Evaluation of Annexins Family as Potential Biomarker for Predicting Progression and Prognosis in Clear Renal Cell Carcinoma

Jiyu Zhao

Chuiyangliu Hospital Beijing

Luchen Chang

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Jianping Tu

The third hospital of Xiamen

Bei Sun

Tianjin Medical University Cancer Institute and Hospital: Tianjin Tumor Hospital

Xi Wei (✉ weixi@tmu.edu.cn)

Tianjin Medical University Cancer Institute and Hospital, <https://orcid.org/0000-0003-4734-3900>

Research

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Abstract

Background: Annexins family (ANXAs), as a Ca^{2+} -dependent, phospholipid-binding protein super family, participated in a wide variety of biological activities and has been reported to be dysregulated in numerous types of human cancers. Evidence from cell lines and human tissues indicates that ANXAs are involved in kidney clear renal cell carcinoma (KIRC) tumorigenesis. However, their prognostic value and expression pattern associated with KIRC remain to be elucidated.

Methods: We visited public databases including ONCOMINE, Gene Expression Profiling Interactive Analysis (GEPIA), Kaplan-Meier plotter, cBioportal and GeneMANIA to conduct comprehensive bioinformatics analysis, and tried to detect basic relationships between each Annexins family member and KIRC.

Results: In the present study, we found that the expression level of ANXA1/2/4/5/6/7/8/13 in clear renal cell carcinoma tissue was higher than that in kidney tissue, while the expression level of ANXA3/9/11 in the former was lower than that in the latter. The expression level of ANXA7/8/13 is related to the stage of the tumor. Survival analysis using the Kaplan-Meier plotter database showed that the high transcription level of ANXA2/5/8/10 is related to the low overall survival rate (OS) in predicting KIRC patients. In contrast, high ANXA3/4/7/9/11/13 levels are associated with high OS in these patients.

Conclusions: Our study implies that ANXA4/8/13 are potential targets of precision therapy for patients with KIRC and that ANXA2/5/8/10 are new biomarkers for the prognosis of KIRC.

Background

Renal cell cancer (RCC) is one of the most common malignant, and about 73,820 patients were diagnosed in 2019¹. Majority of RCC patients have kidney clear renal cell carcinoma (KIRC) histology (75– 80%), and the other common histological types include papillary, chromophobe, hereditary leiomyomatosis associated RCC, collecting duct carcinoma, etc. These are called “non-clear cell cancers”². Despite advancements in diagnostic methods and operative techniques have allowed for more effective treatment of KIRC, the 5-year overall survival rate of metastatic KIRC remains less than 20%¹. Therefore, it is necessary to investigate molecular markers to refine prognostic prediction and identify potential treatment targets.

Annexins are Ca^{2+} -dependent, phospholipid-binding protein super family, with members expressed in the zoology and botany cell. Annexins, including 12 family members, play a major role in the regulation of a broad range physiological processes linked to cellular membranes³. Previous studies have revealed that aberrant expression of ANXAs was frequently observed in various types of cancer, including cervical cancer⁴, bladder cancer^{5,6}, breast cancer^{7–9}, ovarian cancer¹⁰, lung cancer^{11,12}, oral squamous cell carcinoma¹³, hepatocellular carcinoma^{14,15} and Cholangiocarcinoma¹⁶. ANXAs may function as either oncogenes or suppressors depending on tumor biology.

ANXA1/2/3/4/5/7 have been reported to be dysregulated in KIRC^{17–20}. However, the majority of these studies only focused on the changes expression levels and a few prognosis data available. The expression patterns, potential biological functions and the prognostic value of Annexins in KIRC have yet to be fully elucidated.

As far as we know, there is no bioinformatics analysis of Annexins profiles has been applied to investigate in KIRC. The use of large scale DNA/RNA sequencing has undergone a revolutionary development and become an integral part of biomedical research. In the present study, we performed a collective analysis of thousands of gene expression or variation in copy number analyses published online to investigate the expression of Annexin family members in the database to determine its clinical value in KIRC.

Results

Transcriptional levels of ANXAs in patients with KIRC

Twelve ANXA factors have been found in mammalian cells. We used the ONCOMINE database to compare the transcription levels of ANXA in cancer with those in normal samples (Fig. 1). The mRNA expression levels of ANXA4 were significantly upregulated in patients with KIRC in twelve datasets. In Higgins's dataset²¹, ANXA4 is overexpressed compared with that in the normal samples in KIRC with a fold change of 3.662. In Gumz's dataset²², ANXA4 is also overexpressed in KIRC with a fold change of 13.931. The transcription levels of ANXA4 in KIRC are higher than those in normal tissues in Yusenko²³ and Jones's datasets²⁴, and their fold changes are 4.763 and 2.598, respectively. (Table 1)

Table 1

The ANXAs expression between different types of renal clear cell carcinoma and kidney tissues.

GENE	Cancer tissue vs Normal tissue	Fold change	t-test	p-value	Dataset
ANXA1	Renal Clear Cell Carcinoma vs kidney	4.853	11.741	1.18E-12	Yusenko et al
	Renal Clear Cell Carcinoma vs kidney	5.348	11.22	7.71E-10	Gumz et al
	Renal Clear Cell Carcinoma vs kidney	3.26	6.477	4.53E-06	Beroukhim et al
ANXA2	Renal Clear Cell Carcinoma vs kidney	2.358	10.276	3.49E-10	Higgins et al
	Renal Clear Cell Carcinoma vs kidney	2.444	9.038	2.17E-08	Gumz et al
	Renal Clear Cell Carcinoma vs kidney	2.383	10.586	9.99E-14	Jones et al
ANXA3	NA	NA	NA	NA	NA
ANXA4	Renal Clear Cell Carcinoma vs kidney	3.662	12.691	2.99E-12	Higgins et al
	Renal Clear Cell Carcinoma vs kidney	3.577	13.931	2.23E-11	Gumz et al
	Renal Clear Cell Carcinoma vs kidney	4.763	9.538	5.94E-08	Yusenko et al
	Renal Clear Cell Carcinoma vs kidney	2.598	8.071	1.12E-09	Jones et al
ANXA5	NA	NA	NA	NA	NA
ANXA6	NA	NA	NA	NA	NA
ANXA7	Renal Clear Cell Carcinoma vs kidney	2.565	7.775	5.98E-07	Jones et al
ANXA8	NA	NA	NA	NA	NA
ANXA9	NA	NA	NA	NA	NA
ANXA10	NA	NA	NA	NA	NA
ANXA11	NA	NA	NA	NA	NA
ANXA13	NA	NA	NA	NA	NA

Higgins²¹ showed another mRNA expression factor with increased expression; that is, ANXA2 has a fold change of 2.358 in patients with kidney renal clear cell carcinoma compared with that in patients with

normal kidney tissues. ANXA2 overexpression is also found in kidney renal clear cell carcinoma with a fold change of 2.444 in Gumz's dataset²² and 2.383 in Jones's dataset²⁴. (Table 1)

The mRNA expression levels of ANXA1 and ANXA7 were upregulated in patients with KIRC. The transcription level of ANXA1 in KIRC is higher than those in kidney tissues, and their fold changes are 4.853, 5.348 and 3.26 in Yusenko²³, Gumz²² and Beroukhir's datasets²⁵ respectively. In Jones's datasets²⁴, the mRNA expression of ANXA7 in KIRC increases with a fold change of 2.565.

Relationship between the mRNA levels of ANXAs and the clinicopathological characteristics of patients with KIRC

Using GEPIA (Gene Expression Profiling Interactive Analysis) dataset (<http://gepia.cancer-pku.cn/>), we compared the mRNA expression of ANXAs factors between KIRC and kidney tissues. The results indicated that the expression levels of ANXA1, ANXA2, ANXA4, ANXA5, ANXA6, ANXA8, and ANXA13 were higher in KIRC tissues than in kidney tissues, whereas and the expression level of ANXA3, ANXA7, ANXA9 and ANXA11 were lower in the former than in the latter (Fig. 2). We also analyzed the expression of ANXAs with tumor stage for KIRC. ANXA7, ANXA8 and ANXA13 groups significantly varied, whereas ANXA1, ANXA2, ANXA3, ANXA4, ANXA5, ANXA6, ANXA9, ANXA10 and ANXA11 groups did not significantly differ (Fig. 3).

Association of the increased and decreased mRNA expression of ANXAs with the improved prognosis of patients with KIRC

We further explored the critical efficiency of ANXAs in the survival of KIRC patients. We applied the Kaplan-Meier Plotter tool using a publicly available dataset, (http://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq) to analyze 530 KIRC patients for ANXAs correlation between mRNA levels and survival. Analysis by Kaplan-Meier curve and log-rank test revealed that increased levels of ANXA2/5/8/10 mRNA and decreased levels of ANXA3/4/7/9/11/13 mRNA in KIRC patients were significantly associated with decreased overall survival (OS) ($P < 0.05$) (Fig. 4). KIRC patients with higher levels of ANXA1/4/5/6 mRNA or lower levels of ANXA10 mRNA were predicted to have lower relapse free survival (RFS), ($p < 0.05$) (Fig. 5).

Amplification, deletion, mutation, and fusion of ANXAs in KIRC

Genetic variations of Annexins in 317 cases retrieved from four studies, including 35 cases from Dana-Farber Cancer Institute²⁶ (DFCI), 98 cases from Beijing Genomics Institute²⁷ (BGI), 78 cases from Cancer Research UK London Research Institute²⁸ (IRC) and 106 cases from the University of Tokyo²⁹ (Utokyo), were analyzed using the cBioPortal database (Figure. 6). We found that there are varying degrees of genetic variation among 12 ANXA family members, of which ANXA6 has the highest incidence of genetic variation. Most genetic variations in Annexins were amplifications, although ANXA3, ANXA4 and ANXA10

had higher probabilities of mutation events. Deep deletions were found in ANXA5 and ANXA10. In addition, there is no gene fusion event found in four datasets.

Correlations between ANXAs and construction of a gene–gene interaction network

The constructed functional network based on the gene function predictions of the 12 ANXAs through GeneMANIA database, is shown in Fig. 7. The 12 central nodes representing ANXAs were surrounded by genes which greatly correlated with physical interactions, co-expression, predictions, co-localization, and genetic interactions.

Established a gene-gene interaction network of 12 Annexin genes, and analyzed their functions through the GeneMANIA database(Fig. 7). The top 5 genes representing the most relevant ones with the ANXAs including STXBP2 (syntaxin binding protein 2), RACK1 (receptor for activated C kinase 1), S100A10 (S100 calcium binding protein A10), NDRG1 (N-myc downstream regulated 1) and HARS (Histidyl-tRNA synthetase).STXBP2 was correlated with ANXA3 in terms of co-localization, and physical interacted with ANXA3 and ANXA11. RACK1 was physically interacted with ANXA2. S100A10 was correlated with ANXA2 in terms of physical interactions, co-localized with ANXA5 and co-expressed with ANXA11, ANXA2, ANXA6, ANXA7, ANXA8, ANXA9, ANXA10 and ANXA11. NDRG1 was physically interacted with ANXA5 and was co-expressed with ANXA3, ANXA4, and ANXA6. In addition, HARS was correlated with ANXA5 in terms of physical interactions and correlated with ANXA6 in terms of co-expression.

We also found that ANXAs were the greatest correlation with calcium-dependent phospholipid binding. Additionally, they were also correlated with specific granules, phospholipid binding, S100 protein binding, lipase inhibitor activity, secretory granules, regulation of vesicle-mediated transport, postGolgi vesicle-mediated transport, calcium-dependent protein binding, and enzyme-inhibitor activity.

Discussion

ANAXs, as a Ca^{2+} -dependent, phospholipid-binding protein super family, have been reported in numerous types of human cancer, and have participated in a wide variety of biological activities, such as tumorigenesis, progression and resistance to chemotherapeutic agents^{3,30}. However, a further comprehensive bioinformatics analysis of ANAXs in KIRC has yet to be performed. In the present study, we analyzed the relationship between different ANAX factors expression and prognoses (OS and RFS) of KIRC for the first time. We hope that our findings may help create a foundation to better understand and improve current therapies and prognostic accuracy for patients with KIRC.

ANXA1 is known as an anti-inflammatory protein, but recognized to have a broader role in tumor cell biology beyond inflammation alone. ANXA1, located on the human chromosome 9q21.13, is the first characterized member of the super-family¹². Transposition of ANXA1, which was found in Esophagus cancer, could affect the activities of arachidonic acid metabolism³¹. However, It's more likely a double-edged sword due to its numerous and sometimes opposite functions. Yamanoi et al¹⁸ found that

knockdown of ANXA1 inhibits the proliferation, migration, invasion and adhesion of kidney carcinoma cells. We first used the oncomine databases to reveal that ANXA1 mRNA expression level was significantly higher in KIRC tissues than that in normal tissues. However, its expression didn't correlate with specific clinical features in KIRC. Using the Kaplan-Meier Plotter, we estimate the prognostic value of ANXA1 in patients with KIRC. The patients with KIRC with high mRNA level of the ANXA1 was predicted to have a lower RFS.

The ANXA2 monomer exists in cell cytoplasm and heterotetramer complexed with S100A10 which exists on cell membranes. Sadashiv et al³² reported that moderate immune expression of ANXA2 was found in the proximal convoluted tubules which were considered as the origin of KIRC. Yang et al²⁰ demonstrated that the abilities of migration and invasion of the tumor cell were suppressed by silencing the expression of ANXA2, whereas tumor cell proliferation was not affected. In our study, the expression of ANXA2 in KIRC tissues was higher than that in normal tissues. We also demonstrated that high ANXA2 expression was significantly correlated with poor OS in the patients with KIRC. However, its expression wasn't correlated with tumor stage.

Bianchi et al³³ cultured primary cell from human renal cell carcinoma and observed two spliced isoforms of ANXA3. However, one spliced isoform of ANXA3 down-regulated in KIRC and the other up-regulated. The total ANXA3 protein down-regulated in KIRC cultures based on microarray analysis. In our report, we demonstrated that the expressions of ANXA3 were not significantly different between tumor and normal tissue, which seemed inconsistent with the Bianchi et al. But we found a high ANXA3 expression was significantly correlated with favorable OS in the patients with KIRC.

A data-independent acquisition-mass spectrometry proteomic approach demonstrated ANXA4 at the expression levels is higher in KIRC than normal tissue³⁴. In our research, we proved for the first time that mRNA expression of ANXA4 was higher in KIRC tissues than in normal tissues, but this expression did not correlate with tumor progression stage. Meanwhile, low expression of ANXA4 was remarkably associated with poor OS in KIRC patients. This study seems that ANXA4 acts as a tumor suppressor gene.

ANXA5 was upregulated in KIRC, and high expression was associated with the higher clinical stage and histological grade¹⁹. We found the similar result in our study, that is, the expression of ANXA5 in KIRC tissues was higher than that in normal tissues. And a higher ANXA5 expression was significantly related with poor OS in the patients with KIRC.

ANXA6 is found highly expressed in a variety of tumors, such as acute myeloid leukemia³⁵, pancreatic cancer³⁶, and breast carcinoma³⁷. However, its expression and prognostic role in KIRC have not been reported. In the present study, we confirmed that ANXA6 expression was higher in KIRC tissues than in normal tissues, but this expression did not relate to the stage of the tumor. A higher ANXA6 expression was significantly correlated with poor RFS in the patients with KIRC. Moreover, ANXA6 displayed the highest incidence rate of genetic variations among the super family, which might relate to kidney tumor progression.

The ANXA7-GTPase is considered as a tumor suppressor frequently inactivated by genomic alterations at 10q21 in a variety of human malignancies, including KIRC³⁸. In this report, we demonstrated that the expression of ANXA7 in KIRC tissues was higher than that in normal tissues in Jones's dataset²⁴, but we also got opposite result from the GEPIA dataset. It was discovered that high-stage KIRC exhibited a significantly reduced ANXA7 expression. Moreover, the decreased ANXA7 mRNA levels were significantly associated with the lower OS that conformed to its role of a tumor suppressor.

ANXA8 is highly expressed in patients with several malignancies, such as ovarian cancer³⁹ and oral squamous cell carcinoma⁴⁰. Harumi et al⁴¹ found that co-transfection with an expression vector for ANXA8 and a reporter gene vector containing the HIF-1 α promoter enhanced the activity of the HIF-1 α promoter. And it might play a role in calcium fluctuation-mediated HIF-1 α transcriptional activation in pancreatic cancer. However, ANXA8's expression and prognosis role in KIRC have not been reported. In this report, we found that the expression of ANXA8 in KIRC tissues was higher than that in normal tissues, and the high-stage KIRC exhibited a significantly increased ANXA8 expression. The increased ANXA8 mRNA was significantly associated with the poor OS.

ANXA9 and ANXA11's expression and prognosis role in KIRC have not been reported. Yu et al⁴² demonstrated ANXA9 promoted invasion and metastasis of colorectal cancer and predicted poor prognosis. ANXA9 showed high expression in head and neck squamous cell carcinomas and associated to the tumor differentiation grade. Hua et al⁴³ found ANXA11 participated in the gastric cancer proliferation, migration, and invasion via the AKT/GSK-3 β pathway. But in our report, we demonstrated that the expression of ANXA9 and ANXA11 in KIRC tissues were lower than that in normal tissues, and the expression did not correlate with tumor stage. The decreased ANXA9 and ANXA11 mRNA levels were significantly associated with the poor OS.

It has been proved that abnormal expression of ANXA10 is playing a key role in the generation, progress and prognosis of tumor⁴⁴⁻⁴⁶, although its functional role remains to be clarified and has not been reported in KIRC. The decreased expression of ANXA10 probably participates in malignant progression and poor prognosis⁴⁷. Overexpression of ANXA10 could promote apoptosis of hepatocellular carcinoma cell⁴⁸. However, the results of the present study confirmed that high expression of ANXA10 was significantly associated with the poor OS. And the expression of ANXA10 did not associated with clinical stage.

ANXA13 is the latest ANXAs member to be identified. There are limited studies available that focus on ANXA13 in cancer. ANXA13 ramps up cell growth and invasion. It also portended lymph node metastasis and poor prognosis in human colorectal cancer patients⁴⁹. ANXA13 was identified as a regulator of chemotherapy resistance because ectopic overexpression of ANXA13 could increase the sensitivity of malignant breast cancer cells to Rapamycin⁵⁰. In the present study, the expression level of ANXA13 was higher in KIRC tissues than in kidney tissues, and the high-stage KIRC exhibited a significantly decreased

ANAX13 expression. Moreover, the decreased ANXA13 mRNA level was significantly associated with the poor OS.

Cancer cells carry different mutations which leads to wide varieties of clinical manifestation. Many gene show variations in copy number alterations and many may be associated with recurrence and death⁵¹. In present study, we found gene alteration in 9.32% of Annexin family in the database, including an amplification rate of 7.77 %, and a mutation rate of 1.44 %. The incidence rate of genetic variations in the ANXA6 gene was up to 7.0 %, whereas ANXA3, ANXA4 and ANXA10 had higher probabilities of mutation events. However, our finding shown the KIRC has relatively low alteration rate in ANXAs compared with other cancers^{39,52–54}.

We adopted GeneMANIA database to construct a gene-gene interaction network which clarified the mechanisms of function of ANXAs in kidney cancer. The results showed that 20 genes, including STXBP2, RACK1, S100A10, NDRG1 and HARS, were enriched in this network based on their functions associated with physical interactions, co-expression, co-localization, pathway and genetic interaction. It has been demonstrated that RACK1 could regulate the ANXA2 phosphorylation to make it be involved in the invasion and metastasis of drug-resistant carcinoma cells.⁵⁵ NDRG1, like ANXAs involving in plasma membrane repair, has been recognized as a suppressor of carcinoma by decreasing EMT-associated protein expression⁵⁶. Aberrant expression of these interacting genes is related to the tumorigenesis and progression of tumors, but these interactions with ANXAs in kidney cancer are intriguing, and still need substantial experimental confirmation. It has been demonstrated that S100A10, located in the plasma membrane, could unite with ANXA2 to form a heterotetramer which composed of two subunits of S100A10 and ANXA2. The heterotetramer could activate the plasminogen activation pathway played a key role in cellular repair and further promote degradation of the extracellular matrix to increase the invasion capability of carcinoma cells⁵⁷.

Conclusion

In the present study, the expression of ANXAs were systemically analyzed to evaluate their clinical and prognostic value in KIRC and which provided an important molecular biological basis for understanding the complex development of KIRC. The results indicated that ANXA1/2/4/7/13 may be potential therapeutic targets for KIRC treatment, whereas ANXA2/5/8/10 may be potential prognostic biomarkers of KIRC. The results of the present study introduced ANXA4/8/13 as good candidates for future experimental works.

Methods

Ethics statement

This study was approved by the Academic Committee of ChuiYangLiu Hospital affiliated to Tsinghua University, and performed in accordance with the ethical principles expressed in the Declaration of

Helsinki. All data are collected from published literature.

ONCOMINE analysis

The transcription levels of ANXAs in different cancers were analyzed using the online tumor microarray database ONCOMINE databases (<https://www.oncomine.org/>). Comparison of ANXAs mRNA expression levels between tumor and normal samples was performed using Students't-test. The p-value and fold change were used as cut-off points at 0.0001 and 2, respectively.

GEPIA (Gene Expression Profiling Interactive Analysis) dataset

We used the online database Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancerpku.cn>) to analyze ANXAs mRNA sequencing expression data⁵⁸. GEPIA could provide mRNA differential expression analysis according to cancer stage, patient survival data using one-way ANOVA. The analysis setting is $|\log_2(FC)| \geq 1$ and $P \leq 0.01$.

The Kaplan-Meier plotter

We examined the prognostic value (OS and RFS) of ANXAs mRNA expression in KIRC using the Kaplan-Meier plotter database⁵⁹ (www.kmplot.com). And we only select JetSet best probe set and automatically choose best cut-off to take analysis.

The cBioPortal

We examined the mutations, putative copy-number alterations (CNA) of ANXAs in KIRC using cBioPortal (<http://www.cbioportal.org/>), a online database on 30 different cancers collecting from the Cancer Genome Atlas⁶⁰. 317 KIRC cases with pathology reports were selected in our study. The online tools and data sources are provided by cBioPortal.

GeneMANIA

To explore interactive functional association among ANXAs, we use GeneMANIA (<http://www.genemania.org>) to create an interactive functional-association network. The online tools and data sources are provided by GeneMANIA.

Abbreviations

ANXAs

Annexins family

KIRC

Kidney clear renal cell carcinoma

GEPIA

Gene Expression Profiling Interactive Analysis

OS

Overall survival
RCC
Renal cell cancer
CNA
Copy-number alterations
RFS
Relapse free survival
STXBP2
Syntaxin binding protein 2
RACK1
Receptor for activated C kinase 1
S100A10
S100 calcium binding protein A10
NDRG1
N-myc downstream regulated 1
HARS
Histidyl-tRNA synthetase

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Human and Animal Rights

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets generated during and/or analyzed during the current study are available in the ONCOMINE database (<https://www.oncomine.org/>), Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancerpku.cn>), the Kaplan–Meier plotter database AND cBioPortal (<http://www.cbioportal.org/>).

Conflict of Interest

The authors declare that they have no competing interests.

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Figures

Figure 1

The transcription levels of ANXAs in different types of cancers (ONCOMINE). The threshold was set to following parameters: fold change= 2 and P-value= 0.0001. The cell number indicates the number of datasets that meets the thresholds. The color red or blue directly indicates up- or down-regulation, respectively.

Figure 2

The mRNA expression levels of ANXAs in clear renal cell carcinoma and normal kidney tissues (GEPIA). The plots show mRNA expression of Annexins in kidney tumor (red plot) and the corresponding expression in normal tissues (green plot).

Figure 3

Correlation analysis of the ANXAs expression and clinical stages in clear renal cell carcinoma (GEPIA). Spearman's correlation analysis between gene and clinical stages is based on the entire clinical stages.

Figure 4

Prognostic values of ANXAs in clear renal cell carcinoma (overall survival in Kaplan–Meier plotter). The P-values were calculated using the log-rank test.

Figure 5

Prognostic values of ANXAs in clear renal cell carcinoma (relapse free survival in Kaplan–Meier plotter). The P-values were calculated using the log-rank test.

Figure 6

Analyses of genetic variations in ANXAs in clear renal cell carcinoma (cBioPortal). a OncoPrint visual summary of variations on a query of Annexin family members and overview of the analyses of genetic variations in ANXAs. b Analyses of genetic variations in Annexin family members reported in different studies. c Analyses of genetic variations in Annexin family members respectively in different studies.

Figure 7

Gene-gene interaction network among Annexin family members (GeneMANIA). Each node represents a gene. The node size represents the strength of interactions. The connection lines represent the types of gene-gene interactions, and the line color represents the types of interactions. The node color represents the possible functions of respective genes.