The lower methylation of RANK is associated with osteoporosis in male aged general population of Xinjiang: A Case-Control Study

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Abstract

Background

RANK is a candidate gene for osteoporosis on both functional and genetic grounds. The study is to investigate the relationships between the methylation of *RANK* and osteoporosis in aged general population.

Methods

On the basis of an epidemiological investigation, we detect for methylation CpGs in promoter of *RANK* in 32 aged subjects (16 males and 16 females) firstly. Secondly, after considering the relationships among osteoporosis and the methylation rate of identified CpGs in male and female subjects, the selected representatives CpGs were detected in 90 male aged general subjects (43 controls and 47 cases) by bisulfite sequencing. Then a case-control study is conducted.

Results

Age and the prevalence of diabetes were significantly difference between the case patients and control individuals ($P = 0.025$, $P = 0.005$)., There was no statistical significance between the case group and the control group for the following values: systolic blood pressure, diastolic blood pressure, body mass index, 25-dihydroxyvitaminD3, folic acid, testosterone, creatinine, serum calcium concentration, and the prevalence of smoking, drinking and hypertension ($P > 0.05$). The methylation rate of *RANK* in control group was significant higher than that in osteoporosis group ($P < 0.001$). In addition, by covariance analysis to adjust age, prevalence of smoking, drinking, hypertension, and diabetes, the methylation rate of *RANK* in control group was significant higher than that in osteoporosis group in male aged general population of Xinjiang ($P = 0.001$). After adjusting for confounding factors (age, smoking, drinking, and diabetes), multivariate logistic regression analysis also showed that lower methylation of *RANK* gene were significantly associated with osteoporosis (OR = 0.930, 95% CI = 0.886–0.976)

Conclusions

The lower methylation rate of *RANK* was associated with osteoporosis in male aged general population of Xinjiang. This confirms that lower methylation of *RANK* might be involved in the pathogenesis of osteoporosis

Background

Osteoporosis is a kind of bone disease with systemic metabolic disorder, which characterized by bone mass reduction, bone tissue structure degradation, brittleness increase and fracture prone. It affects one

in three women and one in eight men over the age of 50 years all over the world ^[1]. In China, epidemiological survey showed that the prevalence of osteoporosis was 19.2% (6.0% in men, 31.2% in women, 16.2% in urban areas and 20.7% in rural areas) in people over 50 years old; and was 32.0% (10.7% in men, 51.6% in women, 25.6% in urban areas and 35.3% in rural areas) in people over 65 years old ^[2]. Osteoporosis has become a serious health problem for the elderly ^[3]. The risk of fracture in elderly patients with osteoporosis is significantly increased, and fracture can occur under slight violence such as fall and slight impact. Osteoporosis not only makes patients suffer from physical pain, but also causes economic burden ^[4]. The total treatment cost for osteoporotic fractures in China was \$9.45 billion in 2010, and it is expected to increase to \$25.4 billion in 2050^[5].

The pathogenesis of osteoporosis is still unclear. Many factors may increase the risk of osteoporosis, such as sex, age, diet, exercise intensity and drug use ^[6]. Moreover, many studies have shown that epigenetic modification is an important regulatory mechanism of gene expression, including deoxyribonucleic acid (DNA) methylation, histone modification and non-coding ribonucleic acid (RNA) ^[7, 8].

DNA methylation, the most common form of epigenetic modification, is an important mechanism for regulating genomic function ^[9]. DNA methylation is mainly the process of adding methyl to DNA molecules. DNA methylation can change chromatin structure, DNA conformation, DNA stability and the interaction between DNA and protein, thus controlling gene expression. Methylation in eukaryotes usually occurs on cytosine-rich CpG islands in the gene promoter region. The CpG islands that gather cytosine and guanine dinucleotides, are important regions for the formation and regulation of methylation in the human genome. CpG islands are probably related to 60% of human gene promoters ^[10]. DNA methylation plays an important role in the pathogenesis of geriatric diseases.

The signaling pathway, receptor activator of NF- κ B ligand (RANKL)/ receptor activator of NF- κ B (RANK)/ osteoprotegerin (OPG), has been confirmed to be the main regulator of osteoporosis. It has been confirmed that variations of *RANK* were related to the pathogenesis of osteoporosis ^[11]. Wang et al demonstrated that DNA methylation influenced the transcriptional expression of OPG and RANKL, which probably take on a "main switch" role in pathogenesis of primary osteoporosis ^[12]. Baicalin, a well-known traditional Chinese medicine, can improve osteoporosis by regulation of the RANK/RANKL/OPG signaling pathway ^[13]. Nevertheless, there is no consensus whether the methylation of *RANK* plays a role in the pathogenesis of osteoporosis. Thus, this study aimed to investigate the relationships between the methylation of *RANK* and osteoporosis in aged general population.

Methods

Study population

The study was approved by the Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region (Xinjiang, China). Written informed consent was obtained from all subjects before data collection

and measurements.

This is a case-control study based on a cross-sectional epidemiological survey of general populations. A three-stage stratified sampling method was used to select representative samples of subjects from the Xinjiang Uygur Autonomous Region of China. In the first stage, Xijiang was stratified into north and south, as delineated by the Tianshan Mountain. One county was randomly selected from each region: Mulei County in the northern region and Luofu County in the southern region. In the second stage, eight townships were selected randomly from each county. In the third stage, individual participants (who were aged >65 years) were selected for inclusion in all selected areas.

A set of standardized questionnaires ^[14] was completed. The questions were based on: demographics; self-reported history of stroke, myocardial infarction, and congestive heart failure; previous diagnosis and treatment of hypertension, obesity, high blood levels of cholesterol, diabetes and other diseases; family history of hypertension, diabetes and stroke; drug treatment, obesity, or being overweight; education; diet; exercise; alcohol consumption; and cigarette smoking. Data collection was undertaken by trained and certified physicians. A total of 3122 individuals completed the survey and examination.

Subjects

Inclusion criteria: Those subjects signed informed consent with age ≥ 65 years old.

Exclusion criteria: subjects with: 1) sequelae of cerebrovascular disease; 2) edocrine and metabolic diseases or hereditary bone diseases, such as thyroid, parathyroid, gonadal, pituitary diseases, type 1 diabetes, osteomalacia, etc; 3) chronic kidney disease, chronic liver disease; 4) treated with anticancer drugs, glucocorticoids, immunosuppressive agents and anticonvulsant drugs for more than 6 months; 5) systemic lupus erythematosus, rheumatoid arthritis and other rheumatic immune diseases.

Finally, 32 aged subjects (16 males and 16 females) were randomly recruited to detect for methylation CpGs in promoter of *RANK*, and 90 aged male were randomly recruited for this study (because no significant representative CpGs were found in women).

Diagnostic criteria and measurements

For each participant, blood-pressure measurements were obtained by a standardized protocol adapted from procedures recommended by the American Heart Association. Body weight and height were measured by trained observers following a standard protocol. Hypertension was defined as a mean systolic blood pressure ≥ 140 mmHg and/or a mean diastolic blood pressure ≥ 90 mmHg and/or self-reported current treatment for hypertension with antihypertensive medication. Diabetes was defined as having a fasting plasma glucose level ≥ 7.0 mmol/L and/or self-reported current treatment of diabetes. The body mass index (BMI) was calculated using the following equation: $BMI = \text{body weight (kg)} / [\text{height (m)}]^2$.

Venous blood was drawn from all participants who had fasted for ≥ 12 h. Serum was separated immediately and stored at -80°C . All blood samples were examined within one month in the Clinical Center of People's Hospital of Xinjiang Uygur Autonomous Region (Grade 3A Hospital) by special personnel. Serum levels of total cholesterol, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, triglycerides, fasting blood glucose, 25-dihydroxyvitaminD3, folic acid, testosterone, creatinine, and serum calcium were obtained by enzymatic methods.

In light of a common protocol recommended by the ACR Appropriateness Criteria Osteoporosis and Bone mineral Density (2017), the bone mineral density (BMD) measurement was performed by trained and certified observers with portable bone mineral density analyzer (Osteosys EXA-3000, Korea). The bone mineral density of forearm ulna and radius were measured by dual energy X-ray absorptiometry method [15].

The BMD lower than that of healthy adults of the same sex is equal to or greater than 2.5 standard deviations (SD), which is the diagnostic criterion for osteoporosis [16]. Among the selected objects, there were 47 cases of osteoporosis (case group) and 43 cases in control group.

Detecting for methylation CpGs in promoter of *RANK*

Firstly, we detected all CpGs islands in the promoter region of *RANK* by bisulfite sequencing. We sequenced all promoter region of *RANK*. Blood samples were obtained from 16 subjects with osteoporosis (8 males and 8 females) and 16 subjects without osteoporosis (8 males and 8 females), which were randomly chosen from the study population, and genomic DNA was isolated from peripheral blood leukocytes using a PAXgene Blood DNA kit (PreAnalytiX).

The genomic DNA was modified by bisulfite sodium by EpiTect Fast Bisulfite Conversion Kits (QIAGEN, Germany). Then the modified DNA was repeatedly eluted, purified, and stored at -20°C . The qualified genomic DNA samples were obtained for polymerase chain reaction (PCR) amplification.

The primers were designed by methyl primer express v1.0. The primer with upstream primer 5'-GGTGCCTTTCTAGAATTTGGTGG-3', and downstream primer 5'-CCAACCCTAAATTACCCTTCAC-3', the length of PCR product was 376bp, with 20 CpG sites. Then the PCR was carried out and the amplified products were recovered. The products of PCR amplification were sent to SHANGHAI BIOLOGICAL ENGINEERING CO., LTD (China, Shanghai) for sequencing. The standard and sequencing sequences of *RANK* genome were compared by BiQ Analyzer analysis software.

Detecting of representative CpGs in male aged general population

Secondly, after considering the relationships among osteoporosis and the methylation rate of identified CpGs in male and female subjects (Table 1), the selected representatives CpGs were detected in 90 male aged general subjects (43 controls and 47 cases) using the method of bisulfite sequencing (the research

method and process are the same as above). All of the 20 selected representative CpGs were successfully detected in 90 subjects participating in the study.

Statistical analysis

Data analyses were performed by SPSS for Windows (version 17.0; SPSS Inc., Chicago, IL, USA). Values are expressed as the means $\pm$ SD or median (quartile). The counting data are showed by percentage. The distribution of patient characteristics between the case and control groups was analyzed using a Student's *t*-test or a chi-square test. The differences in distributions of methylation rate between case and control individuals were analyzed using a Student's *t*-test or Kruskal-Wallis test. In addition, Covariate variance analysis was performed to compare the methylation rate between case and control individuals adjusting for age, smoking, drinking, blood lipids, body mass index and hypertension, diabetes prevalence. Finally, logistic regression analysis was performed to assess the contribution of the major risk factors for osteoporosis (including smoking, drinking, age, diabetes and methylation rate of *RANK*). Statistical significance was established at *P* values < 0.05.

Results

Screening for CpGs variations in *RANK* in general population

Twenty CpGs islands in the promoter region of *RANK* were successfully detected in 32 individuals. In male, among which there were 4 CpGs with higher methylation in control group than that in case group (CpG7, *P*=0.001; CpG9, *P*=0.009; CpG11, *P*=0.001; CpG14, *P*=0.044). For 20 CpGs, there was no statistical significance between case group and control group in female (Table 1).

Next, we looked for an association between methylation in *RANK* and osteoporosis in the male aged general population.

Clinical characteristics of subjects

Table 2 presents the clinical characteristics of the study participants. The age and the prevalence of diabetes were significantly difference between the case patients and control individuals (*P*=0.025, *P*=0.005)., There was no statistical significance between the case group and the control group for the following data: systolic blood pressure, diastolic blood pressure, body mass index, 25-dihydroxyvitaminD3, folic acid, testosterone, creatinine, serum calcium concentration, and the prevalence of smoking, drinking and hypertension (*P*>0.05).

Relationship between osteoporosis and methylation of *RANK* in male aged general population of Xinjiang

Table 3 presents the relationship between osteoporosis and methylation of *RANK* in male aged general population of Xinjiang. The methylation rate of *RANK* in control group was significant higher than that in osteoporosis group (*P*<0.001). There were 6 CPGs with higher methylation in control group than that in

case group (CpG6, $P=0.009$; CpG7, $P<0.001$; CpG8, $P=0.004$; CpG9, $P<0.001$; CpG11, $P<0.001$; CpG19, $P=0.007$).

In addition, by covariance analysis to adjust age, prevalence of smoking, drinking, hypertension, and diabetes, the methylation rate of *RANK* in control group was significant higher than that in osteoporosis group in male aged general population of Xinjiang ($P=0.001$)(Table 4).

Odds rations and 95% confidence interval for methylation of *RANK* associated with osteoporosis

After adjusting for confounding factors (age, smoking, drinking, and diabetes), multivariate logistic regression analysis also showed that lower methylation of *RNAK* gene were significantly associated with osteoporosis (OR=0.930, 95% CI = 0.886-0.976; Table 5).

Discussion

In this study, by systemically screening CpGs of *RANK*, we investigated the associations of the methylation rate of *RANK* with osteoporosis. We first report that lower methylation rate of *RANK* was significantly associated with osteoporosis in male aged general population. There were 6 CPGs with lower methylation in case group than that in control group (CpG6, $P=0.009$; CpG7, $P<0.001$; CpG8, $P=0.004$; CpG9, $P<0.001$; CpG11, $P<0.001$; CpG19, $P=0.007$).

With the aging of the population, osteoporosis has become a major chronic disease that endangers the health of the elderly ^[1]. The study of the etiology of osteoporosis has been a hot spot, in which, genetic factors play the most important role ^[11]. Gene methylation is considered an important component of epigenetic. It is involved in the regulation of the expression of osteoporosis related genes, and is associated with the pathogenesis of osteoporosis ^[7]. The occurrence of osteoporosis is closely related to bone loss caused by bone reconstruction imbalance. A great deal of data has been reported that methylation of some genes can affect the differentiation and cell activity of osteoblasts and osteoclasts, and affect the occurrence and development of osteoporosis^[17-21].

Determination of whole-cell gene methylation of bone cells in femoral head region of patients with osteoporotic hip fracture and hip osteoarthritis has been reported by Delgado-Calle et al. It was found that there was significant difference in methylation level of 241 CpG loci and 228 genes between the two groups. This indirectly suggests that osteoporosis may be associated with changes in methylation levels of certain specific genes ^[20]. The whole cell RNA sequencing and DNA methylation level of mesenchymal stem cells in young and old women were evaluated by Roforth et al. The results showed that the promoter had different expression of 1528 sequences, and the methylation level of some genes was different. This suggests that age-related decline in bone formation may be associated with the level of methylation of mesenchymal stem cells, which to some extent suggests that osteoporosis due to reduced bone formation may be associated with the methylation level of some genes^[21].

RANK-RANKL-OPG signaling pathway plays an important role in the differentiation and activation of osteoclast. It is an important pathway for the interaction between osteoblasts and osteoclasts^[17–19]. RANK is an important regulatory factor in RANK-RANKL-OPG signaling pathway. The genome-wide association study confirmed the association between osteoporosis and variations in *RANK*^[11]. Therefore, the DNA methylation level of *RANK* may play an important role in the pathogenesis and progression of osteoporosis in the elderly. However, there is no study involved in the relationship between methylation of human *RANK* and osteoporosis. Therefore, we studied the relationship between *RANK* methylation and osteoporosis in aged general population. The results showed that the lower methylation of *RANK* was associated with osteoporosis in this population after adjusting for confounding factors (age, smoking, drinking, and diabetes). Moreover, we found 6 CPGs with lower methylation in case group than that in control group.

Xinjiang is located in the northwest of China. The residence living in Xinjiang pastoral areas is relatively fixed. The environmental factors affecting bone mass, such as living environment, eating habits, lifestyle and occupation, are more consistent^[22]. So it is an ideal population to study the genetic mechanism of complex diseases. Moreover, this study is based on an epidemiological investigation. All subjects were randomly recruited from it. Therefore, the results of this study are highly reliable.

Conclusions

In summary, the lower methylation of *RANK* was significantly associated with osteoporosis in male aged general population. There were 6 CPGs with lower methylation in case group than that in control group. This confirms that lower methylation of *RANK* might be involved in the pathogenesis of osteoporosis.

Abbreviations

ACR: American College of Radiology; BMD: Bone mineral density; BMI: body mass index; DNA: Deoxyribonucleic acid; OPG: osteoprotegerin; PCR: Polymerase chain reaction; RANK, Receptor activator of NF- κ B; RANKL: Receptor activator of NF- κ B ligand; RNA: Ribonucleic acid; SD: standard deviations.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the “People's Hospital of Xinjiang Uygur Autonomous Region (KJ-001-2016011)”. Written informed consent was obtained from all patients prospectively included.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors have no competing interests.

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Authors' contributions

HMW contributed to the study design, data collection, analysis, interpretation of the results and made a critical revision of the manuscript. JJS and HX wrote the article. XB and BH were involved in the analysis and interpretation of the results and made a critical revision of the manuscript. All the authors read and approved the submitted manuscript.

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Tables

Table 1 Screening for CpGs islands in RANK in aged general population						
Methylation rate (%)	Female			Male		
	control group	case group	<i>P</i>	Control group	Case group	<i>P</i>
	(n=8)	(n=8)		(n=8)	(n=8)	
CpG1	0.33±0.191	0.21±0.125	0.184	0.25±0.12	0.28±0.116	0.678
CpG2	0.68±0.26	0.61±0.21	0.606	0.54±0.239	0.55±0.12	0.897
CpG3	0.43±0.198	0.38±0.139	0.568	0.39±0.173	0.33±0.139	0.438
CpG4	0.48±0.198	0.50±0.001	0.727	0.49±0.099	0.43±0.089	0.205
CpG5	0.10(0.00-0.10)	0.05(0.00-0.10)	0.642	0.10(0.10-0.25)	0.10(0.03-0.18)	0.429
CpG6	0.10(0.03-0.20)	0.10(0.03-0.10)	0.513	0.20(0.01-0.28)	0.15(0.03-0.28)	0.662
CpG7	0.05(0.00-0.10)	0.10(0.00-0.18)	0.429	0.15(0.10-0.28)	0.00(0.00-0.00)	0.001*
CpG8	0.00(0.00-0.00)	0.00(0.00-0.00)	0.334	0.00(0.00-0.18)	0.00(0.00-0.00)	0.074
CpG9	0.10(0.00-0.10)	0.10(0.00-0.10)	0.693	0.20(0.03-0.28)	0.00(0.00-0.00)	0.002*
CpG10	0.00(0.00-0.10)	0.10(0.00-0.25)	0.245	0.20(0.00-0.28)	0.05(0.00-0.10)	0.065
CpG11	0.10(0.00-0.18)	0.20(0.10-0.20)	0.120	0.20(0.10-0.28)	0.00(0.00-0.08)	0.001*
CpG12	0.00(0.00-0.00)	0.00(0.00-0.08)	0.149	0.00(0.00-0.08)	0.00(0.00-0.00)	0.149
CpG13	0.00(0.00-0.08)	0.05(0.00-0.10)	0.334	0.00(0.00-0.15)	0.00(0.00-0.08)	0.506
CpG14	0.05(0.00-0.18)	0.05(0.00-0.18)	1.000	0.00(0.00-0.08)	0.10(0.03-0.10)	0.049*
CpG15	0.00(0.00-0.08)	0.00(0.00-0.00)	0.149	0.05(0.00-0.10)	0.05(0.00-0.10)	1.000
CpG16	0.00(0.00-0.10)	0.00(0.00-0.08)	0.619	0.15(0.00-0.28)	0.05(0.00-0.25)	0.575
CpG17	0.00(0.00-0.00)	0.00(0.00-0.08)	0.554	0.00(0.00-0.00)	0.00(0.00-0.00)	0.334
CpG18	0.00(0.00-0.00)	0.00(0.00-0.00)	0.334	0.00(0.00-0.00)	0.00(0.00-0.00)	0.334

CpG19	0.00(0.00-0.10)	0.00(0.00-0.08)	0.619	0.10(0.00-0.18)	0.05(0.00-0.10)	0.303
CpG20	0.05(0.00-0.10)	0.05(0.00-0.10)	1.000	0.00(0.00-0.08)	0.00(0.00-0.08)	1.000
*The methylation rate of <i>RANK</i> in control group was significant higher than that in case group						

Table 2 The clinical characteristics of the study participants				
	control group	case group	t/χ^2	P
	n=43)	n=47)		
Age	68.53±9.143	72.32±6.484	2.246	0.025*
Systolic Blood Pressure	131.72±8.719	130.17±25.747	0.389	0.699
Diastolic Blood Pressure	81.30±14.025	78.72±10.498	0.980	0.330
Body Mass Index	25.33±3.838	24.43±3.672	1.127	0.263
25-dihydroxyvitaminD3	15.63±9.876	11.41±8.179	1.785	0.078
Folic Acid	12.91±6.088	14.51±5.696	1.088	0.281
Testosterone	3.83±1.335	4.14±1.107	0.928	0.358
Creatinine	76.68±18.456	76.44±18.454	0.061	0.951
Serum Calcium Concentration	2.30±0.136	2.31±0.177	0.182	0.856
Smoking☐No/Yes☐	19/24	17/30	0.601	0.438
Drinking☐No/ Yes☐	20/23	16/31	1.455	0.228
Hypertension☐No/Yes☐	15/28	19/28	0.293	0.588
Diabetes☐No/Yes☐	31/12	12/27	7.980	0.005*
*Statistical significance.				

Table 3 Relationship between osteoporosis and methylation of RANK in male aged general population of Xinjiang				
Methylation rate (%)	control group	case group	<i>t</i> (<i>Z</i>)	<i>P</i>
	(n=43)	(n=47)		
total	51.74±14.593	41.17±9.042	4.088	<0.001*
CpG1	0.26±0.169	0.21±0.127	1.536	0.128
CpG2	0.61±0.269	0.58±0.162	0.655	0.514
CpG3	0.38±0.172	0.38±0.160	0.015	0.988
CpG4	0.47±0.152	0.47±0.151	0.023	0.982
CpG5	0.10(0.000.10)	0.10(0.000.10)	1.695	0.090
CpG6	0.10(0.000.20)	0.10(0.000.10)	2.752	0.006*
CpG7	0.00(0.000.10)	0.00(0.000.00)	4.675	<0.001*
CpG8	0.00(0.000.10)	0.00(0.000.00)	2.844	0.004*
CpG9	0.00(0.000.10)	0.00(0.000.00)	4.040	<0.001*
CpG10	0.00(0.000.20)	0.10(0.000.10)	0.062	0.950
CpG11	0.20(0.100.30)	0.10(0.000.20)	3.720	<0.001*
CpG12	0.00(0.000.00)	0.00(0.000.00)	0.519	0.604
CpG13	0.00(0.000.00)	0.00(0.000.10)	0.786	0.432
CpG14	0.00(0.000.10)	0.00(0.000.10)	0.503	0.615
CpG15	0.00(0.000.10)	0.00(0.000.09)	1.178	0.239
CpG16	0.00(0.000.10)	0.00(0.000.10)	1.269	0.204
CpG17	0.00(0.000.00)	0.00(0.000.00)	0.082	0.934
CpG18	0.00(0.000.00)	0.00(0.000.00)	0.953	0.340
CpG19	0.10(0.000.10)	0.00(0.000.10)	2.690	0.007*
CpG20	0.00(0.000.10)	0.00(0.000.10)	1.189	0.235
*The methylation rate of <i>RANK</i> in control group was significant higher than that in case group				

Table 4 Adjusted methylation rate of RANK between case and control group in male aged general population of Xinjiang					
	Adjusted Methylation rate [%]	Standard Error	95%CI	<i>F</i>	<i>P</i>
control group n=43	50.48	1.718	47.067-53.895	11.019	0.001
case group n=47	42.327	1.638	39.071-45.584		

Table 5 odds ratios and 95% confidence interval for methylation of RANK associated with osteoporosis in male after adjusting age, smoking, drinking and diabetes.			
	odds ratios	95% confidence interval	<i>P</i>
age(Year)	1.080	1.0151.149	0.015
smoking	1.141	0.2934.44	0.849
drinking	2.668	0.65610.852	0.170
Diabetes	1.908	0.5836.247	0.285
Methylation rate (%)	.930	0.8860.976	0.003