The Impact of Freezing Temperature and Pretreatment Protocol on Solid Phase HR-MAS-MRS Metabolomes of Colorectal Cancer Tissue

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Abstract

Background: To explore the best pretreatment method of colorectal cancer tissue samples for metabolomics research based on solid-phase nuclear magnetic resonance.

Method: Taking mucosal tissues of colorectal cancer and divide it into 5 groups of 0.2cm*0.2cm*0.2cm. Pretreatment was performed as follows: I. Liquid nitrogen storage; II. Transfer to the -80°C refrigerator after storing in liquid nitrogen for 10 minutes; III. Transfer to the -80°C refrigerator after storing in liquid nitrogen for 20 minutes; IV. Transfer to the -80°C refrigerator after storing in liquid nitrogen for 30 minutes; V. -80°C refrigerator storage. The interval between tumor sample separation to pretreatment is less than 30 minutes. The tissue sample testing process is carried out on Bruker AVII-600 Spectrometer equipped with a high-resolution probe having a $1\text{H}/^{13}\text{C}$ magical angle rotation. The tissue samples were put into the NMR which run at a speed of 5000Hz for 10 minutes. NMR signals were collected and analyzed by Fourier transform, partial least squares discrimination analysis (PLS-DA). Corresponding metabolites and metabolic pathways were found in Human Metabolome Database (HMDB) according to the ppm's with variable importance of projection (VIP) [1].

Results: The content of pelargonic acid, stearic acid, D-Ribose, heptadecanoic acid, pyruvic acid, succinate, sarcosine, glycine, creatine, and L-lactate in liquid nitrogen storage group were significantly lower than the other groups ($P < 0.05$), the content of glycerophosphocholine in liquid nitrogen storage group was lower than the other groups ($P = 0.055$). Pyruvic, succinate and L-lactate are participating in glucose metabolism. Glycerophosphocholine, sarcosine, glycine and creatine are participating in choline phospholipid metabolism. This indicated that the glucose and choline phospholipid metabolism levels of the liquid nitrogen group were significantly lower than those of the other 4 groups.

Conclusion: Liquid nitrogen storage can slow down the glucose and choline phospholipid metabolism process of colorectal cancer tissue samples in vitro; liquid nitrogen can preserve tissue sample's metabolic state in the body. It is therefore the better way to store tissue sample than the other methods. clinical trial registry website: <http://www.chictr.org.cn/index.aspx>. Trial number: ChiCTR1900024640

1. Research Background

Metabolomics is a high-throughput detection technique for investigating metabolites in body tissues and body fluids. The metabolic characteristics of the tissue at a specific time point are called "metabolic fingerprint". Pattern recognition can be used for processing metabolic fingerprints and selecting biomolecules for disease diagnostics or treatment. These biomolecules are called "Biomarkers"[1]. Biomarkers play an important role in the diagnosis and treatment of many diseases, such as cancer, trauma, etc. There are much research regarding cancer based on metabolomics, such as the prediction of cancer stage, the monitoring of metastasis, the prediction of chemotherapy sensitivity, drug efficacy, toxicity evaluation etc[2–19]. Commonly used detection techniques in metabolomics include nuclear magnetic resonance (NMR) and mass spectrometry. The advantages of NMR are that the pretreatment of

samples is very simple and can be used for nondestructive testing[20–21], making it more suitable for clinic research. In addition, NMR equipped with a probe of High Resolution-Magic Angle Spinning Probe (HR-MAS) [22] can be used for the analysis of intact tissue samples. In addition, NMR only require small samples, which can be obtained from surgery[23–25] or hollow needle puncture[26–27]. Currently, most cancer research based on metabolomics take body fluid samples, but body fluids only reflect the metabolic characteristics of the central metabolic pool, not the actual metabolic characteristics at the cellular level.

Colorectal cancer is the third most common cancer in the world and the second most common cause of death from cancer[28–29]. It is of great significance to establish a metabolic model of colorectal cancer for precision treatment[30–33]. It is well known that the metabolic rate of tumor cell is much faster than that of normal cells. Usually, there is a time interval from sampling to detection, and the metabolic processes continue in cancerous tissues that have lost blood supply. From the perspective of the researchers, it would be desirable to get the metabolic messages of tumor tissues in vivo. Ideally, tissue metabolic research should provide a real-time “snap shot” of tissues conditions. However, the delay from pretreatment may lead to metabolic state changes, and the information obtained is no longer accurate. In other word, various enzymatic reactions in cells lead to changes in metabolic characteristics of tissues in vitro[34]. Therefore, freezing delay time and sample pretreatment methods are essential for maintaining specimen quality. Some researchers have discussed and analyzed the concept of freezing delay time. They proposed that delayed freezing leads to metabolic changes and the effect on the metabolic activity of breast cancer within 60 minutes after being isolated from the body is acceptable[34]. In terms of sample pretreatment methods, most researchers choose liquid nitrogen storage, -80°C storage or both based on their experience[35–41]. The metabolic characteristics of colorectal cancer is different from breast cancer, and the biological behaviors between them are also different. The conclusions about breast cancer research cannot be applied mechanically in colorectal cancer research. In this study, we are ready to explore the comparison of various pretreatment methods based on HR-MAS-MRS technology for colorectal cancer.

2. Method

This study is a clinical cohort research. The research protocol was approved by the Ethics Committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital. The clinical trial's registration number is ChiCTR1900024640. All parents who are participating in this research must sign an informed consent.

all authors had access to the study data and reviewed and approved the final manuscript.

2.1 Inclusion and exclusion criteria

All patients underwent planned surgeries at Sichuan Provincial People's Hospital, and the primary cancer nest was completely removed.

Inclusion criteria: 1) colorectal cancer; 2) clinical stage cT3NxMx-cT4NxMx; 3) age 18–80 years.

Exclusion criteria: 1) severe heart, liver, kidney and hematopoietic diseases; 2) history of metabolic and endocrine diseases such as diabetes and hyperthyroidism; 3) pregnant or lactating women; 4) people who suffer from mental illness unable to cooperate with treatment, suffer from psychosis illness, lack of self-control, and unable to express clearly; 5) participating in other clinical trial; 6) not signing informed consent.

2.2 Basic data analysis

First, we do a distribution test of the basic data. If the data is normally distributed, we use the form of median $\pm$ standard deviation to describe, and the Student's T test is used for difference comparison. If the data is non-normally distributed, we use the form of median (interquartile Distance) to describe, and rank sum test is used for difference comparison. For categorical variables, the number of cases and percentage are used to describe, and chi-square test is used for difference comparison. If $P < 0.05$, the difference between the groups is considered to be statistically significant.

2.3 Sample

In this research, we take 0.2 x 0.2 x 0.2cm samples of the mucosal layer of the primary cancer nest and divide these into 5 groups and put them in cryotubes numbered as (A1, A2, A3, A4, A5) (B1, B2, B3, B4, B5), etc... The tissue samples are processed in the following ways:

1. Stored in liquid nitrogen immediately after they are resected;
2. Transferred to the -80°C refrigerator after stored in liquid nitrogen for 10 minutes;
3. Transferred to the -80°C refrigerator after stored in liquid nitrogen for 20 minutes;
4. Transferred to the -80°C refrigerator after stored in liquid nitrogen for 30 minutes;
5. Stored in the -80°C refrigerator immediately after they are resected.

The thawing process of all samples is completed in five minutes.

All samples are clipped with a sterile blade and put into a 4mm zirconium OD rotor (the entire sample volume is 50ul, and the average weight is 8.8mg), then 10ul D2O is added to the vessel for locking and shimming[42–45].

2.4 NMR experiment

The testing process is carried on a Bruker AVII-600 spectrometer under 20°C , which is equipped with a $1\text{H}/^{13}\text{C}$ magic-angle spinning high-resolution probe (Bruker Biospin Rheinstetten, Germany). The rotor which contains the tumor tissue sample is inserted into the NMR instrument and the instrument runs at the speed of 5000 Hz for 10 minutes under room temperature. This instrument collects the NMR signals in the form of a spectrum and generates a document to record it. The detailed testing parameters are as follows: center frequency is 600.11MHz, sampling spectrum width is 20 ppm, accumulation times is 64 times, pre-saturation excitation pulse zgpr, excitation intensity is 5 microseconds (18 watts), and the pre-

saturation power is 1.8×10^{-5} watts for water signal. In this testing process, the sampling interval is 5 seconds.

2.5 NMR data analysis

The collected NMR signal data is saved and imported into MestReNova software (version 6.1.0, Mestrelab Research SL, Spain), which can obtain a one-dimensional NMR spectrum through Fourier transform; the ppm from 0.13 to 8.99 is segmented in unit of 0.01 ppm to obtain 896 chemical shift segments. Finally, we integrate each chemical shift segment to obtain the corresponding integral value, for the purpose of converting the spectrum signal to a digital signal. After data conversion, we import the data matrix into MATLAB R2013b (Mathworks, America), and then call out the least squares discriminant analysis (PLS-DA) of PLS toolbox (Eigenvector Research, America) to discriminate and analyze above digital information. Next, we establish discriminant models through different metabolic pool sources (variable Y) based on the NMR metabolic data (variable X) and discriminate the recognition performance of the model according to the model parameters and score map. If PLS-DA can distinguish different metabolic pools of metabolome data, the important chemical shift value (ppm) that affects the pattern discrimination is determined by the corresponding variable importance in the projection (VIP) value[46, 47]:

VIP value is used for determining how much the primary variables contribute to the principal component; its mathematical expression is following[48]:

$$VIP_i = \sqrt{\frac{P}{R^2(Y; t_1, \dots, t_m)} \sum_{h=1}^m R^2(Y; t_h) w_{hi}^2}$$

L-lactate

If the VIP value = 1, then these primary variables' contribution to the principal component are equal. If VIP value > 1, That means the weight of the variable for the construction of the principal component is more than that of other independent variables, so this independent variable is more important for explaining the outcome variable Y. We imported the information from ppm segment VIP > 1 into the HMDB database (<http://www.hmdb.ca/>, Canada) to identify these chemical shift segments. Finally we obtained a series of important landmark metabolites (biomarkers). The mapping of variable importance (VIP) is used to identify metabolites, and VIP value > 1 is considered having significant difference. Then we checked HMDB (Human Metabolome Database) to identify enzymes that catalyze related metabolism and key metabolites. After obtaining key metabolites from HMDB, the enzyme ID is converted to the enzyme ID of KEGG (Kyoto Encyclopedia of Genes and Genomes)[49]. To explore the potential correlation between metabolites (whether the level of metabolites is up or down), we map the enzyme to the metabolic pathways. Ultimately, the up or down of regulated metabolites are interconnected with seemingly disordered pathways. All data were not normalized and scaled.

2.6 Computing platform and tools

All software and websites (MATLAB R2013b, MestReNova 6.1.1, Human Metabolome Database, <http://www.hmdb.ca/>, Canada) are run or accessed under the Windows 10 operating system.

3. Result

The basic characteristics of patients are shown in Table 1. A total of 20 patients are enrolled into the study; 8 are diagnosed with ascending colon cancer, 6 with Sigmoid colon cancer, and 6 with rectal cancer. Most of patients are elderly, and their BMI fluctuates within the normal range.

As shown in Fig. 1, the effects of different pretreatment methods on metabolic status of samples were significant. Further analysis demonstrates that there were 60 ppms with VIP ≥ 1 and 11 characteristic metabolites related to glucose metabolism, glycerophosphocholine phospholipid metabolism and tissue sample corruption were identified (Table 2). As shown in Fig. 2, the relative amount of nonanoic acid, octadecanoic acid, ribose, heptadecanoic acid, pyruvate, succinic acid, sarcosine, glycine, creatine and lactic acid in the liquid nitrogen group were significantly lower than that in the other four groups ($p \leq 0.05$). Although not statistically significant, the content of glycerophosphocholine in the liquid nitrogen group was lower than the other four groups ($p = 0.055$). There was no significant difference among the other four groups except the liquid nitrogen group.

4. Discussion

After decades of development, metabolomics are now widely used in clinical researches. In particular, solid-phase NMR has become a favorite, with which clinical researchers can obtain accurate information from tissue samples. More and more researchers have begun to engage in solid-phase NMR metabolomics research.

The key to good metabolomics research is to collect metabolic information from tissue samples in vivo as much as possible. However, due to the limited conditions, it is usually impossible to perform NMR testing immediately after the tissue sample is separated. Generally, the metabolic processes continue after tissue samples are removed from the body, and metabolic levels are affected by the sample storage mode. Therefore, tissue samples must be temporarily stored under conditions that minimize the loss of tissue metabolic information and minimize the changes in metabolic state. Inappropriate pretreatment methods of tissue samples may change the metabolic information in vivo, resulting in the acquisition of wrong metabolic information by NMR testing, which would greatly affect the results and even cause trial failure. Therefore, we try to find the optimal pretreatment method for colorectal tissue samples in this research.

The results showed that liquid nitrogen storage had the least impact on the metabolic information of colorectal cancer tissues, mainly by slow down the glucose metabolism and choline phospholipid metabolism process of the tissue samples after in vitro. Compounds with statistically significant

difference included nonanoic acid, octadecanoic acid, ribose, heptadecanoic acid, pyruvate, succinic acid, sarcosine, glycine, creatine and lactic acid. Mapping these compounds into metabolic pathways, we found differences in glucose metabolism, choline phospholipid metabolism and possible corruption metabolic processes.

Glucose is the main source of energy for cell metabolism. It is mainly supplied via three metabolic pathways: glycogen synthesis, glycolysis and pentose phosphate (PPP)[50]. The carbohydrate metabolism process of cancer cells is different from that of normal tissue cells, and their rapid division and proliferation make cancer cells prefer glucose as energy supply materials. Cancer cells have extremely fewer organelles and are unable to carry out complex biochemical reactions. In the process of carbohydrate metabolism in cancer cells, the main metabolic mode is called Warburg effect, also known as aerobic glycolysis[51–53]. The level of Pyruvic and L-lactate, as the downstream metabolites of glucose metabolism, could reflect the speed of glucose metabolism. When the tissue samples is in vivo, glucose metabolism is under way all the time. But after removal from the body, this metabolic process continues or changes to produce some metabolites which is irrelevant to metabolic information in vivo. In this research, we found that the level of pyruvic and L-lactate are lower in liquid nitrogen group than that of the other groups. And as we can see in Fig. 3, pyruvic and L-lactate are down-stream metabolites of glucose metabolism, which means pyruvic and L-lactate are produced continuously along with glucose metabolism after in vivo, and the speed of pyruvic and L-lactate production in liquid nitrogen is slower than that of other groups. We can therefore conclude that the speed of glucose metabolism in liquid nitrogen is slower than that of other groups.

Choline, as the core metabolite of choline phospholipid metabolism, also had a lower level in liquid nitrogen than that of other groups, as shown in Fig. 3. We know that choline continues to decrease with choline phospholipid metabolism progresses. In this research, the level of glycerophosphocholine in liquid nitrogen is lowest among all groups, which means that the speed of choline consumption in liquid nitrogen is the slowest. We can conclude that liquid nitrogen storage could slow down or even stop the speed of choline phospholipid metabolism[54–56].

In addition, we also find that the levels of nonanoic acid, octadecanoic acid, heptadecanoic acid and ribose in the liquid nitrogen group were significantly different from those of the other four groups. Consulting relevant literature, we find that these compounds are related to the metabolic pathway of corruption. However, the specific pathway has not been reported in the literature. Based on the existing research data, we can make the following speculations: nonanoic acid, octadecanoic acid, heptadecanoic acid and ribose may be related to the corruption process of the tissue samples after in vivo. Liquid nitrogen not only can stop the internal metabolic process of the cell, but also the bacterial-related corruption. Specific metabolic processes, specific mechanisms and pathways need to be discussed after the relevant research is available[57–59].

This experiment manually screened 11 compounds with significant differences. However, there are 60 compounds with a VIP value greater than 1, and other compounds may play important roles in other

pathways. Therefore, we used MATLAB to draw heat maps of these compounds to analyze the changing trend of these metabolites visually.

Although we obtained much useful information from this research, the following shortages are noted. The sample size is not large, and it can only be used as a catalyst for methodological innovation. There is no statistical difference in the changes of most metabolites, only if the sample size is expanded in the future can we continue to explore the metabolic discipline of related metabolites and consequently explain the corruption metabolic pathways.

5. Conclusion

This research designed a rigorous, standardized tissue sample processing and analysis process, through a series of statistical calculations, and finally reached the following conclusion: liquid nitrogen preservation tissue samples can freeze the glucose metabolism and choline phospholipid metabolism for resected tissue samples. Among the pretreatment protocols we tested in this research, the loss of metabolic information from tissue samples stored in liquid nitrogen is the minimum, and the impact is the minimum. Whether it is freezing in liquid nitrogen for a short time or stored straightforward into a refrigerator at -80°C, the metabolism of the cancer tissue cannot be shut-down. Thus, the metabolomes obtained from NMR does not represent the status of cancer tissue in vivo because of the biochemical reactions are still processing. In conclusion, our work demonstrated that placing the resected colorectal cancer tissue sample directly into liquid nitrogen can reserve the in vivo metabolome information. We recommend it as the standardized pretreatment procedure of clinical tissue samples for HR-MAS-MRS analysis.

Abbreviations

HR-MAS-MRS

High resolution-Magic angle spinning-Magnetic Resonance Spectroscopy. NMR:Nuclear magnetic resonance. PLS-DA:Partial least squares discrimination analysis. VIP:Variable importance of projection. HMDB:Human Metabolome Database. PPP:Pentose phosphate. PPM:Parts per million

Declarations

Our study adheres to CONSORT guidelines.

Ethics approval and consent to participate:

The study protocol was reviewed and approved by the Sichuan Provincial People's Hospital. All study participants gave their written informed consent.

Trial registration

Chinese Clinical Trial Registry. Clinical trial registry website: <http://www.chictr.org.cn/index.aspx>.

Trial number: ChiCTR1900024640

Consent for publication

not applicable

Availability of data and materials

not applicable

Competing Interests

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service or company that could be construed as influencing the position presented in the manuscript entitled. We promised that authors have not published or submitted the manuscript elsewhere.

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Author's contribution

Conceptualization: HL, YW, HH, PCD and HJ.

Data curation: HL, YQH, GYY, LW

Funding acquisition: HJ, PCD

Methodology: YW, HL, PCD and HJ

Software: YW

Supervision: HH, PCD and HJ

Validation: HH, PCD and HJ

Writing – original draft: HL, YW

Writing – review & editing: HH, PCD, CDL and HJ

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Tables

Due to technical limitations, tables 1 to 4 is only available as a download in the Supplemental Files section.

Figures

Red: liquid nitrogen group

Green: liquid nitrogen 10min group.

purple: liquid nitrogen 20min group.

Blue: liquid nitrogen 30min group.

Pink: -80°C refrigerator group

Figure 1

PLS-DA analysis among 5 groups

Figure 2

Comparison of metabolite content under different treatment methods

Red: Indicates that the content of corresponding metabolites in the liquid nitrogen group is on the rise compared with that of other four groups (liquid nitrogen 10 minutes group, liquid nitrogen 20 minutes group, liquid nitrogen 30 minutes group and -80 C° refrigerator group).

Figure 3

Tissue sample metabolism related pathway.

Red area indicates high abundance, and the green area indicates low abundance.

Figure 4

Heat map of 60 different chemical Metabolites

Supplementary Files

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