ORIGINAL ARTICLE

# Hypermethylation of dihydrofolate reductase promoter increases the risk of hypertension in Chinese

**Guodong Xu[1,2], Zhiyi Wang[2], Lian Li[2], Wenxia Li[2], Jingcen Hu[2], Shuyu Wang[2], Hongxia Deng[3], Bo Li[4], Changyi Wang[4], Zhishen Shen[3], Liyuan Han[5,6]**

[1]Medical Record Statistics Room, Ningbo Medical Center Lihuili Hospital, Ningbo, Zhejiang, PR China, [2]Department of Preventive Medicine, Zhejiang Provincial Key Laboratory of Pathophysiology, School of Medicine, Ningbo University, Ningbo, Zhejiang, China, [3]Department of Otorhinolaryngology Head and Neck Surgery, Ningbo Medical Center Lihuili Hospital, Ningbo, Zhejiang, China, [4]Department of Non-Communicable Disease Prevention and Control, Shenzhen Nanshan Center for Chronic Disease Control, Shenzhen, Guangdong, China, [5]Hwa Mei Hospital, University of Chinese Academy of Sciences, Ningbo, Zhejiang, PR China, [6]Department of Global Health, Ningbo Institute of Life and Health Industry, University of Chinese Academy of Sciences, Ningbo, Zhejiang, PR China

**Background:** DNA methylation was considered to play an important role in hypertension. However, the direct association between dihydrofolate reductase (*DHFR*) promoter methylation and hypertension remains unclear. We thus aimed to investigate the relationship between DNA methylation of *DHFR* promoter and hypertension. **Materials and Methods:** A total of 371 hypertensive patients (diastolic blood pressure ≥90 mmHg and/or systolic blood pressure ≥140 mmHg or a history of antihypertensive treatment) and 320 age- and sex-matched healthy controls from the Hypertension Management Information System in Nanshan Community Health Service Centers were included in this case–control study. Quantitative methylation-specific polymerase chain reaction was used to measure the level of *DHFR* promoter methylation, which was presented as the percentage of methylated reference (PMR). A multivariate logistic regression model was used to explore the risk of *DHFR* promoter methylation. **Results:** Our results indicated that the level of *DHFR* promoter methylation was higher in hypertensive patients (median PMR, 34.32%; interquartile range, 11.34–119.60) than in healthy controls (median PMR, 18.45%; interquartile range, 8.16–35.40) ($P < 0.001$). Multivariable analysis showed that the risk of *DHFR* promoter hypermethylation was significantly higher in hypertensive patients than in healthy controls (odds ratio = 3.94, 95% confidence interval = 2.56–6.02, $P < 0.001$). Furthermore, hypermethylation was positively associated with sex, high blood homocysteine levels, and alcohol drinking. In particular, the area under the receiver operating characteristic curve was 0.688 (0.585–0.668) for the male hypertensive patients, suggesting the potential diagnostic value of *DHFR* promoter methylation in male hypertension. **Conclusion:** Our results demonstrated that *DHFR* promoter hypermethylation is positively associated with the risk of hypertension in Chinese.

**Key words:** Dihydrofolate reductase, hypermethylation, hypertension

**How to cite this article:** Xu G, Wang Z, Li L, Li W, Hu J, Wang S, *et al.* Hypermethylation of dihydrofolate reductase promoter increases the risk of hypertension in Chinese. J Res Med Sci 2020;25:117.

## INTRODUCTION

Hypertension is a major public health burden, and about 31.1% of world's adults had hypertension.[1] According to the Results from the China Hypertension Survey (2012–2015), 23.2% of Chinese adults had hypertension and 41.3% had prehypertension,[2] indicating that the burden of hypertension is also very high in China. Hypertension is influenced by susceptibility genes, environmental factors, and their complex interactions.[3,4] Epigenetic modifications, which provide a link between environment and gene expression, are known to be involved in the development

Access this article online

Quick Response Code:

Website: www.jmsjournal.net

DOI: 10.4103/jrms.JRMS_895_19



**Address for correspondence:** Dr. Liyuan Han, Hwa Mei Hospital, University of Chinese Academy of Sciences, Ningbo, Zhejiang, PR China. E-mail: hanliyuan@nbu.edu.cn

Dr. Zhisen Shen, Ningbo Medical Center Lihuili Hospital, Ningbo, Zhejiang, China. E-mail: szs7216@163.com

**Submitted:** 13-Jan-2020; **Revised:** 03-Mar-2020; **Accepted:** 15-Jul-2020; **Published:** 30-Dec-2020

of hypertension via several mechanisms.[5] For example, DNA methylation, one of the most common epigenetic modifications, usually occurs at CpG dinucleotides in mammals.[6] DNA methyltransferase transfers a methyl group to cytosine nucleotides on the promoter, likely leading to the transcriptional silencing of gene expression.[7] Compelling evidence has indicated that aberrant DNA methylation of the *ADD1*,[8] *GCK*,[9] and *SHMT1*[10] genes contributes to the risk of hypertension.

Hyperhomocysteinemia is a well-known risk factor for hypertension.[11] Dihydrofolate reductase (DHFR) enzyme is a cytosolic protein of 21.3 kDa, and nicotinamide adenine dinucleotide phosphate is required as its coenzyme.[12] DHFR converts folate derivatives to dihydrofolate and then to tetrahydrofolate (THF). Eventually, via catalysis by the enzyme methylene tetrahydrofolate reductase (MTHFR), THF is converted to 5-methyl tetrahydrofolate (5-MTHF), which provides a methyl group to homocysteine and influences its metabolism. The variations in the noncoding regions of the *DHFR* gene affect the blood homocysteine (Hcy) levels: deletion of a 19-bp region in intron 1 can decrease Hcy levels, while variation in a 9-bp repeat in the CpG island may also lead to changes in Hcy levels.[13] However, the direct association between *DHFR* promoter methylation and hypertension or Hcy levels remains unclear. Thus, we aimed to investigate the contribution of *DHFR* promoter methylation to the occurrence of hypertension in a Chinese population.

## MATERIALS AND METHODS

### Subjects

In total, 371 hypertensive patients were recruited from December 2018 to August 2019, all of whom were local residents of Shenzhen (living in Shenzhen for at least half a year) registered in the Hypertension Management Information System in Nanshan Community Health Service Centers [Figure 1]. In addition, 320 age-matched (±3 years) and sex-matched healthy controls were also recruited from the same communities. The data collection of the participants was carried out simultaneously. According to the present sample size, we used the formula: n= (1 + 1/c) $q\_p\_$ (U$\alpha$ + U$\beta$) $^2$/(p1 − p0) $^2$, where $\alpha$ =0.05 and $c$ = 0.863, and the power of this case–control study was 91%, the β value was 0.09. The diagnostic criteria for hypertension were diastolic blood pressure (DBP) ≥90 mmHg and/or systolic blood pressure (SBP) ≥140 mmHg or a history of antihypertensive treatment. Furthermore, individuals with a history of liver or renal insufficiency, secondary hypertension, cancer, or other serious diseases or who previously took folic acid, Vitamin B6, or Vitamin B12, which can directly influence Hcy levels, were excluded. This study was approved by the ethics committee of the Shenzhen Nanshan Center for Chronic Disease Control. Informed written consent was obtained from all participants (1120170008).

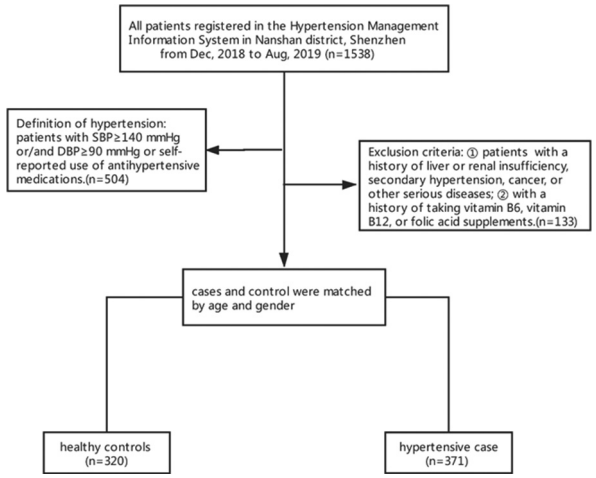


**Figure 1:** The study flowchart

### Physical examination

Physical examination included height, weight, waist–hip ratio, and SBP and DBP measurements. Mercury sphygmomanometers were used to measure the participants' blood pressure on their right arm after 5 min of sitting. Inextensible anthropometric tapes were used to measure the waist and hip circumferences. All physical examinations were performed by professional medical staff.

### Biochemical measurements

After the participants were fasted for 12 h, their blood samples were collected in EDTA anticoagulant tubes and were immediately transported to the laboratory in a cryogenic sampling chamber for further investigations or stored at −80°C in an ultra-low temperature freezer. The plasma levels of fasting blood Hcy, uric acid, triglyceride, total cholesterol, low-density lipoprotein (LDL), and glucose (Glu) were measured using an automatic biochemical analyzer (HITACHI 7080, Tokyo, Japan).

### Detection of DNA methylation levels

SYBR green-based quantitative methylation-specific polymerase chain reaction (qMSP) was used to measure the level of *DHFR* promoter methylation. The DNA extraction, bisulfite DNA modification, and qMSP procedures were followed as described in previous studies.[14,15] The following primers were used for qMSP of the *DHFR* promoter: forward, 5′-TATTTGAGCGGTGGTTAG-3′ and reverse, 5′-TCTACTATAACGAACGAACTC-3′. The thermal cycling conditions were one initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 45 s, and extension at 72°C for 20 s. A melting curve analysis was performed under the following conditions: holding at

95°C for 15 s, cooling to 60°C for 60 s, and slowly heating to 95°C at the rate of 0.11°C/s. The percentage of methylated reference (PMR) was used to represent the level of *DHFR* promoter methylation.

### Statistical analysis

The differences in normal distribution continuous and categorical variables between the hypertensive patients and healthy controls were examined by the *t*-test and Chi-square test, respectively. The levels of *DHFR* promoter methylation are skewed distribution and expressed as the median (interquartile range), and comparison between the levels was performed using the Mann–Whitney U-test. In addition, the Chi-square test was used to explore the relationship between the *DHFR* promoter methylation and clinical characteristics, and *DHFR* methylation was divided into hypermethylation and hypomethylation based on the cutoff value of the receiver operating characteristic (ROC) curve. A multivariate logistic regression analysis model was used to determine the relationship between the level of DHFR methylation and the risk of hypertension after adjusting for potential confounding variables. In addition, the area under the ROC curve (AUC) was used to evaluate the diagnostic value of *DHFR* methylation for hypertension. The multiple imputation method was used to impute the missing data. All statistical analyses were performed using PASW Statistics version 18.0 (IBM SPSS Statistics, ARMONK, USA), and a two-sided $P < 0.05$ was considered as statistically significant.

## RESULTS

### Characteristics of cases and controls

In total, 371 hypertensive patients and 320 age- and sex-matched healthy controls were recruited in the present study. The baseline study flowchart of the patients and controls are shown in Table 1. Figure 2a shows the *DHFR* promoter assay used to determine *DHFR* methylation levels. Figure 2b shows the quality of extractive DNA assay and bisulfite conversion. Significantly higher body mass index (BMI), plasma Glu levels, SBP, and DBP were observed in hypertensive patients than in healthy controls (all $P < 0.001$), whereas the LDL levels were lower in hypertensive patients than in healthy controls ($P < 0.001$). The *DHFR* methylation levels (median [interquartile range]) were significantly higher in hypertensive patients (34.32% [11.34%–119.60%]) than in healthy controls (18.45% [8.16%–35.40%]) ($P < 0.001$). The Hcy levels were also higher in hypertensive patients than in healthy controls (16.49 ± 11.40 μmol/L vs. 14.34 ± 5.97 μmol/L, $P = 0.001$).

**Table 1: Characteristics of cases and controls**

| | Healthy controls | Hypertensive cases | t/$\chi^2$ | *P* |
|---|---|---|---|---|
| Age (years) | 65.39±9.14 | 66.39±9.27 | −1.45 | 0.148 |
| Gender (men/women) | 165/155 | 205/166 | 0.94 | 0.332 |
| BMI (kg/m$^2$) | 23.51±3.09 | 24.36±2.95 | −3.77 | <0.001 |
| WHR | 0.91±0.10 | 0.91±0.10 | −1.25 | 0.212 |
| SBP (mmHg) | 123.90±13.83 | 134.00±16.26 | −8.91 | <0.001 |
| DBP (mmHg) | 77.38±8.50 | 82.45±10.93 | −6.87 | <0.001 |
| Hcy (μmol/L) | 14.34±5.97 | 16.49±11.40 | −3.11 | 0.001 |
| UA (μmol/L) | 360.48±90.67 | 357.39±95.75 | 0.44 | 0.658 |
| TG (mmol/L) | 1.83±1.47 | 1.90±1.50 | −0.59 | 0.556 |
| TC (mmol/L) | 5.11±0.98 | 5.12±0.99 | −0.13 | 0.893 |
| LDL (mmol/L) | 3.19±0.76 | 2.98±0.75 | 3.67 | <0.001 |
| Glu (mmol/L) | 5.05±1.28 | 5.65±1.28 | −6.33 | <0.001 |
| Smoking (no/yes) | 273/47 | 332/39 | 2.75 | 0.097 |
| Drinking (no/yes) | 206/114 | 234/137 | 0.13 | 0.730 |
| PMR-DHFR (%) | 18.45 (8.16–35.40) | 34.32 (11.34–119.60) | −6.03 | <0.001 |

WHR=Waist–hip ratio; Hcy=Plasma homocysteine; UA=Uric acid; TG=Triglyceride; TC=Total cholesterol; LDL=Low-density lipoprotein; Glu=Blood glucose; BMI=Body mass index; SBP=Systolic blood pressure; DBP=Diastolic blood pressure; PMR=Percentage of methylated reference; DHFR=Dihydrofolate reductase


**Figure 2:** Methylation assay of dihydrofolate reductase gene and its quality control.[7] (a) The target sequence is located on the CpG island of dihydrofolate reductase gene (location). F = Forward primer; R = Reverse primer. (b) The fragment length of MSP product is 131 bp

### Association between promoter methylation of dihydrofolate reductase and hypertension and baseline characteristics

As shown in Table 2, the adjusted risk of *DHFR* promoter hypermethylation was significantly higher in hypertensive patients than in healthy controls (odds ratio [OR] =3.94, 95% confidence interval [CI] =2.56–6.02). Men showed a higher risk of hypermethylation than women (OR = 1.78, 95% CI = 1.36–2.33). Individuals with Hcy levels >15 mmol/L and alcohol drinkers also exhibited a higher risk of hypermethylation (OR = 1.27, 95% CI = 1.00–1.60 and OR = 1.66, 95% CI = 1.04–2.64, respectively). However, the risk of *DHFR* promoter methylation was not associated with age, BMI, and LDL levels [Table 2].

### Diagnostic value of dihydrofolate reductase methylation in hypertension

AUC values were used to indicate the diagnostic value of *DHFR* promoter methylation levels for hypertension. The AUC value was 0.628 (0.585–0.668) in total, 0.688 (0.625–0.737) in men, and 0.545 (0.488–0.613) in women [Figure 3].

## DISCUSSION

Our matched case–control study revealed that *DHFR* promoter hypermethylation increased the risk of hypertension. *DHFR* enzyme plays an important role in Hcy metabolism. Hyperhomocysteinemia is a well-known risk factor for hypertension.[11] In a previous study, hyperhomocysteinemia was found to increase the risk of hypertension by approximately 36%.[16] A high level of *DHFR* promoter methylation may reduce the expression of DHFR enzyme,[7] thereby contributing to the decrease in 5-MTHF levels. As a methyl donor of Hcy metabolism, the decrease in 5-MTHF levels leads to significant accumulation of Hcy, further increasing the risk of hypertension. In the present study, after adjusted age, gender, BMI, LDL, and drinking status, we also found that *DHFR* promoter hypermethylation associated with high Hcy levels (OR = 1.27), suggesting that *DHFR* promoter hypermethylation results in hypertension by increasing the Hcy levels in Chinese.

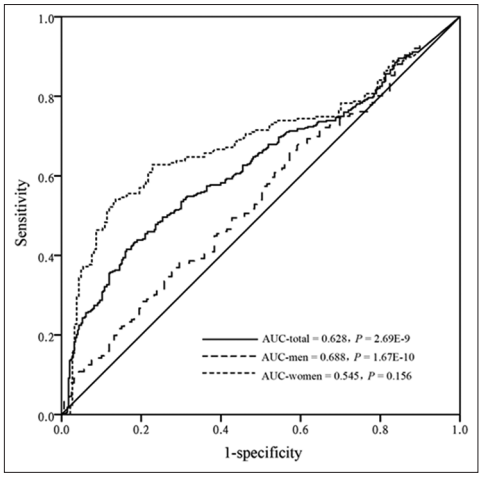


**Figure 3:** Receiver operation characteristics curve of dihydrofolate reductase methylation in hypertension

**Table 2: Association between promoter methylation of dihydrofolate reductase and hypertension and baseline characteristics**

| | *n* | Hypomethylation | Hypermethylation | OR (95% CI) | *P* |
|---|---|---|---|---|---|
| Case | 371 | 214 | 157 | 3.94 (2.56–6.02)* | <0.001 |
| Control | 320 | 269 | 51 | | |
| Gender | | | | | |
| Men | 205 | 97 | 108 | 1.78 (1.36–2.33) | <0.001 |
| Women | 166 | 117 | 49 | | |
| Age (years) | | | | | |
| >65 | 232 | 129 | 103 | 1.26 (0.82–1.93) | 0.295 |
| ≤65 | 139 | 85 | 54 | | |
| BMI (kg/m$^2$) | | | | | |
| >24 | 203 | 115 | 88 | 1.10 (0.73–1.66) | 0.658 |
| ≤24 | 168 | 99 | 69 | | |
| Hcy (mmol/L) | | | | | |
| >15 | 166 | 86 | 80 | 1.27 (1.00–1.60) | 0.049 |
| ≤15 | 205 | 127 | 78 | | |
| LDL (mmol/L) | | | | | |
| >3.12 | 165 | 101 | 64 | 0.77 (0.51–1.17) | 0.218 |
| ≤3.12 | 206 | 113 | 93 | | |
| Drinking | | | | | |
| Yes | 97 | 47 | 50 | 1.66 (1.04–2.64) | 0.032 |
| No | 274 | 167 | 107 | | |

*Adjusted for age, gender, BMI, Hcy, LDL, drinking. The methylation level of DHFR≥51.08% was considered as hypermethylation and<51.08% was considered as hypomethylation. BMI=Body mass index; Hcy=Plasma homocysteine; LDL=Low-density lipoprotein; DHFR=Dihydrofolate reductase; OR=Odds ratio; CI=Confidence interval

We observed that men are more likely to have higher *DHFR* methylation levels and the diagnostic value is higher in men. A possible explanation for this phenomenon is the effects of sex hormones.[17] The DNA methylation level is influenced by sex hormones. Estrogen has been found to increase the *DHFR* gene expression in methotrexate-resistant human breast cancer cells.[18] Blood pressure is also affected by sex hormones. For example, estrogen reduces blood pressure by binding to estrogen receptor alpha expressed by the medial amygdala neurons,[19] and testosterone elevates blood pressure through the renin–angiotensin–aldosterone system.[20,21] Thus, we speculate that the presence of sex hormones interferes with the effect of *DHFR* promoter methylation on blood pressure, but this needs to be evaluated in further studies.

In one study, alcohol consumption was found to increase the level of global DNA methylation.[22] Consistent with that study, we also found that alcohol consumption increased the risk of hypermethylation, thus contributing to hypertension. Alcohol can be viewed as a drug or a potentially poisonous solvent, in which case, the relationship of hypermethylation and alcohol consumption could be explained as the gradated cellular responses to an exogenous toxin.[23]

To the best of our knowledge, this was the first study to evaluate the association between *DHFR* promoter methylation and hypertension in a matched case–control study. However, the following limitations should be noted. First, the participants included in our study were limited to the residents of Shenzhen, which may limit the generalizability of our findings. Second, several genes, such as MTHFR, MS, and MTRR,[24] are involved in Hcy metabolism but were not investigated in this study; thus, methylation of other genes needs to be investigated in future studies. Third, we did not investigate other mechanisms of epigenetic regulation, such as histone modification and miRNA action, which also contribute to gene expression; thus, further studies are warranted to explore other mechanisms of epigenetic regulation in relation to hypertension. At last, antihypertensive drugs could inevitably affect the level of gene methylation expression.

## CONCLUSION

Our matched case–control study suggested that hypermethylation of *DHFR* promoter increased the risk of hypertension, especially in males. The *DHFR* promoter methylation may serve as an indicator for the diagnosis of hypertension. To prevent hypertension, more attention needs to be paid to changes in the methylation levels.

### Acknowledgment

This work was supported by the National Key R and D Program of China (Grant No. 2017YFC1310902, 2018YFC1315305), the Ningbo Health Branding Subject Fund (Grant No. PPXK2018-02), and the Sanming Project of Medicine in Shenzhen (Grant No. SZSM201803080).

### Financial support and sponsorship

This work was supported by the National Key R and D Program of China (Grant No. 2017YFC1310902, 2018YFC1315305), the Ningbo Health Branding Subject Fund (Grant No. PPXK2018-02), the Sanming Project of Medicine in Shenzhen (Grant No. SZSM201803080). Major Program of Social Development of Ningbo Science and Technology Bureau (Grant No. 2019C50094), and Natural Science Foundation of Ningbo Municipality, China (Grant No. 2016A610169).

### Conflicts of interest

There are no conflicts of interest.

## REFERENCES

1. Mills KT, Bundy JD, Kelly TN, Reed JE, Kearney PM, Reynolds K, *et al*. Global disparities of hypertension prevalence and control: A systematic analysis of population-based studies from 90 countries. Circulation 2016;134:441-50.
2. Wang Z, Chen Z, Zhang L, Wang X, Hao G, Zhang Z, *et al*. Status of hypertension in China: Results from the China Hypertension Survey, 2012-2015. Circulation 2018;137:2344-56.
3. Zhang X, Zhao H, Zhang J, Han D, Zheng Y, Guo X, *et al*. Gene environment interaction of GALNT2 and APOE gene with hypertension in the Chinese Han population. Biomed Mater Eng 2015;26 Suppl 1:S1977-83.
4. Egan BM. Collectrin, an X-linked, angiotensin converting enzyme 2 homolog, causes hypertension in a rat strain through gene-gene and gene-environment interactions: Relevance to human hypertension. Circulation 2013;128:1727-8.
5. Levy E, Spahis S, Bigras JL, Delvin E, Borys JM. The epigenetic machinery in vascular dysfunction and hypertension. Curr Hypertens Rep 2017;19:52.
6. Ziller MJ, Gu H, Müller F, Donaghey J, Tsai LT, Kohlbacher O, *et al*. Charting a dynamic DNA methylation landscape of the human genome. Nature 2013;500:477-81.
7. Hu J, Zhu H, Xu G, Chen Z, Li L, Wang S, *et al*. Significant association between DHFR promoter methylation and ischemic stroke in a Chinese hypertensive population. J Clin Lab Anal. 2020;00:e23322.
8. Zhang LN, Liu PP, Wang L, Yuan F, Xu L, Xin Y, *et al*. Lower ADD1 gene promoter DNA methylation increases the risk of essential hypertension. PLoS One 2013;8:e63455.
9. Fan R, Wang WJ, Zhong QL, Duan SW, Xu XT, Hao LM, *et al*. Aberrant methylation of the GCK gene body is associated with the risk of essential hypertension. Mol Med Rep 2015;12:2390-4.
10. Xu G, Wang C, Ying X, Kong F, Ji H, Zhao J, *et al*. Serine hydroxymethyltransferase 1 promoter hypermethylation increases the risk of essential hypertension. J Clin Lab Anal 2019;33:e22712.
11. Sen U, Mishra PK, Tyagi N, Tyagi SC. Homocysteine to hydrogen sulfide or hypertension. Cell Biochem Biophys 2010;57:49-58.
12. Frye RE, Slattery JC, Quadros EV. Folate metabolism abnormalities in autism: Potential biomarkers. Biomark Med 2017;11:687-99.

13. Lin Z, Li Q, Sun Y, Huang J, Wang W, Fu J, *et al*. Interactions between genetic variants involved in the folate metabolic pathway and serum lipid, homocysteine levels on the risk of recurrent spontaneous abortion. Lipids Health Dis 2019;18:143.
14. Li B, Chen X, Jiang Y, Yang Y, Zhong J, Zhou C, *et al*. CCL2 promoter hypomethylation is associated with gout risk in Chinese Han male population. Immunol Lett 2017;190:15-9.
15. Wang C, Xu G, Wen Q, Peng X, Chen H, Zhang J, *et al*. Significant association of methylenetetrahydrofolate dehydrogenase 1 promoter hypomethylation with stroke in a Chinese population with primary hypertension. Ann Clin Lab Sci 2019;49:112-8.
16. Zhong F, Zhuang L, Wang Y, Ma Y. Homocysteine levels and risk of essential hypertension: A meta-analysis of published epidemiological studies. Clin Exp Hypertens 2017;39:160-7.
17. Sebag IA, Gillis MA, Calderone A, Kasneci A, Meilleur M, Haddad R, *et al*. Sex hormone control of left ventricular structure/function: Mechanistic insights using echocardiography, expression, and DNA methylation analyses in adult mice. Am J Physiol Heart Circ Physiol 2011;301:H1706-15.
18. Teo GY, Rasedee A, Al-Haj NA, Beh CY, How CW, Rahman HS, *et al*. Effect of fetal bovine serum on erythropoietin receptor expression and viability of breast cancer cells. Saudi J Biol Sci 2020;27:653-8.
19. Hinton AO Jr., He Y, Xia Y, Xu P, Yang Y, Saito K, *et al*. Estrogen receptor-α in the medial amygdala prevents stress-induced elevations in blood pressure in females. Hypertension 2016;67:1321-30.
20. Basaria S, Coviello AD, Travison TG, Storer TW, Farwell WR, Jette AM, *et al*. Adverse events associated with testosterone administration. N Engl J Med 2010;363:109-22.
21. Maric-Bilkan C, Manigrasso MB. Sex differences in hypertension: Contribution of the renin-angiotensin system. Gend Med 2012;9:287-91.
22. Semmler A, Heese P, Stoffel-Wagner B, Muschler M, Heberlein A, Bigler L, *et al*. Alcohol abuse and cigarette smoking are associated with global DNA hypermethylation: Results from the German Investigation on Neurobiology in Alcoholism (GINA). Alcohol 2015;49:97-101.
23. Philibert RA, Plume JM, Gibbons FX, Brody GH, Beach SR. The impact of recent alcohol use on genome wide DNA methylation signatures. Front Genet 2012;3:54.
24. Lima A, Bernardes M, Azevedo R, Seabra V, Medeiros R. Moving toward personalized medicine in rheumatoid arthritis: SNPs in methotrexate intracellular pathways are associated with methotrexate therapeutic outcome. Pharmacogenomics 2016;17:1649-74.