Original Article

Identification the potential of stool-based *SNCA* methylation as a candidate biomarker for early colorectal cancer detection

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Background: Stool-based DNA methylation are emerging as promising biomarkers for colorectal cancer (CRC), but their capability for detecting curable stages (adenomas) remains unclear. This study aims to explore the potential of selected candidate biomarkers (SNCA, SPG20 and FBN1) in stool samples for identifying patients with adenomas or CRC.

Methods: Thirty-one patients with CRC, 49 patients with adenomas, and 64 normal controls were selected. Stool samples were processed by the stool intestinal mucosal cells collector and analyzed by quantitative methylation-specific polymerase chain reaction (qMSP).

Results: Promoter methylation status of genes was denoted as percentage of methylated reference (PMR) values. The SNCA PMR values were significantly higher in CRC and adenomas groups than normal controls. The area under the ROC curve (AUC) for SNCA was 0.836 and 0.772 for detection of CRC and adenomas, respectively. The sensitivity of stool-based SNCA methylation was 83.9% for CRC and 75.5% for adenomas, with the specificity of 75% for both. The odds ratio (OR) for CRC and adenomas with stool SNCA hypermethylation was 11.291 and 9.234, respectively, after adjustment for patients' age and sex. Though there was a trend of increase of SNCA promoter methylation with disease progression, the difference was not significant. No significant difference in stool-based SNCA methylation was found between proximal and distal lesions. SNCA methylation significantly reduced after tumor resection compared to the initial status. The SPG20 and FBN1 methylation status was not significantly different among groups.

Conclusions: The results validated the capability of SNCA methylation in stool samples for the identification of patients with adenomas or CRC.

Keywords: Colorectal cancer (CRC); early detection; DNA methylation; stool testing

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Introduction

Colorectal cancer (CRC) has become one of the leading causes of cancer-associated death worldwide. According to the pathological study, CRC mainly arises from benign precursors (adenomas) to cancer during a long time interval. Detection of CRC at an early curable stage and removal of precancerous lesions can improve the cure rate (1). At present, the conventional methods, fecal occult blood test (FOBT) and colonoscopy are used most frequently to screen for CRC. However, FOBT might be interfered by diet composition and the measurement repeatability is limited. Colonoscopy is invasive, costly and low adherence rates, although it has highly positive predictive value (1). Therefore, it is imperative to develop molecular biomarkers as a noninvasive screening method for the diagnosis of early CRC.

Hypermethylation of DNA promoter occurs early in the development of CRC (2), suggesting its suitability as a molecular marker in early diagnosis of CRC. Periodic exfoliation of intestinal epithelial cells provides a basis for the analysis of stool DNA to identify methylation variation of intestinal tissue (3-6). But previous studies on stool DNA methylation showed lower sensitivity for detecting adenomas or advanced precancerous lesions which would be the curable stage to avoid the morbidity of CRC (3,5). In addition, another crucial factor influencing test outcomes is stool sample processing to recover analyzable human DNA from stool (7).

Alpha-synuclein (*SNCA*) gene encodes conserved protein that is abundant in neurons, especially presynaptic terminals. Aggregated alpha-synuclein proteins form brain lesions that are hallmarks of neurodegenerative synucleinopathies (8). Previous studies have shown *SNCA* hypermethylation in blood and cerebral cortex of patients with Parkinsons's disease (9), in biliary brush samples with cholangiocarcinoma (10), and in CRC cell lines (11). Spastic paraplegia-20 (*SPG20*) gene encodes the multifunctional spartin protein, which was found to be involved in intracellular epidermal growth factor receptor trafficking (12). *SPG20* was demonstrated to be methylated in 89%, 78% and 1% of the CRC, adenomas and normal mucosa samples, respectively (13). Fibrillin-1 (*FBN1*) encodes the structural fibrillin protein, which is an extracellular matrix glycoprotein that serves as a structural component of calcium-binding microfibrils. *FBN1* was hypermethylated in CRC samples compared to the normal colorectal mucosa (11). Our previous work in tissue samples showed hypermethylation of the promoter of *SNCA*, *SPG20*, and *FBN1* in CRC patients (14).

In this study, we aimed to explore the potential of stool-based DNA methylation of the three genes (*SNCA*, *SPG20*, *FBN1*) as suitable candidate biomarkers to discriminate patients with adenomas and colorectal cancer from normal controls and to illuminate the relationship between validated biomarkers and clinicopathologic factors.

Methods

Clinical samples

Thirty-one patients with CRC, 49 patients with adenomas, and 64 normal controls were recruited in the National Center of Colorectal Surgery, the 3rd affiliated Hospital of Nanjing University of Traditional Chinese Medicine between April 2014 and February 2015. Details regarding the following information are summarized in *Table 1*: gender, age of onset, tumor location, lesion size, histological and TNM staging classifications. There were no age and gender differences between CRC/adenomas groups and normal controls. The adenomas included adenomatous polyp, tubular adenomas (TA), tubulovillous adenomas (TVA), and high-grade intraepithelial neoplasm (HGIN). An advanced precancerous lesion was defined as tubular adenomas 1cm or larger, TVA, and HGIN (15). All participants in the study provided written informed consent and this study were approved by the Ethics Committee of the 3rd affiliated Hospital of Nanjing University of Traditional Chinese Medicine (KY2014018).

Stool processing using the stool intestinal mucosal cells collector

All control/adenomas stool samples and a subset of the CRC stool samples were collected before bowel purgation and colonoscopy. Some CRC stool samples were collected >7 days after colonoscopy. A single stool sample was put into the provided collection container and homogenized in a DNA stabilization buffer and frozen at −80 ℃ immediately. The stored stool samples were further processed within 3 days. All stool samples were handled in a blinded fashion during storage, processing, DNA extraction, and qMSP analysis.

To obtain high quality and quantity of intestinal mucosal cells from stool samples, a stool device called the stool intestinal mucosal cells collector was designed. This device consists of three volumes of 1,000 mL containers containing

Table 1 Clinicopathological characteristics of subjects

Characteristics	CRC (n=31[a])	Adenoma (n=49)	Normal (n=64)	P
Age at diagnosis (mean ± SD)	62.37±11.39	58.43±12.11	57.79±8.88	0.142
Gender				0.062
Male	14	32	28	
Female	16	17	36	
Location[b]				
Proximal	3	14		
Distal	26	29		
Both	1	6		
TNM stage				
I and II	17			
III and IV	13			
Lesion size, cm				
0.5	1	30		
0.5–1	2	8		
>1	27	11		
Lesion histology				
Adenomatous polyp		19		
Tubular adenoma		21		
Tubulovillous adenoma		6		
HGIN		3		

[a], clinicopathological characteristics of one case is not available; [b], proximal lesions included tumors at or proximal to the splenic flexure, and distal lesions were those distal to the splenic flexure. CRC, colorectal cancer.

Figure 1 Stool intestinal mucosal cells collector.

the filter screen on the bottom with pore diameter of 100, 200, and 300 meshes, respectively (*Figure 1*).

The operational steps of the stool processing were as follows: Stool sample (20–30 g) mixed with 500 mL of buffer was poured into the container A. Vibrate the container to let the mixture passes through filter screens of container A, B, and C in sequence. The filter liquor were collected and poured into a 500 mL centrifuge tube and centrifuge at 5,000 rpm for 20 min. Discard the supernatant and transfer the middle layer (intestinal mucosal cells enrichment) into a new 50 mL centrifuge tube and store at −80 ℃.

DNA extraction and bisulfite modification

Genomic DNA in stool extractions was obtained using the TIANamp Genomic DNA kit (Qiagen) according to the manufacturer's instructions. DNA concentration was around 100 μg/mL and purity (OD260/280) was around 1.8, suggesting the stool DNA was achieved for the requirement of DNA methylation detection.

Table 2 Differences in stool *SNCA* methylation status[a] between groups

Characteristics	Numbers	Median of PMR values	P
Groups			
CRC	31	2.06	<0.001[b*]
Adenoma	49	1.45	<0.001[b*]
Normal	64	0	
Lesion stage			
NAA	36	1.41	0.310[c]
APL	13	1.95	
$T_{I, II}$	17	1.98	
$T_{III, IV}$	13	2.88	

[a], stool *SNCA* methylation status were showed as the *SNCA* PMR values (calculated by qMSP data as showed above in Method part); [b], compared with the Normal group by Mann-Whitney U test; [c], analyzed by Kruskal-Wallis H test among the four groups of lesion stage; *, P<0.05. NAA, non-advanced adenomas; APL, advanced precancerous lesion; $T_{I, II}$, TNM stage I and II CRC; $T_{III, IV}$, TNM stage III and IV CRC. CRC, colorectal cancer.

DNA (1–2 μg) was bisulfite modified using CpGenome™ Universal DNA Modification Kit (Millipore) according to manufacturer's protocol and eluted in 20 μL of TE buffer (stool sample). CpGenome™ Universal Methylated DNA (Millipore) was used as a methylation-positive control.

Quantitative methylation-specific polymerase chain reaction (qMSP)

The methylation status of *SNCA*, *SPG20*, and *FBN1* in stool samples was confirmed by qMSP. The primer and probe sequences are listed in *Table S1*. The qMSP was carried out in a 10 μL reaction volume including 0.7 μL each of forward and reverse primers (0.9 μM), 0.4 μL probe (0.4 μM), 3 μL bisulfite treated template, 5 μL TaKaRa Premix Ex Taq™ and 0.2 μL ROX Reference Dye. The PCR program was performed by ABI StepOne Plus real-time PCR system (Applied Biosystems) as follows: 30 sec at 95 ℃, then 55 cycle of 10 sec at 95 ℃ and 30 sec at 55 ℃. The qMSP results were calculated as percentage of methylated reference [PMR, the GENE: ALU ratio of a sample was divided by the GENE: ALU ratio of the positive control (CpGenome Universally Methylated DNA) and multiplied by 100] (11). The most optimal PMR value was determined at the point on the ROC curve at which (sensitivity + specificity) was maximal.

Statistical analysis

This study was planned to get a power of 80% to test the hypothesis that the DNA methylation test would achieve a sensitivity of 75% or more for the detection of CRC under the null hypothesis, at a one sided type I error rate of 0.05. PMR Differences in stool *SNCA* methylation status between two independent groups were analyzed by Mann-Whitney U test, and between paired samples by Wilcoxon signed-rank. PMR Differences among groups more than two were analyzed by Kruskal-Wallis H test. Detection rates differences were compared by Fisher's exact test. ROC curve was generated using PMR values. For the ROC curve analysis, the areas under the curve (AUC) values were used to reflect the diagnostic performance of *SNCA* methylation status. The P values for the ROC curves are generated under the nonparametric assumption with the null hypothesis: true area =0.5. Multivariable logistic analyses were used to calculate the odds ratio (OR) related to CRC or adenomas according to stool *SNCA* methylation levels after adjustment of age and sex cases. The statistical tests were performed by the SPSS software package (version 16). P<0.05 was considered statistically significant.

Results

Significant difference in SNCA methylation between CRC/adenomas groups and normal controls in stool sample

SNCA, *SPG20* and *FBN1* promoter methylation were evaluated by qMSP in 144 stool samples including 31 CRCs, 49 adenomas, and 64 normal controls. The *SNCA* PMR values (calculated by qMSP data as showed above in Method part) was significantly higher in CRC (median 2.06) and adenomas (median 1.45) than the normal control (median 0) (P<0.001 and P<0.001, respectively. *Table 2* and *Figure 2A*). However, there were no significant differences in stool *SPG20* or *FBN1* methylation among CRC, adenomas and normal controls (P>0.05).

ROC curves were used to evaluate the discriminating potential of *SNCA* methylation as a noninvasive biomarker for detecting CRC and adenomas. The area under the ROC curve (AUC) values for *SNCA* were 0.836 (P<0.001) and 0.772 (P<0.001) for the detection of CRC and adenomas, respectively (*Figure 2B*,*C*). The optimal cutoff value for scoring positive methylation was set at PMR of 0.74 using method showed above in Method part. The sensitivity was 83.9% and 75.5% to identify a patient with CRC and

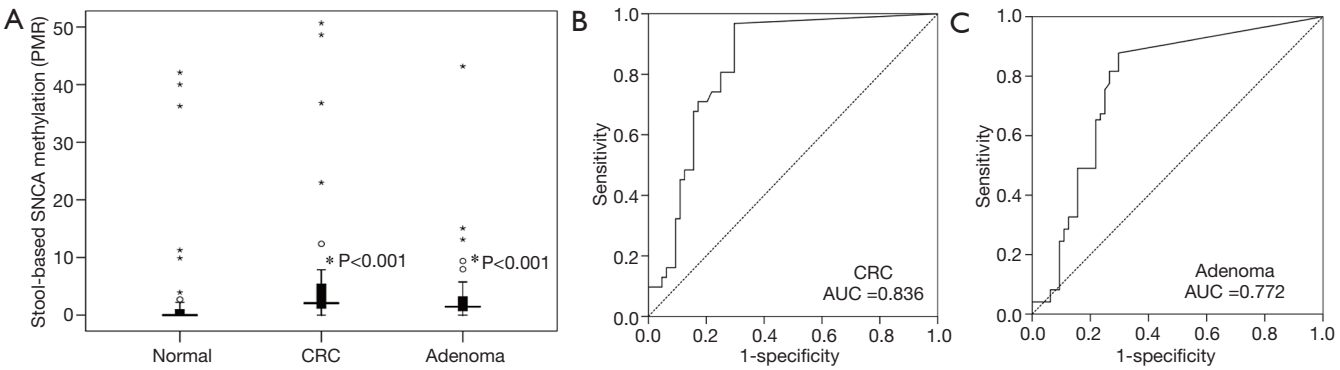

Figure 2 *SNCA* methylation levels in stool samples (n=144) (A) percentage of methylated reference (PMR) values of *SNCA* in subjects of normal controls, adenomas and CRCs. The *SNCA* PMR values was significantly higher in CRC (median 2.06) and adenomas (median 1.45) than the normal control (median 0) (P<0.001 and P<0.001, respectively); (B) receiver operating characteristics (ROC) curve for stool-based *SNCA* methylation in colorectal cancers versus normal controls with an AUC value of 0.836; (C) ROC curve for stool-based *SNCA* methylation in adenomas versus normal controls with an AUC value of 0.772. CRC, colorectal cancer.

Table 3 Sensitivity and specificity of stool-based *SNCA* methylation for CRC and adenoma detection

Groups	Total numbers	Positive numbers	Sensitivity [a] (%)	Specificity [a] (%)
CRC	31	26	83.9	75
Adenoma	49	37	75.5	75
Normal	64	16	–	–

[a], the sensitivity and specificity were calculated by using the optimal cutoff value on the ROC curve (at PMR =0.74). CRC, colorectal cancer; ROC, receiver operating characteristics; PMR, percentage of methylated reference.

adenomas, respectively and the specificity was 75% for both (*Table 3*).

In addition, multivariable logistic regression analyses showed that the OR for CRC and adenomas patients with high stool-based *SNCA* methylation was 11.291 and 9.234, respectively, after adjustment for patients' age and sex (*Table 4*). Thus, it revealed that *SNCA* methylation could be used as a potential biomarker for identifying patients with CRC and adenomas. *Table 4* also showed that the elder age can increase the prevalence risk of CRC.

SNCA methylation showed an increasing trend across the disease transition but the difference was not significant

PMR values of stool-based *SNCA* methylation showed an increasing trend across the disease transition from non-advanced adenomas (NAA, n=36), advanced precancerous lesion (APL, n=13), tumor-node-metastasis (TNM) stage I and II CRC (n=17) to TNM stage I and II CRC (n=13), but the difference was not significant (P=0.310, *Table 2*). When compared PMR of CRC TNM stage I and II with TNM stage I and II, or NAA with APL, the differences were not significant as well (P=0.660 and 0.248, respectively). Detection rates of stool-based *SNCA* methylation according to PMR cut-off showed a stepwise increasing trend across the disease transition (72.2% for NAA, 76.9% for APL, 82.3% for TNM stage I and II CRC, and 84.6% for TNM stage I and II CRC, *Figure 3A*), but the difference was not significant (P=0.788 by Fisher's exact test).

Stool-based SNCA methylation was not associated with the location of CRC or adenomas

To determine the detection rates of CRC or adenomas according to the location within the colorectum, *SNCA* methylation between proximal and distal lesions was compared. The results illustrated that stool-based *SNCA* methylation did not correlate with lesion location from patients with CRC (P=1.000, *Figure 3B*) or adenomas (P=0.213, *Figure 3C*).

Table 4 Multivariable logistic analysis for stool-based *SNCA* methylation and various diagnostic factors in patients with CRC and adenoma

Variables	OR (95% CI)	P
CRC *vs*. normal controls		
Age: ≥65 *vs*. <65 years	5.346 (1.659–17.233)	0.005
Sex: female *vs*. male	1.348 (0.446–4.070)	0.597
Stool *SNCA* methylation: ≥0.74 *vs*. <0.74[a]	11.291 (3.662–34.816)	<0.001
Adenoma *vs*. normal controls		
Age: ≥65 *vs*. <65 years	2.156 (0.752–6.182)	0.153
Sex: female *vs*. male	0.502 (0.205–1.234)	0.133
Stool *SNCA* methylation: ≥0.74 *vs*. <0.74[a]	9.234 (3.769–22.624)	<0.001

[a], the cutoff values of stool-based SNCA methylation in CRC patients or adenoma patients *vs.* normal controls were derived by ROC curves. OR, odds ratio; CI, confidence interval; CRC, colorectal cancer; ROC, receiver operating characteristics.

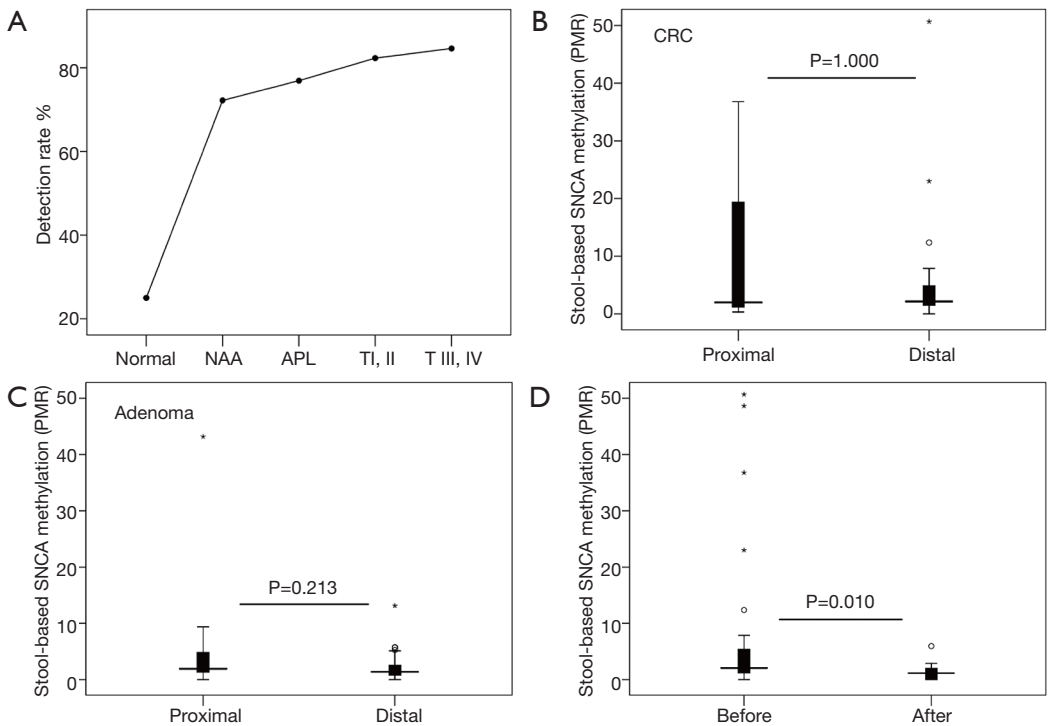

Figure 3 Associations between stool-based *SNCA* methylation and clinical features. (A) The detection rates of stool-based *SNCA* methylation for CRC detection according to lesion stage; (B,C) no significant difference in stool-based *SNCA* methylation between proximal and distal lesions for CRC or adenomas (analyzed by Mann-Whitney U test, P=1.000 and 0.213, respectively); (D) alteration of stool-based *SNCA* methylation after removal of tumors (analyzed by Wilcoxon signed-rank test, P=0.010). *, they are off-group points data.

Stool-based SNCA methylation is reduced after tumor resection

Thereafter, we intended to determine whether stool-based SNCA methylation was altered subsequent to tumor resection in stool CRC sample (31 before *vs*. after). It's interesting to note that *SNCA* methylation significantly decreased after tumor resection compared with the initial status (P=0.010, *Figure 3D*).

Discussion

In this study, we explored the potential of stool-based DNA

methylation as biomarkers for discriminating CRC or adenomas from normal controls.

The challenge of detecting stool DNA is to recover trace amounts of analyzable human DNA. In stool, only a tiny proportion of exfoliation of intestinal mucosal cells are mixed with a large amount of bacteria and diet, which increase the difficulty to get enrichment of stool human DNA. Our data indicated the high quality of stool DNA processed by the stool intestinal mucosal cells collector, suggesting the obtained stool DNA was satisfied for the requirement of DNA methylation detection.

Evidence from our previous and other studies indicated methylation of *SNCA* in >80% of CRC cell lines and the frequency of *SNCA* methylation was significantly higher in CRC tissues compared to adjacent normal ones (11,14). To our knowledge, this study is the first to investigate the potential of *SNCA* methylation in stool samples as biomarkers. The results showed that stool-based *SNCA* methylation had highly discriminatory performance for detecting CRC or adenomas. This is supported by AUC values of 0.836 for the detection of CRC (sensitivity =83.9%; specificity =75%) and 0.772 for adenomas (sensitivity =75.5%; specificity =75%). Moreover, the OR for CRC and adenomas with stool *SNCA* hypermethylation was 11.291 and 9.234, respectively. In addition, logistic analyses showed a high prevalence of risk factors for CRC in persons 65 years or older compared to the ones less than 65 years old.

As we know, to reduce the mortality of CRC, diagnosis of the disease at curable stages, such as adenomas, precancerous lesions and early CRC, is highly important (16). Yet, the previous reports mostly refer to CRC state, and the detecting sensitivity is lower for adenomas (3,5). Our current study showed stool-based *SNCA* methylation had a sensitivity of 80% for early colorectal neoplasm (including advanced precancerous lesion and CRC at stage I and II). This may provide an important opportunity for the ideal stage of early detection of CRC. Another finding of our study was the significant decrease in stool-based *SNCA* methylation levels after tumor resection in patients with CRC, which indicated that *SNCA* methylation is a specific biomarker for CRC. Though a trend of growing PMR of stool-based *SNCA* methylation found through the adenoma-cancer sequence (*Figure 3A*), the difference was not significant. This is perhaps because of the relative small sample size. Or molecular alterations of CRC might occur earlier since the initial of adenoma.

Stool *SPG20* and *FBN1* methylation status did not differ significantly among CRC, adenomas and normal controls in our study, suggesting the two genes are not suitable for CRC diagnosis.

Our present findings have several clinical implications. First, though colonoscopy is a gold standard for detecting CRC, it's invasive. In addition, molecular alterations of CRC might occur earlier than histomorphology changes, which suggest that a test using stool-based DNA methylation may provide noninvasive detection at lesions prior to malignancy. Second, sensitivity is an important feature for screening tests. In our study, the sensitivity (80%) of stool-based *SNCA* methylation for the detection of early colorectal neoplasm exceeded the performance of many previous reports. Third, as the relative incidence of proximal CRC and adenomas is higher than those of distal, effective detection of proximal lesions is of great importance. Previous studies demonstrated that colonoscopy is less effective for proximal than distal colon neoplasms (17,18), while stool-based *SNCA* methylation has comparable efficacy in the proximal and distal colorectum for detection of both CRC and adenomas. In view of this performance, stool-based *SNCA* methylation testing has potential to extend the scope for detecting neoplasms throughout the colorectum.

The limitations of this study include the following. First, patients were from a single center and some were symptomatic, and larger scale validation across multiple centers and different populations is required. Second, the specificity of the stool-based *SNCA* methylation (75%) is inferior to that of the multitarget stool DNA test (86.6–89%) (6). A combined biomarker panel may increase the specificity in screening compared to a single marker (11). Third, the sample size is relative small, especially for the proximal CRC group, and this will reduce the test power of this study.

Conclusions

Our results highlight the capability of stool-based *SNCA* methylation to detect adenomas which provide rationale for further development of the epigenetic biomarker in noninvasive early detection of CRC. Our next step is to detect combing molecular assays with better diagnostic performance for the early detection of CRC.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2017.01.30). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All participants in the study provided written informed consent and this study were approved by the Ethics Committee of the 3rd affiliated Hospital of Nanjing University of Traditional Chinese Medicine (KY2014018).

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Table S1 Primer and probe sequences of qMSP

Gene primer	Forward primer (5' to 3')	Reverse primer (5' to 3')	Probe (5' to 3')	Product size (bp)
SNCA	GCGTTTTGGGCGTTTTTTTAC	CGCTATAAACCGACGACGC	6-FAM-CGCTAACCTATCGTCGAA-TAMRA	143
SPG20	TGACGCGAAGCGTTCGAGAGCGCG	CGCTCGCCGAAAACCGATCCCGAA	6-FAM-CGCGCTTACCGTAACAA-TAMRA	102
FBN1	GAGTTATAGTTGGGATAGTTGCGAGC	AACGACGACTCCGACTCCC	6-FAM-CGCTACAACCACTACTCGA-TAMRA	101

qMSP, quantitative methylation-specific polymerase chain reaction.