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An Observational Study on Aberrant Methylation of Runx3 With the Prognosis in Chronic Atrophic Gastritis Patients

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Abstract: The aim of this study is to discuss whether the methylation levels of Runx3 could be used as the early biomarker for predicting the prognosis in chronic atrophic gastritis (CAG) patients. A total of 200 subjects including 60 controls without CAG (Group 1), 70 patients with mild CAG (Group 2), and 70 patients with moderate and severe CAG (Group 3) were recruited for this cross-sectional investigation in the Department of Gastroenterology in Daqing Oilfield General Hospital from July 2013 to May 2014. The MlALDI-TOF-MS was used to measure the methylation levels of Runx3 in all of the subjects. Real-time quantitative reverse transcription polymerase chain reaction and western blotting were chosen to determine the expression levels of Runx3. The correlations between methylation levels of Runx3 among these CAG patients and their prognosis were shown by logistic regression models. The results demonstrated that the methylation levels of CpG13, CpG14, and CpG15 in Runx3 were higher in Group 3 than those in Groups 1 and 2 ($P < 0.05$), whereas the mRNA and protein expression levels of Runx3 were lower in Group 3 than those in Groups 1 and 2 ($P < 0.05$). There were significantly negative correlations between the methylation levels of Runx3 with its expression and the healing prognosis of CAG patients. In brief, this study proved that the hypermethylation modifications of CpG13, CpG14, and CpG15 in the promoter region of Runx3 could result in the down regulation of Runx3 expression to affect the prognosis of CAG. So the methylation levels of these CpG sites in Runx3 in the peripheral blood can be used as the biomarker for predicting the healing prognosis of CAG patients.

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Abbreviations: CAG = chronic atrophic gastritis, GC = gastric cancer, Hp = *Helicobacter pylori*, MALDI-TOF-MS = Laser Matrix Support Release/Ionization Time of Flight Mass Spectrometry, qRT-PCR = real-time quantitative PCR, WHO = World Health Organization.

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INTRODUCTION

Chronic atrophic gastritis (CAG) is one of the common diseases of digestive system. Its main pathological characteristic is the atrophy accompanied with the intestinal metaplasia (IM), dysplasia, and inflammation in the gastric glands especially the cardiac, fundus and pyloric glands.[1] There is no specific clinical manifestations among CAG patients; however, most patients have the clinical symptoms of pain, fullness, nausea, belching, constipation, and diarrhea in the epigastrium.[1,2] Recently, the incidence, prevalence, and mortality of CAG in China have been found to be growing because of the increasing infection rate of helicobacter pylori (Hp), aggravation of environmental pollution, and unhealthy lifestyles such as high-fat and high-salt diet, shortage of fruits, and vegetables intake.[3] Although the pathogenesis of CAG is still unclear, many research works have proved that long-term changes in gastric mucosa of CAG are one of the risk factors for gastric cancer (GC).[4] CAG has already been identified as the precancerous lesion of GC by the World Health Organization (WHO) in the year 1978. So far, a gastric mucosal biopsy combined with the pathological examination is still the most reliable diagnostic method for CAG, but this cannot be accepted by all of the patients because of the trauma caused by using the gastroscope.[2] Therefore, it is significant to find a new noninvasive biomarker in early stage of CAG, which is beneficial for screening and preventing the incidence of GC.

It has been already confirmed that the occurrence of many diseases, such as CAG, can be regulated by the gene–environment interaction, which demonstrated that the expression of many genes could be influenced by many environmental contaminants and diet through a variety of methods. The abnormal expression levels of the genes can cause physiological, pathological, and histological changes that lead to CAG.[5] Recently, many research works have proved that the expression levels of genes could be regulated by some elements such as the DNA methylation modifications.[6] As we all known, DNA methylation is an important component of epigenetic modification. It has many biological functions, for example, it can be involved in the regulation of gene expression, chromosome stability, DNA conformation, and DNA stability, so the changes in DNA methylation levels can lead to the occurrence of many diseases, even cancer.[6]

Runx3, an important member of Runx family, is located in the short arm of human first chromosome (1P36.1) with two large and high conservative CpG islands in the total 67 KB length.[7] As a new tumor suppressor gene, Runx3 has many roles in the signal transcription, the regulation of cells proliferation, and differentiation.[8,9] The highly conserved region of CpG island can play an important role in regulating the transcription and expression of Runx3. Many references had proved that the hypermethylation of Runx3 was related to the occurrence and

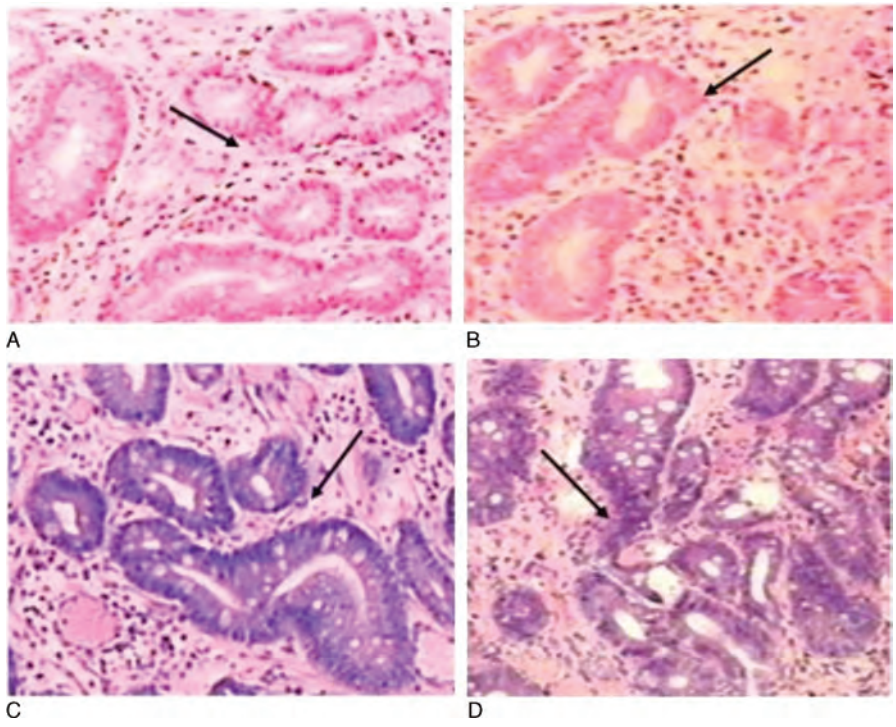


FIGURE 1. The pathological symptoms of gastric mucosa in CAG patients. (A) and (B) indicated the changes of gastric mucosa of mild CAG. As shown by the arrows, the glands in the antrum of the stomach were in the focal atrophy, but the sizes of these glands were not changed. (C) and (D) demonstrated the changes of moderate and severe CAG. As shown by the arrows, most of the glands in the antrum of the stomach were in the focal atrophy or even disappeared, and they were replaced by the metaplasia glands. CAG: chronic atrophic gastritis.

development of some cancer such as the GC, breast cancer, bladder cancer, etc,[10] and the methylation levels of Runx3 also could be used as the biomarker for many diseases. Li et al had proved that the methylation levels of Runx3 could play the roles in the occurrence and development from CAG to the GC,[11,12] but there were few studies on discussing the location of CpG sites of Runx3 in which the methylation levels had changed. So the objectives of this research were to investigate the methylation levels of different CpG sites in Runx3 of CAG patients and analyze the correlations between the methylation levels of these CpG sites and the prognosis of CAG patients to discuss whether the methylation levels of these CpG sites in Runx3 can be used as the early biomarkers for predicting carcinogenesis and providing the theoretical basis for the new therapeutic of CAG.

MATERIALS AND METHODS

Study Design and Population

This cross-sectional survey was conducted in Department of Gastroenterology in Daqing Oilfield General Hospital from July 2013 to May 2014. The outpatients who had the clinical symptoms of CAG were chosen as the subjects. A total of 381 subjects were requested to sign informed consent, complete the questionnaires, and examine the situation of gastric mucosa by the gastroscopy.

Based on the situation of gastric mucosa, these 381 subjects were divided into three groups such as negative group (110 subjects), mild CAG group (144 subjects), and moderate and severe CAG group (127 subject). According to the results from the questionnaires and the inclusion criteria, 60 subjects in the negative group, 70 subjects in the mild CAG group, and 70 subjects in the moderate and severe CAG group were respectively and randomly recruited as the Group 1, Group 2, and Group 3. As shown in Figure 1, the criterion for the gastric mucosa in Group 1 was that there was focal atrophy of the superficial gland in the gastric antrum, whereas the glands in the greater and lesser curvatures of the stomach were normal. The common atrophy of the glands in the gastric antrum and the lesser curvatures of the stomach was seen in Group 2, whereas the range of the atrophy was wider than that in Group 1. The gastric mucosa in the moderate and severe CAG group(Group 3) was that there was wide atrophy or even disappeared of all the glands in the gastric antrum, greater and lesser curvatures of the stomach, and the mucous membrane was significantly thinner or even the generation of IM.

The inclusion criteria of the subjects in this study were as following: firstly, the patients should be first diagnosed as CAG. Secondly, there should be no infection of Hp in the CAG patients. Thirdly, the subjects should not use antibiotics and other drugs in the week before going to the hospital, and also they should not have taken any vitamin and element supplements for a long period (>3 months) in the past year. Fourthly, there should not be any medical history of tumor, allergy, asthma, or allergic rhinitis and no family history of digestive tumors such as the GC, colon cancer, etc.

The questionnaires included a lot of information such as gender, age, body weight and height, hair dye, house decoration, radiation exposure, smoking status, alcohol intake, and the medical history in 4 weeks before this study. The ethical approval was approved by Medical Ethics Committee of Daqing Oilfield General Hospital, Daqing City, Heilongjiang Province, China. Every subject should be given a written informed consent before they agreed to involve in this survey.

TABLE 1. General Information of All the Subjects in These Three Groups

Variables	Index	Group 1 (n = 60)	Group 2 (n = 70)	Group 3 (n = 70)	*P* Value
Gender (%)	Man	43 (71.2)	46 (66.7)	48 (68.2)	0.808
	Woman	17 (28.7)	24 (33.3)	22 (31.8)	
Age (year)	Mean ± Standard	46.0 ± 7.5	46.0 ± 9.3	46.3 ± 6.7	0.990
BMI (kg/m^2)	Mean ± Standard	23.3 ± 3.0	22.9 ± 3.1	22.6 ± 4.2	0.557
Smoking (%)*	Yes	26 (42.5)	21 (30.0)	26 (36.5)	0.319
	No	34 (57.5)	49 (70.0)	44 (63.5)	
Drinking (%)**	Year	24 (40.2)	28 (40.0)	34 (49.2)	0.223
	No	36 (59.8)	42 (60.0)	36 (51.8)	

BMI = body mass index, CAG = chronic atrophic gastritis.
*Smoking referred to smoke at least one cigar per day and last ≥1 year, smoking quit but <1 year was also included.
**Drinking was definitive by weekly drinking no less than three times a day.χ^2 test was used to compare the gender, smoking, and drinking in these three groups. The difference of age and BMI were compared by *t* test.

Collection of Blood Samplings

Based on the criterion of blood sampling, 6 mL peripheral blood was drew from each subject and assigned to the vacuum blood collection tubes (EDTA, Beijing, China). Then these blood samples were used to extract RNA, DNA, and protein. Subsequently, all of the samples were stored at −80°C within 6 hours until used.

Methylation Analysis of Runx3

The methylation levels of many CpG sites of Runx3 were quantified by Laser Matrix Support Release/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) of the MassArray system (Sequenom EpiTYPER assay, San Diego, CA).[17] In this process, bisulfite conversion of DNA was firstly performed using the EZ DNA Methylation kit (Zymo Research, CA) following the manufacturer's instructions. Secondly, PCR and in vitro transcription were carried out in DNA samples by bisulfite conversion. Thirdly, the target regions were amplified using the primer pairs (EpiDesigner software, www.epidesiger.com) including the forward primer (aggaagagagGTTTTTGGGGATGTAGGTTTGG) and reverse primer (cagtaatacgactcacta tagggagaaggctAAAAAACACTTCATAA TAAACCACC), and then treated by Shrimp Alkaline Phosphatase (SEQUENOM, San Diego, CA). Fourthly, the products were used as the template for in vitro transcription and base-specific cleavage with RNase A. Lastly, all of the cleavage products were analyzed by MALDI-TOF-MS according to the manufacturer's instructions; 10% of the parallel samples were done in order to ensure the accuracy of the results.

Expression Levels of Runx3

Following the protocol (Invitrogen), total RNA was extracted from the peripheral blood by the Trizol (Invitrogen™,). The NanoDrop 2000c spectrophotometer (Thermo,) was used to measure the concentration of RNA. Agarose gel electrophoresis was chosen to evaluate the quality of RNA. Absolute quantification was performed using PCR Master Mix for SYBR Green assays (Vazyme) on Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) system (CFX-96, Bio-Rad Company).[12] All samples were run in triplicate, and genes expression data was normalized by β-actin. All proteins were extracted from the peripheral blood. Absolute quantification of Runx3 was performed using Western blotting. All samples were run in triplicate, and protein expression data was normalized using β-actin.[13]

Investigation of the Prognosis

All patients who were included in this study were administered the conventional internal medicine treatment. The prognosis of CAG patients was followed up after 2 weeks of drug treatment. The criterion for the prognosis of CAG patients was determined by the status of gastric mucosa through the combination of endoscopic and pathological examination. Then the status of the gastric mucosa was compared with that before the clinical treatment to judge whether the prognosis was healing.

Statistical Analysis

Epidata software was used to enter data from the questionnaires and experiments into the computers. The whole process utilized double entries and logistical error check to ensure the accuracy.

All analyses were performed by the SPSS17.0 and normality was assessed by *K-S* test. Differences in continuous and categorical parameters were tested using two sample *t* tests (or Mann–Whitney *U* nonparametric test), ANOVA, and χ^2 test. The unconditional logistic regression model was used to evaluate the correlations between the methylation levels of many CpG sites with the prognosis among the CAG patients. Statistical significance for two-sided *P* values was defined as $P < 0.05$.

RESULTS

General Information of the Subjects

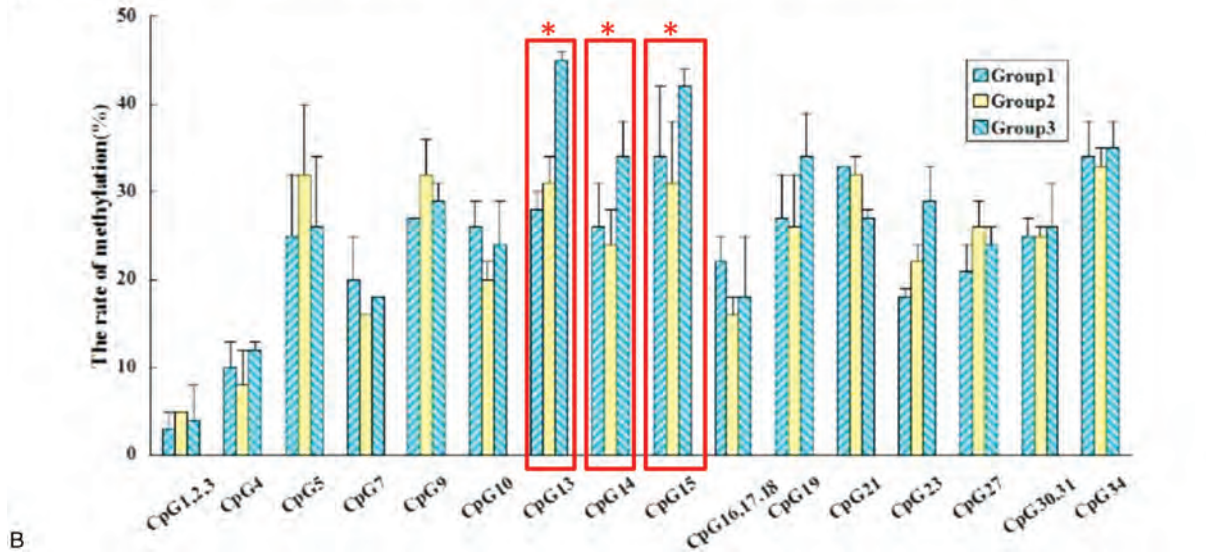
A total of 200 subjects were recruited in this study including 60 subjects in the negative group (Group 1), 70 subjects in the mild CAG group (Group 2), and 70 subjects in the moderate and severe CAG group (Group 3). The demographic characteristics of all of the subjects in this research are presented in Table 1, which showed that there were no significant differences in the distribution of gender, age, body mass index (BMI), smoking and alcohol consumption between these three groups.

Methylation Levels of Runx3

The location information of the CpG Island, target sequence, and CpG sites of Runx3 in this research was shown

>hg38_cpg Island Ext_CpG: 63 range=chr9:133255615-133256444 5'pad=0 3'pad=0 strand=+ repeat Masking=none
CGGGAGGGGGACGGGGCTGCCGGCAGCCCTCCCAGAGCCCCTGGCAGCCGCTCACGGGTTCCGGACCGC
CTGGTGGTTCTTGGGCACCGCAGTGAACCTCAGCTTCCTCAGGACGGCGGGCCAGCCCAGCAGCTGCTGG
TCCCACAAGTACTCGGGGGAGAGCACCTTGGTGGGTTTGTGGCGCAGCAGGTACTTGTTCAGGTGGCTCT
CGTCGTGCCACACGGCCTCGATGCCGTTGGCCTGGTCGACCATCATGGCCTGGTGGCAGGCCCTGGTGAGC
CGCTGCACCTCTTGCACCGACCCCCCGAAGAACCCCCCCAGGTAGTAGAAATCGCCCTC{GTCCTTGGGGA
TGTAGGCCTGGGACTGGGGCCGGCGCTCGTAGGTGAAGGCCTCCCGGCTGCTTCCGTAGAAGCCGGGGTG
CAGGGTGCCGAACAGCGGAGTCAGGATCTCCACGCCCACGTGGTCGCGGAACTCCATGTCCACGTCCACG
CACACCAGGTAATCCACCTCGCTGAGGAAGCGCCGCTCGCAGAAGTCACTGATCATCTCCATGCGGCGCAT
GGACACGTCCTGCCAGCGCTTGTAGGCGCGCACCTCCAGCACTGACAGCTGCCGACCGGTCCCCAGCGTC
ACGCGGGGCACCGCGGCCGGCTGGTCGGTGAAGACATAGTAGTGGACACGGTGGCCCACCATGAAGTGCT
TCTC}CGCCGTCTCCAGGAACAGCTTCAGGAAAGCCACGTATCTGCAAGGCAGGCGGACGGGGGCTGGGG
GAGCCGCCGGCCGTGCACCCCTGGGCTGCAGGAGGCCCGTCCTGCACCCGCCCGCCAGCG

A



B

FIGURE 2. The methylation levels of the CpG sites in Runx3. (A) demonstrated the location information of the CpG island, target sequence, and CpG sites in Runx3; (B) showed the methylation levels of many CpG sites of Runx3. Group 1—negative group, Group 2—mild CAG group, and Group3—moderate and severe CAG group. The target sequences were marked in brackets and the CpG sites were underlined. ∗proved that the methylation levels of these CpG sites in Group 3 were significant higher than those in Groups 2 and 1, $P<0.05$. CAG: chronic atrophic gastritis.

in Figure 2A. The methylation levels of CpG13, CpG14, and CpG15 were significantly higher in Group 3 than those in Groups 1 and 2 (Figure 2B), whereas no differences in the methylation levels of all the CpG sites in Runx3 were observed between the subjects in Groups 1 and 2.

Expression Levels of Runx3

The mRNA and protein expression levels of Runx3 were significantly lower in Group 3 than those in Groups 1 and 2, as shown in Figure 3. However, there was no difference in the mRNA and protein expression levels of Runx3 between the subjects in Groups 1 and 2.

Correlations Between Methylation Levels of Runx3 and the Prognosis of CAG Patients

As shown in Table 2, there were significantly negative correlations between the methylation levels of CpG13, CpG14, and CpG15 sites in the promoter region of Runx3 with the healing prognosis of CAG patients by the multiple regression analysis ($P<0.05$), whereas no correlations between the other factors such as gender, smoking, drinking, age, and BMI and the prognosis were observed among the CAG patients.

DISCUSSION

GC has become one of the serious health problems all over the world. The incidence of GC was in a stepwise manner, and the subjects with precancerous lesions may be at high risk of developing CG, such as CAG, IM, and dysplasia. Subsequently, as the precancerous lesions, improving the prognosis and treatment of CAG is an important method for reducing the incidence of GC.[14,15]

Recently, many studies have indicated that epigenetic mechanism can be involved in the occurrence and development of GC, in which the methylation alteration in many genes such as Runx3 in the serum can be used as the biomarker for the detection of CG.[16,17] Runx3, as one of the significant members in the Runx family, was located in the short arm of human first chromosome (1P36.1) with the total length of 67 KB. It contains

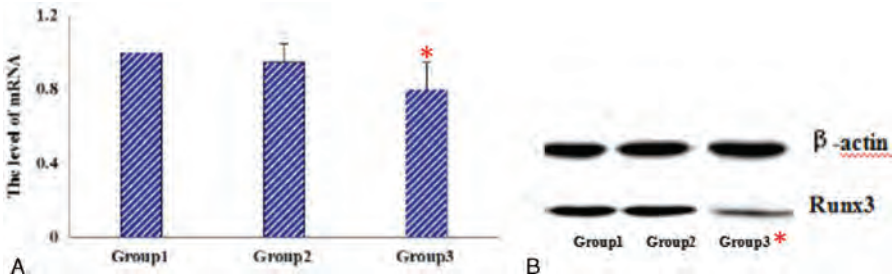


FIGURE 3. Comparison of the expression levels of Runx3 in different groups. (A) indicated the mRNA expression levels of Runx3 in different groups and (B) showed the expression levels of Runx3 protein in different groups; Group 1—negative group, Group 2—mild CAG group, and Group3—moderate and severe CAG group; ∗ proved that the expression levels of mRNA and protein of Runx3 in Group 3 were significantly higher than those in Groups 2 and 1, $P<0.05$. CAG = chronic atrophic gastritis.

TABLE 2. Correlations Between Methylation Levels of CpG Sites in Runx3 With the Prognosis of CAG Patients

	Prognosis (n = 140)				Prognosis (n = 140)				Prognosis (n = 140)		
CpG Site	*r*	r_{std}	*P*	CpG Site	*r*	r_{std}	*P*	CpG Site	*r*	r_{std}	*P*
CpG13	−0.179	−0.156	0.013	CpG14	−0.198	−0.474	0.031	CpG15	−0.639	−0.431	0.014
Gender	−0.099	−0.147	0.078	Gender	−0.026	−0.055	0.795	Gender	−0.231	−0.207	0.072
Smoking	−0.046	−0.039	0.373	Smoking	−0.113	−0.141	0.518	Smoking	−0.078	−0.084	0.106
Drinking	−0.111	−0.100	0.167	Drinking	−0.125	−0.111	0.581	Drinking	−0.049	−0.053	0.226
Age	−0.111	−0.120	0.158	Age	−0.612	−0.424	0.059	Age	−0.138	−0.141	0.056
BMI	−0.107	−0.132	0.101	BMI	−0.143	−0.122	0.539	BMI	−0.051	−0.046	0.130

BMI = body mass index, CAG = chronic atrophic gastritis.
According to the situation of gastric mucosa, the prognosis of good and bad was respectively assigned as one and two.

two high conservative CpG islands, which can play an important role in the regulation of the expression of Runx3.[18] However, little information was available on the methylation levels of Runx3 among the CAG patients. So in this research, the methylation levels of many CpG sites in the promoter region of Runx3 and its expression were measured in serum. Our results had proved there were higher methylation levels of CpG13, CpG14, and CpG15 in the promoter region of Runx3 in Group 3, compared with those in Groups 1 and 2 ($P<0.05$). However, the expression levels of Runx3 were lower in Group 3 than those in Groups 1 and 2 ($P<0.05$). Moreover, there were significantly negative correlations between the methylation levels of CpG13, CpG14, and CpG15 sites in Runx3 with its expression. So the hypermethylation modifications of CpG13, CpG14, and CpG15 in the promoter region of Runx3 can significantly affect its expression.

The appropriate expression of Runx3 can play a tumor suppressor role. The Runx3 protein was an important transcription factor in the TGF-β signal transduction pathway. Its role was to combine with the Smad receptor to form TGF-β /Smad compound and then transfer the TGF-β /Smad compound from the cytoplasm to the specific site in the nucleus to promote TGF-β /Smad compound connection with this specific site. Considering the significant role of Runx3 in the TGF-β signaling pathways, it is reasonable to think that Runx3 can be involved in the regulation of multiple cell functions such as the target gene transcription, cellular regulation, differentiation, and invasion.[19,20] Recently, many research works had also proved that the hypermethylation status of many CpG sites in Runx3 could cause the disorder of TGF-β signaling pathway to increase the occurrence and development of some cancer including GC, breast cancer, bladder cancer, etc by affecting the expression levels of Runx3.[21–23] So the methylation levels of Runx3 can be used as the biomarker for the early stage of tumor diagnosis.[24]

Few research works had discussed the correlation between the methylation levels of Runx3 and the occurrence and development of CAG. So under this condition, the objective of this research was to discuss whether the methylation levels of Runx3 could be used as the early biomarker to predict the prognosis of CAG patients. Our results in this research had indicators that there were significantly negative correlations between the methylation levels of CpG13, CpG14, and CpG15 sites in the promoter region of Runx3 with the prognosis of CAG patients.

In summary, the methylation of CpG13, CpG14, and CpG15 in the promoter region of Runx3 can be used as the biomarker for the diagnosis and clinical treatment of CAG.

CONCLUSIONS

In brief, our study demonstrated that there were hypermethylation modifications of CpG13, CpG14, and CpG15 in the promoter region of Runx3. This methylation status could result in the down regulation of Runx3 expression and affect the prognosis of CAG. So in this condition, the methylation levels of these CpG sites in peripheral blood can be used as the biomarker to provide the criterion for clinical treatment of CAG patients.

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