

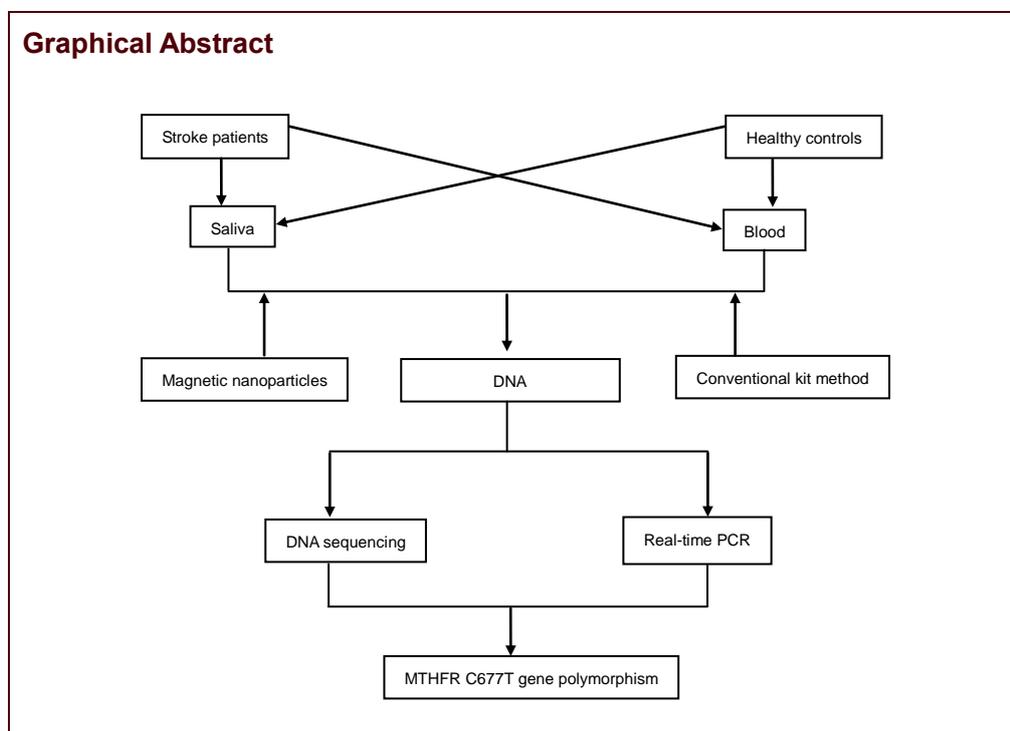
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A magnetic nanoparticles-based method for DNA extraction from the saliva of stroke patients

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Ethical approval: The study protocol was approved by the Ethics Committee at Peking University Shenzhen Hospital in China.

Abstract

C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene is a risk factor for stroke, suggesting that widespread detection could help to prevent stroke. DNA from 70 stroke patients and 70 healthy controls was extracted from saliva using a magnetic nanoparticles-based method and from blood using conventional methods. Real-time PCR results revealed that the C677T polymorphism was genotyped by PCR using DNA extracted from both saliva and blood samples. The genotype results were confirmed by gene sequencing, and results for saliva and blood samples were consistent. The mutation TT genotype frequency was significantly higher in the stroke group than in controls. Homocysteine levels were significantly higher than controls in both TT genotype groups. Therefore, this noninvasive magnetic nanoparticles-based method using saliva samples could be used to screen for the MTHFR C677T polymorphism in target populations.

Key Words

neural regeneration; brain injury; stroke; magnetic nanoparticles; saliva; methylenetetrahydrofolate reductase; homocysteine; gene polymorphism; gene screening; grants-supported paper; neuroregeneration

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

INTRODUCTION

Stroke is a major cause of disability and mortality in China. There are more than 2 million incidents of stroke annually, with 6–7 million stroke survivors every year^[1]. More than one million die from stroke-related causes, and post-stroke disability places a heavy burden on society. Moreover, there are almost no effective treatments for stroke patients besides thrombolytic therapy, which only applies to 1–2% of stroke patients^[2]. Thus, stroke prevention through the modification of risk factors is the best way to reduce the burden of the disease and should be top research priority.

Several traditional risk factors for the occurrence of stroke exist, including age, hypertension, hyperlipidemia, diabetes, and smoking, but these characteristics do not account for all cases. No obvious causes or underlying disorder can be diagnosed in one third of stroke patients^[3]. Gene defects related to stroke may act as risk factors. Frosst *et al*^[4] first presented a candidate genetic risk factor for stroke: a C-T polymorphism at nucleotide 677 in the methylenetetrahydrofolate reductase (MTHFR) gene, because of its role in catalyzing the formation of 5-methylenetetrahydrofolate, a co-substrate for the conversion of homocysteine to methionine. The MTHFR gene is located at 1p36.3 and consists of 11 exons, with the polymorphism occurring at exon 4^[5]. About 10% of the population is homozygous (TT) for a 677 C-T polymorphism of the MTHFR gene^[4]. The TT polymorphism leads to a reduction in enzyme activity and subsequent ~20% elevation of plasma homocysteine^[6], resulting in hyperhomocysteinemia, which is atherogenic and thrombogenic, contributing to the pathophysiologic mechanisms of stroke^[7-9].

The role of MTHFR C677T polymorphism has been widely studied across the world in different populations, but the results remain controversial^[10-11]. Variation in ethnicity and methodological differences may account for this discrepancy^[12]. The population frequency

of C677T homozygosity ranges from 1% or less among Black Americans to 20% among Italians and U.S. Hispanics^[13]. Studies from Asian countries report a homozygosity frequency of 40% in Chinese, clearly higher than the 0–1.2% in Indians^[14-16].

Because the C677T mutation may increase the risk of stroke and its prevalence in the Chinese population, the genotyping of MTHFR C677T polymorphisms and analysis of genetic sequences are critical for the primary prevention of stroke. People with defective genes can be diagnosed earlier and even treated for stroke in advance. Confirming subjects carrying gene mutations increasing the chances of stroke is of clinical importance as hyperhomocysteinemia can be prevented or effectively lowered safely and affordably by folic acid, vitamin B12, and vitamin B6^[17-19]. Lowering total homocysteine by about 3 $\mu\text{mol/L}$ with B vitamins should reduce the odds of stroke by about 20% in the overall population, independent of individual genotypes^[20-22]. Thus, a rapid and efficient screening method for genotype mutations is needed for the screening of large-scale target populations.

Usually, the detection of genetic diseases is rather complicated on a large scale owing to the invasive sampling and a cumbersome DNA extraction process. Blood is currently the most common source of DNA for genetic testing. Unfortunately, blood sampling is invasive, painful, and risks infection, making subjects often reluctant to submit and limiting our ability to enact screening of target populations on a large scale. Moreover, the classic methods for purification of DNA from blood are laborious and time-consuming. Many steps are involved in the purification of DNA from blood, including detergent-mediated lysis, proteinase treatment, extractions with organic solvents and ethanol precipitation, which also increase the risk of infection of the test technicians^[23]. In this study, we report the development of a simple, rapid, reliable, environmentally friendly, and industrially scalable protocol for DNA extraction from saliva samples. The method is based on the use of magnetic nanoparticles, and

the process relies on cell lysis to release DNA and its subsequent adsorption to the surface of the Dynabeads^[24-25]. Magnetic bead-based applications were first developed during the 1980s^[26]. The main advantages of bead-based methods for the small-scale purification of DNA include increased surface area for immobilization of DNA, reduced incubation time, and increased sensitivity^[27-28]. Magnetic bead-based assays have been used in gene detection for forensic casework^[29], and in research fields with animals, plants, and microorganisms^[30-31]. In addition, several studies on the use of magnetic nanoparticles to diagnosis disease based on small sample volumes from serum, hair, and blood^[32-35]. However, to our knowledge, few studies have focused on the application of magnetic bead-based methods to extract DNA from saliva samples for use in clinical medicine^[36]. Saliva samples are a good alternative source of genomic DNA owing to the painless and noninvasive collection^[37-39]. Compared to blood, saliva samples are much more convenient, efficient, and accessible if DNA can be extracted and purified. Thus, magnetic nanoparticles-based methods for DNA extraction from saliva samples are promising.

The purpose of this study was to develop a high sensitivity, high accuracy screening method for fast genetic analysis of DNA extracted from the saliva of stroke patients. A magnetic microbead-based method was developed for the detection of defective genes. The experimental results showed that this magnetic nanoparticles-based method using saliva was more effective and noninvasive than traditional methods using blood samples^[36]. A total of 70 stroke patients and 70 controls were tested for the MTHFR C677T gene polymorphism by the two methods at the same time. The aim of this study was to compare our magnetic nanoparticle-based method using saliva samples with the traditional method using blood samples to determine whether comparable results are obtained for the detection of MTHFR C677T polymorphism in stroke patients.

RESULTS

Quantitative analysis of participants

A total of 70 stroke patients and 70 healthy controls were included in the final analysis.

Baseline data of stroke patients and healthy controls

A comparison of baseline characteristics between cases and controls is summarized in Table 1. There were no significant differences in age, gender, body mass index,

blood pressure, blood lipids, or blood glucose between the two groups (all $P > 0.05$).

Table 1 Comparisons of baseline data between stroke patients and healthy controls

Item	Stroke (n = 70)	Control (n = 70)	P
Age ^a (year)	64.1±5.9	63.7±5.9	0.07
Gender ^b (male/female, n)	42/28	35/35	0.06
Body mass index ^a (kg/m ²)	23.7±5.3	23.3±5.5	0.10
Smoking ^b (n)	38	41	0.66
Alcohol intake ^b (n)	35	33	0.76
SBP ^c (mmHg)	134.7±26.0	131.8±22.8	0.40
LDL-C ^c (mmol/L)	2.5±0.7	2.6±0.7	0.17
BG ^c (mmol/L)	5.5±1.0	5.7±1.0	0.08

Measurement data were expressed as mean ± SD. a for paired sample *t*-test; b for chi-square test; c for independent sample *t*-test; all characteristics between two groups are out of significant (all $P > 0.05$). SBP: Systolic blood pressure; LDL-C: low-density lipoprotein cholesterol; BG: blood glucose.

PCR amplification using saliva and blood samples

The experimental data showed that DNA with an average concentration of 15.67 ± 7.67 ng/L was extracted from saliva samples, which was less than 39.39 ± 4.78 ng/L extracted from blood samples. All DNA obtained from the saliva and blood samples were used for PCR amplification. DNA was isolated from the blood to serve as a control. Figure 1 shows that conventional PCR amplified a band of 462 bp, as expected, in the saliva sample (lanes 1–5; five subjects were randomly shown), indicating that DNA isolated from the saliva was intact. The intensity of bands amplified from the blood samples were slightly lighter than those from the saliva, indicating that the DNA concentration extracted from the saliva was lower than that extracted from the blood. No false positive results were observed.

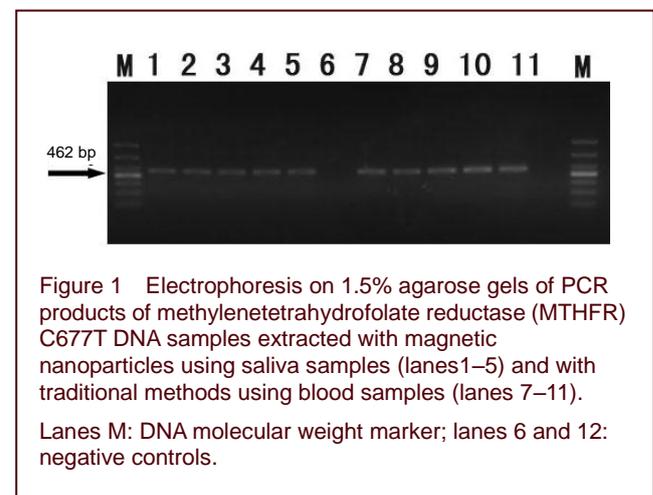


Figure 1 Electrophoresis on 1.5% agarose gels of PCR products of methylenetetrahydrofolate reductase (MTHFR) C677T DNA samples extracted with magnetic nanoparticles using saliva samples (lanes 1–5) and with traditional methods using blood samples (lanes 7–11).

Lanes M: DNA molecular weight marker; lanes 6 and 12: negative controls.

To test the quality of DNA extracted from saliva with magnetic nanoparticles, the DNA was serially diluted and

then amplified by PCR. In all cases, the PCR amplified the expected band of 462 bp (Figure 2, one random sample shown). No false positive results were observed.

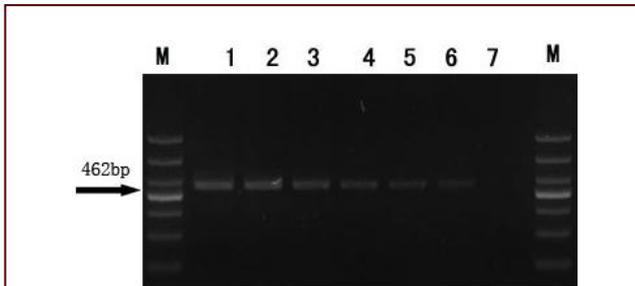


Figure 2 Electrophoresis on 1.5% agarose gels of PCR products of DNA extracted from saliva samples.

Lanes 1–6: DNA concentration is diluted by 10^0 , 10^1 , 10^2 , 10^3 , 10^4 , and 10^5 -fold, respectively. Lanes M: DNA molecular weight marker; lane 7: negative control.

Genotype of MTHFR polymorphism from stroke patients and healthy controls

The genotype of the polymorphism under examination is summarized in Table 2. The TT genotype and T allelic frequencies were significantly higher in the stroke group than in the control group ($P < 0.01$). The patients who are homozygous (TT) for the MTHFR polymorphism had a significantly greater risk of stroke (odds ratio (OR): 3.50, 95% confidence interval (CI): 1.04–3.01) than patients who are unaffected (CC). The patients who are homozygous (CT) for the MTHFR polymorphism had a significantly greater risk of stroke (OR: 2.02, 95%CI: 1.02–4.01) than patients who are unaffected (CC). The stroke odds ratio for the combined CT and TT groups was 2.21 (95%CI: 1.13–4.30).

Table 2 Distribution [n (%)] of methylenetetrahydrofolate reductase (MTHFR) C677T genotypes in stroke patients and healthy controls

Genotype	Control	Stroke	OR (95%CI)	P
CC	32(45.7)	12(17.1)	1.00(reference)	
CT	29(41.4)	41(58.6)	2.02(1.02–4.01)	0.039
TT	9(12.9)	17(24.3)	3.50(1.04–3.01)	0.021
CT+TT	38(54.3)	58(82.9)	2.21(1.13–4.30)	0.016

Subjects in control and case groups with CC genotype served as a references for statistical analysis. Chi-square tests were used to compare the genotype distribution between stroke and control groups. OR: Odds ratio; CI: confidence interval.

The results suggest that the TT and CT genotypes confer susceptibility to increased stroke risk in the Chinese population. The frequency distribution was consistent with the distribution previously reported in the Chinese population^[40] and was in accordance with Hardy-Weinberg equilibrium.

The genotype distribution results showed that the control group was a representative sample of the population.

Polymorphisms of MTHFR C677T detected by real-time PCR using salivary and blood samples

The saliva and blood samples were used in real-time PCR analysis to detect polymorphisms of MTHFR C677T. Three genotypes (CC, CT, and TT) were shown. DNA purified from saliva gave identical results to DNA purified from blood in these tests, indicating that DNA purified from saliva was as effective (Figure 3).

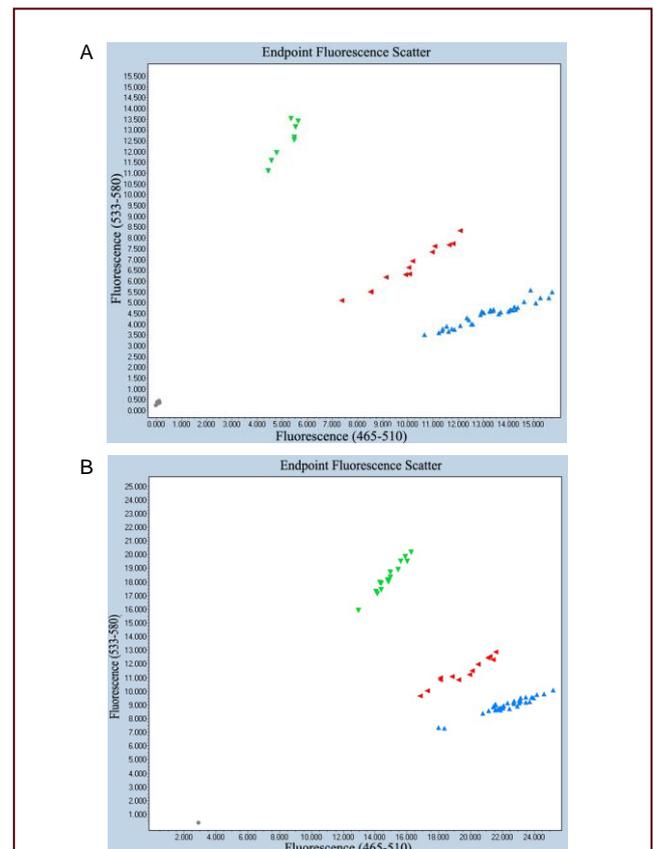


Figure 3 Real-time PCR results of MTHFR C677T polymorphisms from the saliva samples (A) and the blood samples (B).

According to the probe, green stands for TT type, red for CT type, and blue for CC type. The two figures show the same genotype results.

Polymorphisms of MTHFR C677T detected by sequencing of conventional PCR products

To confirm the genotype results of real-time PCR, the conventional PCR products were sequenced. All results from the real-time PCR were in agreement with results from the PCR sequencing (Figure 4, not all data shown).

Plasma homocysteine levels and their association with different genotypes

The mean total homocysteine plasma concentration for

the stroke group was $16.5 \pm 6.78 \mu\text{mol/L}$, while for the control group the mean was $11.68 \pm 4.29 \mu\text{mol/L}$. This difference between the two groups was significant ($P < 0.05$) by two-sample *t*-test, suggesting that elevated homocysteine levels may be one of the mechanisms of stroke.

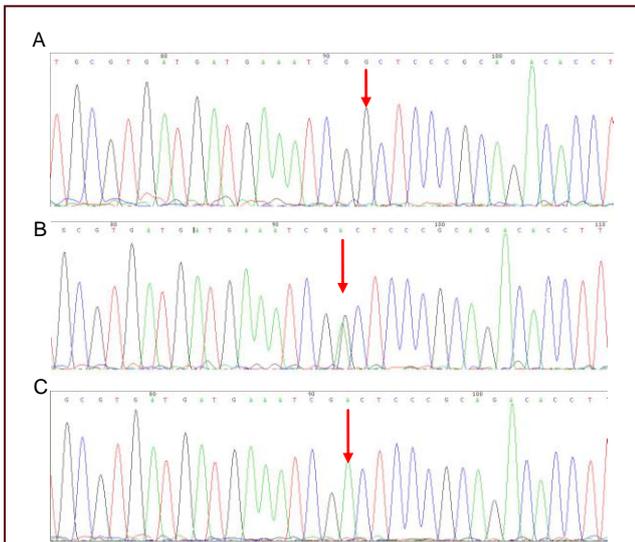


Figure 4 Sequencing results of MTHFR C677T polymorphisms from traditional PCR results.

(A) CC, (B) CT, and (C) TT genotypes. The normal sequence was -ATGAAATCGGCTCCCGCA-. The wild type CC had a normal sequence and a single peak. The heterozygous mutation type CT had mutant G→A and double peaks. The homozygous mutant type TT had mutant G→A and a single peak. All the results were in agreement with the real-time PCR data.

As shown in Table 3, the results indicate increasing total homocysteine levels with addition of TT genotype in both the stroke control groups. Statistical analysis showed significant differences between homocysteine levels of TT and CC ($P = 0.001$), TT and CT ($P = 0.017$) in the stroke group, as well as significant differences between TT and CC ($P = 0.02$) and TT and CT ($P = 0.029$) in the control group.

Table 3 Homocysteine levels ($\mu\text{mol/L}$) with different MTHFR C677T genotypes in stroke patients and healthy controls

Genotype	Stroke group		Control group	
	n	Homocysteine	n	Homocysteine
CC	12	16.0 ± 2.4^b	32	11.8 ± 4.4^a
CT	41	17.5 ± 5.4^a	29	14.0 ± 3.8^a
TT	17	21.1 ± 4.3	9	17.4 ± 4.4

The data are expressed as mean \pm SD. ^a $P < 0.05$, ^b $P < 0.01$, vs. TT genotype in the same group using analysis of variance followed by least significant difference tests.

DISCUSSION

The main findings of this study were: (1) the MTHFR C677T polymorphism is associated with elevated plasma homocysteine, which is a risk factor for ischemic stroke; (2) compared with conventional methods using blood samples, the magnetic nanoparticles-based method using saliva samples is an efficient method for DNA extraction to detect the MTHFR C677T polymorphism effectively and noninvasively.

The results also showed that the risk of ischemic stroke is higher with homozygous TT genotype than in those with wild type CC genotype, which is consistent with a previous meta-analysis^[41]. Frosst *et al*^[4] first showed that MTHFR C677T was a candidate genetic risk factor for vascular disease. The contribution of MTHFR C677T polymorphism to stroke may be associated with increased homocysteine, which leads to arterial endothelial dysfunction and results in stroke^[42-43]. However, such correlation is controversial. Several reasons may account for these conflicts: the difference in genotype frequencies by ethnic group, which is the main reason; selection bias in small sample sizes; environmental factors that contribute to stroke; and methodological limitations in the studies^[2]. The frequency of MTHFR gene polymorphism is variable in different geographic and ethnic groups: 38% in French, 5–15% in Canadians, 40% in Chinese, 32% in Japanese, 38% in Koreans, and 0% in African Americans^[2, 15-16, 44-46]. Meta-analysis of MTHFR C677T polymorphism with susceptibility to stroke in Asian populations, including Chinese, Japanese, Korean, and Indian, revealed a 1.47 times higher risk^[14, 46]. Owing to the high prevalence of MTHFR gene polymorphism in Chinese, it is necessary to screen for the mutation genotype of this target population on a large scale.

The results showed that the mutation genotype is associated with higher homocysteine levels both in the stroke and control groups. Consistent with this result, most previous studies had also showed that hyperhomocysteinemia was recognized as a risk factor for ischemic stroke^[47-48]. The mechanisms underlying the effects of increased homocysteine on ischemic stroke are not yet clear. A murine model showed that elevated homocysteine played a key role in the expression of vascular inflammation, atherogenesis, thrombophilia, and vulnerability to established atherosclerotic plaques. The destructive enzymes, which might promote atherosclerotic plaque instability and rupture, are increased in mice with

elevated homocysteine^[49-51]. Thus, elevated homocysteine could promote ischemic stroke through its hyper-coagulative effects by promoting plaque rupture. Therefore, increased homocysteine levels should be controlled to stabilize atherosclerotic plaques and prevent their rupture.

Because the MTHFR C677T polymorphism contributes to hyperhomocysteinemia and is regarded as an independent factor related to stroke, preventative genetic profiling is a necessity. However, traditional gene screening using blood samples is invasive, with resampling risks and poor compliance by subjects. In the present study, we described an alternative magnetic nanoparticles-based method for the rapid purification of DNA from human saliva samples.

Silica magnetic nanoparticles can bind nucleic acids under the control of three competing effects: weak electrostatic repulsion forces, dehydration, and hydrogen bond formation^[52]. Through the use of detergents, nucleic acids are released from biological samples, such as saliva. Next, the released nucleic acids specially bind to silica magnetic nanoparticles forming DNA-bead complexes under chaotropic conditions. Through magnetic separation, the complexes can be rapidly collected from the biological sample lysis solution. Proteins, other impurities, and any salts are rinsed away during the washing steps with different washing buffers. Finally, pure DNA can be easily eluted from the beads by an elution buffer.

The novel method of nucleic acids isolation based on silica beads can be used to extract DNA with different lengths from a wide variety of biological samples. For example, isolation of genomic DNA has been achieved from plants^[30], microorganisms^[31], blood^[33], and forensic tissues^[29]. However, few studies have focused on the application of magnetic bead-based methods for DNA extraction from saliva samples in clinical medicine.

The magnetic bead-based method relies on cell lysis, DNA adsorption to the beads, magnetic separation of the bead-DNA complexes, and extensive washing to remove contaminants and inhibitors^[24, 53-54]. To demonstrate the feasibility and advantages of this novel method, the conventional DNA extraction method was performed at the same time as a control. Saliva and blood samples from all subjects were used in real-time PCR to detect MTHFR C677T genotypes. DNA extracted from saliva samples gave identical results to DNA extracted from blood samples, indicating that the bead-based method is

acceptable for MTHFR C677T genotype detection. Compared with the traditional DNA extraction method using blood samples, we choose to use the magnetic nanoparticle-based method on saliva samples in the study for the following reasons.

(1) Feasible and precise: The method is sensitive and reproducible. Extracted DNA should be sufficiently pure to allow for PCR amplification and gene screening. The average quantity of DNA extracted from saliva was $15.67 \pm 7.67 \mu\text{g/mL}$; sufficient for the performance of real-time PCR analyses of MTHFR C677T genotypes and the quantity extracted did not affect the results. In addition, as little as a 105-fold dilution of DNA as a template was suitable to produce the expected band during PCR amplification, indicating that the extracted DNA was concentrated enough for the PCR reaction and genotype screening. Complete agreement of PCR typing results was achieved between the saliva and blood samples. All genotype results from real-time PCR were in agreement with those from gene sequencing after conventional PCR. In conclusion, the magnetic nanoparticles method for DNA extraction was feasible and accurate.

(2) Convenient and rapid: The method is simple and rapid. First, it is easy to take clinical saliva samples with buccal swabs, which can be performed by subjects themselves without a physician. The entire collection procedure takes less than 15 seconds. Second, DNA extraction with the magnetic nanoparticle-based method requires only five easy steps and needs no more than 40 minutes. No special laboratory equipment is needed. This method requires only a magnet and a heating block, and can be performed in any laboratory without the need for sophisticated instruments. Third, genotype detection with the BHQ-Taqman real-time PCR procedure is efficient and high throughput, as a large number of samples can be simultaneously tested on a 96-well plate within 45 minutes^[55-56]. Thus, the entire procedure from sample collection to genotype detection is rapid, only taking no more than 1.5 hours. As the method both rapid and requires no specialized equipment or knowledge of biochemistry, it thus allows for DNA purification from a large series of clinical specimens in a routine setting.

(3) Noninvasive and painless: The magnetic nanoparticle-based method is noninvasive and easily acceptable to patients because the specimen used in the method can come from saliva. Although blood is currently the most common source of DNA for genetic testing, blood collection is invasive, painful, expensive, and risks infection, so it is not always patient acceptable or practical. It

also requires specialized consumables (syringes, anti-coagulant-treated tubes) and a trained phlebotomist. Special handling and storage are also required. By contrast, saliva is a potentially useful source of genomic DNA for genetic studies since it can be collected in a painless and noninvasive manner. Saliva collection is simple to perform and requires no special equipment or training. What is more, the use of saliva as a source for DNA extraction has reduced the risk of transmitted infections. In addition, saliva can act as a useful source of genomic DNA even when stored under less than optimal conditions. A previous study^[33] examined various saliva storage conditions, reporting that fresh samples can be stored at room temperature for 1 month, room temperature for 6 months and -80°C for 6 months without negatively impacting DNA quality as shown by results from real time PCR. Although DNA purity is decreased with prolonged storage, this does not affect real-time PCR genotype results. Ease of storage could be a key advantage for large-scale population, in-community gene screening.

In the present study, a magnetic nanoparticle-based method using saliva samples for rapid DNA extraction and MTHFR C677T genotype detection in stroke patients was successfully demonstrated. As MTHFR C677T polymorphism is a risk factor for stroke, genetic screening and prevention is necessary. The magnetic nanoparticle-based method using saliva may be suitable for large, community-based studies of genetic susceptibility to stroke because it is sensitive, efficient, convenient, rapid, noninvasive, and painless.

SUBJECTS AND METHODS

Design

A case-control study.

Time and setting

Experiments were performed at the Department of Neurology and Central Laboratory, Peking University Shenzhen Hospital in China from November 2011 to November 2012.

Subjects

The study included 70 patients who were admitted to the Department of Neurology, Peking University Shenzhen Hospital from October 2011 to January 2012. The inclusion criteria were those with a history of ischemic stroke or those who had stroke confirmed by MRI and/or CT, according to the International Classification of Diseases,

9th revision^[57]. Patients with renal disease, cardioembolic stroke, liver failure, malignancy, and those with exposure to folic acid inhibitors were excluded. Patients who had received B12 or folic acid supplementation in the past year were also excluded.

During the same period, 70 age- and sex-matched healthy subjects attending the outpatient laboratory of the hospital for routine general checkups with no history of venous or arterial thrombosis were recruited as controls. Subjects were excluded if they were on medications or supplements that were known to interfere with homocysteine levels.

The ethical protocol was in accordance with the *Declaration of Helsinki*. No subjects were related to each other. Informed consent was obtained from all subjects.

Methods

Total plasma homocysteine assays

Fasting venous blood (at least 12 hours fasting) was collected in tubes containing trisodium ethylenediamine tetraacetic acid, and were centrifuged and stored at -20°C . Plasma homocysteine was analyzed with high-performance liquid chromatography (HP1100, Hewlett Packard, Taipei, Taiwan, China) coupled with a fluorescence detector (HP1045, Hewlett Packard, Palo Alto, CA, USA) based on previously verified methods^[58].

DNA extraction from blood

About 2 mL of peripheral median cubital venous blood was processed using a standard protocol^[59] and stored in tubes containing trisodium ethylenediamine tetraacetic acid. DNA was extracted from the whole blood using a Blood Genome DNA Extraction Kit (TaKaRa, Dalian, Liaoning Province, China) according to the manufacturer's instructions^[59].

Magnetic nanoparticles DNA extraction method using saliva samples

First, approximately 0.5 mL of saliva was obtained by a buccal swab either before a meal or 10 minutes after the meal with leftover food removed from the mouth with water. The swab stained with saliva was cut by scissors, placed in a sterile Eppendorf tube, and then stored at room temperature.

Second, saliva samples were placed in 1.5 mL Eppendorf tubes and immediately vortexed (approximately 10 seconds), then mixed with 200 μL of cell lysis buffer (Nanotechnology Development Co., Ltd., Wuhan, Hubei Province, China) for 30 minutes at 80°C . The tubes were

vortexed again (15 seconds) and centrifuged (20 seconds) in an Eppendorf microfuge (fixed angle, 24 000 × *g*), and the saliva-lysis buffer mixtures were vacuum aspirated. Buccal cells were lysed and DNA was released.

Third, the saliva-lysis buffer mixtures were transferred to fresh 1.5 mL Eppendorf tubes, and 20 μL of DNA magnetic nanoparticles (Nanotechnology Development Co., Ltd, HeFei, China) were added and centrifuged at room temperature for 20 seconds at 600 RPM. Then mixtures were placed at room temperature for 10 minutes to ensure target DNA was completely bonded onto the surface of the magnetic nanoparticles. After placing the tube beside a magnetic for 20 seconds to attract the nanoparticles, the supernatant was discarded with a pipette and DNA pellet was retained.

Fourth, the DNA pellet was re-suspended in 100 μL of 70% ethanol to purify it, and was then centrifuged at room temperature for 20 seconds at 600 r/min and then returned to the magnetic to attract the beads for 20 seconds. The supernatant was discarded again. The purification procedure was repeated once to remove any residual contaminants. Especially in the first wash, pellets may occasionally be very tight, taking up to 1 minute to resuspend them by vortexing. In the following washes, suspension can then be achieved without difficulty. The tubes were dried at 50°C with open lids in an Eppendorf heating block for 10 minutes. The tubes were briefly vortexed again and centrifuged for 2 minutes at 600 r/min.

Fifth, purified DNA was eluted by the addition of 50 μL of elution buffer and incubated at 70°C for 10 minutes to dissociate the bonded DNA from the magnetic nanoparticles, then vortexed until the pellet was (visually) completely resuspended. After centrifugation (15 seconds at approximately 12 000 × *g*), the supernatant with pure DNA was disposed of by vacuum aspiration to another fresh tube. With this approach, the DNA could be isolated and purified from saliva samples and stored at 4°C until use. The DNA concentration was measured by spectrophotometer (DN-1000 spectrophotometer, NanoDrop, Wilmington, DE, USA) and verified by agarose gel electrophoresis. A schematic illustration of the experimental procedure is shown in Figure 5^[25, 60].

Real-time PCR

Real-time PCR was used to detect the DNA in saliva and blood. A set of primers and probes were designed as follows: Primers: 5'-CCT CAA AGA AAA GCT GCG TGA

T-3' (forward), 5'-CTG ACC TGA AGC ACT TGA AGG A-3' (reverse), and TaqMan BHQ-Probe 1:5'-FAM-TGA AAT CGG (G/C) CTC CCG CAG ACA-3', BHQ-Probe 2: 5'-HEX-TGA AAT CGA CTC CCG CAG ACA CC-3' (GeneCore Biotechnologies, Shanghai, China). The reaction system was set in 10 μL volume containing 5 μL 2 × Premix Ex Taq Mix (Takara), 0.25 μL of each primer (10 μmol/L), 0.125 μL of each probe (10 μmol/L), 2.25 μL RNase free water (Takara), and 2 μL of extracted DNA as the template for each reaction. Real-time PCR was performed using the Roche Light Cyler 480 Software release 1.5.0 (Roche, Mannheim, Germany) with the Endpoint Gene Analysis procedure. The PCR cycling program consisted of an initial activation step at 95°C for 30 seconds, followed by 40 cycles at 95°C for 5 seconds, 60°C for 30 seconds, and 40°C for 60 seconds. Negative controls using sterile distilled water instead of DNA were performed. 96 samples could be detected at one time and genotyping results could be seen in the output visually within 45 minutes.

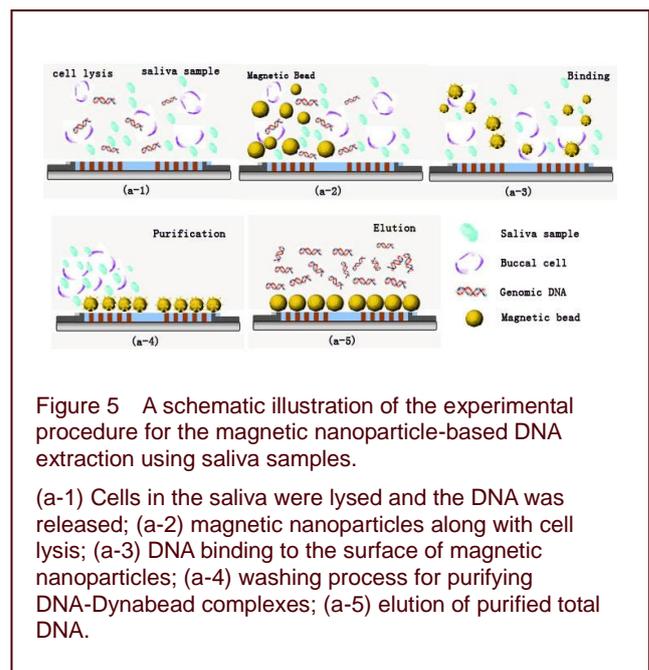


Figure 5 A schematic illustration of the experimental procedure for the magnetic nanoparticle-based DNA extraction using saliva samples.

(a-1) Cells in the saliva were lysed and the DNA was released; (a-2) magnetic nanoparticles along with cell lysis; (a-3) DNA binding to the surface of magnetic nanoparticles; (a-4) washing process for purifying DNA-Dynabead complexes; (a-5) elution of purified total DNA.

Conventional PCR and sequence analysis

To confirm the genotyping results, all the DNA samples obtained from saliva and blood were amplified with conventional PCR, and the PCR products were further sequenced directly. A 462-bp fragment was amplified in a total volume of 25 μL consisting of 12.5 μL of 2 × Premix Ex Taq Mix (Takara), 0.5 μL of each primer (10 μmol/L), 9.5 μL of RNase free water (Takara), and 2 μL of extracted DNA as the template for each reaction. The primers for the PCR were 5'-TCA CCT GGA TGG GAA AG-3' (forward) and 5'-TGT GGG AGT TTG GAG CA-3' (reverse) (BGI, Shenzhen, Guangdong Province, China).

The PCR was performed using an ABI 2720 (Roche, Mannheim, Germany) with a cycling program consisting of 95°C for 30 seconds followed by 40 cycles at 95°C for 5 seconds, 58°C for 30 seconds, and 72°C for 28 seconds. The amplified DNA products were visualized on 2% agarose gels after electrophoresis. Photographs (Tri-X pan, Eastman Kodak, Rochester, NY, USA) were taken by UV transillumination (WEALTEC, Sparks, NV, USA). The direct sequences of the PCR products were tested to confirm the genotyping results.

Statistical analysis

All analyses were performed with SPSS version 16.0 (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered significant (2 tailed). Genotype and allele frequencies between stroke and control groups were compared by chi-square test with Hardy-Weinberg equilibrium. Plasma homocysteine levels between stroke and control groups were compared through two-sample *t*-tests. The plasma homocysteine levels are expressed as mean \pm SD. Plasma homocysteine levels among the three genotypes were compared with analysis of variance followed by least significant difference tests.

Research background: Detection of C677T polymorphism in the MTHFR gene requires time-consuming procedures and invasive blood sampling, making it hard to perform screening for mutant genes in a large scale population.

Research frontiers: Magnetic nanoparticles can be used to extract DNA from saliva samples. However, no studies currently exist that describe methods using magnetic nanoparticles for gene screening in stroke patients.

Clinical significance: Our method, using magnetic nanoparticles and saliva samples, is sensitive, efficient, quick, easy to perform, noninvasive, painless, compatible with DNA extraction, and can be used for the screening of gene mutations.

Academic terminology: Magnetic nanoparticles are a class of nanoparticle consisting of magnetic elements that can be manipulated using magnetic fields. A small saliva sample was pipetted into a reaction vessel containing lysis buffer and magnetic nanoparticles. Lysis of saliva released DNA that bound to the magnetic nanoparticles, forming complexes that could be rapidly sedimented by centrifugation. These complexes were then washed twice with buffer and once with 70% ethanol. The complexes were then dried, and pure DNA was subsequently eluted. DNA could be purified from 10 samples within 40 minutes. The procedure was inexpensive and no special laboratory equipment was needed.

Peer review: This nicely logical method could be beneficial to clinics as a basic research and diagnostic technique for non-invasive DNA detection.

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