG_3) were significantly lower in CHD cases with time of onset ≤ 2.5 years (ORs per −10% methylation from 1.35 to 1.64, $p \le 0.045$ for all, Table 4). As for stroke, cases with onset time ≤ 1.5 years showed significantly lower methylation level of HYAL2_CpG_4 than controls [OR per −10% methylation (95% CI)= 1.76 (1.27–2.44), $p=0.001$, Table 4]. Moreover, significant methylation differences of HYAL2_CpG_2 and HYAL2_CpG_4 were observed in stroke cases with time of onset ≤ 2.5 years compared to controls [for HYAL2_CpG_2, OR per −10% methylation (95% CI)= 0.76 (0.59–0.98), $p=0.033$; for HYAL2_CpG_4, OR per −10% methylation (95% CI)= 1.64 (1.25–2.15), $p=3.98\times10^{-4}$, Table 4].

Table 4 Methylation difference of *HYAL2* between CHD cases, stroke cases and controls with different onset times

CpG sites	Controls (N = 356)	CHD cases (N = 75)	Stroke cases (N = 91)	CHD cases vs. controls		Stroke cases vs. controls	
	Median (IQR)	Median (IQR)	Median (IQR)	OR (95% CI) per − 10% methylation	*p-value*[a]	OR (95% CI) per − 10% methylation	*p-value*[a]
Within 1.5 years							
HYAL2_CpG_1	0.33 (0.29–0.37)	0.32 (0.28–0.36)	0.33 (0.30–0.37)	1.46 (0.95–2.24)	0.082	0.97 (0.70–1.35)	0.852
HYAL2_CpG_2	0.25 (0.20–0.31)	0.22 (0.18–0.28)	0.27 (0.21–0.32)	1.69 (1.17–2.44)	**0.006**	0.80 (0.60–1.08)	0.146
HYAL2_CpG_3	0.37 (0.33–0.40)	0.35 (0.32–0.39)	0.37 (0.33–0.39)	1.55 (0.94–2.56)	0.085	1.29 (0.86–1.93)	0.223
HYAL2_CpG_4	0.54 (0.49–0.59)	0.56 (0.51–0.60)	0.51 (0.47–0.56)	0.88 (0.62–1.24)	0.460	1.76 (1.27–2.44)	**0.001**
CpG sites	**Controls (N = 356)**	**CHD cases (N = 171)**	**Stroke cases (N = 139)**	**CHD cases vs. controls**		**Stroke cases vs. controls**	
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	**OR (95% CI) per − 10% methylation**	***p-value***[a]	**OR (95% CI) per − 10% methylation**	***p-value***[a]
Within 2.5 years							
HYAL2_CpG_1	0.33 (0.29–0.37)	0.32 (0.28–0.36)	0.33 (0.30–0.37)	1.35 (1.01–1.81)	**0.045**	0.93 (0.71–1.23)	0.616
HYAL2_CpG_2	0.25 (0.20–0.31)	0.22 (0.19–0.27)	0.27 (0.22–0.32)	1.64 (1.25–2.15)	**3.46E−04**	0.76 (0.59–0.98)	**0.033**
HYAL2_CpG_3	0.37 (0.33–0.40)	0.36 (0.32–0.39)	0.37 (0.33–0.40)	1.41 (1.01–1.99)	**0.042**	1.14 (0.80–1.62)	0.472
HYAL2_CpG_4	0.54 (0.49–0.59)	0.55 (0.50–0.59)	0.52 (0.47–0.57)	0.94 (0.73–1.20)	0.613	1.64 (1.25–2.15)	**3.98E−04**

[a] Logistic regression adjusted for age, gender, smoking, drinking, hypertension and diabetes. Signifcant *p*-values are in bold

HYAL2 methylation as a potential marker for the preclinical detection of CHD and stroke

To evaluate the potential clinical utility of *HYAL2* methylation as a marker for the preclinical detection of CHD and stroke, ROC curve analyses were conducted by logistic regression model adjusted for age, gender, smoking, drinking, hypertension and diabetes effects. When all four measurable CpG sites were considered, the methylation levels of *HYAL2* showed a moderate discriminatory power to distinguish CHD and stroke cases from controls [AUC = 0.67 and 0.68 for CHD and stroke, respectively, Fig. 3A and B]. Interestingly, the methylation levels of the four CpG sites in the *HYAL2* amplicon demonstrated efficient discriminatory power to distinguish both CHD and stroke cases from matched controls in the subjects without hypertension [AUC = 0.78 and 0.75 for CHD and stroke, Fig. 3C and D, respectively], surpassing that observed for the subjects with hypertension [AUC = 0.65 and 0.68 for CHD and stroke, Fig. 3E and F, respectively].

Discussion

This prospective nested case–control study has revealed a significant association of *HYAL2* methylation in peripheral blood DNA with preclinical CHD and stroke, which was more pronounced in female, younger, and non-hypertensive CHD cases as well as CHD cases with cancer history. The methylation levels of the four CpG sites in the *HYAL2* amplicon showed a discriminatory power to distinguish both CHD and stroke cases from controls at preclinical stage, especially in the subjects without hypertension.

Hyaluronan (HA) is the predominant glycosaminoglycan in the extracellular matrix. The hyaluronidases are classes of enzymes that degrade predominantly hyaluronan. Five Hyals have been identified in humans: *HYAL1*, *HYAL2*, *HYAL3*, *HYAL4, PH20 (SPAM1)*. The human Hyals have significant degrees of sequence conservation, indicative of their presumed common structural and catalytic properties [34]. All the five Hyals were involved in cancers [17, 35–38], but only a few studies reported *HYAL1* and *HYAL2* in vascular diseases so far [39, 40]. *HYAL2* is responsible for hydrolyzing the high molecular weight HA into LMW-HA. During inflammation, there is an increase in HA catabolism and accumulation of LMW-HA, which lead to the activation of monocyte and macrophage activation. As previously mentioned, the most significant pathophysiological manifestations of atherosclerotic diseases are the inflammatory response and lipid aggregation. Previous histological studies have demonstrated the strong positive immunostaining for HA and its receptor CD44 at the interface between the endothelium of the eroded plaque and the luminal thrombus [41]. Therefore, the hyaluronidase encoded by the *HYAL2* gene plays a crucial role in CHD and stroke. Furthermore, population studies have reported an association between hyaluronic acid and acute coronary syndrome (ACS). Daniela et al. collected peripheral blood mononuclear cells from ACS patients, stable angina (SA)

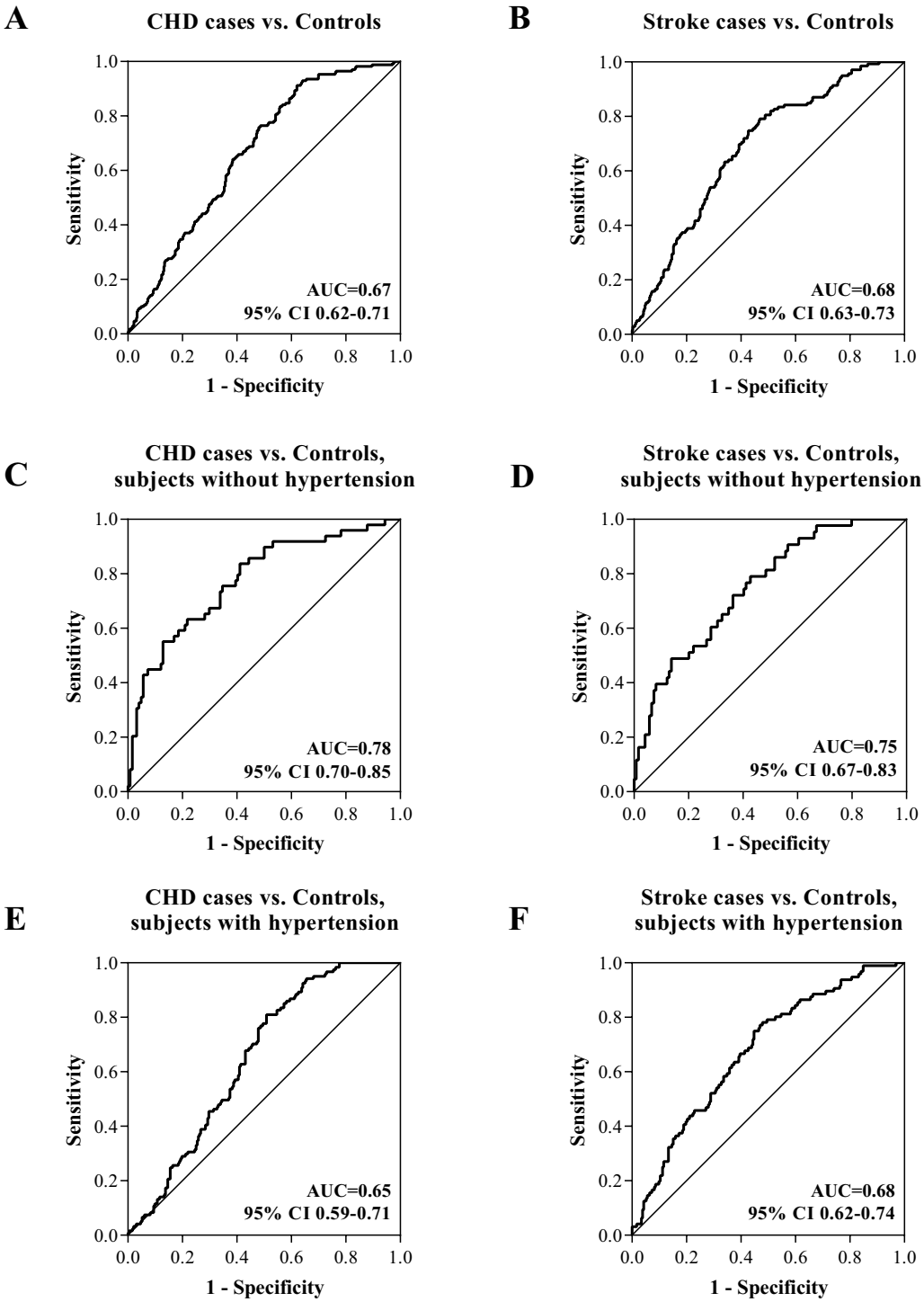

Fig. 3 The power of *HYAL2* methylation (HYAL2_CpG_1, HYAL2_CpG_2, HYAL2_CpG_3, HYAL2_CpG_4 combined) to distinguish CHD cases, stroke cases from controls. **A** The discrimination of CHD cases from controls by *HYAL2* methylation. **B** The discrimination of stroke cases from controls by *HYAL2* methylation. **C** The discrimination of CHD cases from controls by *HYAL2* methylation in subjects without hypertension. **D** The discrimination of stroke cases from controls by *HYAL2* methylation in subjects without hypertension. **E** The discrimination of CHD cases from controls by *HYAL2* methylation in subjects with hypertension. **F** The discrimination of stroke cases from controls by *HYAL2* methylation in subjects with hypertension. The Receiver operating characteristic (ROC) analyses were calculated by logistic regression models, **A**, **B** adjusted for age, gender, smoking, drinking, hypertension and diabetes; **C**–**F** adjusted for age, gender, smoking, drinking and diabetes

patients and controls to explore the involvement of hyaluronan metabolism in ACS. The results demonstrated significantly higher mRNA and protein expression of *HYAL2* in ACS patients compared to SA patients and controls [40]. Recent evidence suggests that atherosclerosis involves the interplay of multiple epigenetics mechanism [42], particularly DNA methylation. Zheng's study disclosed a significant improvement ($p=0.004$) in the identification of coronary artery calcification status using a methylation risk score compared to cardiovascular health score. In addition, the methylation risk score was associated with the risk of coronary artery calcification events occurring 5–10 years later [43]. Here, we investigated the alterations of *HYAL2* methylation in peripheral blood from individuals with CHD and stroke, which share a common characteristic of atherosclerosis. This is the first study that reported the association between Hyals methylation and cardiovascular and cerebrovascular diseases, and it may worth to extend this study from the methylation of *HYAL2* to the methylation of other Hyals in the future.

Although CHD and stroke exhibit highly analogous pathogenesis and pathophysiological characteristics, our data demonstrated distinct or even opposite methylation patterns in CpG sites of the *HYAL2* gene when comparing CHD patients and stroke patients to controls. More specific, three CpG sites (HYAL2_CpG_1, HYAL2_CpG_2 and HYAL2_CpG_4) exhibited opposite methylation trends for the two diseases (Fig. 1). This observation may be attributed to the absence of hyaluronidase 2 in the human brain. A previous study employing Northern plot analysis revealed the presence of *HYAL2* mRNAs in all human tissues, except for the adult brain [44]. In this tissue, the gene is silenced after birth through de novo methylation of CpG islands in the promoter and 5′-untranslated region [45]. Further investigation is required to determine whether alterations in blood-based *HYAL2* methylation levels in the stroke population are associated with the silencing of *HYAL2* expression in brain tissue.

Previous studies have shown that males had a higher incidence of CHD than females [46]. Surprisingly, our results revealed significantly lower levels of HYAL2_CpG_1, HYAL2_CpG_2 and HYAL2_CpG_3 methylation in females compares to males, suggesting that the *HYAL2* methylation contributes more significantly to CHD in females. This discrepancy could be attributed to the influence of gender hormones and gender-related habits and lifestyles [47]. Unfortunately, we do not have the information about the hormone levels in the women. Instead, we stratified the subjects with the age of 50 years old, since it has been reported that the average age of menopause in Asian women is around 48.8 years old [48]. As shown in Table S3, the significantly decreased *HYAL2* methylation only existed in the female CHD cases with age > 50 years old (three CpG sites, all p-values < 0.002), but none in the younger women, implying the hormonal factors as a potential influence on the methylation level of *HYAL2*. Meanwhile, both male and female stroke cases exhibited alterations in the methylation of HYAL2_CpG_4, indicating that the same gene is altered at different methylation sites in distinct diseases. In our previous study on the association between altered *ACTB* methylation and CHD, we observed more significant methylation loci in males [49]. This further supports the notion that, for the same disease, methylation of different genes acts independently in relation to gender. Moreover, age-stratified analysis showed that *HYAL2* presented lower methylation levels and was more pronounced in CHD and stroke cases below the age of 70 years, highlighting the potential of altered *HYAL2* methylation as a novel biomarker for early detection in younger individuals. Thus, gender and age adjustments are necessary when exploring the relationship between *HYAL2* methylation and diseases.

In addition, DNA methylation levels are strongly correlated with clinical characteristics. Numerous studies have identified hypertension and hyperlipidemia as important risk factors influencing the development of CVDs [50–52]. However, our study yielded unexpected findings that pronounced *HYAL2* methylation alterations were observed in CHD cases without hypertension and with higher blood lipid, whereas the correlation between *HYAL2* methylation and clinical characteristics of stroke was relatively weaker. Our results demonstrated that in the CHD population without hypertension, two CpG sites showed significant methylation differences with very high ORs, indicating that hypomethylation of the *HYAL2* gene could be an independent risk factor of hypertension in CHD. This result may be related to the disruption of the vascular permeability barrier by the glycan surface layer composed of HA. The glycan surface layer and its principal glycosaminoglycans facilitate increased access of leucocytes to the arterial intima and alteration of endothelial mechanotransduction mechanisms that protect against disease [53]. However, the precise mechanism still needs to be verified through functional experiments. Likewise, the methylation levels of four CpG sites in the *HYAL2* amplicon exhibited effective discriminatory power to distinguish subjects without hypertension, as demonstrated by the ROC curve analysis. Furthermore, we observed lower methylation levels in CHD cases compared to controls in the subjects with TC ≥ 5.0 mmol/L and LDL-C ≥ 3.0 mmol/L, indicating that *HYAL2* hypomethylation is a predictor for high risk of CHD patients with hyperlipidemia. This finding may explain why individuals with high blood lipid levels have

a higher incidence and mortality of CHD, and expanding our comprehension of methylation profiles. Nevertheless, our study does not demonstrate an association between *HYAL2* methylation levels and traditional CVD risk factors such as smoking and alcohol consumption. Epigenetic modifications have strong associations with CVDs and cancer due to shared risk factors and pathogenesis. Previous study showed that common genes associated with cancer and CVDs are enriched in nucleotide methylation pathway [54]. We found decreased *HYAL2* methylation levels in CHD cases predominantly occurred in subjects with cancer history. Numerous studies have reported clinically significant risk of medically induced cardiotoxicity in oncology patients [55, 56]. What's more, the methylation level of *HYAL2* has been closely related to the development of various cancers [17–19]. It is plausible to infer that cardiotoxicity related to oncology drugs is associated with changes in *HYAL2* methylation levels and the development of cardiovascular disease, providing new prospects for baseline cardiovascular assessment in every cancer patient receiving potentially cardiotoxic therapy [57]. Nevertheless, the sample size of subjects with cancer history were limited in our study and had no details. It will be important to collect more comprehensive information in the future studies.

In this prospective nested case–control study, ORs showed a gradual but not significant decrease with the increasing CHD onset time, suggesting that altered methylation is primarily associated with a higher risk of CHD in individuals with a shorter duration of onset and this risk is further increased with shorter onset time. Therefore, the methylation patterns of *HYAL2* may have the potential as a robust and reliable biomarker for early detection of CHD and stroke.

Conclusion

In summary, this prospective nested case–control study revealed the association of altered *HYAL2* methylation with CHD and stroke. It is worth noting that the GpG loci and direction of *HYAL2* methylation alterations differed between CHD and stroke cases. Meanwhile, the female gender, younger age group and non-hypertensive patients displayed more pronounced *HYAL2* methylation alterations in CHD cases. Cancer history also enhanced the influence of the altered *HYAL2* methylation on CHD patients. However, our study is limited by the sample size of events, multicenter studies based on large prospective cohorts are needed for validation.

Data and code availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-024-01742-7.

Additional file1

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Author contributions

This study was planned, conceived and designed by RY and CS; LB were responsible for analyzing the results and drafting the manuscript; LB, JJ and ML performed the experiments; YL, HX and CC provided the materials and supervised the patient enrollment and acquisition of clinical data; and YF contributed to the collection of biological samples. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Nanjing Medical University, and all individuals involved provided written informed consents.

Consent for publication

All authors give consent for the publication of the manuscript.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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