The role of splicing factor PRPF8 in breast cancer

Difei Cao*a, Jiaying Xuea, Guoqing Huanga, Jing Anb and Weiwei Anb

*aInstitute of Advanced Technology, Heilongjiang Academy of Sciences, Harbin, Heilongjiang, China
*bInstitute of Cancer Prevention and Treatment, Heilongjiang Province Academy of Medical Sciences, Harbin, Heilongjiang, China

Abstract.
BACKGROUND: Alternative splicing is a mechanism to produce different proteins with diverse functions from one gene. Many splicing factors play an important role in cancer progression. PRPF8 is a core protein component of the spliceosome complex, U4/U6-U5 tri-snRNP.
OBJECTIVE: However, PRPF8 involved in mRNA alternative splicing are rarely included in the prognosis.
METHODS: We found that PRPF8 was expressed in all examined cancer types. Further analyses found that PRPF8 expression was significantly different between the breast cancer and paracancerous tissues.
RESULTS: Survival analyses showed that PRPF8-high patients had a poor prognosis, and the expression of PRPF8 is associated with distant metastasis-free survival (DMFS) and post progression survival (PPS). Gene Set Enrichment Analysis (GSEA) has revealed that PRPF8 expression is correlated with TGF-β, JAK-STAT, and cell cycle control pathways. Consistent with these results, upon PRPF8 silencing, the growth of MCF-7 cells was reduced, the ability of cell clone formation was weakened, and p21 expression was increased.
CONCLUSIONS: These results have revealed that PRPF8 is a significant factor for splicing in breast cancer progression.

Keywords: PRPF8, breast cancer, alternative splicing, prognosis

1. Introduction

Breast cancer is a common cancer, which has a higher morbidity rate in women [1]. The clinical outcomes of breast cancer are closely linked to prognostic parameters, such as tumor size, grade, and lymph node and metastasis status. However, genes involved in mRNA alternative splicing are rarely included in the prognosis. By generating multiple mRNAs from a precursor mRNA (pre-mRNA), alternative splicing greatly diversify the genome coding capacity. Most genes are multiple-exon genes and generate more than one functional protein.

Recent studies have confirmed multiple splicing factors affect the splicing of critical breast cancer-related genes [2]. PRPF8 is a core protein component of the spliceosome complex, U4/U6-U5 tri-snRNP and contains several WD repeats, which function in protein-protein interactions. It participates in the two sequential transesterification steps of pre-mRNAs during the cut and link of pre-mRNAs. Loss of PRPF8 can lead to the death of mouse embryonic cells [3] and Drosophila cells [4]. Thus, the study...
Table 1

| Oligo         | Sequence          |
|--------------|-------------------|
| PRPF8-1      | TCACGTAACACATACAGGG |
| PRPF8-4      | ACAACACAAGCACAGACAG |
| Control      | TTACTCTCGCCAAAGCGAG |

aimed to investigate the significance of *PRPF8* in breast cancer. Our present study, the data of Oncomine and TCGA (The Cancer Genome Atlas) were used to analyze the expression levels of *PRPF8* in normal tissue and carcinomas. The clinical significance of *PRPF8* in breast cancer was further explored. GSEA analysis and *in vitro* experiments have disclosed the possible role of *PRPF8* in breast cancer.

2. Materials and methods

2.1. Bioinformatic and statistical analysis

The expression of *PRPF8* in different cancer types, between cancer samples and adjacent tissues, is analyzed from GEO and Oncomine database (https://www.oncomine.org/). P values less than $1 \times 10^{-4}$ were considered statistically significant. UALCAN (http://ualcan.path.uab.edu/index.html) dataset was used to analyze the *PRPF8* protein levels in breast cancer tissue. Km-plot (https://kmplot.com) and TCGA portal (http://www.tumorsurvival.org/index.html) websites were used to analyze the expression of *PRPF8* in breast cancer survival. The GSEA (http://www.broadinstitute.org/gsea/index.jsp) analyze the expression level of *PRPF8* was used as the phenotype label, and “Metric for ranking genes” was set to Pearson Correlation. The downstream pathways (positive and negative) of *PRPF8* are obtained by calculating the enrichment score of data in the breast cancer group ($n = 1106$). The enrichment score, and multiple test corrections reveal the biological characteristics and genetic regulatory network of *PRPF8*.

2.2. Cell culture

MCF-7 and HEK-293T cells were obtained from the National Infrastructure of Cell Line Resource. MCF-7 cells were cultured in α-MEM medium containing 10% fetal bovine serum (FBS) and 10 µg/ml insulin. HEK-293T cells were cultured in DMEM medium containing 10% FBS. All the cells were cultured at 37°C.

2.3. RNA purification and quantitative reverse transcription-polymerase chain reaction

Viral packaging vectors of pMD2 (Addgene, USA) and pPAX2 (Addgene, USA) and pGIPZ (Addgene, USA) were transfected into 293T cells with Lipofectamine 2000. The shRNA sequences were listed (Table 1), for pGIPZ. After 3 days, the medium was purified with 0.45 µm filters and mixed with 1/3 volumes of lentiviral concentration solution. After the mixture solution was incubated at 4°C for 12 h, it was centrifuged at 4°C for 45 min, and pellets were resuspended in PBS. 100 µl viruses were added to $2 \times 10^5$ MCF-7 cells, and the harvested cells were used to extract total RNA. Reverse transcription of RNA with PrimeScript RT kit (Takara, Japan). Quantitative PCR (q-PCR) Detection System using iTaq Universal SYBR Green Supermix. Primers for q-PCR were listed (Table 2). Data were analyzed using the $2^{\Delta\Delta C_T}$ method.
Table 2

| Primer      | Sequence               |
|-------------|------------------------|
| PRPF8-qF    | TGTCAGTTGCGTGTTTTCAT   |
| PRPF8-qR    | AGACAGTAAAACCTCCATCA   |
| P21-qF      | TGTCAGTTGCGTGTTTTCAT   |
| P21-qR      | AAGATGTAGACCCGCTTTT    |

Fig. 1. (A) The mRNA expression of PRPF8 in different cancer cell lines. (B) The protein levels of PRPF8 are higher in breast cancer than in adjacent normal tissue. (C) PRPF8 expression in the subtypes of breast cancer.

2.4. Colony formation and cell proliferation

500 MCF-7 cells per well were grown in six-well plates and maintained at 37°C for 12 days. The medium was replaced every 3 days. After 12 days, the cells were stained with crystal violet for 20 minutes as described in the product manual. The colonies were counted and subject to statistical analyses. In the cell proliferation analysis, the same cells were seeded in 96-well plates and the CCK-8 kits were used to detect cell viability on day 1, 3, 5. Data were normalized to day 1 and presented as mean ± standard deviation.
Fig. 2. (A) PRPF8 protein levels are associated with the OS of breast cancer patients in Kaplan-Meier plots. (B) The mRNA expression of PRPF8 is associated with the DMFS probabilities of breast patients. (C) The mRNA expression of PRPF8 is associated with the PPS probabilities of breast patients. (D) Prognostic analysis of PRPF8 with mRNA expression in luminal A patients.

3. Results

3.1. Characterizing the expression of PRPF8 in breast cancer

There are few reports on the expression of PRPF8 in breast cancer. In this study we analyzed the expression levels of PRPF8 in various human tumors from the Oncomine database and Cancer Cell Line Encyclopedia (CCLE) (fold change of > 4, gene rank of > 10%, and p value < 1 × 10^{-4} was set as the threshold). PRPF8 is expressed in all subtypes of cancers (Fig. 1A). Then we compared PRPF8 expression between normal samples (n = 18) and primary breast tumor samples (n = 125) in UALCAN database (http://ualcan.path.uab.edu/index.html), and has found PRPF8 protein expression is significantly higher in primary tumors (Fig. 1B). (PRPF8 proteomic expression profile based on sample types, and Z-values represent standard deviations from the median across samples. p value = 4.3E^{-14}).
3.2. PRPF8 expression is associated with the survival of breast cancer patients

Subsequently, we investigated the association of PRPF8 expression with breast cancer patient survival. In breast cancer, the PRPF8 protein level was associated with the overall survival (OS) according to Kaplan-Meier plots database. Consistent with this result, PRPF8 mRNA expression is also associated with the OS in the Kaplan-Meier plots cohort. In the cohort of (GSE7390), we also found that PRPF8 expression was associated with the PPS ($p < 0.05$, HR = 1.68), and DMFS ($p < 0.05$, HR = 1.8) (Fig. 2). To further characterize the role of PRPF8 in breast cancer, we analyzed the association of PRPF8 expression with the patient OS in different subtypes of breast cancer using the TCGA dataset. The results have shown that PRPF8 expression is significantly associated with the OS in Luminal A patients, but not in other subtypes of breast cancer, indicating PRPF8 might play a role in Luminal A type cancer (Fig. 2D).

3.3. Inhibition of PRPF8 expression impaired breast cancer cell proliferation

To confirm the function of the PRPF8 in breast cancer, we employed shRNAs to silence PRPF8 expression in breast cancer cells, MCF-7. Western Blot (WB) shown that both shRNAs suppressed the expression of PRPF8 (Fig. 3A). Meanwhile, inhibiting PRPF8 expression, the numbers of colonies decreased (Fig. 3C and D), and the growth curves were significantly inhibited, suggesting that PRPF8 was essential for the maintenance of the proliferation in cancer cells (Fig. 3B).
3.4. PRPF8 regulates the expression of p21 in breast cancer

To further explore the functional mechanism of PRPF8 in the breast cancer cell, we used GSEA to analyze the pathways associated with PRPF8 in breast cancer. The results have shown that PRPF8 positively regulates 153 pathways and negatively regulated 31 pathways. The high expression of PRPF8 was correlated with JAK-STAT signaling pathway (ES = 0.557898, p = 0, FDR = 0.001182), TGF-β signaling pathway (ES = 0.588807, p = 0, FDR = 0.001201), pathway in cancer (ES = 0.551778, p = 0, FDR = 4.19E-04), and cell cycle pathway (ES = 0.625877, p = 0, FDR = 0). As cell cycle pathway is a key pathway that regulates cell growth, we selected p21, a critical component of the cell cycle pathway, to verify the GSEA results. After PRPF8 shRNAs were transduced into MCF-7 cells, the mRNA expression of p21 was detected with quantitative PCR (q-PCR). The result shown that inhibition of PRPF8 expression up-regulated the expression of p21 in MCF-7 cells (Fig. 4E). Therefore, PRPF8 inhibition increased p21 expression in MCF-7 cells to inhibit the cell proliferation.

4. Discussion

In this study, we investigate the role of PRPF8 in breast cancer. PRPF8 mRNA expression is significantly elevated in breast cancer samples compared with the paracancerous tissue. PRPF8 mRNA is differentially expressed among different breast cancer molecular subtypes, and its levels were inversely correlated with the OS in breast cancer patients. Furthermore, we confirmed the role of PRPF8 in breast cancer with in vitro experiments, which have shown that silencing PRPF8 in breast cancer cells repressed cell proliferation by upregulating p21 expression.

Alternative splicing is one of the mechanisms to increase protein diversity [5–9]. Recently, with the better understanding of alternative splicing process [10–15], it has been found that abnormal expression of splicing factors is closely related to many diseases. Many splicing factors play an important role in
cancer [16–23], including in breast cancer [24–26]. PRPF8 is the core component of the ribonucleoprotein (RNP) complexes in the spliceosome and participates in splice-site recognition, branch-point formation and catalysis process [27–29]. Whether PRPF8 plays a role in breast cancer is not known.

In this study, we demonstrate that PRPF8 is critical for breast cancer cell survival. Firstly, PRPF8 is elevated in breast tumors compared with the normal tissue (Fig. 1B). Second, PRPF8 was related to OS, PPS, and DMFS in breast cancer patients (Fig. 2A, B&C). More importantly, silencing of PRPF8 slowed down breast cancer cell growth and reduced the colony formation of MCF-7 cells (Fig. 3). Therefore, we found PRPF8 plays an important role in breast cancer.

p21 is a cyclin-dependent kinase inhibitor [30–34]. It binds to cyclin-dependent kinase 2 complexes and inhibits their activity [35,36]. Previous research has shown that co-expression of p21 and p27 proteins in MCF-7 cells induced cell apoptosis and inhibited cell proliferation [37]. Another study has shown that tumor growth was significantly reduced by transferring p21 into breast cancer mouse model cell lines by inhibiting cell proliferation [38]. Our results indicated that PRPF8 expression was associated with many pathways, such as TGF-β pathway, JAK-STAT pathway, cell cycle control pathway. Recent studies have proved that multiple pathways are related to p21, including cell cycle, TGF-β, and JAK-STAT pathways [39–44]. Our study demonstrated that silencing PRPF8 up-regulated the expression of p21 and inhibited cancer cell survival.

5. Conclusion

Our study provides evidence that splicing factor PRPF8 is critical for breast cancer cell survival and has the potential prognostic value in breast cancer. PRPF8 may achieve its functions in breast cancer by modulating p21 expression.

Conflict of interest

The authors report no conflict of interest.

Funding

This work was supported by funds from the Natural Science Foundation of Heilongjiang Province (Grant Number H2017039).

References

[1] Anastasiadi Z, Lianos GD, Ignatiadou E, Harissis HV, Mitsis M. Breast cancer in young women: an overview. Updates in Surg. 2017; 69(3): 313-317. doi: 10.1007/s13304-017-0424-1.
[2] Cerasuolo A, Buonaguro L, Buonaguro FM, Tornesello ML. The Role of RNA Splicing Factors in Cancer: Regulation of Viral and Human Gene Expression in Human Papillomavirus-Related Cervical Cancer. Front Cell Dev Biol. 2020; 12(6): e474. doi: 10.3389/fcell.2020.00474.
[3] Graziotto JJ, Farkas MH, Kinga B, Deramaudt BM, Zhang Q, Nandrot EF, et al. Three gene-targeted mouse models of RNA splicing factor RP show late-onset RPE and retinal degeneration. Invest Ophthalmol Vis Sci. 2011; 52: 190-8. doi: 10.1167/iovs.10-5194. Print 2011 Jan.
[4] Rämet M, Manfruelli P, Pearson A, Mathey-Prevot B, Ezekowitz RAB. Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E.coli. Nature. 2002; 416: 644-8. doi: 10.1038/nature735. Epub 2002 Mar 24.
[5] Baralle FE, Giudice J. Alternative splicing as a regulator of development and tissue identity. Nat Rev Mol Cell Biol. 2017; 18(7): 437-451. doi: 10.1038/nrm.2017.27. Epub 2017 May 10

[6] Miri DG, Regina GG, Eli E, Keren M, Rotem K, Levanon EY. Identification of recurrent regulated alternative splicing events across human solid tumors. Nucleic Acids Res. 2015; 43: 5130-5144. doi: 10.1093/nar/gkv210. Epub 2015 Apr 23.

[7] Chen L, Tovar-Corona JM, Urrutia AO. Increased levels of noisy splicing in cancers, but not for oncogene-derived transcripts. Hum Mol Genet. 2011; 20: 4422-4429. doi: 10.1093/hmg/ddr370. Epub 2011 Aug 23.

[8] Yoshida K, Ogawa S. Nuclear splicing factor PRPF8 in cancer. Wiley Interdiscip Rev RNA. 2014; 5(4): 445-459. doi: 10.1002/wrna.1222. Epub 2014 Feb 12.

[9] Bialkin AV, Waddell N, Kassaahn KS, Gingras MC, Gibbs RA. Nuclear splicing factor PRPF8 in breast cancer. Cancer Res. 2008; 68: 9525-9531. doi: 10.1158/0008-5472.CAN-08-1769. Epub 2008 May 14.

[10] Dvinge H, Bradley RK. Widespread intron retention diversifies most cancer transcriptomes. Genome med. 2015; 7: e45. doi: 10.1186/s13073-015-0168-9. eCollection 2015.

[11] Davide C, Manley JL. Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. Genes Dev. 2010; 24: 2343-2364. doi: 10.1101/gad.1973010.

[12] CSmart A, Margolis CA, Pimentel H, He MX, Miao D, Adeegbe D, et al. Alternative splicing in Arabidopsis. Plant Journal. 2010; 39(6): 877-885. doi: 10.1111/j.1365-313X.2004.02172.x.

[13] Watermann DO, Tang Y, Zur Hausen A, Jäger M, Stamm S, Stickeler E. Splicing factor Tra2-beta1 is specifically regulated in breast cancer. Cancer Res. 2006; 66: 4774-4780. doi: 10.1158/0008-5472.CAN-04-3294.

[14] Urbanski LM, Leclair N, Anczuków O. Alternative-splicing defects in cancer: splicing regulators and their downstream targets, guiding the way to novel cancer therapeutics. Wiley Interdiscip Rev RNA. 2012; 4(3): 241-250. doi: 10.1002/wrna.1222. Epub 2014 Feb 12.

[15] Dvinge H, Bradley RK. Widespread intron retention diversifies most cancer transcriptomes. Genome med. 2015; 7: e45. doi: 10.1186/s13073-015-0168-9. eCollection 2015.

[16] Simon JM, Hacker KE, Singh D, Brannon AR, Parker JS, Weiser M, et al. Variation in chromatin accessibility in human epithelial and cancer cells. The Journal of Biological Chemistry. 2004; 279(2): 1422-1428. doi: 10.1074/jbc.M309937200.
Giles KM, Daly JM, Beveridge DI, Thomson AM, Voon DC, Furneaux HM, et al. The 3′-untranslated region of p21WAF1 mRNA is a composite cis-acting sequence bound by RNA-binding proteins from breast cancer cells, including HuR and poly (C)-binding protein. J Biol Chem. 2003; 278(5): 2937-2946. doi: 10.1074/jbc.M208439200. Epub 2002 Nov 12.

Wu BQ, Jiang Y, Zhu F, Sun DL, He XZ. Long noncoding RNA PVT1 promotes EMT and cell proliferation through downregulating p21 in pancreatic Cancer cells. Technol Cancer Res Treat. 2017; 16: 819-827. doi: 10.1177/1533034617700559. Epub 2017 Mar 30.

Wang LL, Guo HH, Zhan Y, Feng CL, Huang S, Han YX, et al. Specific up-regulation of p21 by a small active RNA sequence suppresses human colorectal cancer growth. Oncotarget. 2017; 8(15): 25055-25065. doi: 10.18632/oncotarget.15918.

Shu L, Yan W, Chen X. RNPC An RNA-binding protein and a target of the p53 family, is required for maintaining the stability of the basal and stress-induced p21 transcript. Genes Dev. 2006; 20(21): 2961-2972. doi: 10.1101/gad.1463306. Epub 2006 Oct 18.

Jiang D, Wang X, Liu X, Li F. Gene delivery of cyclin dependent kinase inhibitors p21 Waf1 and p27 Kip1 suppresses proliferation of MCF-7 breast cancer cells in vitro. Breast Cancer. 2014; 21: 614-623. doi: 10.1007/s12282-012-0438-y. Epub 2013 Jan 22.

Ibnat N, Kamaruzman NI, Ashaie M, Chowdhury EH. Transfection with p21 and p53 tumor suppressor plasmids suppressed breast tumor growth in syngeneic mouse model. Gene. 2019; 701: 32-40. doi: 10.1016/j.gene.2019.02.082. Epub 2019 Mar 18.

Li CY, Suardet L, Little JB. Potential Role of WAF1/Cip1/p21 as a Mediator of TGF-β Cytoinhibitory Effect. J Biol Chem. 1995; 270: 4971-4974. doi: 10.1074/jbc.270.10.4971.

Hu W, Wang Z, Li Q, Wang J, Li F, Jiang G. Upregulation of lincRNA-p21 in thoracic aortic aneurysms is involved in the regulation of proliferation and apoptosis of vascular smooth muscle cells by activating TGF-β1 signaling pathway. J Cell Biochem. 2019; 120(3): 4113-4120. doi: 10.1002/jcb.27696. Epub 2018 Oct 9.

Diab-Assaf M, Semaan J, El-Sabban M et al. Inhibition of Proliferation and Induction of Apoptosis by Thymoquinone via Modulation of TGF Family, p53, p21 and Bcl-2α in Leukemic Cells. Anticancer Agents Med Chem. 2018; 18(2): 210-215. doi: 10.2174/1871520617666170912133054.

Li B, Mao Z, Wu Z, Wang Z, Expression of Smad4, TGF-beta and P21~(waf1) in esophageal squamous cancer tissue and its biological significance. Oncol Lett. 2015; 9(6): 2847-2853. doi: 10.3892/ol.2015.3146. Epub 2015 Apr 23.

Tu X, Zhang Y, Zheng X, Deng J, Li H, Kang Z, et al. TGF-β induced hepatocyte lincRNA-p21 contributes to liver fibrosis in mice. Sci Rep. 2017; 7(1): e2957. doi: 10.1038/s41598-017-03175-0.

Lui AJ, Geanes ES, Ogonj J, Behbod F, Marques J, Valdez K, et al. IFTM1 suppression blocks proliferation and invasion of aromatase inhibitor-resistant breast cancer in vivo by JAK/STAT-mediated induction of p21. Cancer Lett. 2017; 399: 29-43. doi: 10.1016/j.canlet.2017.04.005. Epub 2017 Apr 12.

Bhunia AK, Piontek K, Boletta A, Liu L, Feng Q, Xu PN, et al. PKD1 induces p21(waf1) and regulation of the cell cycle via direct activation of the JAK-STAT signaling pathway in a process requiring PKD2. Cell. 2002; 109(2): 157-68. doi: 10.1016/s0092-8674(02)00716-x.