Journal of Advances in Medicine Science

2019

Journal of Advances in Medicine Science

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Research on the Interventional Effects of Stress Psychological Nursing Method on Patients with Acute Coronary Syndrome

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ABSTRACT

Objective: To investigate the interventional effects of stress psychological nursing method on patients with acute coronary syndrome (ACS). **Methods:** 100 patients with ACS who were rescued in the emergency department and department of cardiology of our hospital between January 2017 and December 2017 were enrolled. According to the random number method, the patients were divided into control group and observation group. There were 50 patients in each group. The interventions were given as routine nursing and stress psychological nursing, and the results of the two interventions were compared. **Results:** Before the intervention, there was no difference in the general clinical data between the control group and the observation group, which was not statistically significant; after the intervention for anxiety and depression, average hospitalization time, and off-bed time, the difference in data between the control group and the observation group on anxiety and depression was statistically significant. Moreover, the values of all the observation groups were lower than those of the control group. **Conclusion:** Applying stress psychological nursing method to the nursing of the patients with ACS can significantly improve their anxiety and depression, which significantly improves their quality of life, and enables the patients to better grasp the relevant health knowledge, at the same time, it obtains good intervention effects, which is worth promoting in a wider clinical scope.

1. Introduction

Acute coronary syndrome (ACS) is a group of clinical syndromes of coronary atherosclerotic plaque rupture or erosion, secondary to complete or incomplete occlusive thrombosis, including unstable angina (UA), acute myocardial infarction (AMI), and sudden death. If the patient's coronary arteries have developed atherosclerosis and merged with the luminal stenosis with multiple vessels, then the blood supply from vessels to the corresponding blood supply parts will be reduced or even interrupted, which can cause large-scale death of cardiomyocytes, namely acute myocardial infarction (AMI).[1] It is found through the survey that,[2] there are many

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factors that can affect the prognosis of AMI, including the patients' own factors, external environmental factors, etc., but one factor should not be ignored, that is, the patients' psychology. According to the survey,[3] patients with ACS often experience discomfort due to severe precordial pain and wheezing when clinical symptoms occur, after entering the hospital, there are complex psychological changes, the main of which are anxiety and depression. The kind of this negative emotion can have a direct impact on the patient's treatment and prognosis. 100 patients with ACS who were rescued in our hospital between January 2017 and December 2017 were enrolled in this research. The research on the intervention effects of stress psychological nursing method in the treatment of patients with ACS was conducted. The report is as follows:

2. Research Objects and Methods

2.1 Research Objects

A Retrospective analysis of 100 patients with ACS who were rescued in our hospital between January 2017 and December 2017 was performed. The age of the patients ranged from 39 to 87 years old, with an average age of 63± 24 years old. The research has been approved and passed by the Medical Ethics Committee of our hospital, and all patients and their families have signed informed consent forms.

2.2 Case Inclusion Criteria

(1) Patients who meet the diagnostic criteria of ACS;[4]

(2) Patients who were intervened by the stress psychological nursing method for the first time in our hospital.

2.3 Case Exclusion Criteria

(1) Patients with cognitive dysfunction;

(2) Patients with mental illness or other diseases that may affect the research results;

(3) Patients who were unwilling to sign informed consent forms, as the patients and their families didn't agree to the research.

2.4 Research Methods of Patients

2.4.1 Grouping Method and Results of Patients

According to the random number method, 100 patients were divided into a control group and an observation group, and each group had 50 patients.

2.4.2 Treatment Method of Patients

On the basis of the traditional routine nursing model,

the observation group patients added the stress psychological nursing intervention method. The main method is as follows:

(1) The primary emphasis of this method is the nursing and intervention of the patient's psychology, which requires the nursing staff to concern and care for the patients not only from the condition of the patients, but also from their psychology. The nursing staff is aimed at the "person" who is sick, not a "sickness". It is necessary to communicate with the family members of the patient to maintain the observation of the patient's emotional changes and the mutual understanding of each other. At the same time, the patient's questions about the disease should be answered with patience and meticulousness. Due to professional circumstances, patients cannot understand certain complex medical terms and medical knowledge. Therefore, nursing staff are required to teach and communicate with patients and their families in a language style that the patient can understand, which not only gives patients access to knowledge, but also increases patient trust in medical staff. During the work process, the nursing staff should pay attention to the guidance and appeasement of the negative emotions of the patients, when encountering the depression and irritability and other negative emotions of the patients, the nursing staff should not shut down or even ignore. They must communicate patiently with the patients, conduct joint research on the causes of such problems and help patients get out of the shadow negative of emotions, and help patients build self-confidence to overcome the disease.

(2) The patient is required to be in a comfortable environment. Keep the whole patient's ward clean and tidy, and keep the temperature between 22 °C and 24 °C, and the humidity at 50%-60%. The environmental indicators are measured and recorded daily, if the deviation is too large, manual adjustments are made in time. The ward is ventilated once a day in the morning, and it is strictly forbidden to place flowers and other items that are allergic in the ward. Negotiate with the patient's family and the competent physician to scientifically manage the patient's diet, and the dietary requirements are not only low sodium, easy to digest and absorb, but also require adequate nutrient supply; the supply of drinking water should also be carried out in accordance with the scientific quantity, and the cleaning of the patient's mouth should also be kept in mind; keep the patient's stool smooth, and avoid forceful defecation. As a whole, the ward is required to be quiet, and the voice of speech and action is kept as low as possible. The nursing staff also needs to be cautious when performing nursing operations on the patient to avoid the high decibel sound, so as not to irritate the patient and

cause discomforts.

(3) Conduct a comprehensive health education for patients. From the patient's admission to the hospital, the patient and family members should be fully informed about the knowledge related to ACS. The ultimate goal is not only for patients and their families to have a comprehensive understanding of the disease, but also for patients to build trust in the medical staff. The health education process needs to go through the entire process of hospitalization, and it is required to provide different knowledge at different phases of the disease based on the different needs of the patient and his or her family.

(4) The final guidance is given before the patient is discharged from the hospital. Before the ACS patient is discharged from the hospital, the patient should be evaluated for exercise endurance, and according to the results of the examination, each patient's exercise plan during the rehabilitation process after discharge should be made. The general principle is that any physical activity must be done in a way that absolutely avoids symptoms such as angina after exercise. The patient is monitored for BMI and is attempting to control the BMI to a level not exceeding 25 kg/m^2, but it is not recommended to use drugs for control, preferably with a green method of appropriate exercise and diet. After the patient is discharged from the hospital, if there is emotional fluctuation, he or she can communicate with the medical staff at any time, and can use the modern means, such as telephone, WeChat, etc. to keep liaison and communication with the other patients, which is also an effective measure to eliminate negative emotions in patients.

2.4.3 Observation Index

First of all, the general conditions, complications, and courses of disease of all enrolled patients were recorded. Before the intervention and after the intervention and before discharge, the patients were scored using the Self-rating Depression Scale (SDS), the Self-rating Anxiety Scale (SAS) and the physiological function and mental health modules in the SF-36 Quality of Life Scale (QOLS). The higher the scores of the SDS and SAS, the more severe the depression or anxiety of the patients are; and the higher the scores of the physiological function and mental health, the better the situations are. The health knowledge mastery questionnaires prepared by our hospital were used; the patients were scored before and after the intervention. The questionnaire describes the health knowledge assessment of patients from three dimensions, namely, health knowledge, good living habits and self-care awareness, which judges whether the patient is aware of health knowledge in the form of a count variable, and

maintains good habits and self-care awareness. The average hospitalization time and off-bed time of all patients were recorded and compared.

2.5 Statistical Analysis

Descriptive analysis of the data was performed, the results of measurement data were expressed by (x ± s), and statistical processing was performed using SPSS19.0 software. The count data comparison was inspected by x^2-test, and the measurement data comparison was inspected by t-test, $P<0.05$, the difference was statistically significant.

3. Results

3.1 The Comparative Results of General Clinical Data between Control Group and Observation Group

In the comparison of the general clinical data of the control group and the observation group, there was no difference in the gender, age, hypertension, diabetes, and disease duration of the two groups, and there was no statistical difference ($P>0.05$).

3.2 The Comparative Results of the SAS and SDS between Control Group and Observation Group before and after the Intervention

Before the intervention, the SAS scores of the control group and the observation group were respectively 55.64±5.34 and 54.87±4.88, which were not statistically significant ($P>0.05$); after the intervention, the SAS scores of the control group and the observation group were respectively 47.12±4.15 and 40.12±3.58, and the differences were statistically significant ($P<0.05$). Before the intervention, the scores of SDS in the control group and the observation group were respectively 56.37±3.55 and 57.38±4.52, which were not statistically significant ($P>0.05$); after the intervention, the SDS scores of the control group and the observation group were respectively 45.27±4.29 and 38.16±3.96, and the difference was statistically significant ($P<0.05$). Moreover, in the two items of SAS and SDS, the values after the intervention were lower than before the intervention. After the intervention, the difference between the control group and the observation group was also statistically significant ($P<0.05$).

3.3 The Comparative Results of the QOLS between Control Group and Observation Group before and after the Intervention

Before the intervention, the scores of physiological function module in the control group and the observation

group were respectively 60.12±7.24 and 59.87±6.48, which were not statistically significant (*P*>0.05); after the intervention, the scores of physiological function module in the control group and the observation group were respectively 71.54±5.67 and 82.46±8.5, and the difference was statistically significant (*P*<0.05). Before the intervention, the scores of mental health module in the control group and the observation group were respectively 56.66±6.85 and 56.84±7.48, which were not statistically significant (*P*>0.05); after the intervention, the scores of mental health module in the control group and the observation group were respectively 65.49±5.41 and 78.81±6.88. Moreover, in the two items of physiological function and mental health modules, the values after the intervention were higher than before the intervention. After the intervention, the difference between the control group and the observation group was statistically significant (*P*<0.05).

3.4 The Comparative Results of Average Hospitalization Time and Off-bed Time between Control Group and Observation Group

There were significant differences in the data between the control group and the observation group on the average hospitalization time and the Off-bed time (*t*=11.631, 110.199, *P*<0.05), and the values of the observation group were lower than those of the control group. The details are shown in Table 1:

Table 1. The Comparative Results of Average Hospitalization Time and Off-bed Time between Control Group and Observation Group

Items	Control Group (*n*=50)	Observation Group (*n*=50)	*t*-values	*P*-values
Average Hospitalization Time	15.37±2.58	9.38±2.57	11.631	<0.001
Off-bed Time	8.77±1.89	5.17±1.63	10.199	<0.001

4. Discussion

With the advancement of society and the improvement of living standards, there are more and more patients with ACS and their ages have been getting younger and younger in recent years. The classical symptoms of ACS at the time of onset are compression pain in the sternum, increased nausea and reactive sweating, which poses a serious threat to health,[5] and its unfavorable prognosis also makes the disease reduce the quality of life of patients with disease. Therefore, in the medical field, not only the emergency treatment of this disease is regarded as the focus, but also how to effectively care for the disease patients, and to maintain the patients' treatment effects have

become a focus issue.[6]

Stress psychological nursing intervention is a brand-new concept put forward in recent years,[7] and introducing stress psychological nursing intervention into patients' ACS nursing work is another innovation. After a certain theoretical research and practical operation,[8] this method has achieved certain success in nursing, and has gradually become a guideline in the nursing work of this disease. In patients with ACS, their negative emotions tend to be strong. From the time of onset to the recovery process, negative emotions dominated by anxiety and depression generally plague the patient, which ultimately leads to poor treatment outcomes for the patient due to psychological or mental burdens. The stress psychological nursing intervention scientifically plans the patient's rest time, highlights the patient's mental and psychological stress, and requires the nursing staff to treat the patient with empathy. This method has higher requirements for the quality of nursing staff. On the one hand, it requires rich clinical knowledge and practical experience. On the other hand, it requires high emotional intelligence including responsibility and communication skills.

5. Conclusion

From the results of this research, the intervention of stress psychological nursing method made the observation group significantly better than the control group in the indicators of each item. Not only the patients' anxiety and depression were well treated, but also the treatment effects were also significantly improved, which also saved a lot of money for the country and was well received by the patients.

In summary, Applying stress psychological nursing method to the nursing of the patients with ACS can significantly improve their anxiety and depression, which significantly improves their quality of life, shortens the length of hospitalization time, and deserves to be promoted in a wider clinical scope.

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ARTICLE

NAT2 Involed in the Susceptibility to Antituberculosis Drug-Induced Liver Injury

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ARTICLE INFO	ABSTRACT
	Objective: To investigate whether the N-acetyltransferase 2 (NAT2) gene is involved in the development of susceptibility to antituberculosis drug-induced liver damage (ATDLI) in patients with pulmonary tuberculosis in the Han nationality. **Methods:** We retrospectively analyzed 300 cases of tuberculosis patients without liver damage (control group) and 221 cases of tuberculosis patients with liver damage after antituberculosis treatment (case group). After antituberculosis treatment, genetic polymorphisms of NAT2 were analyzed in those patients using MassARRAY method. **Results:** Of the 10 tagged SNPs selected, In the promoter area of NAT2, the frequencies of T allele in rs4646243 and A allele in rs4646246 were significantly higher in the patients with ATDLI than controls (0.569 vs. 0.483, p=0.0062 and 0.567 vs 0.487, p=0.0103). The A allele of rs1115784 in the intron area showed a significant association with the development of ATDLI (0.389 vs 0.305, p = 0.0043). The frequencies of the mutated genes T and A in rs1041983 and rs1799930 in the second exon region were significantly higher than those in the control group (0.491 vs 0.360, p<0.00001 and 0.336 vs 0.212, respectively; p<0.00001). Two monomer domains were found in the 10 tag SNP sites, haplotype ht [TGAA] in monomeric domain 1 and haplotype ht [TAG] in monomeric domain 2, both were significantly more likely to be detected in the liver injury group than in the control group(p=0.0038, p<0.001, respectively). Two haplotypes were also found on the NAT2 gene: haplotype ht [CGGG] in monomeric domain 1 and ht [CGG] in block 2, and their presence means a lower risk of liver damage. **Conclusion:** NAT2 genotypes might have significant association with the risk of ATDLI in the Chinese Han nationality. By detecting the NAT2 gene and its haplotype, we can screen patients with a higher risk of liver damage before anti-TB treatment and take measures for the protection of patients.

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1. Introduction

Overall, 5-15% of the estimated 1.7 billion people infected with M. tuberculosis will develop TB disease. However, among people infected with HIV, the probability of developing TB disease is much higher, other risk factors include under-nutrition, diabetes, smoking and alcohol consumption, etc. Currently, short-course chemotherapy for tuberculosis with isoniazid, rifampicin, ethambutol and pyrazinamide as the core is adopted[1]. A large number of clinical studies have shown that isoniazid, rifampicin and pyrazinamide are all likely to cause liver damage. Especially when they are used in combination, the incidence and severity of liver damage increase significantly[2-5]. Many risk factors are associated with the occurrence of hepatic damage caused by antituberculosis drugs, such as females, advanced age, HIV infection, hypoalbuminemia, alcoholism, hepatitis B or hepatitis C virus infection, and severe lung diseases with tuberculosis, etc[6-9]. Hepatic damage is the result of the complex interaction of these risk factors. However, when these risk factors are removed or balanced the risk of liver damage between different individuals is still very different, indicates that the individual's susceptibility may be the most important factor. In recent years molecular epidemiological studies have found a link between polymorphisms in certain drug-metabolizing enzyme genes and susceptibility to antituberculosis drug-induced liver damage [4,5,10], of which, phase II enzyme N-acetyltransferase 2 (NAT2) , the first enzyme in isoniazid metabolism, was considered as a target enzyme in the study of genetic polymorphisms and antituberculosis drug-induced liver damage[11,12].

In this report, using MassARRAY technology, a case-control study was conducted to investigate the relationship between NAT2 gene polymorphism and susceptibility to antituberculosis drug-induced liver damage in the Chinese Han nationality.

2. Research Objects and Methods

2.1 Research Objects

A total of 528 patients with primary and recurrent tuberculosis who met the inclusion criteria from May 2010 to March 2016 were recruited. After a retrospective analysis, the patients were divided into two groups, including 228 patients in the case group, who had liver damage after receiving first-line antituberculosis treatment (2HRZE/4HR). Antituberculosis drug-induced liver damage is defined as asymptomatic or hepatic symptoms after taking antituberculosis drugs, such as loss of appetite, nausea, and vomiting, etc., and includes at least one of the following con-

ditions: (1) Serum AST and/or ALT is more than 2 times the upper limit of normal (ULN) (or > 80 U/L), (2) Any increase in ALT, AST is accompanied by progressively elevated bilirubin (>2.5 mg/dl). A total of 300 tuberculosis patients taking the same antituberculosis drugs without drug-induced liver damage were selected as the control group. All patients included in the study were required to meet the following criteria: normal liver function tests at the beginning of chemotherapy and exclusion of any other factors that may cause liver damage, such as malnutrition, HIV infection, alcohol abuse, viral hepatitis, liver disease, cardiac insufficiency, and no use of other drugs that may cause liver damage, etc. During the course of treatment, the enrolled patients were asked to be closely monitored for changes in their liver function.

2.2 Methods

2.2.1 DNA Extraction from Human Peripheral Blood Mononuclear Cells

A peripheral blood sample of the above-mentioned tuberculosis patient was collected. DNA of the mononuclear cells in those samples was extracted using the whole genome DNA extraction kit (Tiangen Biotech, Beijing,China) according to the instructions and were immediately stored in a refrigerator at -20°C.

2.2.2 Selection of NAT2 Gene SNPs and Detection of Their Polymorphisms

According to the gene polymorphism published in the public SNP database and related literature reports, 10 tag SNP loci from the NAT2 gene of the Chinese Han nationality, were screened using haploview4.2 software. See Table 1 for details. Mass spectrometry (MassARRAY, Sequenom, USA) was used to detect the genotype of each SNP locus.

2.2.3 Statistical Analysis

Using SPSS 13.0 and haploview 4.2 software, t-test, x2 test and other statistical methods were used to analyze the basic parameters of the case group and the control group. Pearson's chi-square test or Fisher's exact test was used to analyze the distribution of genotypes and alleles at each site in the case and control groups. The test level is α=0.05.

3. Results

3.1 Analysis of the Basic Characteristics of the Two Groups

There was no statistically significant difference in gen-

der and age between the case group and the control group. The values of alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin (DBIL) and total bilirubin (TBIL) before administration were all within the normal range. Hardy-Weinberg equilibrium in control group, MAF test of all data, the percentage of non-deletional genotypes in the locus, and other gene locus related information are shown in Table 1.

Table 1. The basic of the 10 SNP loci

Name	Position	ObsH-ET	Pred-HET	HWpval	%Geno	MAF	Alleles
rs4646243	18291889	0.498	0.499	0.2496	99.4	0.48	T:C
rs4271002	18292548	0.281	0.286	1	100	0.173	G:C
rs4646246	18292941	0.495	0.499	0.3619	99.2	0.479	A:G
rs1115784	18299690	0.466	0.45	0.5332	99.8	0.341	G:A
rs1041983	18302075	0.483	0.486	0.0191	99.8	0.416	C:T
rs1801280	18302134	0.087	0.084	1	99.8	0.044	T:C
rs1799929	18302274	0.083	0.08	1	100	0.042	C:T
rs1799930	18302383	0.382	0.39	0.1749	99.8	0.265	G:A
rs1208	18302596	0.084	0.08	1	99.8	0.042	A:G
rs1799931	18302650	0.247	0.247	0.9113	100	0.144	G:A

Notes:

• Position: Position of the locus on the chromosome;

• %Geno: The percentage of non-deleted genotypes on the locus for all samples (the minimum value is 75%, less than this value is considered to have failed the test);

• MAF: The frequency of the last allele at this site (minimum value is 0.001, less than this value is considered to have failed the test);

• Alleles: major and minor alleles at the locus.

3.2 Analysis of Individual SNP and ATDLI

The differences in the distribution of genotypes and alleles at each site between the case group and the control group were analyzed using the Pearson chi-square test or Fisher's exact test. The results are shown in Table 2. As can be seen from the chart, there are statistically significant differences in the alleles of the NAT2 gene at the five SNP loci in the case group and the control group: rs4646243, rs4646246, rs1115784, rs1041983, and rs1799930. Among the five SNPs in NAT2, the T allele in the promoter region rs4646243 and the A allele in rs4646246 were significantly more frequently expressed in patients with antituberculous drug-induced hepatic impairment than in the control group (0.569 vs 0.483, p=0.0062 and 0.567 vs 0.487, p=0.0103, respectively), indicating that they both increased the risk

of liver damage. In rs1115784, which was located in the first intron region, the frequency of its A allele in the case group was also higher than that in the control group (0.389 vs 0.305, p = 0.0043). The frequency of T allele in rs1041983, located in the second exon region, was significantly higher in the case group than in the control group (0.491 vs 0.360, p<0.00001). However, this SNP change does not cause codon changes, and the expression product is a tyrosine-containing protein. Rs1799930 is also located in the second exon region of the NAT2 gene. The frequency of A allele is not only significantly higher in the case group than in the control group (0.336 vs 0.212, p<0.00001), but also this mutation makes the 197th code of the NAT2 gene changed from arginine to glutamine, The tertiary structure of proteins may therefore be altered.

Table 2. Differences in distribution of genotypes and alleles between case group and control group

Site	Alleles	Number of cases (case,control)	Frequency (case,control)	χ^2	P
rs4646243	T	257:195, 288:308	0.569, 0.483	7.504	0.0062
rs4271002	C	79:375, 103:497	0.174, 0.172	0.01	0.9206
rs4646246	A	255:195, 290:306	0.567, 0.487	6.59	0.0103
rs1115784	A	176:276, 183:417	0.389, 0.305	8.165	0.0043
rs1041983	T	222:230, 216:384	0.491, 0.360	18.247	1.94E-05
rs1801280	T	436:18, 570:28	0.960, 0.953	0.318	0.573
rs1799929	C	436:18, 574:26	0.960, 0.957	0.088	0.767
rs1799930	A	152:300, 127:473	0.336, 0.212	20.543	5.83E-06
rs1208	A	437:17, 571:27	0.963, 0.955	0.382	0.5363
rs1799931	A	68:386, 84:516	0.150, 0.140	0.2	0.6545

3.3 Analysis of Linkage Disequilibrium

In a certain population, the frequency at which two alleles at different loci appear on the same chromosome is higher than the expected random frequency. This phenomenon is defined as linkage disequilibrium. A non-random combination of certain alleles of this different locus is often inherited together. By using D'/r^2 and other methods to investigate the linkage disequilibrium between loci, it was found that there are 2 monomer domains in 10 sites of NAT2 gene, and there is a linkage disequilibrium in some sites in each monomer domain. The specific results are shown in Fig. 1. Above the monomer domain, we marked the rs number of the locus, which makes it easy to see which specific rs-sites have haplocells.
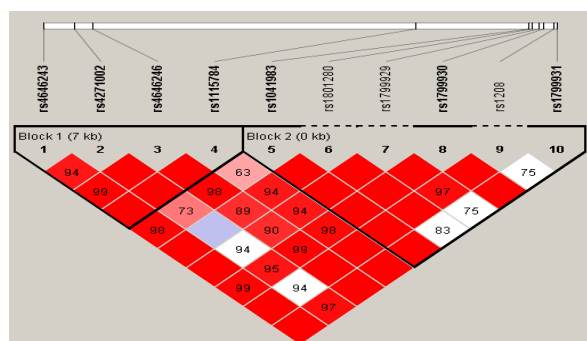
Figure 1. Pairing LDs between SNPs in a gene cluster

The value of each square represents the correlation coefficient of the tag's top-left and top-right SNPs. No data on the grid represents D'=1. A bold square represents haplotype formation in that particular SNP site.

3.4 Haplotype Analysis

A set of single nucleotide polymorphisms that are related to each other in a specific region of a chromosome and tend to be inherited globally to offspring, were known as haplotypes. For the monomer domain detected in the above linkage disequilibrium analysis, The distribution ratios of haplotypes in each monomer domain in the case group and the control group were calculated separately, Pearson's chi-square test was used to examine the association between overall genetic haplotype and disease (p<0.05). The results are shown in Table 3. Four haplotypes closely related to antituberculosis drug-induced hepatic impairment were found in two monomer domains consisting of 10 SNPs. Among them, the haplotype ht [TGAA] located in monomeric domain 1 and the haplotype ht [TAG] located in monomeric domain 2 appeared more often in the case group than in the control group (p=0.0043 and p<0.0001, respectively). Two haplotypes were also found on the NAT2 gene and were associated with a lower risk of liver damage: haplotype ht [CGGG] in monomeric domain 1 and ht [CGG] in monomeric domain 2.

Table 3. Relationship between haplotype and liver damage

Haplotypes	Frequency	Number of cases (case,control)	Frequency (case,control)	χ^2	P
Domain 1					
CGGG	0.475	193.0 : 259.0, 307.0 : 293.0	0.427, 0.512	7.409	0.0065
TGAA	0.341	176.0 : 276.0, 183.0 : 417.0	0.389, 0.305	8.165	0.0043
TCAG	0.168	77.0 : 375.0, 100.0 : 500.0	0.170, 0.167	0.026	0.8726
Domain 2					
CGG	0.584	231.4 : 222.6, 384.0 : 216.0	0.510, 0.640	18.083	2.11E-05
TAG	0.265	152.6 : 301.4, 127.0 : 473.0	0.336, 0.212	20.553	5.80E-06
TGA	0.144	68.0 : 386.0, 84.0 : 516.0	0.150, 0.140	0.2	0.6545

4. Discussion

In the process of isoniazid metabolism, isoniazid is first acetylated to acetyl isoniazid by NAT2 and then hydrolyzed to produce monoacetyl hydrazine, which is then catalyzed by cytochrome P450 2E1 and oxidized to a hydroxylamine form, and this intermediate metabolite can cause liver damage[13]. In the study of pharmacokinetics, the phenotypes of NAT2 were divided into fast acetylated genotypes, mid-acetylated genotypes, and slow acetylated genotypes depending on the rate of acetylation. This classification is mainly related to genetic polymorphisms, and studies have shown that the different NAT2 genotypes have a significant effect on their metabolic capacity[14-16]. The fast-acetylated genotypes convert isoniazid to acetylcholine significantly faster than slow-acetylated genotypes, and by further acetylation, acetyl hydrazine can be converted to diacetyl hydrazine, which makes acetyl hydrazine, an intermediate metabolite with strong hepatotoxicity, not accumulates, thereby increasing detoxification efficiency. On the contrary, in the slow acetylation type, the formation of a non-toxic derivative of diacetyl hydrazine from acetyl hydrazine may be hindered, which favors the formation of more toxic monoacetyl guanidine derivatives through cytochrome P450 2E1-mediated metabolism, causing liver damage.

Studies have evaluated the relationship between NAT2 genotypes and the risk of hepatitis. Through the study of 224 tuberculosis patients treated with antituberculous drugs, Huang et al.[17] reported that the incidence of drug-induced liver damage was 14.7%, and the incidence of severe liver damage was 6.3%. In the slow acetylation genotypes, the risk factors for drug-induced liver damage in the NAT2*6/6 and NAT2*6/7 genotypes were as high as 4.02. Compared with fast-acetylated genotypes, slow-acetylated genotypes have a high incidence of drug-induced liver damage and are more prone to severe drug-induced liver damage. The study concluded that NAT2 slow acetylation genotypes are important susceptibility factors for the development of antituberculosis drug-induced hepatic impairment. In India, Bose et al.[5] studied 218 patients with tuberculosis, including 41 patients with hepatic impairment after treatment, and the results showed that the appearance of NAT2*5/*7 and NAT2*6/*7 genotypes was significantly higher in patients with hepatic impairment than in patients without liver damage. The investigation of Cho et al.[18] pointed out that

in the study of 132 patients with tuberculosis in South Korea (18 cases of drug-induced hepatotoxicity), the risk of liver damage in patients with the NAT2 slow acetylation genotype is 3.8 times higher than in patients with fast acetylation, suggesting that the NAT2 genotype can be used as an effective predictor of antituberculosis drug-induced liver damage. Through the study of 50 tuberculosis patients in Iran, the results of Khalili et al.[19] also confirmed that the frequency of NAT2 slow acetylation genotypes in antituberculosis drug-induced hepatic impairment is much higher than that of fast acetylation genotypes. Wang Jinhe et al.[20] explored NAT2 gene polymorphisms in 32 patients with antituberculous drug-induced hepatic impairment and 35 patients without liver damage. The results of the study also proved that the NAT2 genotype is highly related to isoniazid and rifampin-induced liver disease, and moreover, the 857 codon mutation may be one of the susceptibility genotypes of hepatic toxicity in TB patients. However, the above studies only emphasize the relationship between the acetylation phenotype and liver damage in NAT genotypes, and most studies only verified the relationship between base mutations in the exon region of the NAT2 gene and liver damage.[21,22]

In this study, we screened out the target locus according to the genotype frequency in the SNP of the Chinese population reported in the public SNP database. Most of these sites are in the promoter and exon regions, and a few are in the intron region. Then, the frequency of each SNP locus allele in the case group and the control group was measured by the MassARRAY method. Further, we analyzed the association of these SNP loci and haplotypes with antituberculous drug-induced hepatic impairment. When analyzing NAT2, we found -9905C>T (rs4646243), -8853 G > A (rs4646246) in the promoter region, and -2098G>A (rs1115784) in the first intron, and in the second exon, 282C>T (rs1041983), 590G>A (rs1799930), are all significantly associated with antituberculosis drug-induced liver damage. The relationship between the first three SNP polymorphisms and antituberculosis drug-induced liver damage has not been reported before. Kim et al.[23] proposed that in the Korean population, if A replaces T at -9796 in the promoter of NAT2 gene, is closely related to antituberculosis drug-induced hepatitis, it is closely related to the occurrence of antituberculosis drug-induced hepatitis. Furthermore, in vitro experiments showed that the activity of luciferase containing the A allele was reduced, so it is believed that carrying this variant allele in the promoter will reduce NAT2 transcriptional activity. We speculate that the -9905 C>T, -8853 G>A mutation also reduces the expression of the NAT2 gene by reducing its transcriptional activity, thereby increasing the risk of liver damage. The reason for

the correlation between the first intron -2098 G>A mutation and drug-induced liver damage has yet to be confirmed. The mutation of 282 C>T, 590 G>A in the second exon (according to the international term consensus, also known as NAT2*6) increased the risk of occurrence of antituberculosis drug-induced liver damage, which is consistent with the results of Possuelo et al.[24]. In the study by Possuelo et al., 282 T genotypes and NAT2*6/6 genotypes were more susceptible to antituberculosis drug-induced liver damage, with risk factors of 4.3 and 5.7, respectively (P<0.01). The research of Huang et al.[17] also supports this view. However, the change of 282 C>T in coding region does not cause codon change, and the exact mechanism of the TT genotype prone to antituberculosis drug-induced hepatic impairment is still unclear. It may be because this base change causes a spatial conformational change in the NAT2-encoded protein, resulting in a change in protein function. The change in coding region 590 G>A caused the 197 codon of the NAT2 gene to be changed, and the amino acid at this position in the encoded protein was changed from arginine to glutamine. This leads to changes in the structure and function of functional proteins, resulting in a reduction in the ability of NAT2 enzymes to metabolize, thereby increasing the risk of drug-induced liver damage[15,23]. In addition, we also found 4 haplotypes closely related to antituberculosis drug-induced hepatic impairment in NAT2, of which 2 haplotypes increased the risk of occurrence of hepatic impairment in antituberculosis drugs: haplotypes in monomeric domain 1 ht [TGAA] and haplotype ht [TAG] located in monomer domain 2. While the other two haplotypes: haplotype ht [CGGG] in monomeric domain 1 and ht [CGG] in monomeric domain 2 are suggestive of a lower risk of liver damage, and they may play an important role in predicting the risk of drug related liver damage.

5. Conclusion

In summary, we used MassARRAY method to analyze 10 SNP sites in NAT2 and found 5 SNP sites and 4 haplotypes closely related to antituberculosis drug-induced liver damage. Therefore, we should be able to screen for NAT2 genotypes and haplotypes in TB patients using the MassARRAY method to predict the possibility of liver damage in that particular patient before taking antituberculosis drugs, and thus provide a scientific basis for individualized use of liver protection drugs.

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ARTICLE

The Application of Realtime Fluorescence Quantitative PCR for Prenatal Screening of Group B Streptococcal Infections

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ARTICLE INFO	ABSTRACT

Objective: In the prenatal screening, several different methods were used to detect the presence of group B streptococcus (GBS) infection, in this assay, the diagnostic value and clinical significance of the application of realtime fluorescent PCR were explored. **Methods:** A total of 86 women with 35-37 weeks pregnancy were enrolled, vaginal secretion samples were collected. Fluorescence PCR, bacterial culture and gene sequencing were used to detect whether there was GBS infection, and the results obtained were compared and analyzed. **Results:** 10 subjects were detected to be positive for GBS by fluorescence PCR (the positive rate was 11.6%), however, only 4 cases were positive for GBS by bacterial culture method (the positive rate was 4.7%). There was a statistically significant difference in the positive rate between the two methods (P<0.01). Compared with the results of gene sequencing, the detection of GBS infection by fluorescence PCR has an accuracy of 95.2%, and the sensitivity was 90.9% with 100% specificity. **Conclusion:** The application of realtime fluorescence quantitative PCR for the detection of GBS infection is significantly better than the use of bacterial culture method. Compared with the gold standard method (gene sequencing method), its detection efficiency, accuracy, sensitivity and specificity are relatively high. In summary, PCR for prenatal screening of GBS is worthy of promotion in clinical practice.

1. Introduction

Under normal circumstances, women have a variety of flora in the vagina, which interact with each other to maintain the balance of micro-ecology in the vagina. Once the vaginal flora is dysregulated, it may lead to the invasion of a variety of pathogenic microorganisms, which may induce genital tract infections. Especially for pregnant women, genital tract infections not only seriously endanger their physical wellbeing, but also become a major risk factor causing premature rupture of membranes, miscarriage, premature delivery, fetal growth restriction, stillbirth and other adverse events[1]. At present, GBS inspection has been listed as a mandatory item for prenatal screening of pregnant women in many countries. In China, many doctors have also paid great attention to the harm of this bacterium, and its inspection is gradually becoming one of the important contents of

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prenatal examination[2]. Gene sequencing is often used as a gold standard for the diagnosis of GBS infection. However, this method requires special equipment and is complicated to operate (the operation of which requires expertise), so it cannot be widely used[3]. Realtime PCR has the characteristics of high accuracy and specificity when used for gene analysis. Here, we analyzed and compared the role of PCR and bacterial culture in the prenatal screening for GBS infection, trying to find an efficient and simple method.

2. Materials and Methods

86 pregnant women who received prenatal screening were included. Inclusion criteria include: (1) no use of antibiotics 1 week prior to the collection of specimens; (2) Subjects were pregnant for 34 to 37 weeks. The vaginal secretion samples of those subjects were collected, and 3 samples were collected from each pregnant woman. GBS infections were detected using different methods within 48 hours, including bacterial culture, gene sequencing and PCR method. In the bacterial culture, the specimens were uniformly inoculated on a specific medium, placed under aseptic conditions, cultured at 37 °C, 5% CO_2 for 24h, and the strains were identified according to the morphology, staining, CAMP, etc. The PCR steps were detailed below, and as for gene sequencing, specimens are sent to a third-party testing center and tested according to their specific methods.

2.1 Fluorescence Quantitative PCR

DNA extraction was performed according to the manufactures instructions (Guangzhou biotron Biotechnology Co., Ltd.), and was briefly described as follows:

(1) Samples, 50ul of GBS positive or negative control were placed in 2 ml centrifugal tubes respectively. 350 μl of STE buffer, 30 μl of lysozyme solution, and 1 μl of GBS internal standard solution were added to the sample. The bacteria were fully resuspended in a vortex and then incubated at 37 ° C for 20 min. (STE buffer and lysozyme can be mixed in advance when processing multiple samples, and If the bacteria to be tested are of the genus Staphylococcus, 1 μl of staphylococcal lysin was needed.)

(2) 30 μl of buffer SDS and 10 μl of proteinase K solution were added to the bacterial suspension, vortexed and mixed, and then digested in a 65 °C water bath for 20 min. (When dealing with bacteria that are difficult to lyse, a further digested in a 95 °C water bath for 20 minutes was needed for the lyse of the bacterial cell wall.) 400 μl of lysate DL buffer and 400 μl of absolute ethanol were added to the above lysate, and vortexed for 20s.

(3) The mix was transferred into adsorption column placed in a collection tube, followed by centrifugation for 1 min at 10,000 x g each time the sample was applied. The column was then washed 500 μl GW1 and then with 600 μl GW2 buffers (diluted with ethanol before use), centrifuging each time for 1 min at 10,000 x g, an additional centrifuge at 10,000 x g for 2 min was performed to remove residual ethanol. The column was then placed in a sterile Eppendorf tube, 30 μl EB buffer (Preheated to 65°C) were added to the column and 3 minutes later it was centrifuged for 1 min at 10,000 x g to elute purified bacterial DNA.

(4) Columns were discarded, the DNAs were stored at 2-8 °C for immediately use, or stored at -20 °C for long-term storage.

The protocols for PCR amplification were as follows:

The GBS PCR reaction solution and GBS enzyme solution were thawed at room temperature, mixed on a vortex for 10 s, and then rapidly centrifuged for 10 s. The GBS reaction solution was mixed with the enzyme solution on a volume ratio of 44:1 on ice. 45 ul of the above reaction solution was mixed with 5 ul of the test sample DNA, GBS negative control DNA, and GBS positive control DNA, respectively. DNA amplification was performed using ABI2000 (USA) and the setting was listed in the table below, FAM was selected as the fluorescent channel of the target gene and VIC as the fluorescent channel of the internal standard gene.

Table 1. Fluorescence PCR amplification setting

Phase	Temperature	Time	Cycles	Results
1	50 ℃	2min	1 cycle	/
2	95 ℃	20s	1 cycle	/
3	95 ℃	5s	45 cycles	/
	60 ℃	34s		Fluorescence reading

2.2 Data Analysis

GBS infection positive by PCR test was defined as a typical amplification curve at FAM channel with a Ct value ≤ 40 and Ct value ≤ 35 at VIC channel. GBS infection negative was defined as Ct value > 42 or "undet" at FAM channel and Ct value ≤ 35 at VIC channel. Using gene sequencing test as the gold standard, bacterial culture and fluorescent PCR were compared for accuracy, sensitivity, and specificity in screening for GBS infection. The data were analyzed by statistical software SPSS22.0. Count data were represented by rate, $\chi2$ test was used for comparison between groups, P<0.05 was considered statistically significant.

2.3 Results

The positive rate of Group B streptococcal infection detected by fluorescent PCR method and bacterial culture method was statistically significant (P<0.05), the former had a positive rate of 11.6% (10 cases), while the latter had a positive rate of only 4.7% (4 cases). The accuracy, sensitivity and specificity of detection of group B streptococcal infection by fluorescent PCR method were significantly higher than those of bacterial culture method (P<0.05), as shown in Table 2.

Table 2. Results of different methods for detecting GBS infection (%)

Method	Positive (N)	Negative (N)	Accuracy	Sensitivity	Specificity
Fluorescent PCR	10	76	98.8	90.9	100
Bacterial Culture	4	84	91.8	36.4	87.2
Gene Sequencing	11	75	100.0	100.0	100.0

3. Discussion

There are many microbial species in the vagina constituting the normal flora. The balance of the vaginal microbial ecosystem was achieved through coordinating and mutually restricting between those microorganisms and the host. Bacterial dysregulation can lead to GBS infection[4]. Infection with GBS, in the third trimester of pregnancy, can lead to premature rupture of membranes, chorioamnionitis, amniotic fluid contamination, postpartum hemorrhage, cesarean section, as well as premature birth, infection, asphyxia and fetal distress[5]. Therefore, GBS infection screening should be considered as a routine prenatal testing to reduce the incidence of adverse pregnancy outcomes. The routine diagnosis of group B streptococcal infection is usually bacterial culture, However, the limitation of this method is that some infected pregnant women cannot be effectively detected, leading to poor prognosis.

In this study, the positive rate of group B streptococcal infection detected by fluorescent quantitative PCR and bacterial culture was statistically significant (P<0.05); the accuracy, sensitivity and specificity of fluorescence quantitative PCR in detection of group B streptococcal infection were significantly higher than that of bacterial culture (P<0.05). According to Yan Yanqun et al.[6], fluorescence quantitative PCR was an effectively method in the detection of group B streptococcal infection, which is consistent with this study. Compared to bacterial culture methods, fluorescent quantitative PCR has many advantages. For pregnant women, the time to wait for diagnosis

is shortened because the tests were done very fast. For laboratory personnel, it was easy to operate and quite simplified and does not require inoculation, cultivation and identification operations, which significantly improves work efficiency and repeatability[7]. Although, when using the realtime PCR method, no subculture is performed in the process of detecting bacterial infection, and without the live strain of the tested sample, subsequent bacterial susceptibility testing cannot be performed, makes it difficult to choose a reasonable antibacterial drug for treatment. Generally speaking, GBS is highly sensitive to ampicillin, penicillin and vancomycin, with no 'intermediate' or 'resistant' reports for those medicines in drug resistance tests. Therefore, these drugs can be used as first-line drugs for the treatment of GBS infection[8-11]. Relying on the unique nucleic acid sequence of GBS, and by fluorescent labeling and amplification, PCR can make it detectable for very small amounts of pathogens. The sensitivity of PCR detection is very high that the test results cannot be affected by the environment in which the specimen is stored and even dead GBS bacteria can be detected. The above-mentioned advantages of the PCR method for detecting GBS infection enable pregnant women with infection to have early drug interventions, thereby improving the safety of pregnancy and childbirth.

4. Conclusion

In summary, fluorescence quantitative PCR has a very advantageous accuracy and sensitivity for the diagnosis of GBS infection, and therefore, should be considered as a routine for prenatal screening.

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REVIEW

Comparison and Correlation Study of Polar Biomarkers of CKD Patients in Saliva and Serum by UPLC-ESI-MS

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ARTICLE INFO	ABSTRACT
	There are abundant reports on the use of aromatic amino acids and creatinineas biomarkers in serum and urine forCKD(chronic kidney disease). However, investigationsinto these bio-species in bio-fluids, such as saliva and sweat, are rarely reported. Increasing interest in non-invasive methods for medical diagnosis advocates for the testing of these bio-fluidstoidentify potential biomarkers for prompt clinical andpreliminary screening using advanced analytical equipment. Unstimulated whole saliva samples were obtained from twenty-seven CKD patients and an equivalent number of healthy individuals. Saliva was assayed with ultra-performance liquid chromatography coupled with electro-spray ionization tandem mass spectrometry (UPLC-ESI-MS) in hydrophilic interaction chromatography mode. The data were analyzed using a student's t test and receiver operatingcharacteristic (ROC) to evaluate the predictive power of several potential biomarkers (P<0.01) in saliva for preliminary CKD screening. Through testing of salivary samples between CKD patients and healthy individuals, we found three possible salivary biomarkers that demonstrated significant differences (P<0.01) from the nine reported species in serum and/or urine. The area under the curve (AUC) values for control vs CKDpatientsfor on L-phenylalanine, L- tryptophan, and creatinine were 0.863, 0.834, and 0.916, respectively. This is the first report to compare serum and urine biomarkers in saliva between CKD patients and healthy people. This study explores the potential of CKD diagnosis by saliva, and demonstrates a positive correlation between salivary and serum creatinine.

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1. Introduction

Chronic kidney disease (CKD) is considered to be a part of the rising non-communicable disease burden worldwide. Prevalence of CKD is estimated to be 8-16% world-wide[1]. Important risk factors for CKD include hypertension, diabetes mellitus, and obesity. Treatment of CKD is often neglected by patients because of a delay in diagnosing indicators in common clinical experiments when compared totheprogression of the actual pathological process. Recently, a comparison between control group and healthy group has becomeanincreasingly attractive and important goal.

Currently, biomarkers for CKD are often detected in serum and/or urine using various types of equipment, but this is not ideal becausethereisdiminishedcompliance of patients for these methods of sample collection. Moreover, frequent collection of blood during dialysis is particularly adverse for the elderly and infants. Thus,it is advantageous for investigators to developadditional means of a non-invasivemethod. One such method that should be tested for potential diagnostic ability is the use of biofluidssuch as saliva and sweat. Undoubtedly, saliva is the best option for a diagnostic alternative because of its safety and comfort in collection process. Sincemany compounds in blood make their way into saliva,it is a suitable indicator of the current state of the blood. A number of biomarkers that are used as indicators of pathophysiological states to differentiate between patients and healthy people can be readily found in saliva[2].For example, decreased eGFR (estimated glomerular filtration rate) indicates a reduction in renal clearance of a variety of proteins and nitrogenous byproducts that could be detrimental to the vascular system[3].

Upon activation of apoptosis, the endothelium sheds micro-particlesthat are regarded asbiomarkers of endothelial dysfunction[4] and are believed to behave as bioactive vectors[5]. The metabolism of amino acids is related to the function of kidney, therefore regarding abnormalities of amino acids discovered in CRF can show the loss of renal function[6]. Therefore, we can compare biomarkers identified byultraperformance liquid chromatography coupled to a electrospray ionization mass spectrometry(UPLC-ESI-MS) with diagnostic standard compounds and determine whether saliva could be an alternative medium to serum and urine for patients with CKD. The components of blood that are exchanged in the kidney can also be exchanged in salivary glands, so the concentrations of biomarkers in blood and urine may correlate with those in saliva. Furthermore, it would be a quicker, easier, and cheaper alternative for medical testing and diagnosis. However, factors such as the collection method, collection time, sex, body mass index, weight, and smoking can affect the global metabolite profiles of patients[7]. Diseases that have been previously reported to benefit from the application of saliva as a diagnostic tool range from cancer to other metabolic diseases, including periodontal disease[8], oral and breast cancers[9-12], and Sjögren's syndrome[13].

Nevertheless, salivary biomarkers of CKD certainly need to be compared with those discovered in serum and urine samples although saliva seems to have an attractive future in clinical research, pathology, and therapy. Since most of the compounds of these biomarkers are hydrophilic, we utilized UPLC-ESI-MS to focus on these compounds with a metabolic approach.

UPLC which uses high pressure and small diameter porous particles and is combined with electrospray ionization time of flight mass spectrometry enables us to obtain the properties of compounds with a higher peak capacity, better resolution, increased sensitivity, and greater selectivity at a high acquisition rate. This detection method is especially advantageous for detecting accurate molecules of polar compounds.

While genomics, transcriptomics, and proteomics can provide diagnostic support for various diseases, metabolomics can generate the greatest differentiation of compounds. Currently, there are few investigations reporting metabolites of saliva from CKD patients using UPLC-ESI-MS. Therefore, in this study, we aimed to validate salivary biomarkers of CKD patients using UPLC-ESI-MS for a general screening of CKD biomarkers currently utilized in serum and urine samples, and thus, confirm the potential of saliva for CKD diagnosis.

2. Materials and Methods

2.1 Materials

Acetonitrile (HPLC grade) was provided by Burdick & Jackson (USA). Distilled water was produced "in-house" utilizing an ULUPURE system (Chengdu Ultrapure Technology Co., Ltd, Chengdu, China). Ammonium formate was UPLC grade obtained from Adamas (Switzerland). Creatinine was from CNW(Shanghai).

L-phenylalanine and L-tryptophan were bought from Sangon(Sangon Biotech Co., Ltd, Shanghai, China). The stock standard solutions 0.2mg/mL of L-phenylalanine, L-tryptophan, and creatinine were prepared by dissolving L-phenylalanine, L-tryptophan, and creatinine in acetonitrile/water with 0.05% formic acid, respectively. These solutions were stored at 2-8 °C and the mix solvents acetonitrile/water prior to use were employed through diluting the stock solutions to obtain the respective concentra-

tions with.

2.2 Subjects

Patients suffered from CKD were foundin the West China Hospital of Nephrology. All the volunteers included 27 CKD patients and 27healthy volunteers. Patients (non-smokers) who have cancer, cardiac insufficiency, alimentary tract hemorrhage, respiratory failure or other diseases are excluded due to the fact that may have an impact on the biological indicators and clinical observations. The Ethics Committee of Sichuan University approved the study protocol which was carried out according to the Helsinki Declaration of 1975. All patients are Chinese, and a consent form was obtained and concurred to be responsible as the saliva donors of the experiments.

For patients with CKD without dialysis, participation was allowed if the serum creatinine clearance was below than 59 mL/min/1.73m^2 (measured directly by clearance technique). Twenty-seven healthy adults without history of diabetes mellitus, kidney disease or other diseases consisted of subjects, and were tested clinically at recruitment.

2.3 Saliva Collection and Sample Preparation

After fasting overnight, all the subjects were required to avoid eating, drinking, smoking, and oral hygiene procedures for at least several hours before sample's collection.

We obtained 2-4 mL of clear un-stimulated whole saliva between 9:00 and 11:00 a.m. venous blood samples were drawn from all patients from the antecubital vein (for CKD patients). Saliva was then sent to the laboratory for isolation. We added 400μL of the processed saliva to a mixture of methanol/ acetonitrile (1:3 v/v, 800 μL) to precipitate the proteins, before vigorous shaking for about 60s. Samples were then vortexed for at 13500 rpm for 21 min at 4 °C. After allowing samples to stand at the same temperature for 10 min, the supernatant was taken for UPLC-ESI-MS' analysis.

2.4 UPLC-ESI-MS Analysis

The identifications were performed on the UPLC-ESI-MS system consisting of a Waters ACQUITYTM Ultra Performance Liquid Chromatography system and a MicromassLCT Premier TM orthogonal accelerated time of flight mass spectrometer (Waters, Milford, USA).

The chromatographic separation was achieved with an ACQUITY UPLC BEH Amide column (100mm*2.1mm i.d, 1.7um, Waters, Milford, USA) with the sample temperature set to 8 °C.

The column is eluted utilizing with the solvent system as below with the flow rate as 0.15mL/min: (A) 95:5 acetonitrile-aqueous ammonium acetate (10mmol/L), (B) 50:50 acetonitrile-aqueous ammonium acetate (2mmol/l). After every injection, the auto-sampler was cleaned with a strong/weak wash cycle to remove residue. Each run took 20 minutes.

TOF/MS was performed using an ESI source, operating in positive ion mode. The data profile of positive ions ranges from m/z 50-1000. The capillary voltage was 3.2 kV in positive mode; the cone voltage was 30 V. The desolvation gas flow was set to 650 L/h at a temperature at 350 °C. The cone gas flow was obtained as 30L/h, and the source temperature was 110 °C.

In the recent study, intact mass data can be acquired using the TOF mass spectrometer fitted with alock spray enabled Z-spray ion source. A lock mass of leucine enkephalin (Sigma-Aldrich, L9133, lot 095K5109, Steinheim,Germany, [LE+ H]+, m /z, 556.2771) for positive ion mode was utilized to correct lock mass. The solution of LE was ivia a lock spray interface at the flow rate of 0.05 mL/min with the help of a second LC pump (Waters).

2.5 Data Processing and Analysis

A multivariate data can be identified by integrating and deconvoluting the mass spectral data matrix incorporating an integrated peak area for measured features (unique mass-retention time pair) by alignment, peak picking and deisotoping employing MassLynx 4.1 (WatersCorp, Milford, MA).The raw data were found correspounding to a multivariate matrix containing aligned peak areas with matched retention times and mass-to-charge ratios (m/z). The distinctions of metabolites in both saliva biomarkers' intensity in two groups were analyzed with the Students' T test.

The Students' T test was utilized to examine vital distinctions in the distributions between control group and CKD group. Then to estimate the diagnostic effectiveness of both possible biomarkers such as L-phenylalanine, L-tryptophan and creatinine with 95% confidence interval (CI) for CKD recognition and to find out a best cut-off based on the maximum corresponding specificity and sensitivity receiver operating characteristic (ROC) curves were also used. Two-tailed p<0.01 was taken to be remarkably significant.

This equipment's relative standard deviations (RSD) of peak areas and retention times about the sample of saliva were qualified, and so were the standard materials. The system stability is shown from the predicting data of QC sample. It proved that the developed method had good repeatability and stability. The healthy group was comprised of 27 healthy volunteers.Therewere17 males and 10 fe-

males. Patient group comprised 27 CKD patients,andthere were 16 males and 11 females.

3 Results and Discussions

3.1 Patients Descriptions

All 27 patients recruited in this research were pathologically and clinically diagnosed with CKD. No significant distinctions between CKD patientsand the control group can be seen in measurements of 7-ketolithocholic acid, choline, creatine, adenine, guanine, and betaine (7-ketolithocholic acid: P=0.022; choline: P=0.036; creatine: P=0.229; adenine: P=0.010; guanine: P=0.427; betaine: P=0.012). SIM (selected ion monitoring) showed that accurate molecular weights were provided for the 9 salivary biomarkers found in serum and urine and were fully separated with the correct gradient program.
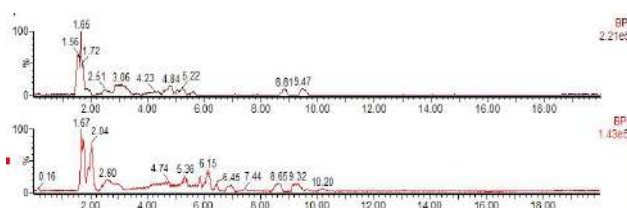

Figure1. Raw BPI of UPLC-MS data obtained from the saliva sample of health and CKDgroup at positive ESI mode

3.2 Linearity and Limit of Quantitation

In order to accurately evaluate the linearity of L-phenylalanine, L-tryptophan, and creatinine, each type of them was added to salivary sample at the concentrations of 0, 10, 30, 50, 150, 200, 300 ng/mL. The calibration profile was established by endogenous L-phenylalanine, L-tryptophan, and creatinine levels' subtraction from added saliva specimen. This assay was found to be linear up to 1000 ng/mL for all three biomarkers. The linear equations of calibration curves of L-phenylalanine, L-tryptophan, and creatinine in human saliva were y=2.003*x+555.367 with r^2=0.990, y=0.482*x+19.358 with r^2=0.999, andy=0.835*x-31.094 with r^2=0.994, respectively (y, peak area; x, concentration (ng/mL) of L-phenylalanine, L-tryptophan and creatinine; r, correlation coefficient). Under our UPLC-ESI-MS conditions, the limits of detection (LOD, S/N=3) of L-phenylalanine, L-tryptophan, and creatinine were 3.39, 15.54, and13.32ng/mL, respectively. The limit of quantification (LOQ) was explained as the level when the S/N ratio is 10. Therefore, LOQ was 11.31, 51.78 and 44.40 ng/mL for L-phenylalanine, L-tryptophan, and creatinine, respectively.
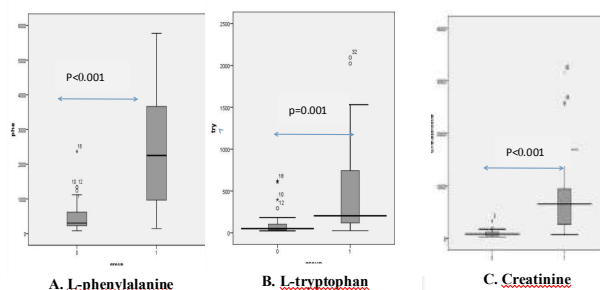

A. L-phenylalanine   **B. L-tryptophan**   **C. Creatinine**

Figure2. Box plots of the three biomarkers in differentiating CKD group from healthy group and the P values of the comparing of CKD with control were marked
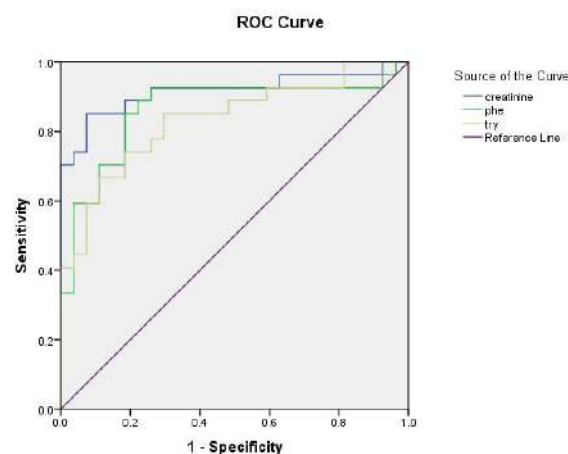

Figure 3. ROC analysis for three biomarkers in diagnosis of CKD

Table 1. Identification and Quantification of biomarkers of CKD patients found in saliva and reported[23] in serum and/or urine

Name	Retention Time	Pattern [M+H]	Actual Mass	Exact Mass	Mass Error	P
7-Ketolithocholic acid	1.56	C24H39O4	391.2884	391.2848	3.6	0.022
Phenylalanine	7.54	C9H12NO2	166.0860	166.0868	-0.8	<0.001
Tryptophan	7.21	C11H13N2O2	205.0966	205.0977	-1.1	0.001
Choline	7.02	C5H15NOd	104.1060d	104.1061	0.7	0.036
Creatine	9.11	C4H10N3O2	132.0761	132.0767	-0.6	0.229
Creatinine	4.33	C4H8N3O	114.0667	114.0660	-1.6	<0.001
Adenine	6.23	C5H6N5	136.0607	136.0623	0.1	0.010
Guanine	10.71	C5H6N5O	152.0573	152.0572	-0.5	0.427
Betaine	4.84	C5H12NO2	118.0863	118.0868	-0.7	0.012

Notes: [a]Selected peak [M-H2O+H]

Table 2. Remarkable salivary biomarkers' ROC curve analysis

| Metabo-lites	Aucs	95%Confidence Interval	Sensitivity	Specificity	Std. Error[a]
Phe	0.863	0.725-0.970	0.926	0.741	0.055
Trp	0.861	0.762-0.961	0.815	0.815	0.051
Creati-nine	0.916	0.832-1.000	0.926	0.741	0.056
LR mod-el	0.936	0.856-1.015	0.889	0.926	0.040

Notes: [a]Under the nonparametric assumption

3.3 Human Saliva Analysis

Saliva samples from 27 healthy individuals and 27 CKD patients were analyzed. We first measured the levels of the three metabolite biomarkers used to distinguish CKD from control. Figure 1 shows a typical BPI (base peak ion) of a saliva sample from both a healthy individual and a CKD patient (T_{RA}=7.86min; cT_{RB}=7.35min; T_{RC}=4.63min). Figure 2 demonstrates that the concentrations of these 3 biomarkers in CKD subjects were significantly higher than that of healthy individuals. Students' t-Tests were employed to study the distinctions' statistical significance in the distributions between the control group and CKD group and P<0.01 was thought to be remarkably significant (two-sided). The Student's t-Test is a parametric method and the p-values of these independent groups were marked in the figures. As the result of the p-values in Figure 2, we discovered that the distributions of L-phenylalanine, L-tryptophan, and creatinine in saliva samples were all significantly different (p<0.01). However, we found that the p-values of 7-ketolithocholic acid, choline, adenine, and betaine were all in the range of 0.01 to 0.05. In addition, the p values of creatine and guanine were larger than 0.05 in these two groups. Therefore, the differentiation power of creatine and guanine is weakest among all the biomarkers, and L-phenylalanine, L-tryptophan, and creatinine were the most promising biomarkers using this analytical technology. We will get further diagnostic power for the most prominent three to compare their ability in discriminating two groups.

To further investigate the diagnostic power of these 3 biomarkers, their ROC curves were evaluated. The ROC curves were constructed using SPSS and can indicate a diagnostic test's overall accuracy. In an excellent test, the relevant AUC value is 1.0 and with the angle's top at the apex left of panel the ROC curve is a right angle.

When AUC ranges from 0.5 to 0.7, which shows a relatively low diagnostic value. An AUC above 0.9 is considered to be highly accurate. Figure 3 shows the curve of the remarkably significant salivary biomarkers, and a detailed

AUC (95%CI), cutoff values, sensitivity and specificity are illustrated in Table 2. We found that L-phenylalanine, L-tryptophan, and creatinine have sensitivities and specificities of 0.926 and 0.815, 0.92 and 0.741, and 0.815 and 0.741, respectively. Furthermore, the AUC values for L-phenylalanine, L-tryptophan, and creatinine were 0.863, 0.861, and 0.916, respectively. These results indicate that amino acids including L-phenylalanine, L-tryptophan have a similar efficiency as a biomarker as creatinine in the diagnosis of CKD. The AUC value for these 3 significant biomarkers in combination were 0.936 (sensitivity=0.889, specificity=0.926).
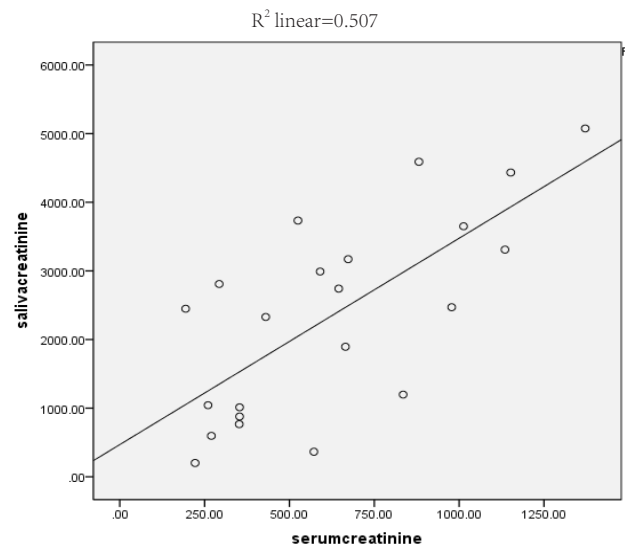
Figure 4. Correlation between saliva creatinine and serum creatinine
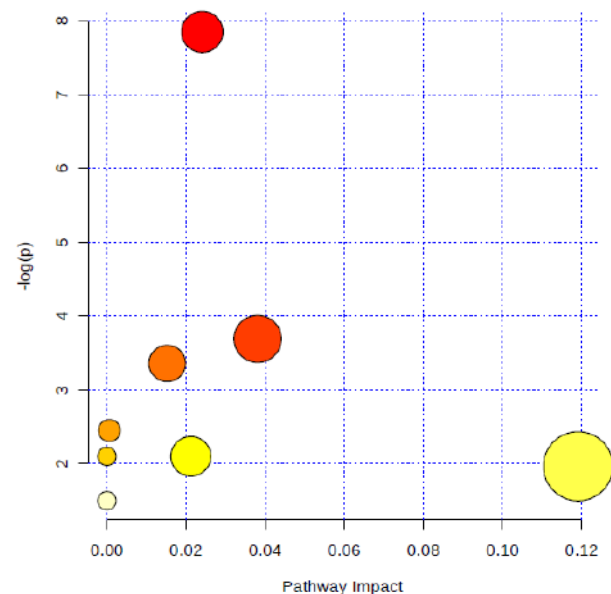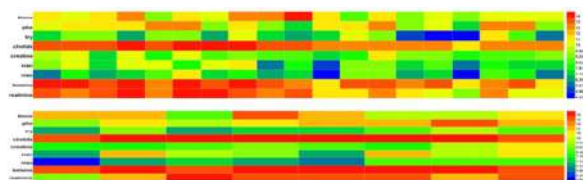

Figure 5. Summary of pathway analysis with MetPA

Figure 6. Hierarchical clustering analysis of metabolites identified by standard substances between males and females
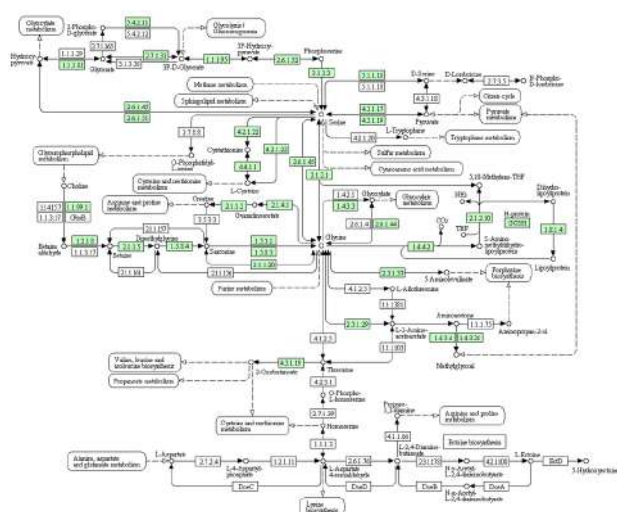

Figure 7. Construction of Glycine, serine and threonine metabolismmetabolism pathways in human CKD disease generated by KEGG.

3.4 Correlation between Saliva Creatinine and Serum Creatinine

To understand the relationship between serum and salivary creatinine and how to change it, we conducted a correlation analysis of the case groups. As shown in Figure 4, a significant correlation was indicated between serum creatinine levels and salivary creatinine concentrations($r=0.433$, $p<0.05$). Low levels of salivary creatinine are associated with a high serum creatinine levels. Additionally, the mean serum and the salivary creatinine concentration were discovered to be significantly higher in CKD subjects in comparison with those in the controls. Serum creatinine is positively correlated with that of saliva in CKD patients with $r = 0.507$ (Figure 4).

3.5 Heatmap Analysis

Heatmap(Figure 6) directly observed the variation in men and women which reflects some biomarkers induced by the sex. Further evaluation is needed to validate the compound in these groups to provide a better diagnosis approach in patients.

3.6 Biochemical Analysis

Chenodeoxycholic acid is the main bile acid, and 7-keto-lithocholic acid is regarded as the main intermediate in the intestinal bacterial transforming from chenodeoxycholic acid to ursodeoxycholicacid[14]. Zhao[15] et al found that in adenine-induced CRF(chronic renal failure) rats, the down-regulation of the 7-ketolithocholic acid is mostly based on the overgrowth of small-intestinal bacteria which causes deconjugation of bile acids and less efficient absorption.

Choline is a quaternary amino cationic alcohol that can be oxidized to betaine in the mitochondria of kidney cells. However, betaine might play a role reducing the risk of atherosclerosis in patients with CRF by decreasing the post methionine load hyper homocysteinemia[16]. Henricus[17] et al discovered the level of betaine's oxide was elevated in stage 3-4 CKD patients.

For phenylalanine and tryptophan, daily diet can be the only way because these two necessary amino acids cannot be synthesized in vivo. LewenJia[18] et al demonstrated that and phenylalanine and tryptophan's concentrations of CRF patients altered dramatically when compared with the control group due to the pathophysiology of CRF disease[19].

Slominska[20] et al indicated a close correlation between anincrease of adenine concentration in plasma from CRF patients and the adenine incorporation rate into erythrocyte adenine nucleotide pool. Furthermore, plasma adenine concentration and its erythrocyte incorporation rate had a strong correlation with erythrocytes' ATP levels. Moreover, Hayashie[21] et al found that guanine levels were decreased in both serum and urine of patients among the serum and urine biomarkers of CKD individuals. In addition to phenylalanine, tryptophan and creatinine are also found in rats[22-24]. Thus, Larsen[25] et al even investigated phenylalanine in dogs and rats with CKD. As shown in Table 1, previous research[26] about the biomarkers are included. The distribution of creatinine (P=0.229) and guanine (P=0.427) were not significantly different.

Other compounds that are important for the pathways of metabolomics include phenylalanine metabolism, glycine, serine and threonine metabolism, arginine and proline metabolism, purine metabolism except 7-Ketolithocholic acid (P=0.022). Both choline and betaine generate glycine, serine, and threonine metabolism. In serum andurine they are statistically significant, and thus, are possible surrogate biomarkers for subjects with CKD. Phenylalanine belongs to necessary amino acids, and phenylalanine hydroxylation activity in the kidney appears to be similar or even greater than that across the splanchnic area. In

addition, phenylalanine hydroxylation of the whole body rates also markedly decreased in renal patients[27,28].

Tryptophan is different from other amino acids because it is largely protein bound. The mechanisms for abnormalities in tryptophan metabolism in kidney disease remain complex, but decreased absorption of intestine and increased catabolism of liver have been suggested[29]. Tryptophan metabolism may act significantly in the progression of CKD except nutritional consequences because changes of tryptophan level are potentially vital[30].

Tryptophan and phenylalanine can be absorbed only from diet. Our results illustrates that the significant level changes in both phenylalanin and tryptophan compared with the controls.

It is reported that this had relation to CRF disease's pathophysiology[19]. The kidney is included in tryptophan metabolism in these ways:1) tryptophan derivatives is eliminated by it and 2) numerous enzymes involved in tryptophan metabolism mainly through the kynurenine pathway are produced by it[31]. Thus it is unknown whether tryptophan exchange in organs involves bound or free tryptophan. However, the uptake of the brain's tryptophan originated from blood seems to be dependent on bound ones[29]. When a meal containing meat or when a large amount of tryptophan is administered, the total concentration of blood tryptophan do not rise as high as that in normal condition[32]. Uremia is another important bio-fluid and altering in tryptophan metabolism appearing in uremia may be considered as several causes: (1)Tryptophan play a critical role in the protein turnover's regulation as it is an essential amino acid which has to be synthesized by absorbing protein; (2) The formation of neurotoxins may be included in tryptophan metabolism (i.e., quinolinic acid); and (3) It has been concluded that, except nutritional results tryptophan, tryptophan metabolism may be a vital part in the progression of CKD.[30] Furthermore, other amino acids such histidine, arginine, taurine and valine are also required [33-35]. In contrast, our results differ from other serum and urine studies, which demonstrate growing levels of L-phenylalanine[36]. Recently, Jie Wei[37] et al showed that this discrepancy between saliva and serum may originate from different metabolite speeds in different biological compartments. Each biomarker has a specific abundance concentration in a given compartment that is affected by both in-vivo and in vitro patho-physiological stimuli. Despite the fact that these markers are from different metabolic pathways, they are able to share certain metabolites with the same path of perturbation partly because they have similar metabolic enzymes that associated with the development of kidney disease. There may also be other causes that this type of instrumentation is highly sensitive and various factors may affect the performance of the apparatus and he sensitivity[38].

Therefore, abnormalities in amino acids of uremia caused by CRF have been ascribed to toxic effects of uremia on the amino acids' intermediary metabolism, low protein intake, deficient renal excretory and metabolic functions, loss of protein and amino acids by the dialysis procedure and changing distribution of some amino acids between the extra- and intra-cellular compartments[39].

These data in this essay may be valuable in understanding the mechanism(s) of the changes in phenylalanine and tryptophan metabolism occurring in disease state. It may also provide a possible cause for poor nutritional status and reduced blood flow that is often observed in uremia.

Creatinine's lipid solubility is very low excreted by the kidney and it is a waste product of metabolism[40] although its molecular mass is large. Virtually all the creatinine that is filtered at the glomerulus is excreted without reabsorption in the tubules. Thus, the level creatinine in the blood is used as an index of renal function[41]. To determine if there is an association between serum and salivary creatinine, a correlation analysis of the case groups were performed. We observed a positive correlation between serum and salivary creatinine in CKD patients (r=0.507). A positive correlation can be explained by the permeability of salivary gland cells between the membranes[42], CKD patients have increased serum creatinine levels, which creates a concentration gradient that facilitates increased diffusion of creatinine from serum to saliva[43]. In fact,Xia[44] et al found a similar positive correlation in both their cases (r=0.971). Our investigation demonstrates that the level of salivary creatinine correlates with that of serum creatinine. As this may be due to different sources, the results of the present study imply a correlated relationship between serum creatinine and saliva creatinine rather than a causation[37].

The basic measurements of diagnostic test accuracy are sensitivity and specificity. Hence, ROC analysis was produced to determine the diagnostic potential of saliva as an alternative medium (Fig. 3).Accuracy is measured by the area under the ROC curve. The highest area under the curve (0.916) got in our study was for salivary creatinine. This suggests that the screening of salivary creatinine is a good alternative diagnostic test to discriminate CKD patients from healthy individuals.

To the best of our knowledge, it is firstly to investigate creatinine (P<0.01) and adenine (P=0.01) in saliva detected by UPLC-MS. This novel method of detecting metabolite salivary biomarkers of CKD is noninvasive, simple, fast, and comfortable.

These results advise that the results of salivary bio-

markers of CKD has the possibility to contribute to ground breaking advancements in molecule-oriented monitoring and screening of CKD since its accuracy seems similar to traditional approaches. Thus, the proposed approach could complement conventional diagnostic method in preliminary screening. Moreover it may assist a quick, early and noninvasive diagnosis of CKD. However, obstacles still remain, as age, sex of the samples and collection time might contribute to changes in the markers measured.

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