Screening and identifcation of differentially expressed microRNAs in diffuse large B-cell lymphoma based on microRNA microarray

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Abstract

We aimed to screen and identify differentially expressed microRNAs (miRNAs) between diffuse large B-cell lymphoma (DLBCL) and control (lymph node reactive hyperplasia, LRH) groups, investigate whether miRNAs associated with DLBCL and could serve as potential therapeutic targets. Five DLBCL experimental samples and five control samples were obtained from fresh tissues. The exclusion criteria were expressed as follows: patients with other lymphoid diseases and patients undergoing chemical treatment. Firstly, fresh samples were analyzed using microRNA microarray to identify differentially expressed miRNAs. Next, three databases (Targetscan, microRNAorg, PITA) were used to intersection predict the potential target gene of the 204 differential miRNAs and performed Venn diagram of the results. Then, target genes of differential miRNAs were performed by Gene ontology (GO) and Kyoto Encyclopedia of Gene and Genome (KEGG) pathway analysis. Finally, to validate the microRNA microarray data, we performed Real-time reverse transcription PCR (qRT-PCR) for eight differentially expressed miRNAs (miR-193a-3p, miR-19a-3p, miR-19b-3p, miR-370-3p, miR-1275, miR-490-5p, miR-630 and miR-665) using DLBCL and LRH fresh samples. Two hundred and four miRNAs showed differential expression, including 105 down-regulated and 54 up-regulated miRNAs. The cut-off criterion was set as P value ≤ 0.05 and fold change (FC) ≥ 2. A total of 7522 potential target genes for the 204 miRNAs were predicted. Potential target genes were enriched in pathway in cancers, MAPK signal pathway, regulation of actin cytoskeleton, focal adhesion, endocytosis, Wnt signal pathway, axon guidance, calcium signal pathway, and PI3K/AKT signal pathway. Eight miRNAs validation by qRT-PCR, four miRNAs (miR-19b-3p, miR-193a-3p, miR-370-3p and miR-490-5p) were low expression in DLBCL ($P< 0.05$). The high expression of miR-630 in DLBCL was statistically significant ($P< 0.05$). Our results of identification of differentially expressed miRNAs, predicted the target gene of the differential miRNAs, enrichment of target gene biological functions and signaling pathway, will provide basis for researchers in identifying the pathogenesis of DLBCL, could serve as reliable biomarkers for precise diagnosis and as therapeutic target for improvement of therapeutic efficacy in DLBCL in the future.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of B-cell non-Hodgkin lymphoma in adults, and is known to be heterogeneous in clinical manifestations, tissue morphology, immune typing, prognosis[1, 2]. The sub-classification of DLBCL based on cell-of-origin by using the Hans algorithm[3], which can be classified into germinal center B-cell like (GCB) and non-GCB DLBCL subtypes. The five years overall survival is approximately 60-70% with standard chemotherapy of rituximab plus cyclo-phosphamide, doxorubicin, vincristine, prednisone (R-CHOP). However, the disease is known to have a recurrence rate of 30-50% shortly after treatment and progresses to the advanced stage[4]. Studies have pointed out that multiple targets, abnormal signaling pathways lead to DLBCL development, recurrence, and drug resistance, so potential therapeutic targets related to signal pathways become the key to improve outcomes for patients. The pathogenesis of DLBCL and effective therapeutic drugs for the disease are still under active exploration.

According to the central principle, DNA is transcribed into RNA, and RNA is translated into proteins. Abnormal expressed proteins lead to diseases or tumors. Therefore, treatment of abnormal proteins has become a research hotspot. However, without changing the regulation of gene expression in the upstream of the protein, abnormal proteins will still be produced. Since DNA is usually not easy to change, RNA becomes the target of regulation. However, in the upstream of mRNA and downstream of DNA, there are numerous non-coding RNAs (ncRNAs). Accumulating evidence suggests that ncRNAs play important roles in many aspects[5].

MiRNAs is a large family of the ncRNAs, the structure length is 19-22 nucleotides, and dysregulation of miRNAs is involved in cancer development and progression. Since the first miRNA (lin-4) was discovered by Victor Ambros in 1993, the role of miRNAs in tumor has been studied widely, which could regulate approximately 1/3 of mammalian expressing genes at the post-transcriptional level, and they can inhibit translation and/or degrade targeted mRNA[6].

The pathogenesis of DLBCL is multifactorial and complex, understanding the molecular mechanisms involved is important to identify new therapeutic targets. So our team wanted to explore the possible pathogenic factors for the occurrence and development of DLBCL at the three levels, including miRNA, mRNA and protein levels, so as to provide new ideas for deepening the understanding of the development and provide therapeutic targets of DLBCL. In our previous study, we used proteomics methods (iTRAQ) to explore the differentially expressed proteins in the DLBCL. P value < 0.05 and the expression multiple was more than 1.2 fold, a total of 335 differentially expressed proteins were identified. Through the KEGG pathway enrichment analysis, we found that the pathways in cancer, PI3K/AKT signaling pathway, alcoholism were significantly changed. The PI3K/AKT signaling pathway occupied large differentially expressed proteins, elucidating DLBCL associated with the PI3K/AKT signaling pathway proteins is required for the comprehensive treatment of DLBCL and for providing new insights into the pathogenesis of the disease[7]. Furthermore, we used formalin-fixed paraffin-embedded DLBCL tissue samples via immunohistochemistry to verify the expression of the differentially expressed proteins which explored by iTRAQ and associated with the PI3K/AKT signaling pathway, and we found that this signaling pathway plays an essential regulatory role in DLBCL[8].

In the current study, we employed microarray methods via Agilent Human miRNA Array to detect differentially expressed miRNAs between DLBCL and control (lymph node reactive hyperplasia, LRH) groups. 10 fresh tissue samples, including 5 samples of DLBCL and 5 samples of LRH were analyzed. Databases were used to predict the potential target gene of the differentially expressed miRNAs. The potential target gene was analyzed by gene ontology (GO) including biological process (BP), molecular function (MF), cellular component (CC) as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. Results of proteomics and MicroRNA array were comprehensively analyzed, 8 differentially expressed miRNAs, which potential target gene may be regulates the PI3K/AKT signaling pathway were verified by real-time reverse transcription PCR (qRT-PCR) in DLBCL and LRH. We showed and identified differentially expressed miRNAs, predict the potential target gene of the differentially expressed miRNAs, and key pathways, so our results provide a basis for further study on the causes, underlying molecular mechanisms, identifying molecular biomarkers for diagnosis, prevention, and effective treatment of DLBCL are critically important.

Materials And Methods

2.1. Patients samples

A total of 30 fresh tissues were collected from untreated patients that none of them received radiation or chemotherapy prior to surgery at the First Affiliated Hospital of Xinjiang Medical University, between January 2012 and December 2019, including 15 cases of DLBCL (5, GCB; 10, non-GCB) and 15 cases of LRH. All fresh lymph node tissues were collected according to standard operating procedures during the operation, and the samples were washed with isotonic saline. The liquid nitrogen surface was slowly frozen within 8 minutes, and then stored in a refrigerator at -80℃.The complete clinical and pathological data, HE sections and paraffin-embedded tissue samples of all patients were obtained, and the histological diagnosis of the tissues was confirmed and classified by two senior hematologists. The pathological classification was based on the 2016 revised 4th edition of the World Health Organization classification and was further subtyped using the Hans algorithm[3] by senior hematopathologists. Ten fresh frozen tissues (5 DLBCL and 5 LRH) containing adequate material for RNA extraction and next-generation miRNA Array. The validation cohort consisted of the above mentioned 30 fresh frozen tissues containing adequate material for RNA extraction for qRT-PCR. Written informed consent was also obtained from all patients and their families. All procedures involving human participants in this study comply with the ethical standards of institutions and/or national research councils and with the Helsinki declaration and its subsequent amendments or similar ethical standards. This study was approved by the ethics committee of the department of medicine, the First Affiliated Hospital of Xinjiang Medical University.

2.2. Screening and Identifcation of differentially expressed microRNAs

A brief workflow of microRNAs analysis is depicted in Figure 1.

2.2.1. RNA extraction

Fresh frozen tissue is between 0.5-250mg, removed from the refrigerator at -80℃, and transported in liquid nitrogen. Smaller tissues are best stored with RNA later and transported to the laboratory with dry ice for subsequent experiments. Total RNA was extracted from fresh frozen tissue by using mirVanaTM RNA Isolation Kit (AM1561) according to the operation instructions. Total RNA was quantified by the NanoDrop ND-2000 (Thermo Scientific) and the RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies).

2.2.2. miRNA microarray analysis

The Agilent Human miRNA Array（8*60K，Design ID:070156） was experimenting of the fresh samples. The sample labeling, microarray hybridization and washing was performed based on the manufacturer's standard protocols. Briefly, total RNA was dephosphorylated, denaturated and then labeled with Cyanine-3-CTP. After purification the labeled RNAs were hybridized onto the microarray. After washing, the arrays were scanned with the Agilent Scanner G2505C (Agilent Technologies).

2.2.3. miRNA microarray data analysis

Feature Extraction software (version10.7.1.1, Agilent Technologies) was used to analyze array images to get raw data. Next, Genespring software (version 12.5; Agilent Technologies) was employed to finish the basic analysis with the raw data. To begin with, the raw data was normalized with the quantile algorithm. The probes that at least 75.0 percent of samples in any 1 condition out of 2 conditions have flags in "Detected" were chosen for further data analysis. Differentially expressed miRNAs were then identified through fold change as well as P value calculated using t-test. The threshold set for up- and down-regulated genes was a fold change≥2.0 and a P value≤0.05. Target genes of differentially expressed miRNAs were the intersection predicted with 3 databases（Targetscan、microRNAorg、PITA）. GO analysis and KEGG analysis were applied to determine the roles of these target genes. Hierarchical Clustering was performed to show the distinguishable miRNAs expression pattern among samples.

2.2.4.Quantitative real-time PCR (qRT-PCR)

Based on the array results, eight differentially expressed miRNAs, including miR-193a-3p, miR-19a-3p, miR-19b-3p, miR-370-3p, miR-1275, miR-490-5p, miR-630 and miR-665, which potential target gene maybe regulates the PI3K/AKT signaling pathway were verified by qRT-PCR in DLBCL and LRH. Total RNA was isolated using Trizol Reagent (15596026ambion), and reversely transcribed into cDNA using the miRNA first strand cDNA kit (tail-adding method) (B532451,Sangon Biotech, Shanghai).The sequence of the Forward Primer for miRNAs is shown in Table 1, and Reverse Primer was provided by miRNA fluorescence quantitative PCR kit(dye method) (B532461, Sangon Biotech, Shanghai). Quantitative real-time PCR analysis was performed with the Applied Biosystems Prism7500 Fast PCR instrument, using miRNA fluorescence quantitative PCR kit. U6 was used as an internal control gene in miRNA PCR assays[9]. The miRNA expression level was determined by qRT-PCR and calculated using the $2^{-\Delta\Delta Ct}$ method in paired tissue samples[10]. The conditions of the PCR amplification were 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 30 sec.

2.3. Statistical analysis

All quantitative data are expressed as means ± standard deviation and were analyzed by the SPSS 18.0 software (SPSS Inc., USA). T-test and one-way ANOVA were used for statistical analysis. A *p* value less than 0.05 was defined as statistically significant.

Results

3.1. Differential miRNAs screening

A total of 2550 microRNAs were detected by Agilent Human miRNA Array. The comparison between the experimental group and the control group, probes marked 100% samples' Detected 'in at least one group were selected for step 2 differential screenings. For the group with biological repeats, FC≥2 and $P \leq 0.05$ were used as the screening criteria, 204 differential miRNAs were screened, among which 54 were up-regulated and 150 were down-regulated (Figure 2).

3.2. Target gene prediction

Target genes of differentially expressed miRNAs were the intersection predicted with 3 databases（Targetscan、microRNAorg、PITA）. TargetScan predicted 121174 target genes, PITA predicted 13401 target genes, and microRNAorg predicted 117485 target genes. 67875 overlapping genes (117485 in microRNAorg, 121174 in Targetscan), 3523 overlapping genes (117485 in microRNAorg, 13401 in PITA), and 2353 overlapping genes (121174 in Targetscan, 13401 in PITA) were identified. The intersection of the target genes predicted by the three databases predicted 7522 target genes (Figure 3).

3.3. GO functional enrichment analysis

Target genes of differentially expressed miRNAs were particularly enriched in molecular function (MF), and they were enriched in protein binding, metal ion binding, zinc ion binding. For biological processes (BP), they were enriched in regulation of transcription, DNA-dependent, signal transduction, multicellular organismal development. In addition, GO cell component (CC) analysis indicated that the Target genes were enriched in the nucleus, cytoplasm, and plasma membrane (Figure 4).

3.4. KEGG pathway functional enrichment analysis

According to the KEGG enrichment analysis, Target genes of differentially expressed miRNAs were enriched in Pathway in cancers, MAPK signal pathway, Regulation of actin cytoskeleton, Focal adhesion, Endocytosis, Wnt signal pathway, Axon guidance, Calcium signal pathway, and PI3K/AKT signal pathway(Figure 5).

3.5. Hierarchical Clustering

Through hierarchical clustering analysis, we found that the same samples can appear in the same cluster through clustering, and miRNAs clustered in the same cluster may have similar biological functions (Figure 6).

3.6. Validation of the differentially expressed miRNAs

Compared with DLBCL and control (Table 2), DLBCL showed statistically differences in the low expression of miR-19b-3p, miR-193a-3p, miR-370-3p and miR-490-5p ($P < 0.05$). The expression of miR-630 in DLBCL was high and the difference was statistically significant ($P < 0.05$) (Figure 7A).

Compared with three groups of GCB, non-GCB and control (Table 3), GCB and non-GCB showed statistically differences in the lower expression of miR-19b-3p, miR-193a-3p, miR-370-3p and miR-490-5p ($P < 0.05$), and in the high expression of miR-630 than LRH. There was no significant difference in miRNA expression between GCB and non-GCB (Figure 7B).

Discussion

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults, despite better therapeutic options and improved survival of the patients, and treatment resistance is a major clinical challenge of DLBCL where approximately 40% of the patients have refractory disease or relapse[11]. Therefore, it is urgent to find new drug targets and effective therapeutic drugs to improve the survival of DLBCL patients.

Nowadays, microarray analysis has become a widely used tool for generating gene expression data on a genomic scale and emerged as a promising and efficient tool for screening significant genetic or epigenetic alterations in carcinogenesis. MiRNAs are known as a large family of short ncRNAs. Its structurally consist of 19-22 nucleotides in length and functionally as one of the main regulators of gene expression in important biological and physiological environment like cell growth, apoptosis, proliferation, differentiation, cell motility, angiogenesis as well as disease formation, progression importantly in cancer cell invasion, migration, and metastasis[12]. In the present study, we analyzed the differentially expressed miRNAs of DLBCL and LRH two groups via Human miRNA Array. A total of 2550 microRNAs were detected, FC≥2 and $P \leq 0.05$ were used as the screening criteria, 204 differential miRNAs were screened, among which 54 was up-regulated and 150 were down-regulated. Studies have shown that miRNAs play an important role in the development of tumor. MiRNAs are also involved in the B cell development, such as B cell receptor, B cell migration/adhesion, the production of follicles, plasma cells, and memory B cells and so on[13]. With the extensive development of miRNAs in various fields, miRNAs are expected to receive more and more attention, such as molecular targets for diagnosis, prognosis prediction and treatment of DLBCL. Beheshti et al discovered circulating miRNA signature in a Smurf2-deficient mouse model that spontaneously develops DLBCL. They investigated this 10-miRNA signature (miR-15a, let-7c, let-7b, miR-27a, miR-10b, miR-18a, miR-497, miR-130a, miR24, and miR-155), and derived let-7b, let-7c, miR-18a, miR-24, and miR-15a with a classification rate of 91% for serum from patients with DLBCL versus normal controls. These circulating miRNAs seemed to distinguish between DLBCL subtypes and disease characteristics for clinicopathological diagnosis[14]. Ting et al found that miR-155, miR-17/92, miR-21, miR-224, and mir-146b-5p have value in predicting treatment response to chemotherapy in DLBCL. They suggested that miRNAs can be employed as an indicator to predict relapse or refractoriness after treatment with DLBCL[15]. So our study screen 204 differential miRNAs will provide a basis for researchers in identifying the pathogenesis of DLBCL, could serve as reliable biomarkers for precise diagnosis, and as therapeutic targets for improvement of therapeutic efficacy in DLBCL in the future.

MiRNAs are an important regulator of gene expression, because they will eventually lead to a decrease in the observed mRNA expression of target genes. Therefore, we predicted target genes of the 204 differentially expressed miRNAs with 3 databases（Targetscan（microRNAorg） PITA）. We found 7522 target genes of 204 differential miRNAs. Our results will provide a theoretical basis for other researchers to study the occurrence and development of DLBCL.

MiRNAs regulated target genes or itself to activate or inhibit signaling pathways, have also become a research hotspot for tumor development and therapeutic targets in DLBCL. Furthermore, KEGG analysis was implemented to determine the roles of these target genes. We found that target genes were enriched in Pathway in cancers, MAPK signal pathway, Regulation of actin cytoskeleton, Focal adhesion, Endocytosis, Wnt signal pathway, Axon guidance, Calcium signal pathway, and PI3K/AKT signal pathway. Shim H et al pointed out that miR-124 is decreased in DLBCL, and that miR-124 is a tumor suppressor by targeting NF-κB p65 in B-cell lymphoma[16]. Zhao CC et al indicated that SMAD5-AS1 inhibits DLBCL proliferation by sponging miR-135b-5p to up-regulate adenomatous polyposis coli expression and inactivate classic Wnt/β-catenin pathway[17]. Yoon S et al found that the PI3K/Akt signaling pathway is strongly enriched with targets of a few miRNAs in DLBCL[18]. In our study, some of the signaling pathways, such as pathway in cancers, MAPK signal pathway, Wnt signal pathway, PI3K/AKT signal pathway, which we found are consistent with relevant studies in DLBCL. But we also found a number of novel signaling pathways, such as Regulation of actin cytoskeleton, Focal adhesion, Endocytosis, Axon guidance, Calcium signal pathway, which are speculated to be related to the occurrence and development of DLBCL. The role of these newly discovered signaling pathways in DLBCL needs to be further studied.

According to our previous studies and literature review, the PI3K/AKT signaling pathway plays an important regulatory role in the occurrence and progress of DLBCL. So in this study we verified 8 differentially expressed miRNAs, which potential target gene maybe regulates the PI3K/AKT signaling pathway by qRT-PCR in DLBCL and LRH. We found that miR-19b-3p, miR-193a-3p, miR-370-3p and miR-490-5p are low expression in DLBCL (Figure 7A), and showed statistically differences ($P < 0.05$). The expression of miR-630 in DLBCL was high and the difference was statistically significant ($P < 0.05$). Compared with three groups of GCB, non-GCB and LRH, GCB and non-GCB showed statistically differences in the lower expression of miR-19b-3p, miR-193a-3p, miR-370-3p, miR-490-5p, and in the high expression of miR-630 in DLBCL ($P < 0.05$). There was no significant difference in miRNA expression between GCB and non-GCB DLBCL (Figure 7B).These differentially expressed miRNAs play a significant regulatory role in a variety of tumors, but there are few or no relevant studies in DLBCL.

MiR-19b-3p has been declared to be associated with favorable or unfavorable events in several cancers, its role is controversial depending on the tumor, and could be good non-invasive biomarkers for cancer detection. Tang Y et al indicated that miRNA plays an important role in the occurrence and development of intrahepatic cholangiocarcinoma (ICC). They collected 94 pairs of specimens of ICC tissues and adjacent

tissues, and 5 ml of peripheral blood of 342 ICC patients who underwent ICC resection before and one week after surgery. Luciferase activity assay was confirmed the regulation of miR-19b-3p on coiled-coil domain containing 6 (CCDC6). The results showed that miR-19b-3p levels were significantly higher in ICC tissues compared with adjacent tissues. Serum miR-19b-3p levels of ICC patients tended to decline after surgery, and were related to lymph node metastasis and histological grading of ICC. They confirmed that miR-19b-3p promoted the ICC cell proliferation, epithelial-mesenchymal transition (EMT), inhibited apoptosis, and knockdown of CCDC6 reversed these effects. These results suggested that serum mir-19b-3p level is an important biomarker for ICC diagnosis, and targeting the miR-19b-3p-CCDC6 axis may be a promising strategy for ICC treatment[19]. Song M et al confirmed that the roles of miR-19b-3p in pancreatic cancer. In this study human pancreatic cancer cell line was transfected with miR-19b-3p mimic and inhibitor. They found that miR-19b-3p overexpression promoted pancreatic cancer proliferation while miR-19b-3p inhibition decreased that. Flow cytometry analysis of cell cycle indicated that miR-19b-3p overexpression increased the percentage of pancreatic cancer in S phase while miR-19b-3p inhibition decreased that. The study demonstrates that miR-19b-3p promotes pancreatic cancer cells proliferation[20]. Park EJ et al indicated that the transfection of the miR-19b-3p impeded breast cancer cell migration. They also found Aquaporin-5 (AQP5) plays a role in breast cancer cell migration, and using bioinformatic analyses identified miR-19b-3p as putative regulator of AQP5 mRNA. Finally, it was confirmed that miR-19b-3p can inhibit the migration of breast cancer cells through exosome-mediated delivery by targeting AQP5[21]. Marcuello M et al showed that a plasma 6-miRNA signature (miR-15b-5p, miR-18a-5p, miR-29a-3p, miR-335-5p, miR-19a-3p and miR-19b-3p) could distinguish between colorectal cancer (CRC) or advanced adenomas (AA) and healthy individuals (controls). The study was included 213 individuals (CRC, 59, AA, 74, controls, 80). MiRNA expression was quantified by real-time RT-qPCR and data analysis was performed by logistic regression. They described in plasma, serum from patients with AA or CRC presented significant differences in the 6-miRNA signature compared to controls. The serum 6-miRNA signature could be a useful strategy to improve diagnostic performances of current CRC screening programmes[22]. We can see that miR-19b-3p has been implicated in some cancers, but its role is controversial, and no relevant studies were noted in DLBCL.

Many studies recently presented the crucial role of the miR-193 family, which comprise miR-193a-3p, miR-193a-5p, miR-193b-3p, and miR-193b-5p in health and disease biological processes by interaction with special target gene and signal pathway, which mainly act as a tumor suppressor[12]. Wang SS et al validated that miR-193a-3p is an anti-oncogene that plays an important role in health and disease biology by interacting with specific targets and signals, and it also inhibited the propagation and facilitated the apoptosis of hepatocellular carcinoma (HCC) cells. HCC patients with a higher level of miR-193a-3p expression had a favorable overall survival. They suggested that miR-193a-3p can be used as a promising biomarker for the diagnosis and therapeutic target of HCC in the future[23]. Lin M et al aimed to explore the role and mechanism of miR-193a-3p in CRC. They found that the expression levels of miR-193a-3p in human CRC cell lines were significantly decreased compared with that in normal colonic epithelium cell line. Then, plasminogen activator urokinase (PLAU) was verified as a direct target gene of miR-193a-3p. Over-expression of miR-193a-3p inhibited proliferation, migration and angiogenesis of CRC cell, however, forced expression of PLAU could rescue the inhibitory effects[24]. Chen ZM et al found that miR-193a-3p expression in pancreatic ductal adenocarcinoma (PDAC) tissue was significantly lower than in non-cancerous tissue. When overexpressing miR-193a-3p in PDAC cells, their multiplication ability was significantly inhibited, apoptosis was accelerated, and cell cycle was blocked in the G1 and G2/M phases. MiR-193a-3p may function as a tumor inhibitor in PDAC advance[25]. In our study, we found that miR-193a-3p is low expression in DLBCL, and showed statistically differences compared with LHR ($P < 0.05$). We speculated the functional role of this miRNA in DLBCL considered as a tumor suppressor, however, according to our knowledge. There is no relevant report in DLBCL.

MiR-370-3p plays an important regulatory role in a variety of tumors, and growing evidence has suggested that it is down-regulated and acts as a suppressor in numerous cancers. Many studies indicated that it plays a regulatory role on tumors through the regulation of target genes, can increase the sensitivity to chemotherapy drugs, and can be also used as a biomarker or a therapeutic tool in tumors. Li LM et al investigated that the role of miR-370-3p in chronic myeloid leukemia (CML) inhibits cell proliferation and induces CML cell apoptosis by suppressing PDLIM1/Wnt/β-catenin signaling. They concluded that the expression of miR-370-3p has markedly decreased in the peripheral blood mononuclear cells of patients with CML and in cell lines. The miR-370-3p in CML cells up-regulated proliferation, and down-regulated apoptosis[26]. Nadaradjane A et al investigated whether miR-370-3p can be used in vivo to increase the anti-glioblastoma multiforme (GBM) effect of temozolomide (TMZ). They used the model of LN18-induced GBMs in mice. The data indicated that the miRNA-370-3p/TMZ treatment was two times more efficient than the TMZ treatment for decreased the tumor volume. They supported that miR-370-3p could be used as a therapeutic tool for anti-GBM therapy[27]. Leivonen SK et al profiled miRNAs of matched primary and relapsed DLBCL by next-generation sequencing. Thirteen miRNAs showed significant differential expression between primary and relapse specimen. MiR-370-3p was markedly down-regulated in most relapsed DLBCL samples, and over-expression of miR-370-3p regulated target genes MAP3K8, PIK3R1, PIK3CG, PI3KCD, SYK, and resulted in down-regulated mRNA levels. They validated that miR-370-3p down-regulate genes on the PI, MAPK, and BCR signaling pathways, and enhanced chemosensitivity of DLBCL cells in vitro. The results demonstrated that differentially expressed miRNAs promote disease progression by regulating key cell survival pathways and mediating chemical sensitivity, thus represented potential novel therapeutic targets[28]. Our current experiment found that miR-370-3p was under-expressed in DLBCL, suggesting that it was may be inhibited the occurrence and development of DLBCL. Research of miR-370-3p in patient with DLBCL was rare, which calls for further study.

Recent studies have found that miR-490-5p is related to the occurrence and development of tumors, and play an important role in a variety of tumors. Wang J et al found that the expression of miR-490-5p was dramatically down-regulated in neuroblastoma (NB) tissues and cell lines using qRT- PCR. They found that significantly decreased miR-490-5p levels were correlated with lymph-node metastasis, and poor survival prognosis in NB patients, by used the Pearson Chi-square test and Kaplan-Meier analysis. The significantly over-expression of the miR-490-5p suppressed cell proliferation migration, invasion, induced cell cycle G0/G1 arrest, and cell apoptosis in NB cell lines. Myeloma overexpressed gene (MYEOV) was confirmed as a target gene of miR-490-5p by luciferase reporter assay. The results were demonstrated for the first time that miR-490-5p functions as a tumor suppressor in NB by targeting MYEOV[29]. Nevertheless Xiang M et al shown that miR-490-5p promoted proliferation of bladder cancer cells, inhibited apoptosis of the cells. They suggested that miR-490-5p has potential to become a new target for the future treatment of bladder cancer[30]. Yu Y et al explored targeting relationship between miR-490-5p and epithelial cell transforming sequence 2 (ECT2) in HCC, and influences of miR-490-5p and ECT2 on the stemness of HCC cells. They found that miR-490-5p was remarkably downregulated, and ECT2 was upregulated in HCC tissues compared with adjacent tissues. The expression of miR-490-5p was positively correlated with OS and DFS in patients with HCC. Over-expression of miR-490-5p restrained the proliferation, sphere formation ability, and stemness of HCC cells. MiR-490-5p inhibited the stemness of HCC cells through repressing the expression of ECT2[31]. In our study, differential expression of miRNAs between DLBCL and LRH was screened, and low expression of miR-490-5p was found in DLBCL with a statistical difference (p=0.001). We speculated that miR-490-5p might be involved in the occurrence and development of DLBCL, but no relevant studies were conducted on its regulatory mechanism in DLBCL.

Evidence has demonstrated that miR-630 is involved in multiple processes in cancer development and progression. Valera VA et al performed miRNA profiling in young prostate cancer (EO-PCa) patients and compared with PCa in older men (LO-PCa). They found that compared with EO-PCa and its normal counterpart, miR -630 was significantly up-regulated. Differentially expressed miRNAs provided insights into the molecular mechanisms involve in this PCa subtype[32]. Chen L et al observed that up-regulation of miR-630 inhibited oxaliplatin uptake in renal cell carcinoma by targeting organic cation transporter[33]. Pan X-M et al firstly provided the evidence that miR-630 inhibited papillary thyroid carcinoma(PTC) cell growth, metastasis, epithelial-mesenchymal transition by suppressing JAK2/STAT3 signal pathway, and that a potential therapeutic strategy through enhancing miR-630 expression might benefit PTC patients[34]. Li GW et al found that the expression of miR-630 was decreased in osteosarcoma (OS)tissues and cell lines. A low level of miR-630 was associated with late clinical stage and distant metastasis. Clinical assay indicated that down-regulation of miR-630 is closely linked to poor prognosis, and was an independent prognostic indicator for overall survival of OS patients. Functional studies showed that over-expression of miR-630 inhibited cell growth, colony formation, migration, invasion, EMT pathway, and promoted apoptosis in OS[35]. In this experiment we screened of DLBCL and LRH differentially expressed miRNAs, found that miR-630 for differentially expressed miRNAs. Used fresh frozen tissue by qRT - PCR test, verification miR-630 with high expression in DLBCL (P = 0.007). We hypothesized that miR-630 may be involved in the occurrence or development of DLBCL, but no relevant studies have been conducted on DLBCL. Since it plays an important role in other tumors, it is worth further study in DLBCL.

Conclusions

In summary, the present study screened 204 miRNAs showed differential expression between DLBCL and control groups via microRNA microarray. Altered expression levels of five miRNAs, including miR-19b-3p, miR-193a-3p, miR-370-3p, miR-490-5p and miR-630 may contribute to development in DLBCL. These findings provide valuable information to understand the pathogenesis of DLBCL, and may lead to the development of therapeutic strategies for using miRNAs for the treatment of patients with DLBCL.

Tables

Table 1. The sequence of the primer for miRNAs.

Primer	Sequence (5' to 3')
miR-193a-3p-F	GCAGAACTGGCCTACAAAG
miR-19a-3p-F	GCAGTGTGCAAATCTATGCAA
miR-19b-3p-F	AGTGTGCAAATCCATGCAA
miR-370-3p-F	CCTGCTGGGGTGGAA
miR-1275-F	GGTGGGGGAGAGGCT
miR-490-5p-F	GCAGCCATGGATCTCC
miR-630-F	CAGAGTATTCTGTACCAGGGAA
miR-665-F	ACCAGGAGGCTGAGG

F=Forward Primer

Table 2. Analysis of miRNA levels in DLBCL VS control groups .

Groups	miR-19a-3p	miR-19b-3p	miR-193a-3p	miR-370-3p	miR-1275	miR-490-5p	miR-630	miR-665
Control(LRH) (n=15)	1.134±0.576	1.132±0.503	1.200±0.722	1.172±0.728	1.186±0.788	1.164±0.655	1.095±0.524	1.085±0.430
DLBCL (n=15)	0.882±0.648	0.578±0.382	0.548±0.252	0.236±0.192	1.299±0.753	0.456±0.326	2.145±1.235	1.440±0.948
T value	1.126	3.397	3.299	4.817	-0.401	3.742	-3.030	-1.319
P value	0.270	0.002	0.004	0.000	0.692	0.001	0.007	0.198

DLBCL = diffuse large B-cell lymphoma, LRH = lymph node reactive hyperplasia.

Table 3. The analysis of miRNA levels in GCB, Non-GCB DLBCL subtypes and control groups.

Groups	miR-19a-3p	miR-19b-3p	miR-193a-3p	miR-370-3p	miR-1275	miR-490-5p	miR-630	miR-665
Control(LRH) (n=15)	1.134±0.576	1.132±0.503	1.269±0.904	1.172±0.728	1.186±0.788	1.164±0.655	1.095±0.524	1.085±0.430
GCB (n=5)	0.682±0.308	0.573±0.397△	0.582±0.263△	0.230±0.111△	0.975±0.568	0.437±0.406△	1.860±1.628△	0.949±0.372
Non-GCB (n=10)	0.982±0.760	0.580±0.396△	0.531±0.259△	0.239±0.228△	1.461±0.807	0.466±0.303△	2.287±1.061△	1.685±1.067

DLBCL = diffuse large B-cell lymphoma, LRH = lymph node reactive hyperplasia, GCB = germinal center B-cell like, △ compared with the GCB/Non-GCB and control groups, P<0.05, ▲ compared with the GCB and Non-GCB groups, P<0.05.

Abbreviations

DLBCL= diffuse large B-cell lymphoma, LRH= lymph node reactive hyperplasia, miRNAs= microRNAs, GO= Gene ontology, KEGG= Kyoto Encyclopedia of Gene and Genome, qRT-PCR= Real-time reverse transcription PCR, FC= fold change, GCB= germinal center B-cell like, ncRNAs= non-coding RNAs, iTRAQ= proteomics methods, BP= biological process, MF= molecular function, CC= cellular component, ICC= intrahepatic cholangiocarcinoma, CCDC6= coiled-coil domain containing 6, EMT= epithelial-mesenchymal transition, AQP5= Aquaporin-5, AA= advanced adenomas, HCC= hepatocellular carcinoma, CRC= colorectal cancer, PLAU= plasminogen activator urokinase, PDAC= pancreatic ductal adenocarcinoma, CML= chronic myeloid leukemia, GBM= glioblastoma multiforme, TMZ= temozolomide, NB= neuroblastoma, MYEOV= myeloma overexpressed gene, ECT2= epithelial cell transforming sequence 2, PTC= papillary thyroid carcinoma, OS= osteosarcoma

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analysed during the current study are available on reasonable request.

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Authors' contributions: Hai-Xia Gao wrote the paper, edited the original figures and tables. Xin-Xia Li, Wei Zhang and Wen-Li Cui reviewed this article. Other authors collected paraffin-embedded tissue samples, fresh tissue samples, and clinicopathological information.

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Figures

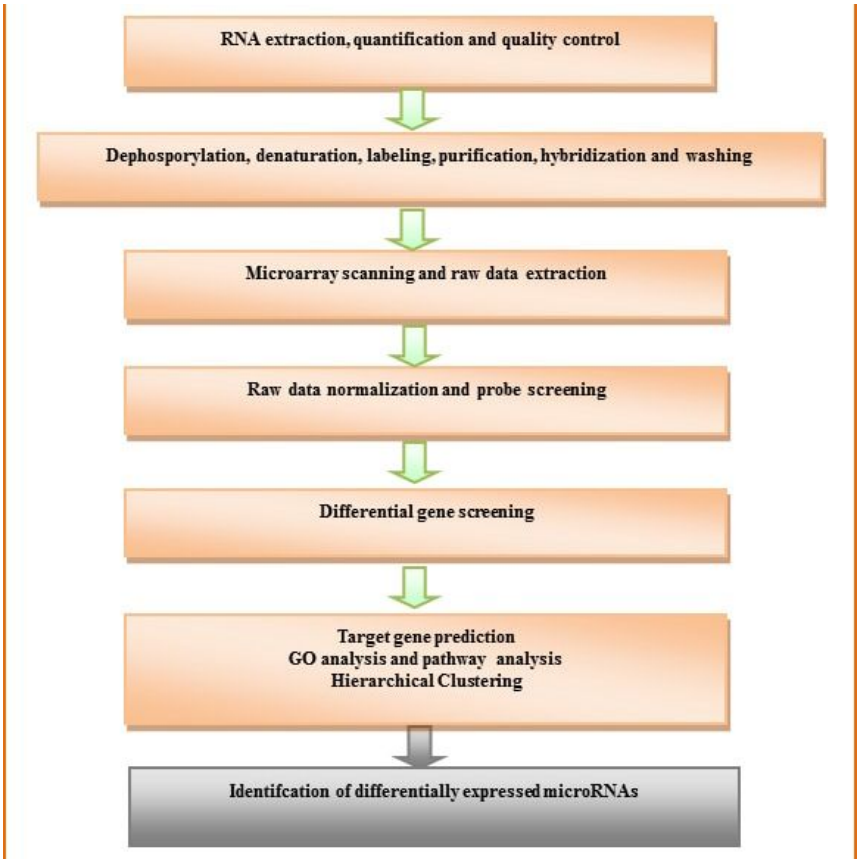

Figure 1

A brief workflow of microRNAs analysis is depicted.

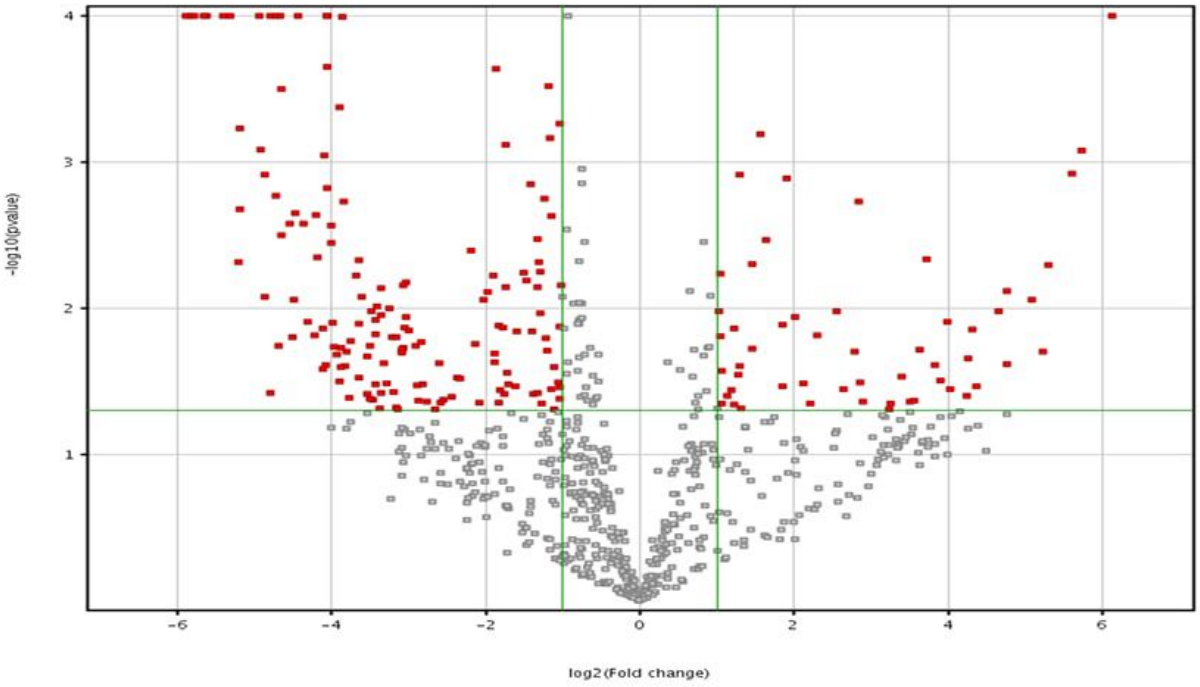

Figure 2

Differential analysis of microRNA microarray.

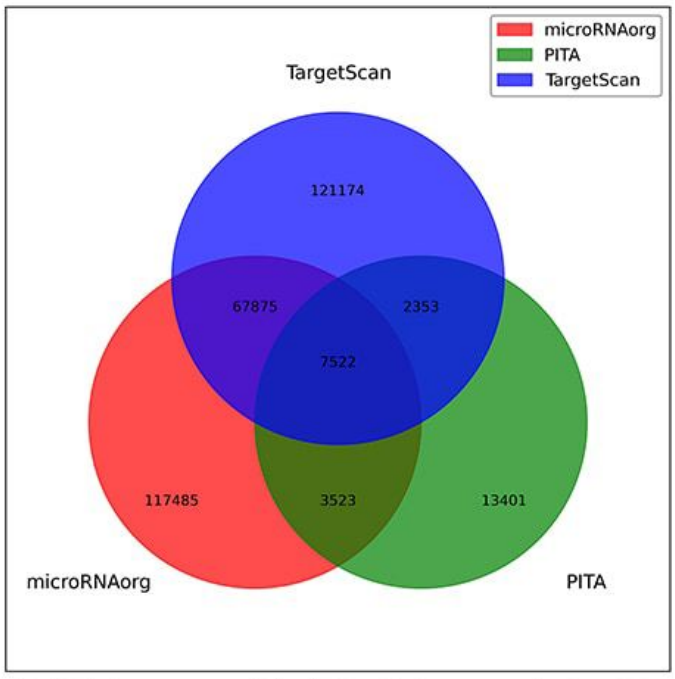

Figure 3

The target genes of the differentially expressed microRNAs predicted by the three databases.

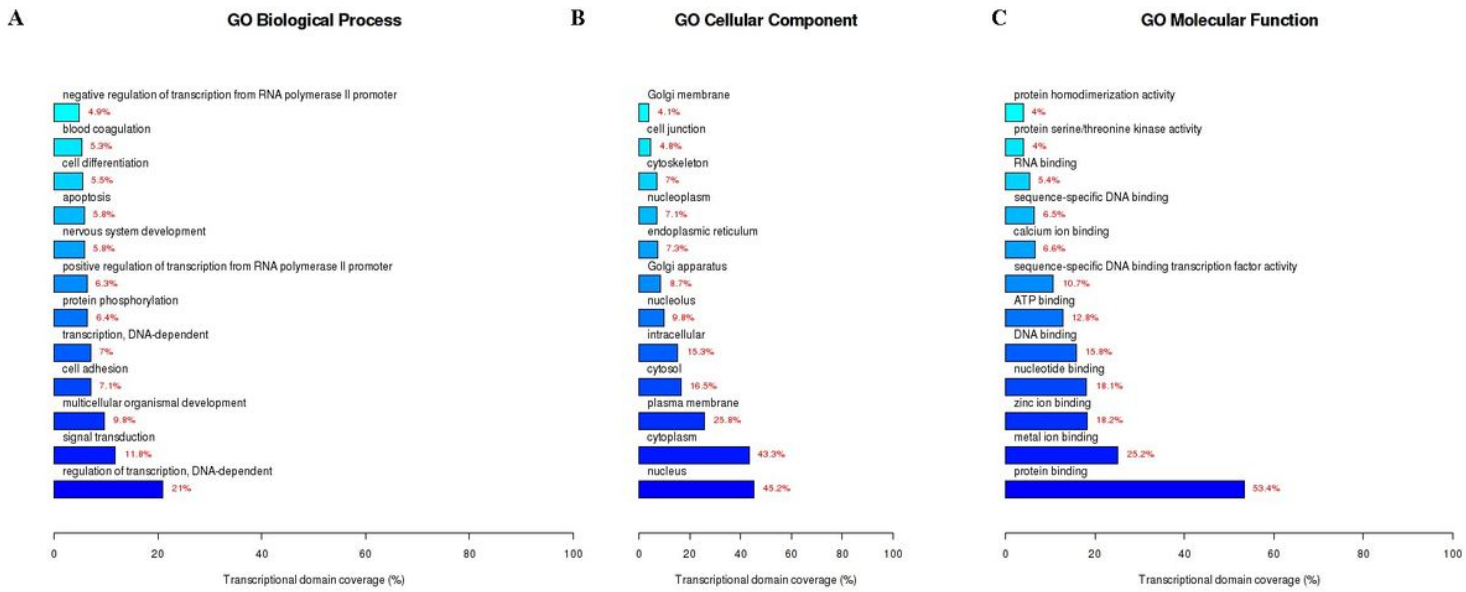

Figure 4

The target genes of the differentially expressed microRNAs performed by GO functional enrichment analysis. A. GO biological process (BP). B. GO cellular component (CC). C. GO molecular function (MF).

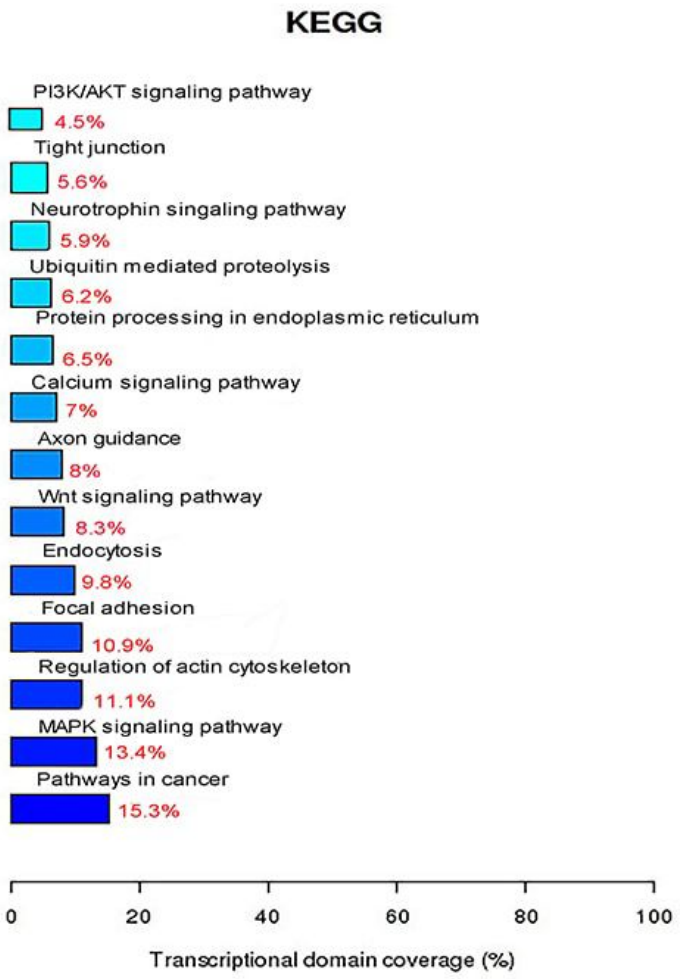

Figure 5

The target genes of the differentially expressed microRNAs performed by KEGG pathway functional enrichment analysis.

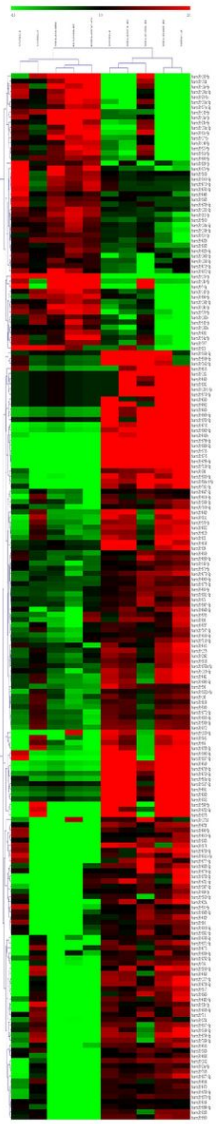

Figure 6

Hierarchical Clustering

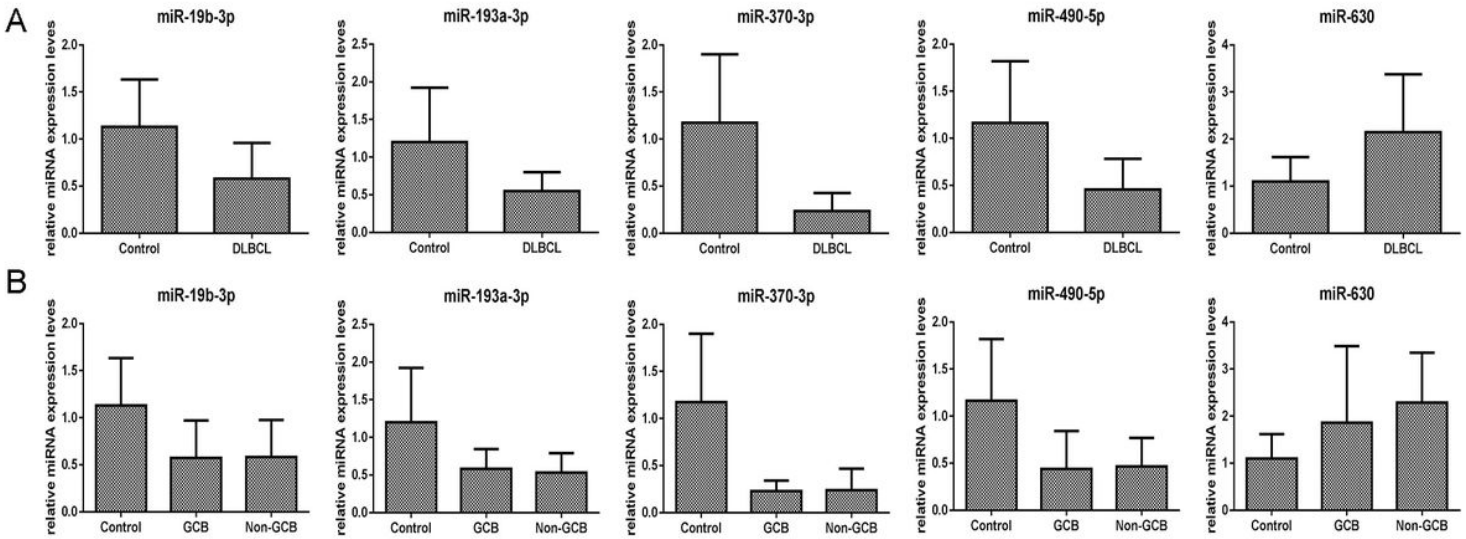

Figure 7

The expression level of the differentially expressed miRNAs (P < 0.05). A. Compared with DLBCL and control groups. B. Compared with GCB DLBCL subtype, non-GCB DLBCL subtype and control groups.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CaseVSControldifferentiallyexpressedmiRNAs.xlsx