Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Identification of key molecules for castration resistance prostate cancer by bioinformatic analysis

Jieping Hu (hu_jieping@163.com)
The First Affiliated Hospital of Nanchang University
Yue Yu
The First Affiliated Hospital of Nanchang University
Wei Liu
The First Affiliated Hospital of Nanchang University
Haibo Xi
The First Affiliated Hospital of Nanchang University

Research Article

Keywords: castration resistance prostate cancer, bioinformatic analysis, lipid metabolism, PMEPA1, SLC45A3

Posted Date: April 18th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1555079/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Objectives

Castration resistance prostate cancer (CRPC) is a complex tumor associated with highly mortality. To discover key molecular in CRPC, we dissected the data of CRPC based on arrays by bioinformatic analysis.

Methods

The gene expression profiling datasets of prostate cancer were analyzed online. The Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) was used to perform Gene Ontology (GO) functional and KEGG pathway enrichment analyses. Molecular Complex Detection (MCODE) in Cytoscape software (Cytoscape_v3.6.1) was applied to screen hub genes. Gene expression data were obtained from ONCOMINE website (https://www.oncomine.org/). The gene expression and survival data were download and analyzed.

Results

4 datasets (GSE104935, GSE120005, GSE78201, and GSE21887) were included for analysis. The 15 overlap up regulated genes and 27 overlap down regulated genes were identified and analyzed by DAVID. 11 proteins corresponding to genes (ELOVL6, ABCA1, FOXO3, and TNRC6B; ALDH1A3, OSBPL8, ACSL3, SLC45A3, KLK2, FKBP5, and PMEPA1) were identified to be key genes. Gene expression suggested OSBPL8, PMEPA1 and SLC45A3 were different expressed in CRPC patients, these genes were also associated with survival.

Conclusions

OSBPL8, PMEPA1 and SLC45A3 were involved with CRPC. Their functions needed more detailed research to reveal.

Introduction

Antiandrogen therapy was the essential treatment for prostate cancer, however, castration resistance prostate cancer (CRPC) eventually developed after 24–36 months[1]. Significant progress has been made in the past several years in the mechanisms of CRPC, androgen receptor (AR) independence or indifference that prostate cancer (PCa) no longer require activation of the AR axis was proposed, AR axis activation mediated by pathways that bypass the AR was discovered[2], novel biomarkers were demonstrated to play critical roles in CRPC progression, such as *ZBTB46*, *SPDEF*, and *ETV6*[3, 4]. Non-hormonal targets that small molecule inhibitors of signaling, DNA repair, or epigenetic pathways, and

immunotherapy are emerging for the treatment of patients with advanced prostate cancer[5]. These progresses depend on the key molecular identifying in CRPC progression.

For last decades, next-generation sequencing (NGS) and gene array has drastically increasing the understanding of PCa, and provided diagnostic and therapeutic implications for PCa. Novel molecules and pathways were found to be involved with PCa progression[2]. However, CRPC was much different from PCa, it was refractory to antiandrogen therapy. Tumor cells suffered from a sequence of alteration, details could be found elsewhere[6, 7]. The mechanisms driving progression from androgen-dependent PCa to CRPC are still largely unclear. NGS or gene array comparing androgen sensitive prostate cancer and androgen resistance prostate cancer was limited and heterogeneity. Here, we concluded the available NGS and gene array data to identify key molecules for CRPC by bioinformatic analysis.

Materials And Methods

Data acquisition

Online database was searched on GEO datasets. Search details was (("orchiectomy"[MeSH Terms] OR "castration"[MeSH Terms] OR castration[All Fields]) AND resistance[All Fields] AND ("prostatic neoplasms"[MeSH Terms] OR prostate cancer[All Fields])) AND "Homo sapiens"[porgn]. The gene expression profiling datasets of prostate cancer were analyzed online (GEO; https://www.ncbi.nlm.nih.gov/geo/geo2r/).

Identify differentially expressed genes

To analysis the microarray data, we compared the gene expression between androgen sensitive prostate cancer and castration resistance prostate cancer to identify genes involved with androgen resistance. Differentially expressed genes were screened by p value and fold change (FC). Differentially expressed genes (DEGs) were restricted by p value < 0.05 and |FC|>2. Duplicated were deleted by software EXCEL.

Data integration

Four DEGs were obtained from four datasets, conditional formatting in software EXCEL applied and duplicates were highlighted between two DEGs. Then, conditional formatting applied again between duplicates and another DEG. The genes upregulated and downregulated were measured respectively.

Gene Ontology and KEGG pathway analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) was used to perform Gene Ontology (GO) functional and KEGG pathway enrichment analyses. $p < 0.05$ was considered as statistically significant.

Retrieve interacting genes

Search Tool for the Retrieval of Interacting Genes (STRING) is an online tool (https://string-db.org) designed to integrate information by consolidating known and predicted protein-protein association data.

Molecular Complex Detection (MCODE) in Cytoscape software (Cytoscape_v3.6.1) was applied to screen key genes.

Gene expression data

Gene expression data were obtained from ONCOMINE website (https://www.oncomine.org/). Cancer type was restricted by prostate cancer, the expressions of ALDH1A3, FKBP5, OSBPL8, ABCA1, PMEPA1, KLK2, SLC45A3, FOXO3, TNRC6B, ACSL3, and ELOVL6 were obtained.

Survival analysis

The protein expression and survival data were download from OncoLnc (http://www.oncolnc.org/), these data were obtained for cox regression analysis. Disease Free Survival and overall survival were analyzed by Gene Expression Profiling Interactive Analysis (GEPIA, online website: http://gepia.cancer-pku.cn/detail.php?gene).

Statistical analysis

Clinical information was analyzed by SPSS 18.0 (IBM Corporation, Armonk, NY). A Cox regression model was conducted to perform a univariate and multivariate analysis. The gene expressions were analyzed by GraphPad Prism 7.0. $p < 0.05$ is considered to reveal a statistically significant difference.

Results

Characteristics of included datasets

A total of 68 potentially relevant datasets were identified through the literature search. 4 datasets (GSE104935, GSE120005, GSE78201, and GSE21887) were included for further analysis[8–11]. For these four datasets, one was public in 2010, and detected the DNA expression profiling in a novel xenograft model by array[11]. Other three datasets detected RNA expression profiling in PCa cell lines[8–10]. The characteristics of included datasets were listed in Table 1.

Table 1
The characteristic of included GEO datasets

GEO datasets	Year	Objective	Experiment type	Platforms	Samples	Treatment
GSE104935	2019	RNA	microarray	GPL10558	LNCaP and C42B	MDV3100
GSE120005	2018	RNA	microarray	GPL570	C42B	MDV3100 or abiraterone
GSE78201	2016	RNA	microarray	GPL10558	CWR-R1, LAPC-4, LNCaP, and VCAP	MDV3100
GSE21887	2010	DNA	microarray	GPL570	Xenograft model tissues	Castration

DEGs analysis

1151 up regulated and 1113 down regulated gene were found in GSE104935, 828, 894, and 2293 up regulated gene were identified in GSE120005, GSE78201, and GSE21887, respectively (Fig. 1A). 1113, 814, 960, and 2027 down regulated gene were screened in GSE104935, GSE120005, GSE78201, and GSE21887, respectively (Fig. 1B). Finally, 15 common up regulated genes (ENPP5, NUDT11, LAMP2, C16orf45, ATP9A, LINC00623, ABCA1, TNRC6B, SUSD4, FOXO3, ELOVL6, BASP1, GALNT10, TRIM45, and ENC1), and 27 common down regulated genes (MBOAT2, RAB3B, IQGAP2, LPAR3, MALT1, FKBP5, KLK15, SLC45A3, BEND4, KLK2, AFF3, ACSL3, ALDH1A3, CAMKK2, PNKD, PMEPA1, PRKCH, KIAA1524, BMPR1B, MPC2, LAMA1, FAM174B, OSBPL8, L3MBTL3, HES6, PDLIM5, UGT2B28) were identified. These genes were the key candidates.

Gene Ontology and KEGG pathway analysis

The 15 overlap up regulated genes and 27 overlap down regulated genes were analyzed by DAVID, GO function analysis discovered overlap DEGs are mainly enriched in endoplasmic reticulum membrane and perinuclear region of cytoplasm. These genes are mainly involved with phospholipid transporter activity, protein domain specific binding, and long-chain fatty-acyl-CoA biosynthetic process (Table 2). KEGG pathway analysis indicated the overlap DEGs are not enriched in a pathway.

Table 2
Gene Ontology analysis of differentially expressed genes

Category	Term	Genes	Count	%	PValue
GOTERM_CC_DIRECT	endoplasmic reticulum membrane	OSBPL8, FKBP5, MBOAT2, ELOVL6, ABCA1, ACSL3, UGT2B28	7	16.7	9.40E-03
GOTERM_MF_DIRECT	phospholipid transporter activity	OSBPL8, ABCA1	2	4.8	2.20E-02
GOTERM_CC_DIRECT	perinuclear region of cytoplasm	RAB3B, ATP9A, MALT1, ABCA1, ACSL3	5	11.9	4.30E-02
GOTERM_BP_DIRECT	phospholipid translocation	ATP9A, ABCA1	2	4.8	4.90E-02
GOTERM_BP_DIRECT	metabolic process	ENPP5, ACSL3, UGT2B28	3	7.1	5.50E-02
GOTERM_MF_DIRECT	protein domain specific binding	LAMP2, BASP1, ACSL3	3	7.1	7.60E-02
GOTERM_MF_DIRECT	cholesterol binding	OSBPL8, ABCA1	2	4.8	8.60E-02
GOTERM_BP_DIRECT	long-chain fatty-acyl-CoA biosynthetic process	ELOVL6, ACSL3	2	4.8	9.10E-02

BP: biological process; CC: cellular component; GO: gene ontology; MF: molecular function.

Protein interaction

We used STRING for investigating and integrating interaction between proteins. Data was exported for further analysis by Cytoscape. 11 proteins corresponding to genes were identified to be key genes (Fig. 2). Of these eleven genes, 4 were up regulated (ELOVL6, ABCA1, FOXO3, and TNRC6B), and 7 were down regulated (ALDH1A3, OSBPL8, ACSL3, SLC45A3, KLK2, FKBP5, and PMEPA1). The relative expression of these genes in four datasets were showed in Fig. 3.

Gene expression in PCa

The relative expression in hormone sensitive and refractory patients of these eleven genes were obtained from ONCOMINE. Statistic difference were found for ALDH1A3 ($p = 0.038$), OSBPL8 ($p = 0.028$), PMEPA1 ($p = 0.039$), KLK2 ($p = 0.033$), SLC45A3 ($p = 0.012$), TNRC6B ($p = 0.036$); FKBP5 ($p = 0.312$), ABCA1 ($p = 0.861$), FOXO3 ($p = 0.277$), ACSL3 ($p = 0.233$), and ELOVL6 ($p = 0.127$) were not significant different between hormone sensitive and refractory patients (Fig. 4). The six difference expression genes were further analyzed.

Clinical analysis

Kaplan-Meier analysis were performed online for the six genes. Only PMEPA1 was found associated with disease free survival ($p = 0.017$, Fig. 5). No of these genes was associated with overall survival. The expression of KLK2 was deficiency in OncoLnc. Thus, the remaining five genes (ALDH1A3, OSBPL8, PMEPA1, SLC45A3, and TNRC6B) were processed cox regression analysis. OSBPL8 and SLC45A3 were associated with survival (supplement Table 1).

Discussion

In this research, key molecules were identified by bioinformatic analysis for castration resistance prostate cancer. DEGs were obtained from four GEO datasets, overlaps were screened by soft EXCEL. 42 overlap genes included 15 common up regulated genes and 27 common down regulated genes. STRING was used to analysis the protein interaction between 42 overlap genes, 11 genes presented interaction. Further analysis found 6 of 11 genes were differently expressed in hormone refractory patients. These genes included ALDH1A3, OSBPL8, PMEPA1, KLK2, SLC45A3, and TNRC6B. Among them, PMEPA1 was found associated with disease free survival. Cox regression analysis indicated OSBPL8 and SLC45A3 were associated with survival. According to the gene expression, OSBPL8 was up regulated in hormone refractory PCa, PMEPA1 and SLC45A3 were down regulated. Thus, our analysis suggested OSBPL8 conferred to castration resistance, PMEPA1 and SLC45A3 played as drug sensitivity gene in CRPC.

Gene array contains massive information; it is crucial to identify key genes. The discrepancy between gene array make it harder. Heterogeneity derive from difference cell lines, specimens, platforms, and treatments. To integrate the information among arrays, overlap DEGs were identified. These genes were considered to be functional regardless of cell line, treatment or else. Gene ontology analysis suggested overlap DEGs are mainly enriched in endoplasmic reticulum membrane and perinuclear region of cytoplasm. Recent studies emphasized that proteins mediated endoplasmic reticulum (ER) stress and nucleus-targeting drug delivery systems involved in the castration resistance[12]. Biological process analysis suggested overlap DEGs are mainly involved with phospholipid transporter activity, protein domain specific binding, and long-chain fatty-acyl-CoA biosynthetic process. This highlighted the dysregulation of lipid metabolism in prostate cancer progression[13].

Protein interaction was analyzed by STRING, a link between two proteins that both contributed jointly to a specific biological function was established[14]. 11 proteins encoding by responding genes were screened out. These genes may be backfired or coordination, there was no doubt that they played important role in CRPC. For example, ELOVL6, FOXO3 and FKBP5 were discovered and validated conferred to prostate cancer progression[15, 16] .

Clinical analysis was performed to verify our research. Gene expression levels in patients were obtained from ONCOMINE. The data profiles were published in two research, one analyzed gene expression profiles of 25 clinical hormone-refractory prostate cancers and 10 hormone-sensitive prostate cancers by genome-wide cDNA microarrays[17], the other compared the gene expression profiles of 10 androgen-

independent primary prostate tumor biopsies with 10 primary, untreated androgen-dependent tumors[18]. Survival analysis were performed for different expression genes, single gene expression and survival was analyzed by Kaplan-Meier plots, cox multivariate survival analysis identified the two key genes.

OSBPL8, PMEPA1 and SLC45A3 were identified the important gene for CRPC. OSBPL8 was a novel gene we found associated with prostate cancer, this gene encodes protein containing an N-terminal pleckstrin homology domain and a highly conserved C-terminal oxysterol-binding protein-like sterol-binding domain. It binds multiple lipid-containing molecules, including phosphatidylserine, phosphatidylinositol 4-phosphate (PI4P) and oxysterol, and act as an endoplasmic reticulum sterol sensor implicated in cellular lipid metabolism[19]. PMEPA1 was highly expressed in various solid tumors and is known to play important roles in the transforming growth factor-beta (TGF-beta) signaling pathway[20]. It was demonstrated that knockdown of PMEPA1 resulted in increased proliferation, migration and invasion of PCa cells[21], PMEPA1 also guarded against TGF-β-mediated prostate cancer bone metastasis[22]. In our work, PMEPA1 was implied involved with castration resistance. SLC45A3 protein, also known under the name of prostein, its expression is restricted to prostate normal tissue, it was differentially and more reactive in advanced compared to early stages of PCa. SLC45A3 was also accounted for erythroblast transformation-specific (ETS) family member gene fusions which was responsible for prostate cancer cell growth. Nevertheless, the role of SLC45A3 in CRPC was remained unknow.

There were some limitations worth noting. First, this research was based on the theory that CRPC was a homogeneity disease, but CRPC may be heterogeneity according to AR/GR expression[23, 24]; Second, the genes were not verified by biological methods, some experiments knocked down or over express these gene would be performed to confirm this research; Thirdly, a major drawback of this study is the lack of evidence to suggest protein-protein interaction, protein interactions were predicted by STRING, there may be some important genes in the DEGs that were not analyzed.

In conclusion, our study suggested OSBPL8, PMEPA1 and SLC45A3 were involved with CRPC. These candidate genes were rarely researched in CRPC, the functions needed experiments in vivo and vitro to verify.

Declarations

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

Funding: This study was funded by the Project of the Natural Science Foundation of Jiangxi, China (No: 20202BAB216033).

Ethics approval and consent to participate: Not applicable.

Authors' contributions: H.J.P and X.H.B designed the study, H.J.P, Y.Y and L.W performed the research, H.J.P, Y.Y analyzed data, and H.J.P wrote the paper.

Acknowledgment: Not applicable.

Conflicts of Interest: None declared.

References

1. Huang J, Lin B, Li B. Anti-Androgen Receptor Therapies in Prostate Cancer: A Brief Update and Perspective. Frontiers in oncology. 2022;12:865350. doi: 10.3389/fonc.2022.865350.
2. Graham LS, Schweizer MT. Mismatch repair deficiency and clinical implications in prostate cancer. The Prostate. 2022. doi: 10.1002/pros.24343.
3. Fararjeh AS, Liu YN. ZBTB46, SPDEF, and ETV6: Novel Potential Biomarkers and Therapeutic Targets in Castration-Resistant Prostate Cancer. Int J Mol Sci. 2019;20(11). doi: 10.3390/ijms20112802.
4. Qin S, Gao H, Kim W, Zhang H, Gu Y, Kalari KR, et al. Biomarkers for predicting abiraterone treatment outcome and selecting alternative therapies in castration-resistant prostate cancer. Clinical pharmacology and therapeutics. 2022. doi: 10.1002/cpt.2582.
5. Saad F, Shore N, Zhang T, Sharma S, Cho HK, Jacobs IA. Emerging therapeutic targets for patients with advanced prostate cancer. Cancer Treat Rev. 2019;76:1–9. doi: 10.1016/j.ctrv.2019.03.002.
6. Laudato S, Aparicio A, Giancotti FG. Clonal Evolution and Epithelial Plasticity in the Emergence of AR-Independent Prostate Carcinoma. Trends Cancer. 2019;5(7):440–55. doi: 10.1016/j.trecan.2019.05.008.
7. Hoang DT, Iczkowski KA, Kilari D, See W, Nevalainen MT. Androgen receptor-dependent and -independent mechanisms driving prostate cancer progression: Opportunities for therapeutic targeting from multiple angles. Oncotarget. 2017;8(2):3724–45. doi: 10.18632/oncotarget.12554.
8. Kregel S, Chen JL, Tom W, Krishnan V, Kach J, Brechka H, et al. Acquired resistance to the second-generation androgen receptor antagonist enzalutamide in castration-resistant prostate cancer. Oncotarget. 2016;7(18):26259–74. doi: 10.18632/oncotarget.8456.
9. Li S, Fong KW, Gritsina G, Zhang A, Zhao JC, Kim J, et al. Activation of MAPK Signaling by CXCR7 Leads to Enzalutamide Resistance in Prostate Cancer. Cancer Res. 2019;79(10):2580–92. doi: 10.1158/0008-5472.CAN-18-2812.
10. Liu C, Lou W, Yang JC, Liu L, Armstrong CM, Lombard AP, et al. Proteostasis by STUB1/HSP70 complex controls sensitivity to androgen receptor targeted therapy in advanced prostate cancer. Nat Commun. 2018;9(1):4700. doi: 10.1038/s41467-018-07178-x.
11. Terada N, Shimizu Y, Kamba T, Inoue T, Maeno A, Kobayashi T, et al. Identification of EP4 as a potential target for the treatment of castration-resistant prostate cancer using a novel xenograft model. Cancer Res. 2010;70(4):1606–15. doi: 10.1158/0008-5472.CAN-09-2984.
12. Fontana F, Moretti RM, Raimondi M, Marzagalli M, Beretta G, Procacci P, et al. delta-Tocotrienol induces apoptosis, involving endoplasmic reticulum stress and autophagy, and paraptosis in prostate cancer cells. Cell Prolif. 2019;52(3):e12576. doi: 10.1111/cpr.12576.
13. Zadra G, Ribeiro CF, Chetta P, Ho Y, Cacciatore S, Gao X, et al. Inhibition of de novo lipogenesis targets androgen receptor signaling in castration-resistant prostate cancer. Proceedings of the National

Academy of Sciences of the United States of America. 2019;116(2):631–40. doi: 10.1073/pnas.1808834116.

14. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Research. 2019;47(D1):D607-D13. doi: 10.1093/nar/gky1131.
15. Shang Z, Yu J, Sun L, Tian J, Zhu S, Zhang B, et al. LncRNA PCAT1 activates AKT and NF-kappaB signaling in castration-resistant prostate cancer by regulating the PHLPP/FKBP51/IKKalpha complex. Nucleic Acids Res. 2019;47(8):4211–25. doi: 10.1093/nar/gkz108.
16. Lv S, Wen H, Shan X, Li J, Wu Y, Yu X, et al. Loss of KMT2D induces prostate cancer ROS-mediated DNA damage by suppressing the enhancer activity and DNA binding of antioxidant transcription factor FOXO3. Epigenetics. 2019:1–15. doi: 10.1080/15592294.2019.1634985.
17. Tamura K, Furihata M, Tsunoda T, Ashida S, Takata R, Obara W, et al. Molecular features of hormone-refractory prostate cancer cells by genome-wide gene expression profiles. Cancer Res. 2007;67(11):5117–25. doi: 10.1158/0008-5472.CAN-06-4040.
18. Best CJM. Molecular Alterations in Primary Prostate Cancer after Androgen Ablation Therapy. Clinical Cancer Research. 2005;11(19):6823–34. doi: 10.1158/1078-0432.ccr-05-0585.
19. Ma H, Zhang S, Zhang K, Zhan H, Peng X, Xie S, et al. Identifying Selection Signatures for Backfat Thickness in Yorkshire Pigs Highlights New Regions Affecting Fat Metabolism. Genes (Basel). 2019;10(4). doi: 10.3390/genes10040254.
20. Koido M, Sakurai J, Tsukahara S, Tani Y, Tomida A. PMEPA1, a TGF-beta- and hypoxia-inducible gene that participates in hypoxic gene expression networks in solid tumors. Biochem Biophys Res Commun. 2016;479(4):615–21. doi: 10.1016/j.bbrc.2016.09.166.
21. Feng S, Zhu X, Fan B, Xie D, Li T, Zhang X. miR19a3p targets PMEPA1 and induces prostate cancer cell proliferation, migration and invasion. Mol Med Rep. 2016;13(5):4030–8. doi: 10.3892/mmr.2016.5033.
22. Stone L. Prostate cancer: TGF-beta signalling regulator PMEPA1 halts metastases to bone. Nat Rev Urol. 2015;12(7):362. doi: 10.1038/nrurol.2015.136.
23. Hu J, Chen Q. The role of glucocorticoid receptor in prostate cancer progression: from bench to bedside. Int Urol Nephrol. 2017;49(3):369–80. doi: 10.1007/s11255-016-1476-8.
24. Serritella AV, Shevrin D, Heath EI, Wade JL, Martinez E, Anderson A, et al. Phase I/II trial of enzalutamide and mifepristone, a glucocorticoid receptor antagonist, for metastatic castration-resistant prostate cancer. Clinical cancer research: an official journal of the American Association for Cancer Research. 2022. doi: 10.1158/1078-0432.Ccr-21-4049.

Figures

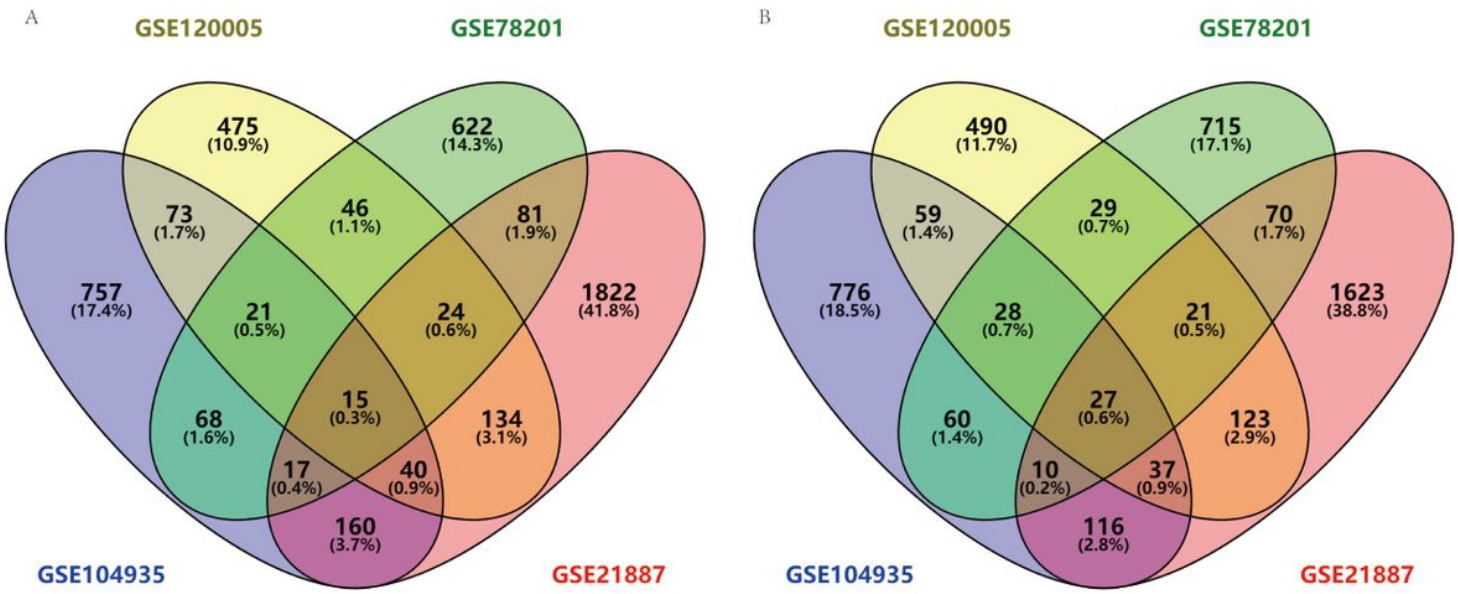

Figure 1

Differentially expressed genes (DEGs) were identified in four datasets. A. the number of up regulated DEGs were noted in each dataset, overlaps were also showed. B the number of down regulated DEGs and overlaps were showed.

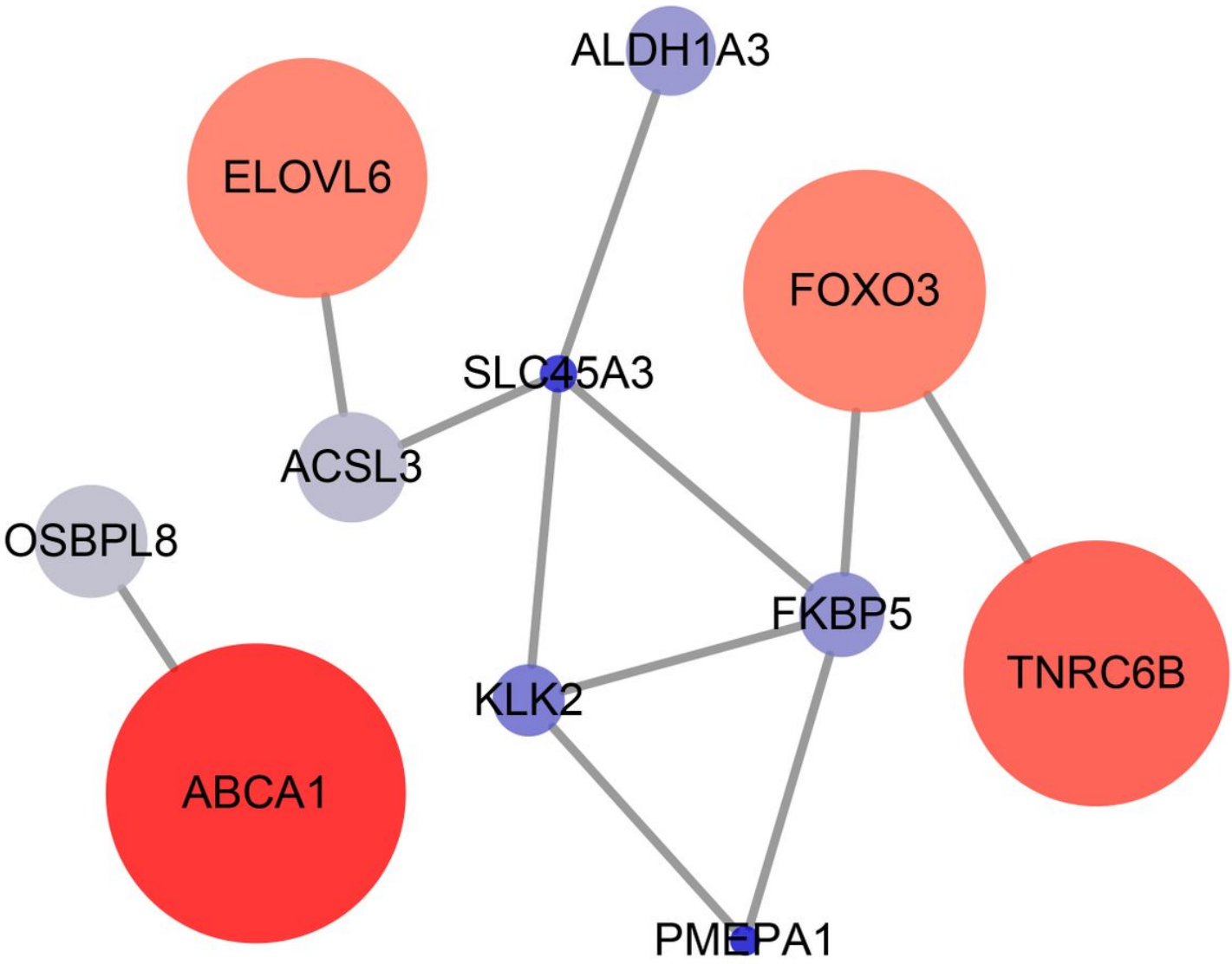

Figure 2

11 proteins corresponding to genes were identified to be key genes by Cytoscape. Red indicated up regulated genes, and blue indicated down regulated. The darker of the color and the bigger of the size indicated the greater of fold change value.

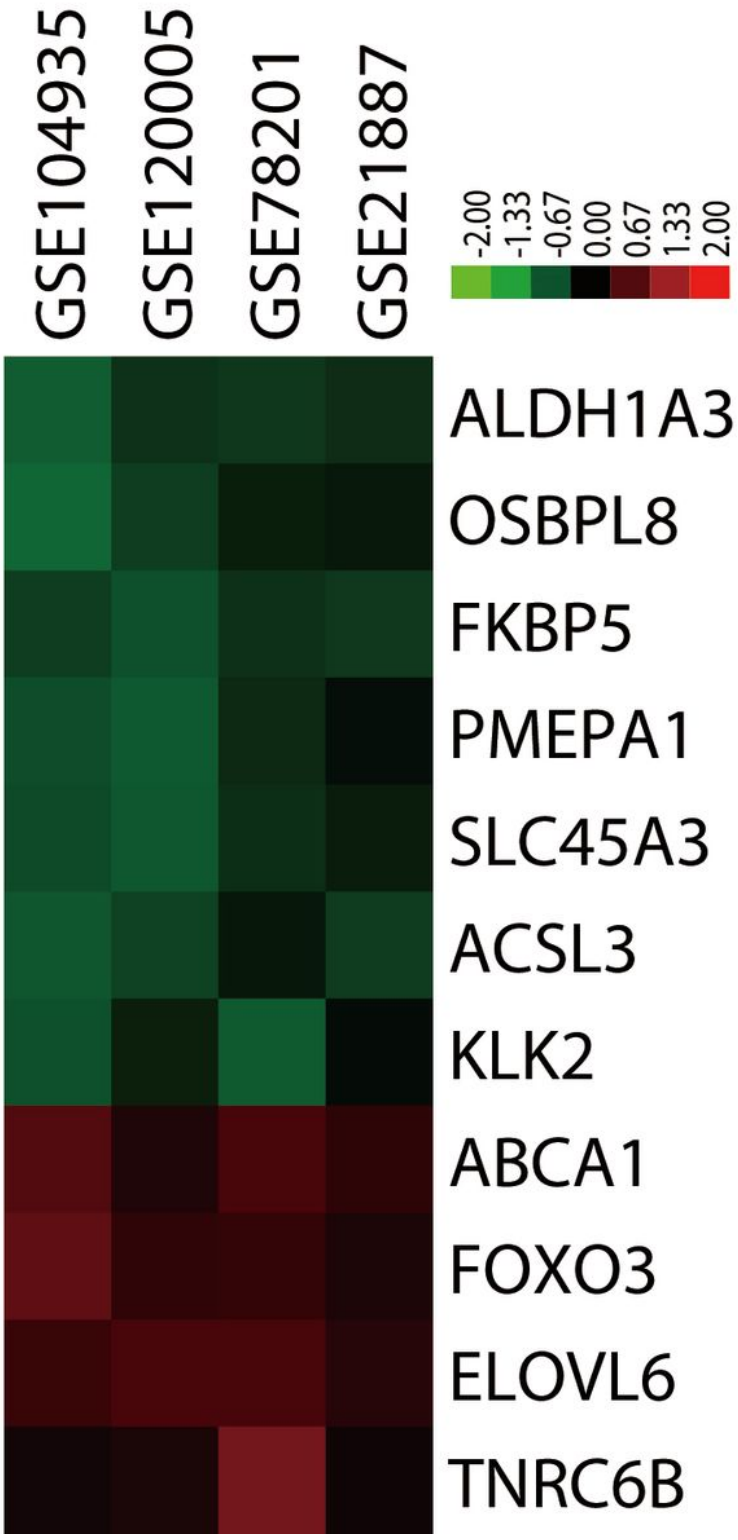

Figure 3

Heatmap showed the fold changes of genes in each dataset. The bar was noted in upper right quarter.

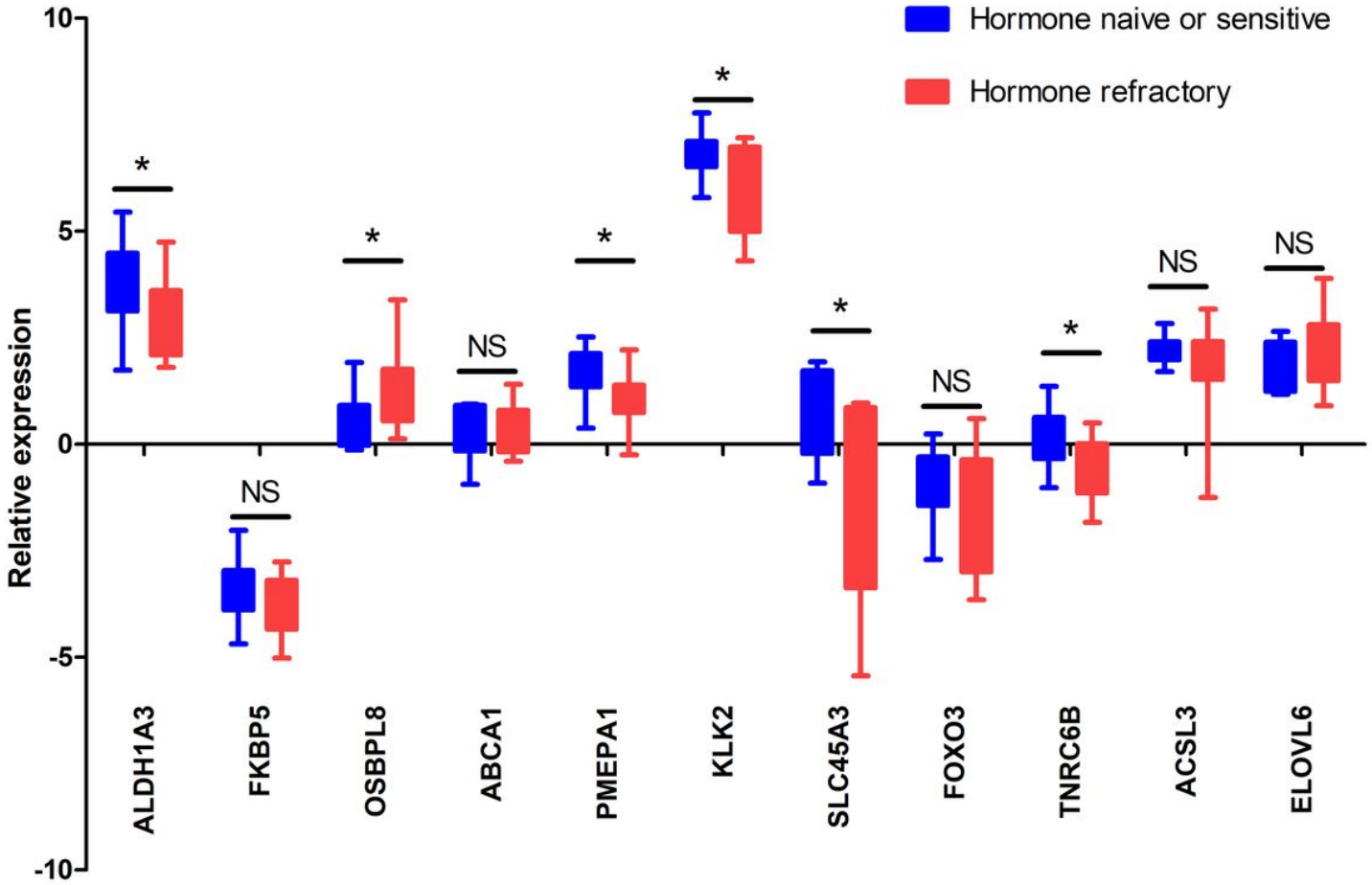

Figure 4

The expressions of genes were obtained from ONCOMINE. * $p<0.05$, NS, not significant.

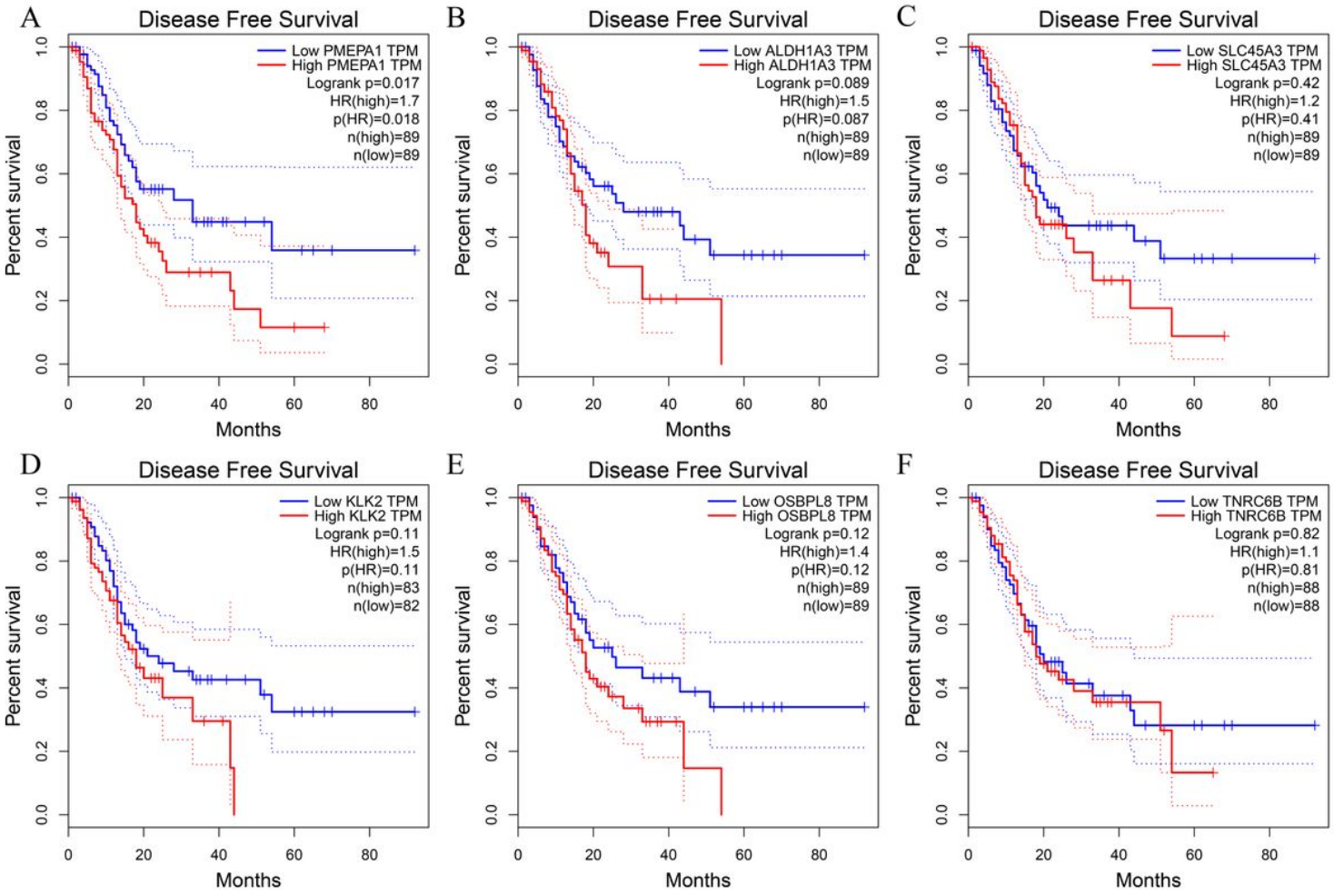

Figure 5

Disease free survival analysis for the six genes were obtained (A. PMEPA1, B. ALDH1A3, C. SLC45A3, D. KLK2, E. OSBPL8, F. TNRC6B).

Supplementary Files

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

- SupplementTable1.docx