LncRNA XIST acts as a microRNA-520 sponge to regulate the cisplatin resistance in NSCLC cells by mediating BAX through ceRNA network

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Abstract

Background

In recent years, LncRNA acts as a member of competing endogenous RNA (ceRNA), playing an important role in drug resistance of lung cancer. The aim of this study was to identify potential biomarkers about cisplatin resistant lung cancer cells using a comprehensive ceRNA network.

Methods

GSE6410 (GPL-201) analyzed gene expression changes about cisplatin resistance in A549 NSCLC cells. GSE43249 (GPL-14613) included noncoding RNA expression profiling derived from the cisplatin resistant A549 lung cells. GEO2R, an online analysis tool, analyzed the differentially expressed mRNAs and miRNAs (DEmRNAs and DEmiRNAs). To explore the functional enrichment implication of differentially expressed mRNAs, we used the GO and KEGG pathway analysis. Through miRDBTargetsCanStarbaseirWalk, we found targeted miRNAs. The Kaplan-Meier curve method was used to show clinical survival analysis of targeted RNAs ($P < 0.05$). The Starbase database predicted potential IncRNAs mediated targeted miRNAs. Eventually, the novel ceRNA network of IncRNAs, miRNAs, mRNA was constructed by cytoscape3.7.2.

Results

118 differentially expressed mRNAs were the basis of the mediated ceRNA network. DAVID and Kaplan-Meier picked out BAX, an apoptosis regulator. Venn Diagram demonstrated 8 miRNAs commonly regulating Bax. Starbase predicted IncRNA XIST mediated miRNAs. Finally, IncRNA XIST maybe a useful biomarker regulating cisplatin resistance in lung cancer cells.

Conclusions

LncRNA XIST competitively bound to miRNA 520 in the regulation of cisplatin resistance by BAX, participating apoptosis in the p53 signaling pathway.

Background

Lung cancer is considered to take a significant role in the cancer death around the world. It is well known that NSCLC comprises approximately 85% of lung cancer. Lung adenocarcinoma (LAC) accounts for 40% of all lung cancers all over the whole world[1].

As for NSCLC, many treatment strategies are effective including surgical operation, chemotherapy, radiotherapy. NSCLC patients, who are treated with cisplatin, also termed cisdiamminedichloroplatinum (CDDP) or diamminedichloroplatinum (DDP), may develop chemoresistance[2]. Cisplatin has been adopted for about 30 years[3], primarily acting by causing DNA damage[4]. Chemoresistance is a major problem for cancer therapy[5]. There are many resistant mechanisms examined in cancer cells, such as P53 signaling pathway, apoptosis[6], cell cycle. At present, chemotherapy has been limited because of resistance[7]. However, relative biomarkers are considered absent[8]. Thus, it is high time that we should establish effective network of biomarkers to predict gene changes in cisplatin resistant NSCLC cells.

LncRNAs have been considered as oncogenes mediating tumorigenesis and chemoresistance. They may have lower expression and exist in the cytoplasm or nucleus[9]. They inhibit effects on miRNAs and mRNAs[10]. In different kinds of cancers, a large number of lncRNAs are explored to mediate cellular processes and drug-resistance, such as NSCLC, ovarian cancer[11], gastric cancer[12], pancreatic cancer[13], breast cancer[14]. LncRNA acts as a member of competing endogenous RNAs (ceRNAs) by competitively binding targeted microRNAs (miRNAs)[15, 16]. In our study, lncRNA XIST locates on chromosome 8q24.21 and combines with the microRNA-520 to regulate the cisplatin resistance by mediating BAX through ceRNA network.
BAX[17] (ENSG00000087088), an apoptosis regulator molecule[18], which locates on the Chromosome 19, having 13 transcripts. BAX is a member of Bcl2 family[19]. DAVID database and Kaplan-Meier curve were used to analyze the GSE6410 DEmRNAs. Next, through four databases, miR-525-5p, miR-4640-3p, miR-214-3p, miR-520a-5p all regulated the BAX. Comparing with GSE43249 (GPL-14613), we decided to explore miR-520 (survival curve $P < 0.01$). In this present novel, the ceRNA network of lncRNA-miRNA-mRNA about cisplatin resistance in NSCLC were created through RNA sequencing data from the GEO database.

**Methods**

**Data Collection And Microarray Analysis**

We used GSE6410 (6 samples mRNAs ) and GSE43249 (6 samples miRNAs )[20] about cisplatin resistant non-small cell lung cancer (NSCLC) cells from the GEO database (https://www.ncbi.nlm.nih.gov/gds/?term=). First, DEmRNAs were analyzed by online software GEO2R. There were 8793 DEmRNAs in GSE6410, and $|\text{Log } 2 \text{ FC}| > 1$ and the $P < 0.05[21]$ were two screening criteria. These 118 DEmRNAs were the basis of ceRNA network. we used the GraphPad-Prism 8.4.0 (https://www.graphpad.com)[22] to show volcano maps and heat maps. To normalize the samples, we showed Box diagram vividly. The Independent two-samples T-test proved that samples were representative by SPSS Statistics 17.0.

**Functional Enrichment Analysis Of DEmRNAs**

We used the DAVID database 6.8 (https://david.ncifcrf.gov/) and Metascape (http://metascape.org) to find the function and pathways of 118 DEmRNAs. As to GO-BP (biological process), sorted by $P$ value < 0.05, 5 counts (CDKN1A, BTG2, BAX, MDM2, GADD45A ) were most significant in participating DNA damage response, p53 signaling pathway. To GO-CC (Cellular component), calcium channel complex was numerous. Steroid binding accounted most in the GO-MF(molecular function). All genes in the genome have been used as the enrichment background. Through the Kaplan Meier-plotter software (https://kmplot.com), we finally decided to study the targeted mRNA BAX, an apoptosis regulator molecule, which participated in the P53 signaling pathway[23].

**Explore Potential miRNAs**

According to the targeted molecule BAX, we found miRNAs, which commonly regulated BAX from miRDB [24](http://www.mirdb.org), Targetscan [24](http://www.targetscan.org), Starbase(https://starbase.sysu.edu.cn/), miRWalk (http://mirwalk.umm.uniheidelberg.de/). Then, we showed 8 miRNAs regulating BAX in only four databases by Venn software, such as hsa-miR-3681-3p, hsa-miR-766-5p, hsa-miR-525-5p, hsa-miR-4640-3p, hsa-miR-128-3p, hsa-miR-216a-3p, hsa-miR-520a-5p, hsa-miR-214-3p. But these miRNAs were not all related to the survival of patients. Later, 4miRNAs (hsa-miR-4640, hsa-miR-520a-5p, hsa-miR-214-3p, hsa-miR-525-5p) were obviously related to overall survival[25] ($P < 0.05$) by the Kaplan Meier plotter software (https://kmplot.com).

**The discovery of LncRNA XIST in cisplatin resistant NSCLC cells**

Identification of potential lncRNA about 4 miRNAs(hsa-miR-4640, hsa-miR-520a-5p,hsa-miR-214-3p, hsa-miR-525-5p) was the critical step. We used the database starbase (https://starbase.sysu.edu.cn/) to separately predict potential lncRNAs for 4 miRNAs. Two lncRNAs XIST, MIR29B2CHG were found to regulate common 4 miRNAs by the Dram-Venn-Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) database. According to the present articles, lncRNA XIST has some researches in the cisplatin resistant NSCLC cells. The expression of lncRNA-XIST was significantly higher in NSCLC tumor tissues and cisplatin resistant A549 LAC cells[26]. At the moment, a research has found that lncRNA XIST was upregulated and induced chemoresistance by combing with miR-29c participating in the DNA respair pathway[27]. However, the ceRNA network mediated by lncRNA XIST remained poorly understood. Thus, our study explored lncRNA-XIST to predict the prognosis of NSCLC patients. This molecule may be a potential target for the cisplatin resistant lung cancer cells.

**Construction of IncRNAs-miRNAs-mRNAs network in cisplatin resistant NSCLC cells.**

PPI networks can provide information on the molecular mechanism. The same to us, IncRNA-miRNAs-BAX network
were established by cytoscape 3.7.2 software obviously ([http://www.cytoscape.org/](http://www.cytoscape.org/)). Every type of RNAs all represented different nodes and the relationships between these genes were considered to be edges. In this circumstance, BAX, 4miRNAs, IncRNA XIST were all visualized in this ceRNA family. LncRNA XIST regulated the miRNAs by BAX, participating in P53 signaling pathway in cisplatin resistant NSCLC (Fig. 1).

**Database Validation Of The Most Likely miRNA**

Contrarily, through the database miRcode ([http://www.mircode.org](http://www.mircode.org)), we predicted potential miRNA by IncRNA XIST. A miRNA family including miRNA 520, miRNA 214, which were also expressed in the ceRNA network. To recognize the differentially expressed miRNAs, GEO2R, an onling software was used to conclude the primary data of microarray in GSE43249 (P value < 0.05 and |Log2FC| >1). Besides, according to the differentially expressed miRNAs in the GSE43249 (GPL14613), we found out miRNA 520 in NSCLC. This further proved the importance of miRNA 520 in the cisplatin resistant NSCLC cells. Thus IncRNA XIST, miRNA 520, BAX were considered to be the key molecules, mediating the apoptosis in the P53 signaling pathway.

**Results**

**Differentially Expressed mRNAs In NSCLC**

Through comparison and screening, we chose GSE6410(GPL201) including mRNAs about cisplatin resistant cancer cells. GEO2R, an online software analyzed 8793 DEmRNAs from the samples (absolute Log Fold change > 1 and p-value < 0.05). There were 118 DEmRNAs, including 80 up-regulated genes, 38 down-regulated genes. In order to show DEmRNAs, we used the volcano maps and heat maps(Fig. 2a, 2b). We chose top 25 down regulated and up regulated mRNAs (Table 1).

| Gene.symbol   | logFC       | P.Value      |
|---------------|-------------|--------------|
| IGLL1         | -1.5812651  | 0.00002736   |
| KCNMA1        | -1.1513622  | 0.00048796   |
| COL15A1       | -1.2280541  | 0.00129318   |
| SEPT5-GP1BB   | -1.2577165  | 0.00158965   |
| PPBP          | -1.4687682  | 0.00202229   |
| RFX4          | -2.0975544  | 0.00189187   |
| SLIT3         | -1.387397   | 0.0022444    |
| NR1H4         | -1.4536816  | 0.00299161   |
| F2RL3         | -1.6589632  | 0.00308639   |
| GMDS          | -1.0053824  | 0.00390183   |

Table 1

The differentially expressed mRNAs in the NSCLC, top 25 down and up regulated mRNAs.
| Gene   | Log2 Fold Change | p-value    |
|--------|-----------------|------------|
| ATXN1  | -1.1879763      | 0.00431833 |
| HTR7   | -1.8627858      | 0.00541648 |
| IL12A  | -2.289003       | 0.00596437 |
| REG1CP | -6.3655607      | 0.00114347 |
| ZBTB48 | -1.8627858      | 0.00714396 |
| GREM2  | -3.9641684      | 0.00679631 |
| UGT1A3 | -1.1995722      | 0.00804717 |
| SLC25A21| -1.5112204     | 0.01079803 |
| HTN1   | -2.2367784      | 0.01239841 |
| TNS1   | -1.431632       | 0.01284794 |
| IFIH1  | -1.380793       | 0.01468609 |
| CELA2B | -2.0812906      | 0.01586124 |
| IL5    | -2.1994291      | 0.0172905  |
| MYOT   | -1.9866327      | 0.01536891 |
| CDKN1A | 2.4718188       | 0.00000126 |
| FAS    | 2.1834746       | 0.00000654 |
| SESN1  | 1.289292        | 0.00000226 |
| FDXR   | 1.965755        | 0.00002315 |
| PPM1D  | 1.6388142       | 0.00005177 |
| BTG2   | 1.4970197       | 0.00006986 |
| DDB2   | 1.0777424       | 0.00014334 |
| CYP39A1| 1.0008984       | 0.00025609 |
| XPC       | 1.056704 | 0.00037856 |
|-----------|----------|------------|
| MDM2      | 1.6441189| 0.00084947 |
| GADD45A   | 1.3567254| 0.00123572 |
| COL8A1    | 2.6565612| 0.00139461 |
| CYP4F11   | 2.8957083| 0.00033948 |
| TNP1      | 1.2582715| 0.00236534 |
| ATF3      | 1.3156861| 0.0024596  |
| RGS9      | 1.2250779| 0.00281468 |
| BAX       | 1.4006089| 0.00303574 |
| ZNF670-ZNF695| 1.1936179| 0.00394263 |
| ADGRE2    | 1.3658147| 0.00435895 |
| NRXN3     | 1.0653136| 0.00465841 |
| RYR1      | 1.4004822| 0.00621905 |
| SYNGR4    | 1.0586675| 0.00599735 |
| MLNR      | 3.2174996| 0.00169798 |
| FOXE3     | 1.4357355| 0.00719558 |
| SPIN2A    | 1.4427327| 0.00800466 |

**Enrichment Analysis And Pathway Of DEmRNAs**

We used the Metascape (http://metascape.org), an online software, to figure out the functions and pathways of 118 DEmRNAs. The most significant term was showed to represent the cluster (Fig. 2c). The p53 signaling pathway had numerous counts, including the targeted biomarker BAX. To show pathways of DEmRNAs, the enriched terms were constructed as a network. The different colors were classified by cluster ID, and the common cluster ID was closely related to each other (Fig. 2d).

At the same time, the top 5 terms of enrichments were listed as follows (Table 2). Through the GO and KEGG analysis, the terms of P53 signaling pathway were typical. DEmRNAs may took part in this pathway to enhance cisplatin resistance in NSCLC cells. We selected the terms having the best \( p \)-values from each of the 20 clusters to show the relationships between the terms. Through the Cytoscape, we constructed the network where each
node was an enriched term. Firstly, the network was colored by cluster ID (Fig. 3a) and then by p-value (Fig. 3b). We can identify that DEmRNAs were interrelate with each other. PPI networks can provide information on the molecular mechanism underlying cellular activity. In our study, a PPI network of differentially expressed mRNAs in NSCLC provided 4 mRNAs (CDKN1A, BAX, MDM2, GADD45A), which were associated with each other tightly (Fig. 3c). They were all key biomarkers in the P53 signaling pathway.

Table 2
Representative enriched terms ."Log10(P)" represents the value of different terms

| GO         | Category       | Description                        | Log10(P) |
|------------|----------------|------------------------------------|----------|
| M145       | Canonical Pathways | PID P53 DOWNSTREAM PATHWAY           | -8.99    |
| M5885      | Canonical Pathways | NABAMATRISOME ASSOCIATED             | -5.58    |
| GO:0009991 | Biological Processes | response to extracellular stimulus | -5.09    |
| GO:0006367 | Biological Processes | RNA polymerase II promoter           | -4.5     |
| GO:0046651 | Biological Processes | lymphocyte proliferation             | -4.32    |

Identification Of Targeted Molecule BAX

Through the function enrichment analysis and pathway, we found that the counts in the P53 signaling pathway were most typical. Four mRNAs, CDKN1A, BAX, MDM2, GADD45A attracted our interest deeply. BAX, may regulated the cisplatin resistance in NSCLC. We utilized the t-test to illustrate that the samples were representative (P-value = 0.013) by SPSS17.0[28]. We used the box diagram to prove that GSE6410 was normalized (Fig. 4a). In the NSCLC, the expression of BAX was highly showed in resistant lung cancer cells (Fig. 4b). The Kaplan Meier-plotter software (https://kmplot.com) was used to find the survival analysis of BAX (P < 0.01) (Fig. 4c). By the GEPIA database (http://gepia.cancer-pku.cn/index.html), BAX’s expression Profiling was higher in the LAC and LUSC cancer cells (Fig. 4d, 4e). As for cancers, different stages represent people’s prognosis. Through our study, we proved that BAX had little impact on patients’ staging (Fig. 4f). Some genes which were similarly with BAX, maybe up-regulated or down-regulated on chromosome (Fig. 4g). From the graph, some genes were considered to be over-expressed typically on the chromosome 14.

Searching Of Potential IncRNA XIST-miRNAs-BAX

As is shown above, the BAX maybe a useful molecule to cisplatin resistance. We chose the BAX as the basical molecule to find miRNAs through different databases, including miRDB(http://www.mirdb.org), Targetscan (http://www.targetscan.org), Starbase(https://starbase.sysu.edu.cn/), miRWalk(http://mirwalk.umm.uni-heidelberg.de/). 8 miRNAs, miR-3681-3p, miR-766-5p, miR-525-5p, miR-4640-3p, miR-128-3p, miR-216a-3p, miR-520a-5p, miR-214-3p were expressed commonly by the Dram-Venn-Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) (Fig. 5b). The ceRNA network of IncRNA XIST-8 miRNAs-BAX were showed in the map (Fig. 5a) and we used the cytoHubba to choose the Hub gene. In the NSCLC, we predicted the survival of 8 miRNAs by Kaplan-Meier-Plotter, but only 4 miRNAs significantly affected quality of people’s life and longevity (Fig. 5d). Using the starbase, we found IncRNAs of these 4 miRNAs and selected out together expressed IncRNA (Fig. 5c). IncRNA XIST and MIR29B2CHG maybe the targeted ceRNAs. Combining with some articles reported in recent years, IncRNA XIST has some researches in cisplatin sensitive-resistant NSCLC cells. We considered that IncRNA XIST may act as a miRNA sponge to lead cisplatin resistance. At the same time, we combined the GSE43249’s differentially expressed miRNAs, 2 miRNAs were included, such as miR-520, miR-
525. miRcode database was used to verify miRNAs from IncRNA XIST. MiR-520, miR-525 were targeted miRNAs of IncRNA XIST. In the present study, IncRNA XIST acted as a member of ceRNA, competitively bound miRNA520 to regulate the BAX, apoptosis associated X, in the P53 signaling pathway.

Discussion

Lung cancer represents a common cancer all over the world and lung adenocarcinoma (LAC) has most numerous type with the high diversity[29]. At present, there are many treatments for different types of cancer, such as surgical operation, chemotherapy, and so on. With the development of molecule-targeted drugs, the molecule-targeted treatment of tumors has been widely agreed. However, due to various reasons, a large number of patients are not sensitive to drugs. Because of complex molecular mechanisms, multiple drug resistance may lead chemotherapeutic failure for lung cancer patients[30].

DDP is a significant chemotherapeutic drug in lung cancer. Thus, the potential mechanism of chemical resistance remains necessary[31]. Therefore the further understanding of resistant theory is helpful for choosing chemotherapy drugs.

In recent years, IncRNAs have been explored through functional analysis[32]. More importantly, IncRNA acts as a member of ceRNA, participating in many functions in the field of cancer, such as protein modification, cell proliferation. Cell apoptosis, a classic signaling pathway, participates in cancer Proliferation and drug resistance.

Our study explored the role of potential IncRNA XIST, acting as a ceRNA of miR520 by regulating BAX involving in the P53 signaling pathway. Through the enrichment analysis of 118 DEmRNAs, we found that the targeted molecule BAX had higher expression in DDP. Four online databases were used to identify potential miRNAs mediated BAX. LncRNA XIST regulated four valuable miRNAs by starbase database.

The present findings clearly demonstrate that the expression of long non-coding RNA (IncRNA XIST) is up-regulated in lung cancer cells, and it maybe involve in the cell proliferation and TGF-β1-mediated apoptosis[33]. And IncRNA XIST may have some functions by downregulating miRNA-144. Compared with previous studies, the present study eventually constructed ceRNA[34] network of IncRNA XIST-miRNAs-BAX. We selected top 10 hub RNAs with higher degree through the cytoHubba (Fig. 6a, 6b, 6c, 6d).

For different tumors, the relations of long noncoding RNA (IncRNA) and microRNA influence cell process, cell growth, and drug resistance[35, 36]. We all know that miRNAs regulate their genes to perform different functions[37]. For lung cancer, miRNAs have numerous functions, such as tumor cell proliferation and progression, inflammation[38]. A previous study has showed that miR1284 influences apoptosis of lung tumorigenesis[39]. BAX is associated with apoptosis in cancer cells through some reports, and it participates in the classic P53 signal pathway. We predicted different molecules through individual databases. This study firstly explored 8 potential miRNAs. Later after analysis, only 4miRNAs were associated with patients’ living (P < 0.05). Combining with the GSE43249 and miRcode database, miR520 was a potential molecule by regulating BAX in cisplatin sensitive-resistant NSCLC cells.

Through our study, potential molecules maybe provided for DDP. In some way, this can reduce cisplatin resistance to chemotherapy and be benefit of clinical NSCLC patients. Of course, there are certain limitations in present study. These connected molecules were predicted only in theoretical aspects. At present, the corresponding experimental verification is lacking. More importantly, the present novel indicated that IncRNA XIST-miRNA 520-BAX influence cisplatin resistance in NSCLC cells.

Conclusions

In conclusion, BAX and LncRNA XIST were up-regulated in NSCLC patients. LncRNA XIST may act as a miRNA520 sponge by regulating BAX, associated apoptosis X, through activating in the P53 signaling pathway to effect
cisplatin resistance. To summary, based on our data, this may enrich the effective therapeutic methods for cisplatin resistant NSCLC patients.

List Of Abbreviations

LncRNA Long non-coding RNA
GO Gene ontology
KEGG Kyoto Encyclopedia of Genes and Genomes
DAVID The annotation, visualization and integrated discovery database
NSCLC Non-small cell lung cancer
LAC Lung adenocarcinoma
|Log2FC| Absolute Log Fold change

Declarations

Ethics approval and consent to participate
RNA sequencing data origined from GEO database, thus, no ethics committee certification was required.

Consent for publication
Not applicable.

Availability of data and materials
All data are available from the sources listed in the manuscript—GEO database (https://www.ncbi.nlm.nih.gov/gds/?term=).

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
TTL and RL, XL conceived and designed the study. XJZ, CH and JPL collected the literature. TTL draft the manuscript, YQQ revised the manuscript. All authors read and approved the final manuscript.

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GSE6410(GPL201) \textsc{SPSS}

\textbf{GEO2R DEmRNAs} \\
|Log2 FC| >1, \( p < 0.05 \)

\textbf{GEPIA, MetaScape, DAVID} \\
\textbf{GO KEGG} PPI network

\textbf{miRDB, Targetscan} \\
\textbf{Starbase, miRWalk} \\
\textbf{miRNAs Kaplan Meier-plotter}
The detailed process of constructing a ceRNA network about lncRNAs- miRNAs - mRNA.
Figure 2

The map and enrichment analysis of DERNAs. (a) Volcano map of differentially expressed mRNAs in cisplatin sensitive-resistant NSCLC, red represents up-regulated mRNAs, red triangle is the most obvious in mRNAs, blue triangle is considered to be down-regulated significantly; (b) Heat map of DEmRNAs in NSCLC. The red represents more expression in samples. (c) GO enrichment results of 114 (118) DEmRNAs, P53 pathway is remarkable. The size of the rectangle represents the number of enrichment analysis participants. (d) Network of enriched terms: colored by cluster ID, the red is the molecule BAX. All nodes are typically close to each other.
Figure 3
Network of enriched terms by metascape software. (a) colored by cluster ID, nodes are different terms. (b) colored by p-value. The significant p-value represents that terms contain more genes. (c) PPI network and MCODE components identified in the gene lists. The red represents mRNAs have good correlation and mediate in the P53 signaling pathway, including the targeted molecule BAX.
The expression of targeted molecule BAX. (a) The box diagram demonstrates the samples of cisplatin sensitive-resistant samples are standardized. (b) BAX is up-regulated obviously in cisplatin resistant NSCLC cells. Detailed research of BAX in some online databases. (c) BAX is associated with overall survival ($P<0.05$) in NSCLC. (d,e) The expression of BAX in LAC and LUSC is higher than normal cells. (f) BAX has little impact on patient’s staging. (g) On the chromosome 14, some genes, associated with BAX are considered to be over-expressed typically.
Figure 5

The construction of ceRNA in NSCLC. (a) The ceRNA network of lncRNA-8miRNAs-BAX, red is the BAX, blue is miRNAs, pink is lncRNAs. (b) The combination of miRNAs and lncRNAs. Four kinds of databases are mixed to explore miRNAs. (c) Looking for the common lncRNA of 4 miRNAs. Two common lncRNAs are XIST and MIR29B2CHG. (d) The OS (overall survival) analysis of 4 miRNAs in NSCLC patients using Kaplan Meier curve (P<0.05).
Figure 6

The ceRNA network of IncRNA XIST-miRNAs-BAX by cytoscape. (a, b, c) The red represents the BAX, the yellow is miRNAs and the blue is IncRNAs. (d) Through the cytoHubba, we select top 10 RNAs with higher degree. Different colors show different RNAs.