MNCR and JPX lncRNAs have a significant oncogenic and biomarker role in the Isfahan Breast cancer population by regulating the MYC expression level: An integrated bioinformatics and experimental approach

Nasrin Nazempour
Zist Fanavari Novin Biotechnology Institute, Isfahan, Iran
Maryam Shakarami
Zist Fanavari Novin Biotechnology Institute, Isfahan, Iran
Fatemeh Tavakolikia
Zist Fanavari Novin Biotechnology Institute, Isfahan, Iran
Hadis Heidari
Zist Fanavari Novin Biotechnology Institute, Isfahan, Iran
Shadi Omidghaemi
Zist Fanavari Novin Biotechnology Institute, Isfahan, Iran
Mansoureh Azadeh (mazadeh@phd.iaurasht.ac.ir)
Department of Biology, Rasht Branch, Islamic Azad University, Rasht, Iran

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Abstract

long non-coding RNAs play essential roles in the regulation of the gene's expression level. The abnormal difference in the gene expression and transcriptome amount in the cells can make the various diseases in the human, including cancer. In this study, the expression of MYC and the two relevant and co-expressed lncRNAs were analyzed in the breast cancer (BC) samples as the potential BC biomarkers. An integrated bioinformatics analysis – including Microarray, RNA interaction, Pathway enrichment, and Gene ontology analyses – was performed to find novel differentially expressed genes in the BC patients. A real-time PCR experiment evaluated the expression of potential BC biomarkers found in the bioinformatics analyses. Bioinformatics and experimental analyses revealed that MINCR and JPX have a remarkable up-regulation in the BC samples and can be the two BC oncogene. Also, it is demonstrated that MYC could act as a tumor suppressor in BC patients by low-expression in the BC samples. All in all, the changes in the expression of MYC – affected by MINCR and JPX – can promote breast cancer pathogenicity. These three coding and non-coding RNAs can act as the acceptable prognostic biomarkers in BC.

2. Introduction

In the past years, it is demonstrated that long non-coding RNAs have a significant essential role in biological regulation. In the past decades, various studies revealed that lncRNAs act as important gene expression and biological regulators. Modifying the expression of lncRNAs can lead the typical physiological situations into several abnormal pathological statuses, including cancer. These non-coding RNAs have a remarkable role in the repression and activation of transcription, remodeling of chromatin, and enhancers. The abnormal changes in the expression of lncRNAs can lead to malignancy and cancer in mentioned approaches [1], [2].

In the past decades, the number of breast cancer patients has been increased remarkable [3]. Breast cancer is the most common cancer in the women population, and it is the second cause of cancer-related death in the woman [3], [4]. Due to the limitations of breast cancer diagnostic methods, it is vital to detect useful biomarkers which will overcome these limitations and reduce mortality. The early detection of breast cancer is essential for choosing the appropriate treatment and responding to it. Our knowledge of MINCR and JPX LncRNAs in breast cancer are primarily based on minimal, very limited data, and the fact that lncRNAs can be dysregulated in tumors has made them high potential candidates for our research in breast cancer [5]–[7]. JPX is one of the most well-known LncRNAs located on the X chromosome, and it can disable the X chromosome by activation of XIST [8]. This LncRNA can promote cancer, and it has been reported in multiple malignancies such as lung tumor, NSCLC, ovarian cancer, and HCC [9]–[11]. MYC induced long noncoding RNA (MINCR) takes part in the initiation, cell cycle progression, and metastasis of various tumors [12]–[14]. It is located on chromosome 8q24.3, which is upstream of the MYC gene that they are about 15000 kbp apart [15]. MYC regulates cell cycle development, and MINCR, by affecting the expression of these genes, can control the cell cycle process. Probably all cancers that depended on MYC expression can rely on MINCR expression, too [16]. Some of the cancers related to MINCR expression are colon cancer, gallbladder cancer, hepatocellular carcinoma, and OSCC, tumor

suppressors, liver neoplasms, and lung cancer can operate [2], [13], [16], [17]. MYC, originally named c-myc, is a fundamental transcription factor and proto-oncogene that regulates the expression of human genes, and becomes active in 20% of all human tumors [4], [18], [19]. The MYC gene is located on chromosome 8q24.1 [15]. protein comprises a basic helix–loop–helix and leucine zipper structural motifs, first found as the cellular homolog of the viral oncogene of the avian myelocytomatosis virus. This oncogene plays several roles in cells, such as transcription factors, cell cycle control, multiplication, development, distinction, apoptosis, cell metabolism. This gene stimulates tumor growth in various types of cancer, including breast, lung, intestinal, brain, skin, blood, prostate, pancreas, stomach, and bladder cancer [19]. Several studies revealed that the amplification of MYC is related to breast cancer development and even affects advanced breast cancers [19], [20]. The genetic and epigenetic variation affects breast cancer development, and it could propel normal breast cells to highly malignant ones because of neoplasm metastasis potential [19].

Based on mentioned studies, it is reasonable to determine the expression pattern of MYC, lncRNA MINCR, and lncRNA JPX in the different populations. This study evaluates the likelihood of prognostic and diagnostic biomarkers for these three RNAs in the Isfahan breast cancer patient population.

3. Materials And Methods

3.1. Microarray analyses

The GSE42568 [21] dataset ([HG-U133 Plus 2] Affymetrix Human Genome U133 Plus 2.0 Array) was chosen and processed to identify differentially expressed genes (DEGs) in breast cancer samples compared to control samples. The GEO database was used to find this dataset. The limma [22] and GEOquery [23] packages were used to conduct the DEG analysis. Microarray analysis plots and figures were created using the ggplot2 and pheatmap packages. Using the quantiles normalization technique, the *normalizeQuantiles* command in R was used to normalize the raw data. A total of 121 samples were analyzed (17 control and 104 breast cancer samples). Based on the distribution of relative expression data of all genes studied in this experiment, genes with logFC more than 1 were classified as up-regulated, whereas genes with logFC less than − 1 were designated as low expressed genes. The significance level was set at an adj.p.value of less than 0.05.

3.2. Bioinformatics analyses

Pathway enrichment analysis was performed by KEGG [24]–[26] and Reactome [27], [28]. miRNA-mRNA interaction analysis was performed by miRWalk 3 [29], [30]. lncBase3 was used to investigate miRNA-lncRNA interactions [31]. lncRRIsearch was used to investigate the relationship between the lncRNA and the gene [15]. Cytoscape 3.8.0 was used to visualize the interaction network. QuickGO online program (https://www.ebi.ac.uk/QuickGO/) was used to perform gene ontology analysis. To visualize the common micro-RNAs interacted to gene and lncRNAs, *VennDetail* package was used. Survival analysis was performed by GEPIA2 online software, based on the TCGA RNA-seq data.

3.3. TCGA data analysis

Breast cancer RNA-seq dataset was downloaded from The Cancer Genome Atlas (TCGA) online database (https://portal.gdc.cancer.gov). 113 control samples and 1109 BC samples were analyzed. The raw data was downloaded by TCGAbiolinks package, downloaded from Bioconductor. EdgeR and Limma (Voom method) packages were used to normalization, batch effect removal, statistical analyses and DEG analysis. TMM (trimmed mean of M-values) method was used for normalization of raw data. All of RNA-seq data analysis process was performed by R Studio (4.0.2).

3.4. Subjects

The Ethics Committee of Al-Zahra Hospital, Isfahan University of Medical Science, authorized all methods for the research in this study that involved human subjects. All of the patients signed written consent documents. Breast cancer and normal breast tissue samples from 24 individuals were analyzed in a case-control study. Clinicopathological features of the samples is indicating in the Table I.

Table 1
The clinicopathological features frequency of samples.

Characteristic	Status	Number of Patients
Stage	I	0
	II	8 (33.3%)
	III	7 (29.2%)
	IV	6 (25%)
	Unknown	3 (12.5%)
Age	< 45	11 (45.8%)
	> 45	11 (45.8%)
	Unknown	2 (8.3%)
Tumor size (TS)	< 5 cm	16 (66.7%)
	> 5 cm	5 (20.8%)
	Unknown	3 (12.5%)
Menopausal status	Yes	13 (54.2%)
	No	11 (45.8%)
	Unknown	0
Lymph node	Yes	17 (70.8%)
	No	4 (16.7%)
	Unknown	3 (12.5%)
ER receptor	Positive	11 (45.8%)
	Negative	7 (29.2%)
	Unknown	6 (25%)
PR receptor	Positive	8 (33.3%)
	Negative	10 (41.7%)
	Unknown	6 (25%)
HER2/neu receptor	Positive	10 (41.7%)
	Negative	8 (33.3%)
	Unknown	6 (25%)

3.5. Real-time PCR experiment

The RNA content of breast cancer tissue samples and standard breast tissue equivalents from the same people was collected and extracted using Trizol reagent, as directed by the manufacturer (Promega Co., Madison, WI, USA).

The first-strand cDNA synthesis package was given by Thermo Fisher Scientific, Waltham, MA, USA, and was used to synthesize cDNA according to the manufacturer's instructions. The expression of MYC, lncRNA MINCR, and lncRNA JPX, as well as GAPDH as a control gene, was investigated using cDNA products treated at 20°C. Using oligo software, unique primers were generated for each gene (Version 7, Table II).

Table 2
The primer sequences of MYC, MICNR, JPX, and GAPDH.

Gene	Primer Sequence
myc	Forward: 5'-TGTCCAAGCCACCTCTCAGA- 3'
	Reverse: 5'-GGGAACTGATGCCTGACCA-3'
mincr	Forward: 5'-TAGAGACCTCAGACCGCAG – 3'
	Reverse: 5'-TGTCACAGACGCACTCTTCC – 3'
jpx	Forward: 5'-CAAGGCGTCCGAAGTATGAG – 3'
	Reverse: 5'-GCAACAAGAGCGAACTCCATC – 3'
GAPDH	Forward: 5'- TACAGCAACAGGGTGGTGG – 3'
	Reverse: 5'- ATTCAGTGTGGTGGGGGAC-3'

3.6. Statistical analysis

GraphPad Prism software (version 8.0) was used to create the Graphs. qRT-PCR data were analyzed utilizing the -ΔΔCT method. Paired t-test and Wilcoxon test were used for statistical analysis to analyze and compare expression data among normal and breast cancer tissue samples by GraphPad prism v.8 and GenEx (version 6). The Kruskal-Wallis test was performed for clinicopathological analysis. DGE analysis was assessed using the RStudio software (version 4.0.2). ROC curves were generated by GraphPad Prism software. The clinicopathological frequency was determined by IBM SPSS Statistics 26.

4. Results

4.1. Microarray data analysis: Quality control

The GSE42568 microarray dataset was selected to find the differentially expressed genes in the BC samples. An integrated quality control analysis was performed to check the quality of samples and the dataset (Fig. 1–4). After checking the quality of samples, three control samples and four tumor samples

were deleted from the analyses as the bad quality samples. Finally, the DEG and statistical analyses selected 14 control samples and 100 breast cancer samples (Fig. 5).

4.2. Microarray analysis: DEG analysis

After integrating quality control analyses and removing bad quality data and normalization, the differential expression analysis was performed. 54675 genes were analyzed in the 14 control and 100 tumor samples. Among all of the genes, 3876 genes were found as significantly down-regulated genes and 3570 genes demonstrated up-regulated ones. Among the down-regulated genes of this analysis, MYC has been selected as a hub significantly down-regulated genes as a potential biomarker for BC (logFC: -1.107384, adj. P. Value: 0.036). Finally, this gene was selected for future investigations (Fig. 5).

4.3. RNA interaction analyses

Based on the lncRRIsearch analysis, it is demonstrated that the lncRNA MINCR directly interacts with the MYC (Fig. 7). Also, based on the mRNA-miRNA interaction analysis by miRWalk and miRNA-lncRNA interaction analysis of lncBase 3, a ceRNA interaction network was performed, showing in Fig. 8. According to this network, lncRNA JPX has an endogenous competition with the MYC by binding to the intersected miRNA. The intersect of the MYC, lncRNA MINCR, and lncRNA JPX miRNA interactome is showing in Fig. 9. So, based on the co-expression with the MYC, these two lncRNAs were selected for future investigations.

4.4. Next generation sequencing data analysis

RNA-seq data analysis was performed to evaluate the expression level of two lncRNAs JPX and MINCR, in the BC samples. 26760 RNAs were analyzed in this analysis. According to the analysis, lncRNA JPX (logFC: 4.42, adj. P. Value: 0.000) and lncRNA MINCR (logFC: 1.87, adj. P. Value: 0.000) significantly high-expression in the BC samples compared to control samples. So, these two ncRNAs could act as the BC oncogenes correlated to MYC.

4.5. Pathway enrichment, GO, and survival analysis

Based on the KEGG online software and QuickGO online database, pathway enrichment and GO analyses was performed (Fig. 9). The results are showing in the Table III.

Table 3
The gene ontology analysis of *MYC* and lncRNA *JPX* and the pathways that *MYC* involves in.

QUALIFIER	GO TERM	GO NAME	Pathway	Gene
Involved in	GO:0006355	regulation of transcription, DNA-templated	Bile secretion	MYC
enables	GO:0003700	DNA-binding transcription factor activity	mTOR	MYC
enables	GO:0046983	protein dimerization activity	Transcriptional misregulation in cancer	MYC
enables	GO:0003677	DNA binding	Breast cancer	MYC
Located in	GO:0005730	nucleolus	Central carbon metabolism in cancer	MYC
Located in	GO:0005654	nucleoplasm		MYC
Involved in	GO:0010628	positive regulation of gene expression		JPX

Based on TCGA RNA-Seq data, survival analysis was performed on the expression level of JPX in two high-expressed and low-expressed groups by the GEPIA2 online software. According to this analysis, high expression of JPX lncRNA positively correlates with the lower survival rate of patients. By analyzing the survival rate, we found that patients who have low JPX expression levels lived longer than patients with high JPX expression levels

4.6. Real-time PCR experiment

The gene expression analysis of MYC, lncRNA JPX, and lncRNA MINCR was performed to evaluate the changes in mentioned RNAs' expression in the Isfahan Breast cancer patients. Based on this experiment, MYC was significantly low-expression in the BC samples (logFC: -2.504, P. Value: 0.0035). Also, lncRNA JPX (logFC: 3.650, P. Value: 0.0126) and lncRNA MINCR (logFC: 3.325, P. Value: 0.0151) have a significant down-regulation in the Isfahan Breast cancer samples (Fig. 11).

ROC analysis was performed to evaluate the potential biomarkers in this research by GraphPad prism. The ROC analysis revealed that MYC, MINCR, and JPX are the three acceptable significant biomarkers in the Isfahan BC samples (P. Value $\leq$ 0.05, AUC > 0.7). So, these RNAs can introduce as the potential prognostic biomarkers for Isfahan BC (Fig. 12).

4.7. Clinicopathological analysis

The expression of MYC, lncRNA MINCR, and lncRNA JPX in the patients with different clinicopathological statuses was analyzed. Eight following pathological variables were analyzed: Stage, Age, Lymph Node Metastasis, Menopausal status, Tumor Size, ER receptor, PR receptor, and HER2 receptor. Based on the

mentioned analyses, it is demonstrated that MYC has a significant decrease in the patients who reached menopause compared to patients who have not reached menopause (Fig. 13).

Also, the expression of lncRNA MINCR had a significantly high expression in the patients younger than 45 years and patients with a tumor size of more than 5 cm, and patients who have not reached and HER2 positive patients with normal persons (Fig. 14).

5. Discussion

We design a reasonable experiment based on integrated bioinformatics analysis and select three hub genes involved in the BC development. Based on the microarray and RNA interaction analyses, MYC, lncRNA JPX, and lncRNA MINCR were selected for the expression and correlation analyses. We demonstrated that MYC has a significantly low expression in the Isfahan BC samples as a significant tumor suppressor and biomarker (Fig. 11, 12). Also, we find that the two MINCR and JPX lncRNAs have a significant up-regulation in the Isfahan BC samples. Also, there was a significant correlation in the expression of MYC and MINCR with the Age, Menopausal status, Tumor Size, and HER2 receptor status of Isfahan BC patients (Fig. 13, 14).

In recent studies, several LncRNAs are identified as regulators in gene expression and play a role in tumorigenesis and metastasis by the actin biological functions and cancer, especially breast cancer.

Some of the LncRNAs involved in cell growth, cell migration, apoptosis, invasiveness, and metastasis of breast cancer [1], [32]–[34]. In the previous investigations, ceRNAs have been reported as one of the post-transcription regulators. LncRNAs are one of the units of ceRNAs that block miRNA from connecting to mRNA through sponging to miRNAs [35], [36]. We first used MINCR and JPX LncRNAs in order to find their effects in BC.

These peruse determined the connection between MINCR lncRNA and breast cancer. On the other hand, MINCR plays a remarkable role in the metastasis and multiplication of various cancer types, such as hepatocellular carcinoma, gallbladder neoplasm, and colon carcinoma by interacting with miRNA. Knockdown of MINCR in OSCC and NSCLS5 cells decline oncogene MYC and represses its downstream genes [2], [13], [14], [37]–[39].

MYC is a transcription factor that combines with a similar molecule to form a dimer with MAX to link DNA and control gene expression [40], [41]. MYC gene regulates its downstream genes and is involved in activating, increase or decrease expression of them [42]. According to what was said, MYC is an essential gene that plays a role in breast cancer, and MINCR also up-regulates it by attaching it to MYC binding site at the transcription start site [43]. MYC overexpressed in basal-like breast tumor, upregulation of MYC genes has also been noticed in carcinoma of the cervix, colon, lung, ovarian, stomach, and prostate carcinomas [19], [20], [44]. Signaling pathways such as Wnt, Notch, and TGF-β have significant functions in regulating MYC expression in breast cancer. Recent evidence proposes that MINCR operated the Wnt/β-

catenin signaling pathway, too [2]. Wnt/β-catenin signaling catalyzes tumor development in nasopharyngeal neoplasms, esophageal squamous carcinoma, and colon cancer [36], [45].

Wnt proteins in the Wnt signaling pathway activate β-catenin, and as a result, specific target genes are activated. Mutations in β-catenin cause a wide range of human cancers [46], [47]. MYC and both LncRNAs that we studied are associated with Wnt/ β-catenin pathway. The previous study showed that Wnt has a significant effect on MYC transcription by using the TCF/β catenin transcription factor complex, which is located on the MYC promoter but in recent peruses, they focused on the role of Wnt in different subtypes [47], [48]. Recent reviews determined the importance of MINCR in the Wnt/β catenin pathway, which by suppressing MINCR, the activity level of the Wnt/β catenin pathway decrease [2], [38]. It has been suggested that JPX could raise β-catenin to activate the Wnt signaling pathway, therefore catalyzing the malignant processes in cancer, and this seems to be a reliable approach [49].

JPX is a LncRNA that plays a role as a tumor suppressor in HCC. It is an inhibitor that can disable the X chromosome by activation of XIST [9], [49]. More recent evidence shows that there was a correlation between JPX and twist level. JPX and twist1 both are upregulated in lung carcinoma tissue, but JPX was enhanced in lung cancer by the Wnt/β-catenin signaling pathway [49]. A low expression level of JPX/twist1 in HCC tissues as compared to surrounding normal liver tissue was noticed [11]. It was also increased in NSCLC and ovarian.

As a result, we determined that MYC was downregulated and was closely related to menopause. lncRNA MINCR was upregulated in BC tissues and was significantly correlated with tumor size, age, menopause, and her2 receptor, confirming previous results. The other LncRNA, JPX, was significantly upregulated in BC tissue, and there was no clear relation with pathological status.

We also used bioinformatic analysis to find common miRNA sponging to MYC, MINCR, and JPX and reach these miRNAs, including hsa-miR-296-3p, hsa-miR-584-5p, hsa-miR-525-5p, and hsa-miR-520a-5p, and all of them are related to the mammary gland. All these miRNAs except hsa-miR-584-5p were associated with the Wnt signaling pathway, hsa-miR-584-5p, hsa-miR-525-5p, and hsa-miR-520a-5p involved in the TGF-β signaling pathway, only hsa-miR-296-3p plays a role in the Notch signaling pathway, future studies on these miRNAs are suggested.

Previous similar studies mentioned some novel lncRNAs as the potential prognostic and diagnostic biomarkers of cancer [50], [51]. As a suggestion, we offer the same experiments on the two lncRNAs JPX and MINCR, in the different clinicopathological subgroups by the larger samples size. Also, the different mutations and single nucleotide polymorphisms in the MYC and the two relevant lncRNAs can be investigated to evaluate the different micro-RNA binding affinity of each RNA and the relation of these SNPs on the BC development. Furthermore, the interaction of each RNA (direct interaction and ceRNA interactions) can be validated by more accurate experiments.

6. Conclusion

MYC has a significant down-regulation in the Isfahan BC patients as a prognostic biomarker and tumor suppressor. lncRNAs JPX and MINCR have high expression in the Isfahan BC samples as the two biomarkers and potential oncogene.

Declarations

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7.3. Availability of data and materials: For accessing the raw data of this experiment, please contact the corresponding author.

7.4. Authors' contributions: Conceptualization: MA, ShO; data analysis: NN, MSh, FT, HH; validation: MA, ShO; writing—original draft preparation: NN, MSh, FT, HH; supervision, ShO; project administration, MA.

7.5. Ethics approval and consent to participate: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the Ethics Committee of Isfahan University of Medical Sciences (approval number: 3838988).

7.6. Competing interests: The authors have no competition of interest in this study.

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Figures

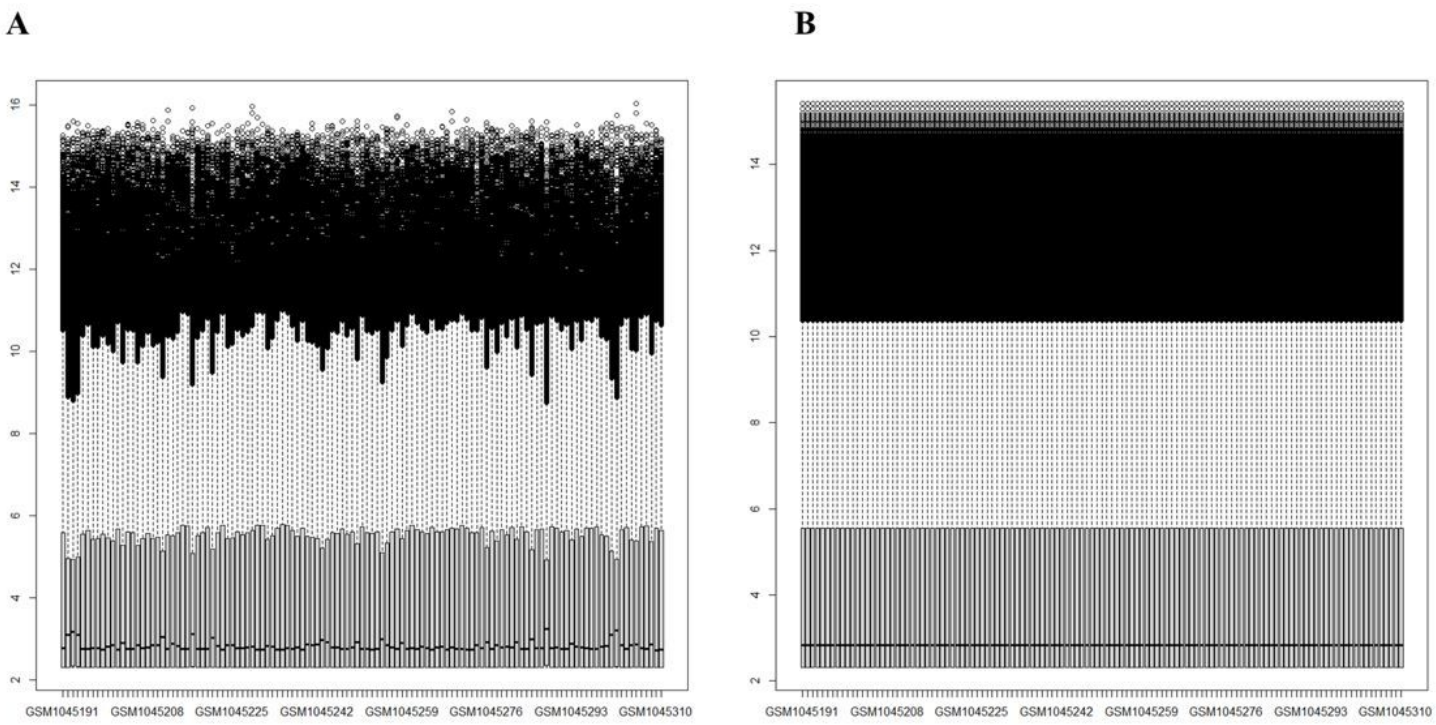

Figure 1

Boxplot of 104 Breast cancer and 17 control samples, based on the distribution of expression data. A) Boxplot of raw data. B) Boxplot of normalized data.

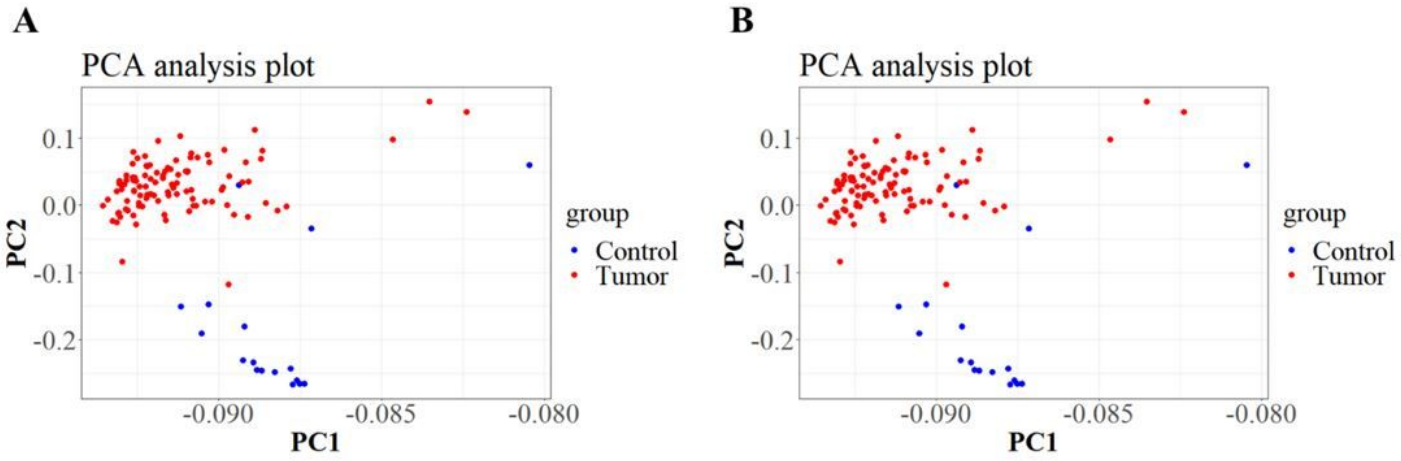

Figure 2

Principal component analysis of the raw and normalized samples. A) PCA from raw data. B) PCA of normalized data.

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Figure 3

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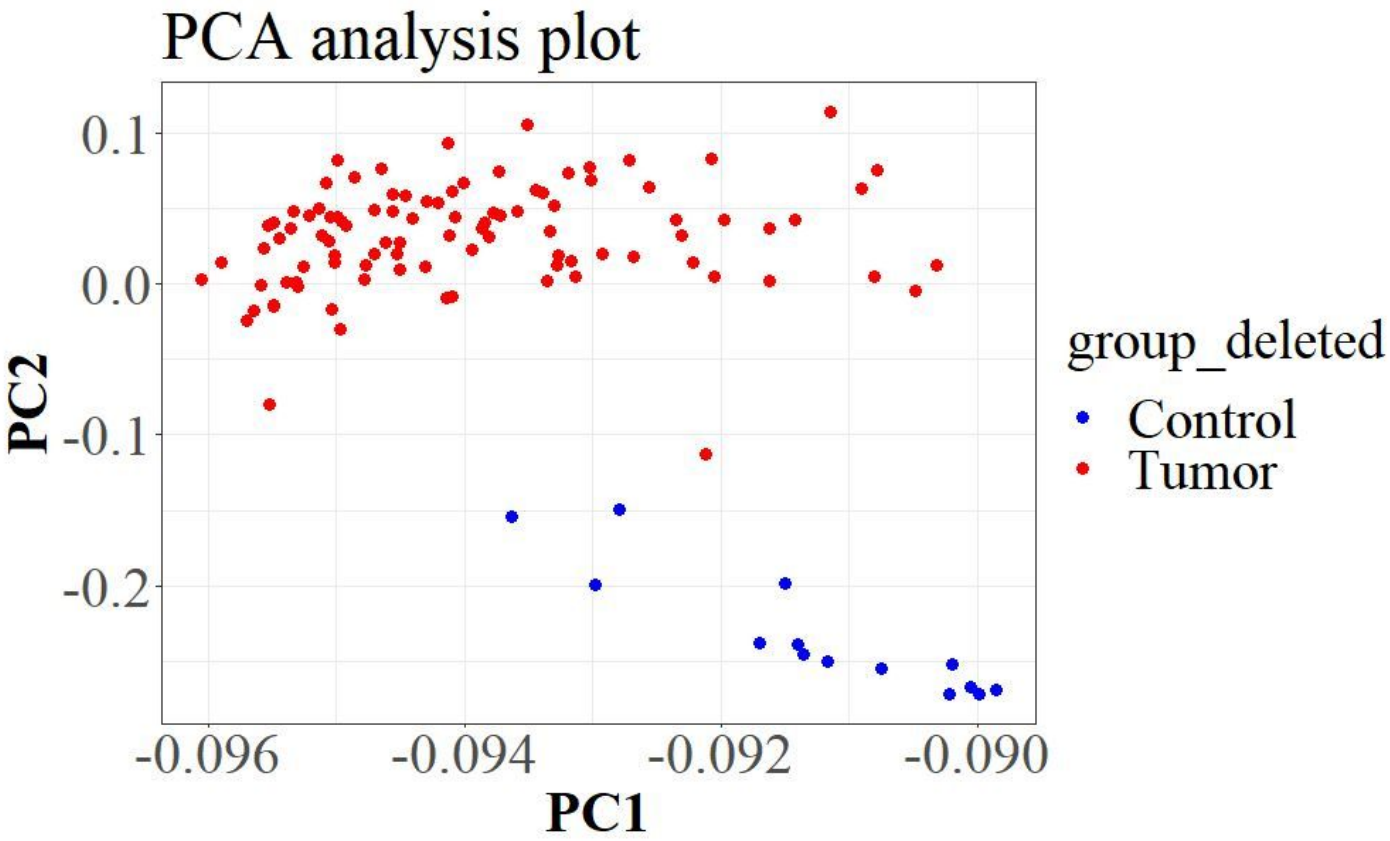

Figure 4

PCA analysis of normalized data, with removing the bad quality samples.

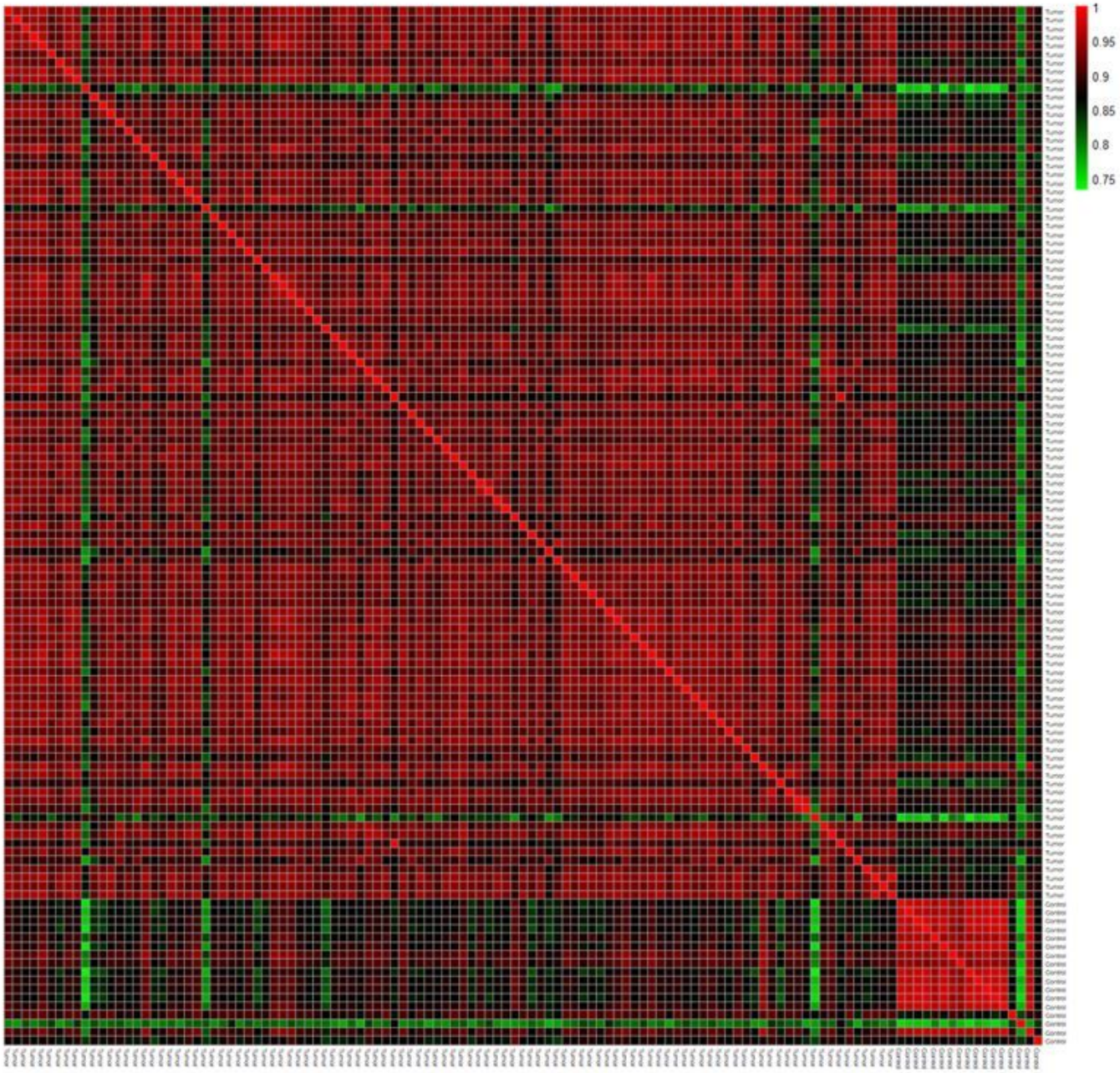

Figure 5

The Pearson correlation heatmap of control and tumor samples.

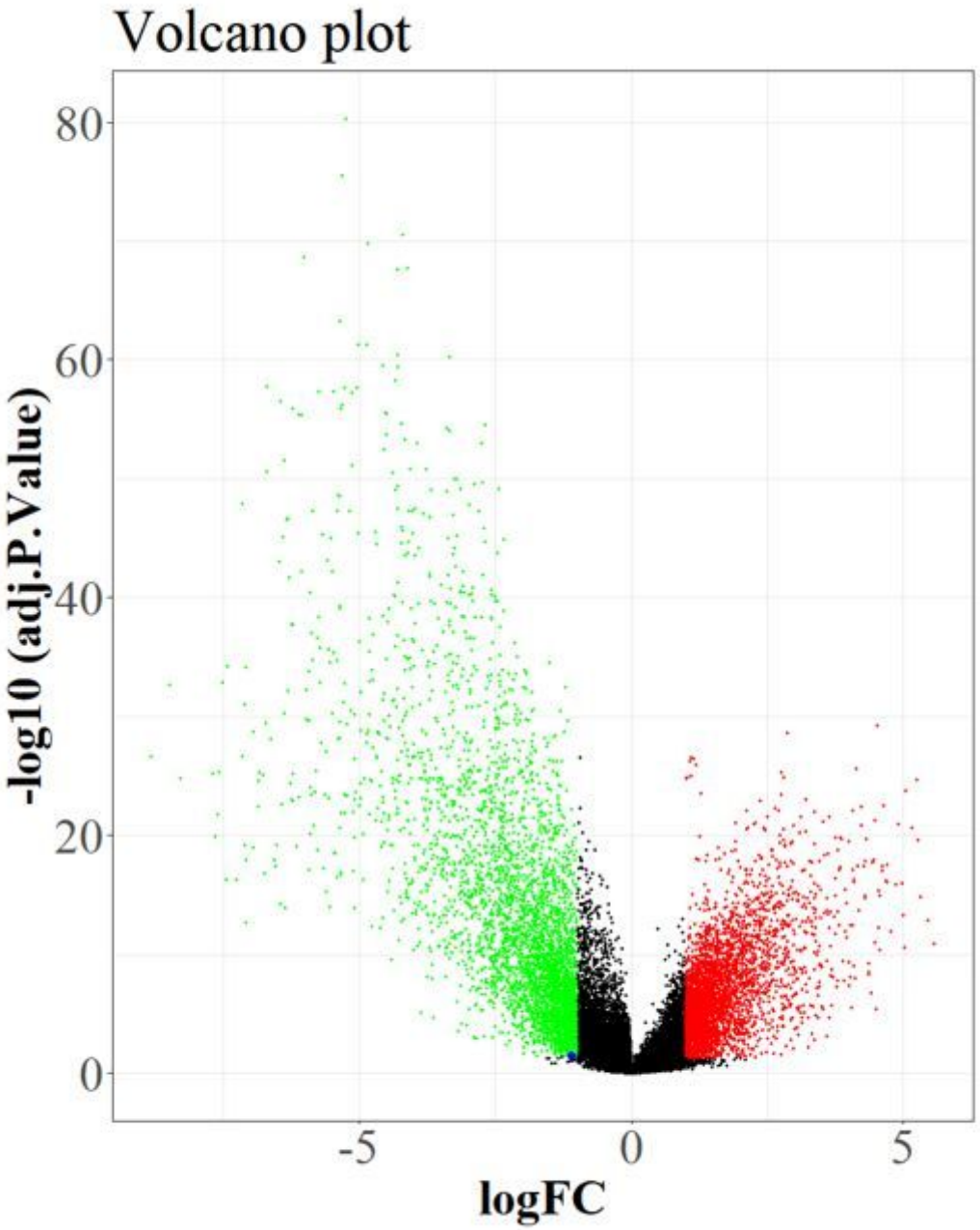

Figure 6

Volcano plot indicating the differentially expressed genes in the GSE42568 dataset. The red color represented the high-expressed (logFC > 1, adj. P. Value < 0.05) genes and the green color represents the low-expressed (logFC < -1, adj. P. Value < 0.05) genes in this dataset. *MYC* is indicating by a blue point in the plot as the significantly low expressed gene.

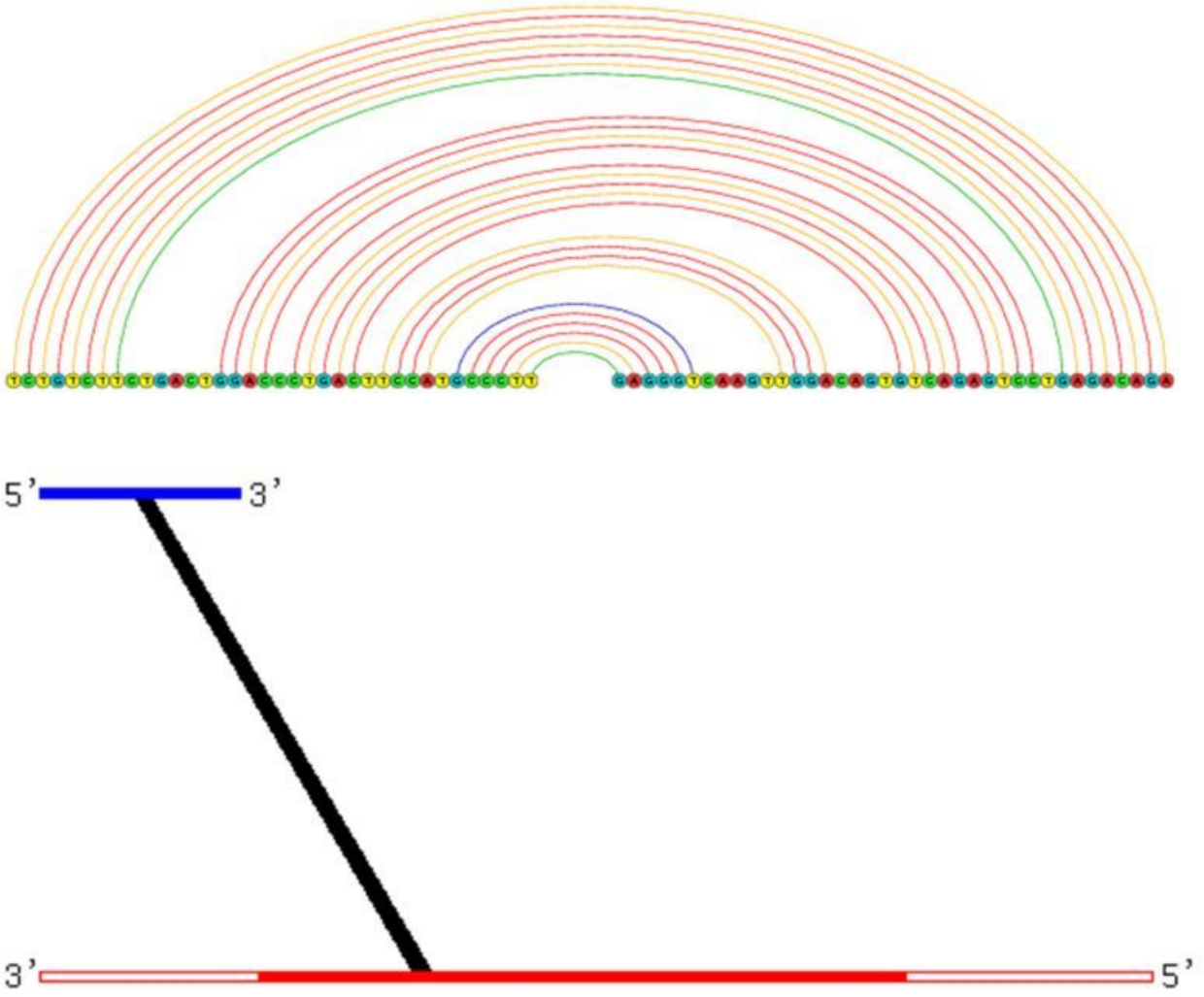

Figure 7

The direct interaction of lncRNA *MINCR* and *MYC*, based on lncRRIsearch.

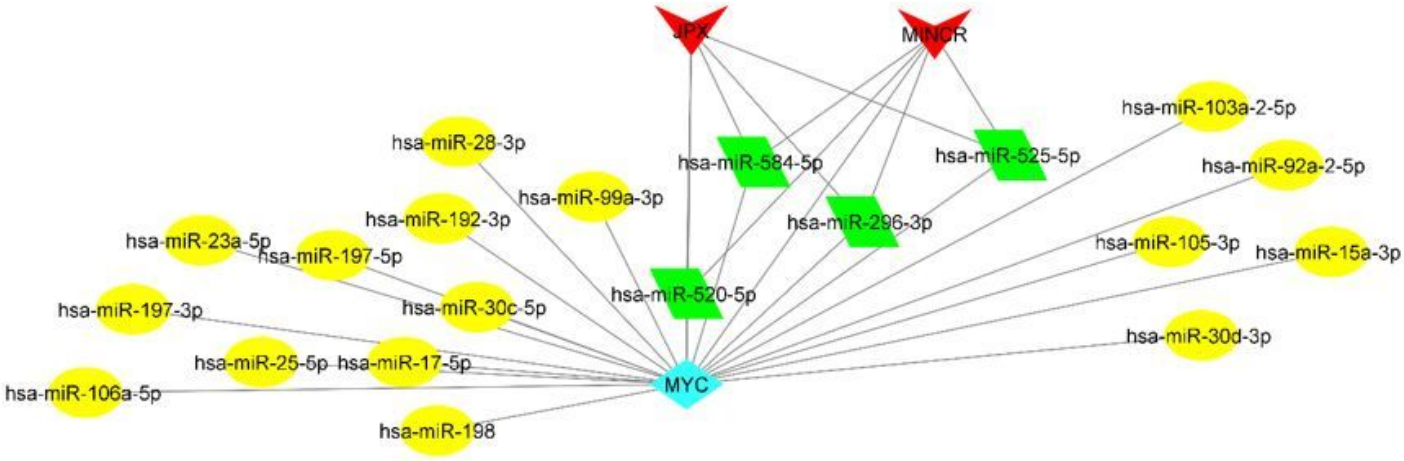

Figure 8

The ceRNA network of MYC interactome. The red nodes indicate the lncRNAs. The green nodes indicate the common miRNAs between gene and the two lncRNAs.

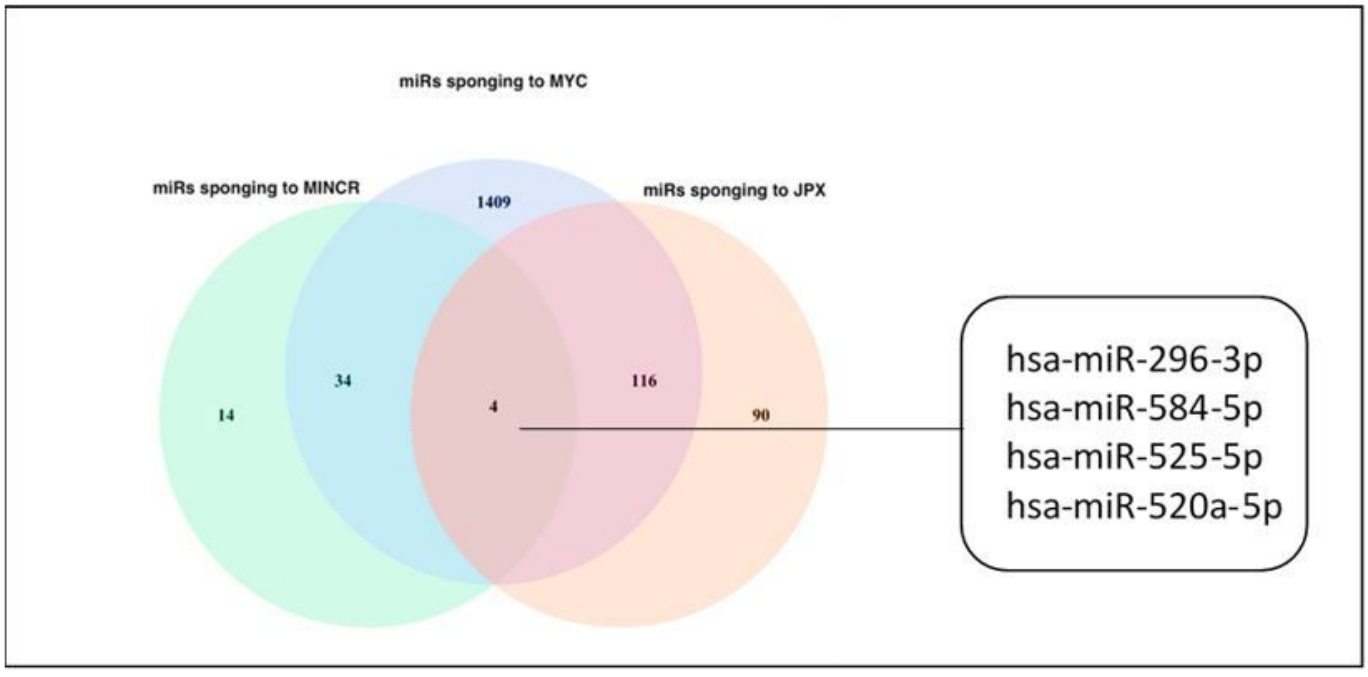

Figure 9

First "Figure 9" in manuscript. The intersection of micro-RNAs that have an interaction with MYC, MINCR, and JPX.

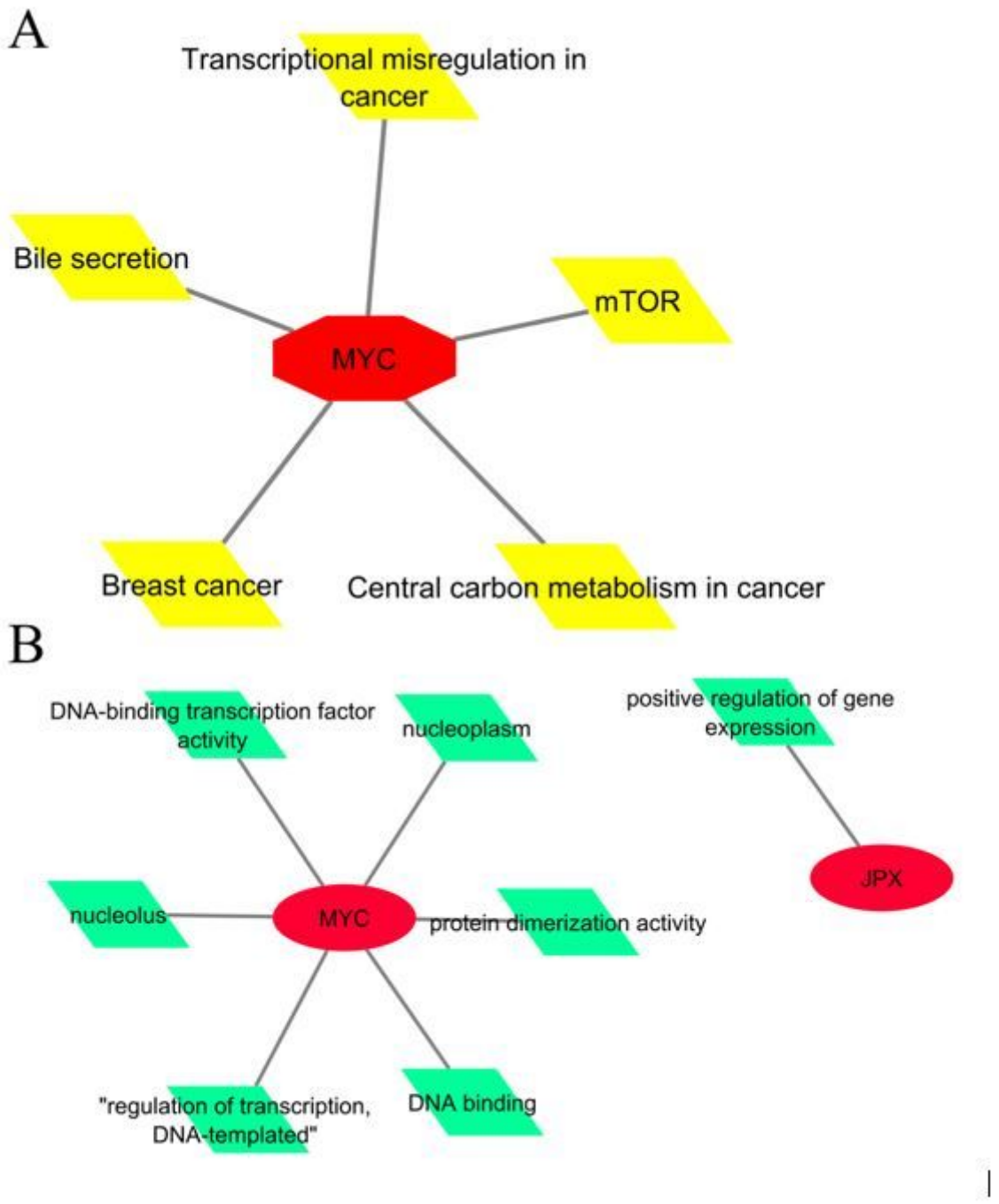

Figure 10

Second "Figure 9"in manuscript. Pathway enrichment analysis and Gene ontology analysis. A) Pathway enrichment network. B) Gene ontology analysis.

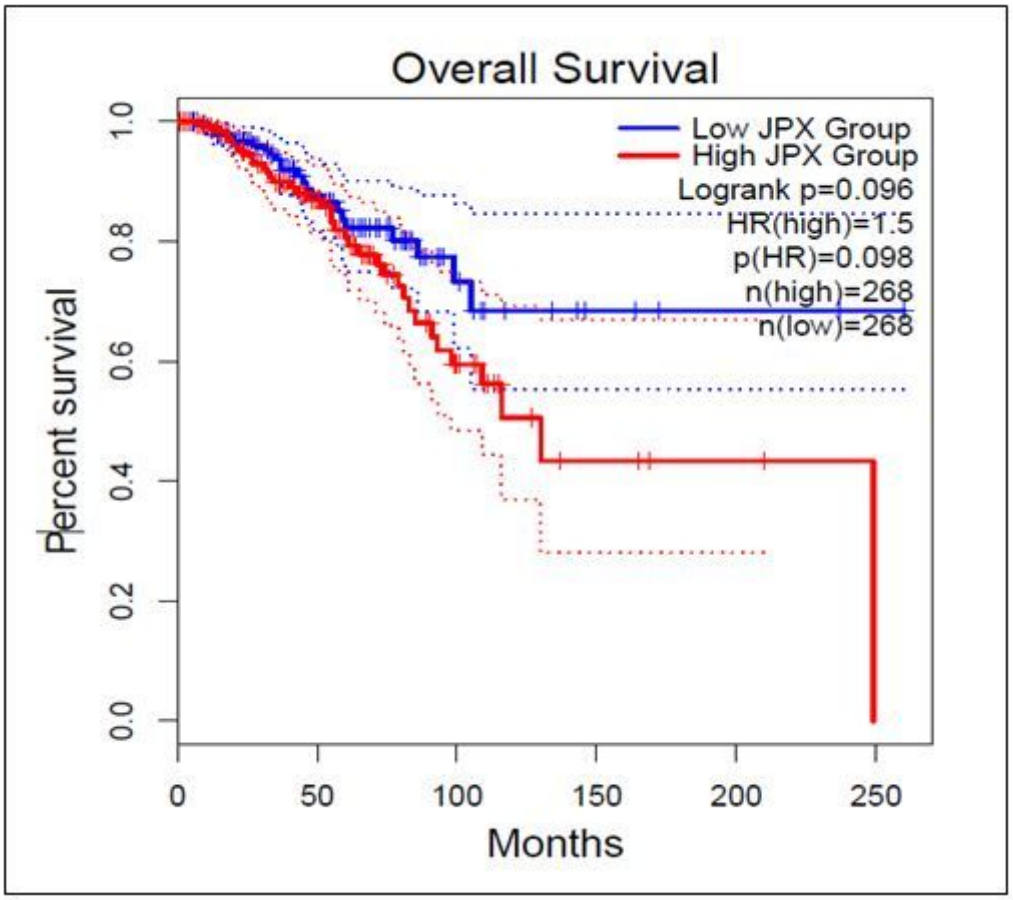

Figure 11

Figure 10: Survival analysis of JPX expression level, based on TCGA datasets.

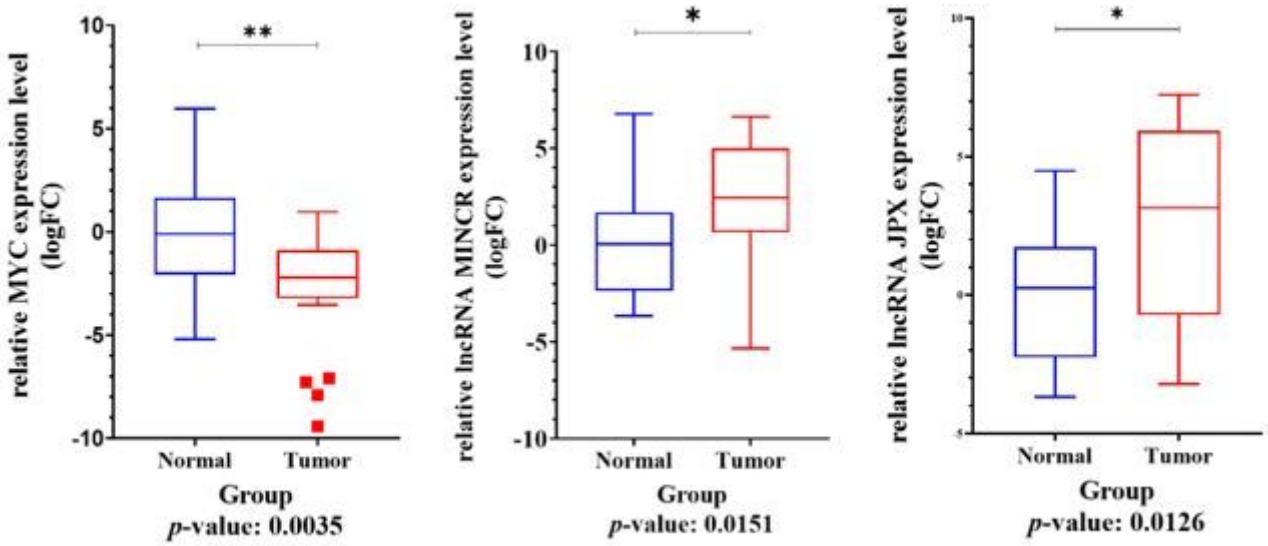

Figure 12

Figure 11: Relative expression analysis of MYC, lncRNA JPX, and lncRNA MINCR in the Isfahan BC samples, compared to control.

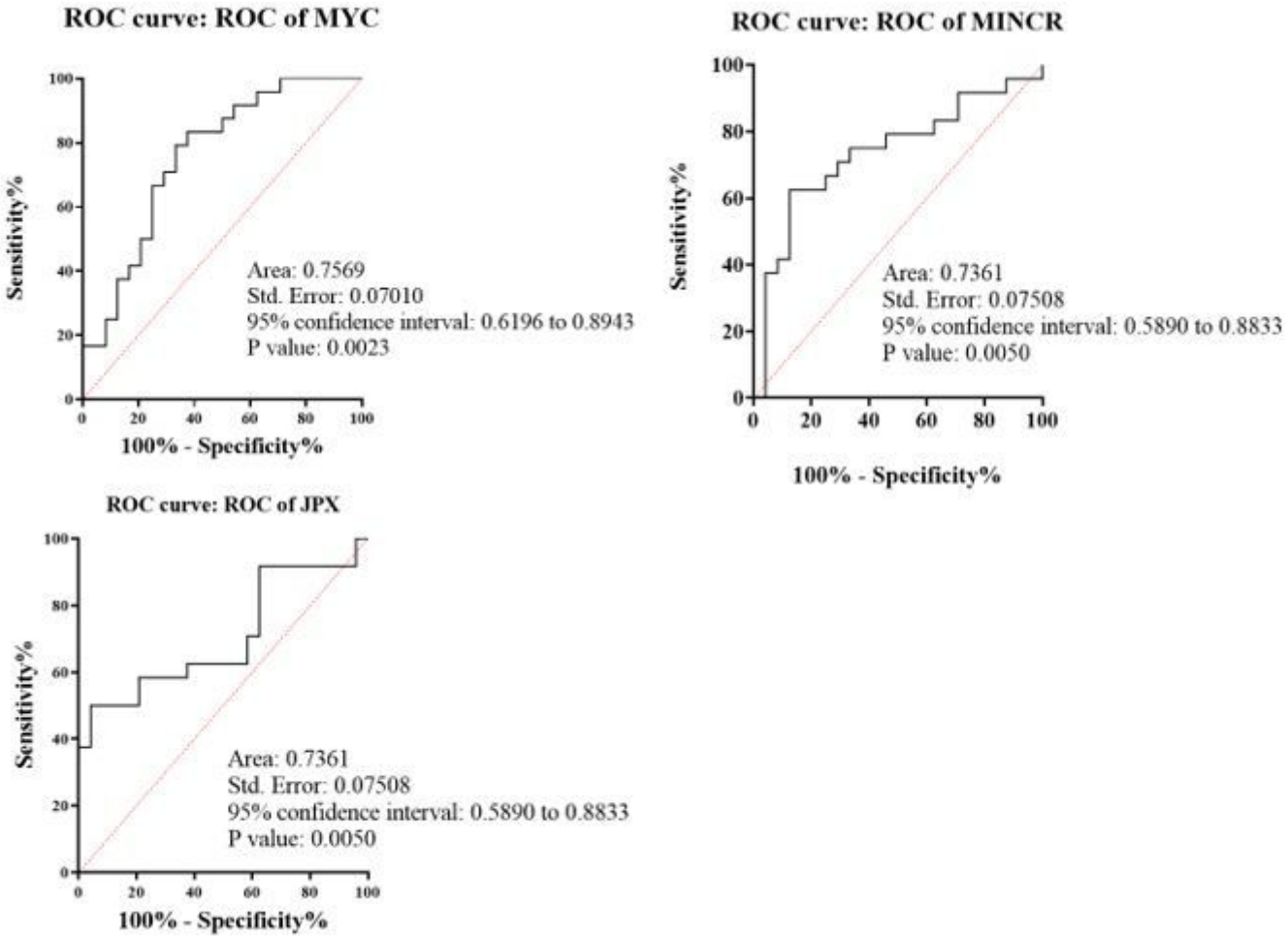

Figure 13

Figure 12: ROC analysis of MYC, MINCR, and JPX.

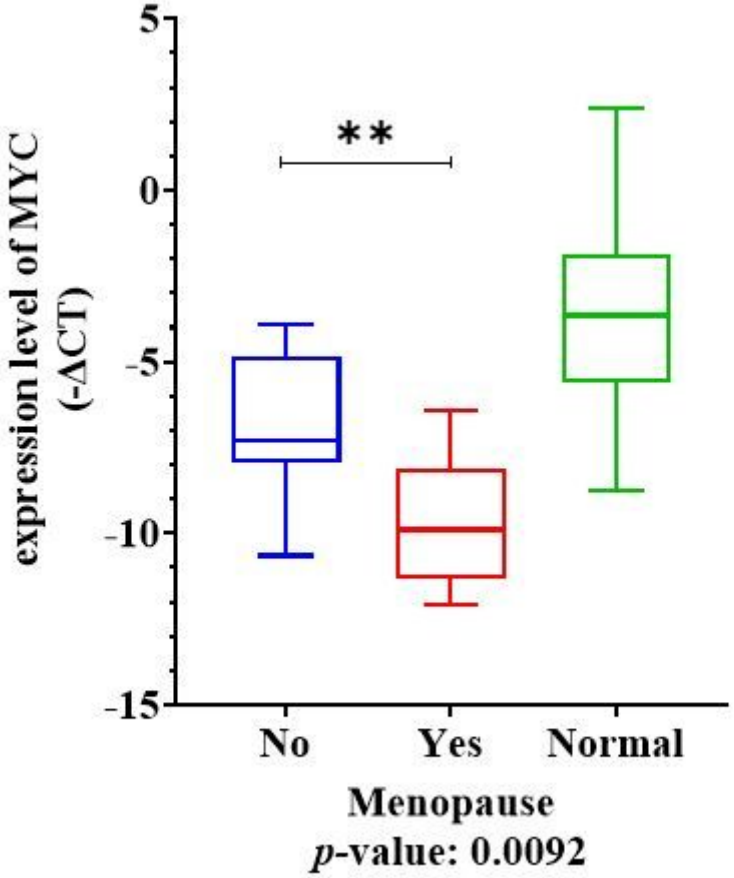

Figure 14

Figure 13: There was a significant difference between the expression level of MYC in persons who reached menopause and the patients who haven't reached menopause.

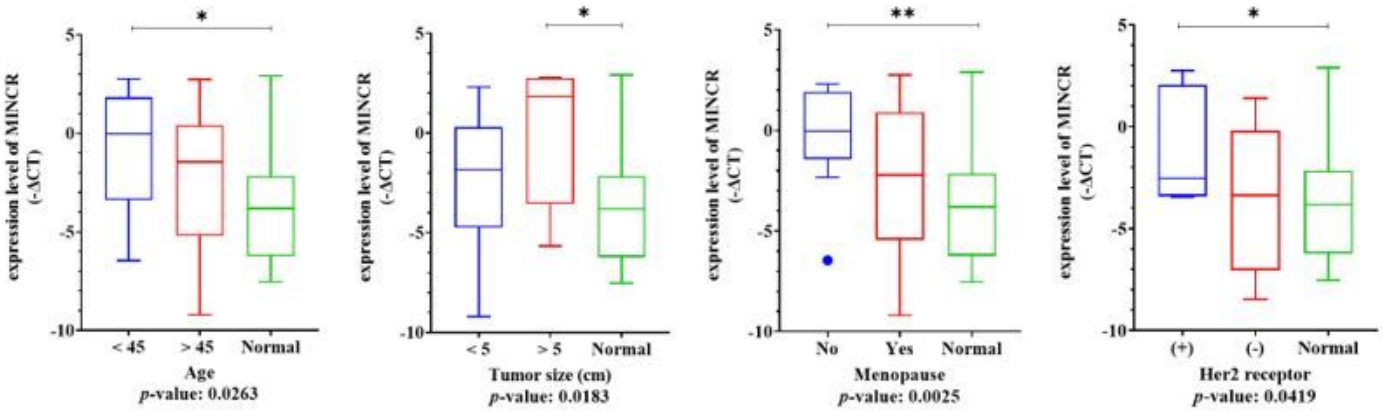

Figure 15

Figure 14: There was a significant difference between up-regulation of MINCR in patient younger than 45 years and patient with a tumor size of more than 5 cm and patient who have not reached menopause and Her2 positive patients with normal persons.