Vault RNAs partially induces drug resistance of human tumor cells MCF-7 by binding to the RNA/DNA-binding protein PSF and inducing oncogene GAGE6

Jianjun Chen¹, Hui OuYang², Xuemei An³, Shixi Liu¹*

¹ Department of E.N.T., West China Hospital, Sichuan University, Chengdu, China, ² Department of E.N.T., the First People’s Hospital of Neijiang, Neijiang, China, ³ Department of neurology, Chengdu University of TCM, Chengdu, China

* liushixi1983@163.com

Abstract

Background

Vault is the largest nonicosahedral cytosolic nucleoprotein particle, which is widely involved in induction of chemoresistance and lead to failure in long-term chemotherapy. Vault contains three different major vault proteins (MVPs) and four vault RNAs paralogues (vtRNAs, vtRNA1-1, vtRNA1-2, vtRNA1-3 and vtRNA2-1). Disruption of the MVPs do not induce hypersensitivity while expression of vtRNAs contributes to cells’ drug resistance, indicates that vtRNAs, but not MVPs play an important role in causing drug resistance. Polypyrimidine tract binding protein associated splicing factor (PSF) contributes to cell sensitivity to chemotherapy by its transcriptional activity, promotes us to figure out its potential association with vtRNAs.

Methods

We investigate the interaction between PSF and vtRNAs by electrophoretic mobility shift assays (EMSA) and RNA-immunoprecipitation (IP), and showed the binding between PSF and vtRNAs. Chromatin Immunoprecipitation (ChIP) was performed to detect the effects of vtRNAs on the interaction of PSF with GAGE6 promoter. The role of vtRNAs on chemoresistance in MCF-7 was detected by CCK-8 and EdU staining. The independent role of vtRNAs with MVP is detected by MVP or vtRNAs knockdown.

Results

The complex with vtRNA1-1 releases PSF, allowing transcription of GAGE6 to proceed. Then we showed that induction of GAGE6 caused drug resistance by promoting cell proliferation and colony formation in soft agar. Ectopic expression of shRNA targets to vtRNA1-1 further confirmed the role of vtRNA1-1 in regulating PSF transcriptional activity independent with the expression of MVP. By vtRNA1-1 or MVP knockdown, it is revealed that vtRNA1-1 caused chemoresistance independent of MVP. Furthermore, knockdown of GAGE6 does...
not cause drug resistance, indicates the GAGE6 is directly involved in cell proliferation, but not the drug resistance.

**Conclusion**

These results suggest that vtRNAs regulates cell proliferation, drug resistance, and possibly other physiological processes of humans, by complex formation with PSF.

**Introduction**

Vaults are identified as a barrel-shaped particles by scanning transmission electron microscopy, which presents the largest ribonucleoprotein complex identified in eukaryotic cells [1]. They function as the intracellular and nucleocytoplasmic transporters through shuttling between cytoplasm and the nucleus [2, 3]. They are widely found in eukaryotic species and present considerable abundance, which indicates their important role in intracellular detoxification processes in normal tissues, and even in cancer cells [4]. By analyzing the vaults particles isolated from the microsomal fraction (100,000×g pellet) by a series of sucrose gradients [5, 6], the particles were found to be composed of 100-kDa major vault protein (MVP, accounts for >75% of the particle mass), 290-kDa telomerase-associated protein 1 (TEP1), 193-kDa vault poly (ADP-ribose) polymerase (VPARP) three proteins and noncoding vault RNA [7–12]. Although the RNAs represents<5% of the mass of the vault complex, in human, the existed four vault RNAs (vtRNA1-1, vtRNA1-2, vtRNA1-3 and vtRNA2-1), which share ~84% sequence identity and similar secondary structure arrangements, may play the critical roles.

vtRNAs have the ability to recognize chemotherapeutic compounds, such as mitoxantrone [13]. The binding of vtRNAs to chemotherapeutic compounds prevents the drugs from reaching their target sites, and consequently causes the chemoresistance in cancer cells. Gopinath et al. found that, the overexpression of vtRNA1-1 in osteosarcoma cell line, U2OS, promoted its chemoresistance [13]. Interestingly, long-term chemo-treatment upregulated the expressing level of vtRNA1-1 in an unknown manner. All these data make it seems that a higher level of vtRNA1-1 expression is involved in their chemoresistant phenotype, at least to mitoxantrone, indicating the association of vtRNAs to chemoresistance. Meanwhile, it is also found that, the overexpression of MVP, TEP1 or VPARP in cancer cells failed to affect the cellular resistance to mitoxantrone [13], which means the poor correlation between their roles in causing mitoxantrone resistance.

The human protein PSF, or SFPQ (PTB-associated Splicing Factor/Splicing Factor Proline-Glutamine rich), was originally identified as a protein component of spliceosomes mediated by its two independent RNA-binding domains (RBDs) of the molecule [14]. Following studies found its association with various functions, including RNA splicing, virus replication, genetic recombination and cancer [15]. It was subsequently also found contains a DNA-binding domain (DBD), and functions as a human tumor-suppressor protein through the binding to the promoter region of multiple oncogenic genes in human tumor cell lines and causes the transcriptional inhibition, consequently, leads to the inhibition of proliferation and colonization of the tumor cells [16]. For example, the oncogenic gene, GAGE6, which is reported to be a critical inducer of doxorubicin resistance in cancer cells, is transcriptionally inhibited by PSF-binding to its promoter region [16–18].

Interestingly, the tumor-regulating property of PSF through its DNA binding activity is reported to be tightly modified by its RNA binding activity to long non-coding RNAs (lncRNAs). Xu et al. revealed a reversible mechanism of gene regulation involving formation of a complex between PSF protein and mouse IncRNA, VL30 retrotransposon RNA in mouse
It presents that PSF represses transcription of GAGE6 by binding to the promoter, and VL30 RNA forms a complex with PSF that dissociates from the gene, activating transcription. In human, several lncRNAs were also found to interact with human PSF (hPSF) and thus controls tumorigenesis in human cells, involving repression of proto-oncogene transcription by hPSF protein and the reversal by lncRNAs binding to hPSF’s RBDs [19]. Ji et al reported that overexpression of Metastasis associated with lung adenocarcinoma transcript-1 (MALAT-1) lead to the promotion of cell proliferation and migration in vitro and in vivo. The underlying mechanism was associated with the PSF and proto-oncogene PTBP2. Overexpressed MALAT-1 binds to PSF’s RBD, thus releasing PTBP2 from the PSF/PTBP2 complex. Consequently, the released PTBP2 promoted cell proliferation and migration [20]. All these data indicates the tight association between PSF and lncRNAs in regulating the physiological processes of tumors.

The vault particle components including vtRNAs are upregulated in cancer cells and contributes to the resistance capacity to several chemotherapeutic compounds [7, 21, 22]. But only the overexpressed vtRNAs can induce chemoresistance, indicating the critical role of vtRNAs, but not the protein components. A well-known mechanism of induction of chemoresistance mediated by vtRNAs is binding to the chemotherapeutic compounds directly and competitively inhibiting the translocation in subcellular target region. The potential novel mechanism is still largely unknown.

In this study, we found that vtRNA1-1 could bind to the RBD on PSF protein, and when the PSF expression was compromised, the cancer cells became sensitive. On the other hand, the binding of vtRNA1-1 to PSF released its target gene, GAGE6, and thus affects its expressing level and caused the sensitization to chemoagent. Together, our present results decline the importance of vtRNA1-1 for conferring drug resistance to the cancer cells.

Material and methods

Cell lines

Doxorubicin resistant cell line MCF-7/DOX (breast cancer cell line) was maintained in the presence of 1 μg/mL doxorubicin, once a week [23–26]. MCF-7/DOX was commercially established and bought from KeyGen Biotech Co., Ltd. (Nanjing China). MCF-7 were kept in ATCC-formulated Eagle’s Minimum Essential Medium (MEM) with 0.01mg/ml human recombinant insulin, 10% heat-inactivated fetal bovine serum (FBS). All cells were grown in a humidified 5% CO2 atmosphere at 37°C.

Cytotoxicity assay

A CCK-8 Cell Counting Kit (CCK8; Dojindo, Kunnamoto, Japan) was engaged to evaluate the change of sensitivity to drugs. Briefly, cells were seeded at a density of 5×104 cells per well in 96-well plates and maintained O/N. After seeding, drugs (IC50 and IC30 concentrations, respectively) for 72h at 37°C and performed CCK8 assay according to the instructions. Absorbance at 450 nm was recorded in a EnSpire® Multimode Plate Readers (PerkinElmer, China). Cell survival rate was represented as relative survival percentages and appeared to reflect the sensitivity to drugs. The formula: relative survival (%) = OD samples/ OD negative control. The experiments were done in reduplicating and repeated three times.

Transfection with plasmid DNA by electroporation

Plasmids were prepared using a standard anion-exchange method according to the manufacturer’s instructions (Life Technologies, US.). 2×106 cells were freshly prepared before electroporation and resuspended in 200 ul electroporation buffer containing 90 mM phosphate.
buffer, pH 7.2, 10 mM MgCl2 and 50 mM glucose. After incubation with plasmids, cells were subjected to a single HV pulses (125 us) 4mm electroporation cuvette (BTX Genetronics, San Diego, CA) was used and electroporated with five pulses of 99 μsec at 820 V over 1.1 sec in an ECM 830 (BTX Genetronics). Following a 10 min recovery period, cells were mixed with complete growth medium and plated.

**In vitro transcription and electron mobility shift assay (EMSA)**

In this study, vtRNA1-1 was employed for further analysis. Template DNA of vtRNA1-1 for in vitro transcription of RNA was amplified from cDNA of SW1573/2R120 cells using primer pairs P1: 5’–GGCTGGCTTTAGCTCAGCGG–3’ and P2: 5’–AAAAGGACTGGAGAGGCC–3’; template DNA of probe negative control, EGFP coding sequence fragment, was amplified from pcDNA3-EGFP using primer pairs P3: 5’–GGCTACGTCCAGGAGGCC–3’ and P4: 5’–CTGCTTGTCGGCCACTGATAT–3’. Primers were designed to include the full length of vtRNA1-1. Sense primers P1 and P3 included the T7 RNA polymerase promoter sequence.

For in vitro transcription of biotinylated vtRNA1-1 or EGFP mRNA fragment, 50 ng of template DNA was incubated with 0.5 mM rNTPs, T7 RNA polymerase and supplemented reaction buffer at 37˚C for 2h. Then the purified RNA was involved the biotinylation procedure followed the commercial instruction of biotin 3’ end RNA labeling kit (Pierce, US.)

For EMSAs, RNAs were refolded by heating (98˚C for 3min), chilling on ice for 5min, and standing at 25˚C for 5 min. 20 μl reactions were set up containing 10% glycerol, 150 mM NaCl, 10 mM KCl, 2.5 mM MgCl2, 1 mM DTT and 0.5 U/μl RNase Inhibitor, 10 pmol RNA probe and 50 ng protein. Reactions stand for 20 min at room temperature and then be loaded on a 4% polyacrylamide RNA Retardation Gel (Life Technologies, US.). Electrophoresis was performed in 0.5×TBE at 220 V for 60min at 4˚C. The bands were transferred to positively charged nylon membranes (Roche) by tank blotting in 0.5×TBE at 15V for 30 min at room temperature and cross-linked at 150 mJ/cm2 at 254 nm. For the detection of RNAs, the Chemiluminescent Nucleic Acid Detection Module (Pierce, US.) was utilized according to the manufacturer’s instruction.

**Cloning of PSF protein, truncated RBD and DBD protein, expression and purification of recombinant proteins**

The PSF open reading frame was PCR amplified from HEK293 cDNA with primers: P5: 5’–CTAGTATGTCTCGGGATCGGTTCC–3’ and P6: 5’–CAGCTACTCGAGTTGTTTGGT–3’; Primers for truncated DBD (1–97 aa): P9 5’–CTAGTATGTCTCGGATCGGTTCC–3’ and P10: 5’–CAGCTACTCGAGTTGTTTGGT–3’; Primers for truncated RBD (98–366 aa): P7 5’–CTAGTATGTCTCGGAGGTTTGGT–3’ and P8: 5’–CAGCTACTCGAGGTTTGGT–3’. All sense and antisense primers contains restriction sites for Nde I and Xho I, respectively. After purification, PCR product was digested with Nde I and Xho I, then cloned into pBluescript II KS+ to produce pBS-PSF. After confirmation by sequencing, the PSF full-length open reading frame was excised from plasmids with Nde I and Xho I, then cloned into similarly digested pET30b (Novagen) to produce pET30b-PSF, RBD or DBD separately. The plasmid was transformed into Escherichia coli strain BL21 (Novagen). His-tagged PSF protein was expressed in 200 ml Overnight Express medium (Novagen) for 24h at 25˚C. Expressed protein was purified according to the standard protocol [27].

**Northern blot**

For each sample, 10 μg of total RNA were separated on 10% denaturing polyacrylamide gels (7M urea, 0.5×TBE buffer), transferred onto nylon membranes, UV crosslined and probed.
with 5’ biotin-labeled antisense DNA probe in ULTRAhyb™ Ultrasensitive Hybridization Buffer (Cat. No.: AM8669, Thermo Scientific, Waltham, MA, USA) following the manufacturer’s instructions. The sequences of probes for each RNA were described as follows: for vtRNA1-1, 5’-GCTTGTTTCAATTAAAGAACTGTCG-3’; for vtRNA1-2, 5’-AGGTGGTTCAATGTACTCGAAG-3’; for vtRNA1-3, 5’-GAGGTGGTTGATGACACGCGAAG-3’; for vtRNA2-1, 5’-TCGCCGATCAGGGGTAACCCGC-3’. 5.8S rRNA was employed as an internal control and the sequence of its probe is 5’-TTCTGCAATTCAATTAATTCCTCGAGCTAGC-3’. For imaging, BrightStarBioDetect™ Kit (Cat. No.: AM1930, Thermo Scientific, Waltham, MA, USA) was employed following the manufacturer’s instructions.

vtRNA1-1, PSF or MVP knockdown
For RNAi construct the stem-loop vector pSLComp1 was used [28]. The target sequence on vtRNA1-1 was the following: 5’-GGCTGGCTTTAGCTCAAGCG-3’; the target sequence on PSF was the following: 5’-CTTTCTGTTCGTAATCTTTCA-3’; the target sequence on MVP was the following: 5’-CCCATACCACTATATCCATGT-3’. The whole shDNA sequence for the vtRNA1-1, PSF or MVP was ligated into pSLComp1 between Bam HI and Bgl II. The transfection of knockdown vector was followed by the manufacturer’s instruction of Lipofectamine™ 2000 (Thermo Scientific, Waltham, MA, USA).

Chromatin immunoprecipitation (ChIP)
Approximately 2×10^7 interest cells were resuspended in 1×PBS and crosslinked by adding a final concentration of formaldehyde to 1% for 10 min’s incubation at room temperature. Nuclear lysates were sonicated to generate 200–800 bps DNA fragments. Chromatin immunoprecipitation was then performed with mouse B92 antibody (Sigma, US.) or rabbit IgG antibody control. To detect enrichment fold of GAGE6 promoter or DHFR 5’UTR bound by PSF protein, PCR amplification of the resulting DNA was performed using the following primers: P11: 5’-GCCTTCTGCAAAGAAGTCTTGCGC-3’ and P12: 5’-ATGCGAATTCGAGGCAGG CGAGACAAT-3’; P13: 5’-CTGATGTCCAGGAGGAGAAAGG-3’ and P14: 5’-AGCCC GACAAATGTCAGGACTG-3’;

RT-qPCR and qPCR analysis
RT-qPCR was utilized to determine relative messenger RNA expression, qPCR was utilized to determine the DNA amount in ChIP products, as described previously [29, 30]. For quantitative detecting vtRNAs, the primers are followed: vtRNA1-1, 5’-GGCTGGCTTTTAGCTCAGC GG-3’ and 5’-AAAAAGGACTTGGAGGCGGC-3’; vtRNA1-2, 5’-GGCTGGCTTTAGCTCAGC GG-3’ and 5’-AAAAAGGACTTGGAGGCGGC-3’; vtRNA1-3, 5’-GGCTGGCTTTAGCTCAGC GG-3’ and 5’-AAAAAGGACTTGGAGGCGGC-3’; vtRNA2-1, 5’-GGCTGGCTTTAGCTCAGC GG-3’ and 5’-AAAAAGGACTTGGAGGCGGC-3’. 5.8S rRNA was considered as internal control and the primers are followed: 5’-CGACTCTTCTGGCTGAGATCA-3’ and 5’-GCAAGTGCGTTCGATAGT-3’.

Cell proliferation assay
Cell proliferation was detected using 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT). Cells were seeded into 12-well plates (2x10^4 cells/well) and allowed to attach overnight. After 24, 48, 72 and 96h, cell viability was assessed using MTT assay. The absorbance at 490 nM (A490) of each well was construed on a spectrophotometer. Three independent experiments were conducted in quadruplicate.
Colony formation assay
Roughly $2 \times 10^3$ cells respectively were placed in a 6-well plate in DMEM containing 10% FBS for 3 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 30min. Each assay was conducted in triplicate.

Data analysis
All results were presented as average±SEM. Student’s t-test was used for comparison between two groups. The difference is considered as statistically significant when p values are less than 0.05.

Results

VtRNAs binds to PSF on its RNA binding domain (RBD) and releases GAGE6 promoter from PSF in vitro

It is found that four vtRNAs paralogs exist in human cells, for confirming this, northern blot was performed to detect the existence of vtRNA1-1, 1–2, 1–3 and 2–1 in MCF-7 cells. As shown in Fig 1A, endogenous vtRNA1-1 is detectable while vtRNA1-2, 1–3 and 2–1 are undetectable. Detected by performing qRT-PCR, vtRNA1-1 presented a comparatively higher expressing level compared with three other paralogues (Fig 1B). It is reported that PSF contributed to cell sensitivity to DOX by inhibiting cell proliferation [31], and it’s transcriptional regulation activity is regulated by RNA binding, these understanding promotes us to examine the possible binding of PSF to vtRNAs by employing RNA native electrophoretic mobility shift assays (EMSA), using a native polyacrylamide gel electrophoresis (PAGE) system. VtRNA1-1, 1–2, 1–3 or 2–1 was transcripted and labeled with biotin on its 3’ end, incubated with His-tagged PSF protein, expressed in E. coli, and analyzed by PAGE. As illustrated in Fig 1C, we detected a band-shift when using 20 nM biotinylated vtRNA1-1, 1–2 or 1–3, but not vtRNA2-1, indicates the potential interaction between them with PSF in vitro. By considering the significant higher expressing level, vtRNA1-1 is mainly being focused on in further research.

We next examined the binding site on PSF to vtRNA1-1 by generating HIS-tagged DNA binding domain (DBD, 1–97 aa) and RNA binding domain (RBD, 98–366 aa) domains. Biotinylated vtRNA1-1 when electrophoresed with RBD gave rise to a migrating band that corresponds to the binary complex of vtRNA1-1 and RBD, but not with the DBD fragment (Fig 1D). Because PSF RBD forms complex with certain RNAs, releasing PSF from a regulated gene GAGE6 and reversing repression, we further determined the role of vtRNA1-1 by binding to PSF. Biotinylated DNA and unlabeled RNA was incubated with His-tagged RBD or His-tagged PSF, and fractionated by a PAGE gel. As expected, binding to the DNA occurred with the His-tagged PSF but not with the His-tagged RBD (Fig 1E), whereas binding to vtRNA1-1 occurred with the RBD but not with the DBD (Fig 1D). Addition of unlabeled vtRNA1-1 inhibited binding of GAGE6 promoter probe to His-tagged PSF, demonstrating the inhibition of vtRNA1-1 to the function of PSF as a DNA-binding tumor suppressor.

Interaction of PSF protein and vtRNA1-1 effects on GAGE6’s transcription in vivo

To further confirm the interaction of PSF protein and vtRNA1-1 in vivo, we transfected plasmids expressing vtRNA1-1 either with FLAG-tagged PSF, DBD or RBD into HEK293. RNA-IP was done 48 hour later. The results showed the strong binding of full-length PSF and RBD to exogenous vtRNA1-1, but not to DBD, which is consistent with our in vitro experiments (Fig 2A).

GAGE6 is strongly transcripted in MCF-7/DOX cells, but repressed significantly in original MCF-7 cells (Fig 2B and 2C). However, no detectable changes of vtRNA1-1 between these two
cells (data not shown). This result promotes us to test the effects of expression of vtRNA1-1 on PSF’s transcriptional regulation activity. We Immunoprecipitated PSF protein from stably transfected MCF-7 cells and detected the GAGE6 promoter fragment in co-purified precipitate. Transfection of Vault cDNA releases the PSF from GAGE6 promoter region (Fig 2D and 2E) and enhanced GAGE6 transcription in MCF-7 (Fig 2F and 2G). Oppositely, the ectopic expression of AS vtRNA1-1, which was supposed to form double-stranded vtRNA1-1 with endogenous vtRNA1-1, significantly enhanced the binding of PSF to GAGE6’s promoter
vtRNA1-1 expression tightly regulates the expressing level of GAGE6 in MCF-7 and MCF-7/MR. (A) RNA-IP assay confirmed the specific binding of PSF to vtRNA1-1 through its RBD in vivo. RT-qPCR (B) and semi-quantitative western blot (C) presents the expressing changes in the mRNA and protein level of GAGE6 in MCF-7 and MCF-7/MR. Chromatin immunoprecipitation assays present that the binding capacity of PSF to GAGE6’s promoter region affected by vtRNA1-1 in MCF-7 (D) or MCF-7/MR (E). RT-qPCR (F) and Semiquantitative western blot (G) present the mRNA and protein levels affected by the ectopic expression of vtRNA1-1 in MCF-7 or MCF-7/MR. *P<0.05.

https://doi.org/10.1371/journal.pone.0191325.g002
region (Fig 2D and 2E), and consequently caused the repression of GAGE6’s expressing levels in mRNA and protein levels (Fig 2F and 2G).

The effects of vtRNA1-1 in cell proliferation and colony formation in a PSF-dependent way

We then asked if ectopic expression of vRNA affected the proliferation and colony formation. In the presence of PSF, MCF-7 showed significantly increased proliferation rates and colony formation when vtRNA is introduced in (Fig 3A, 3B & 3C). In PSF-knockdown MCF-7 cells, ectopic expression of vtRNA1-1 showed undetectable affections on cell proliferation and colony formation in soft agar. Expectedly, introduction of RBD, but not DBD into MCF-7-vRNA cell line inhibits the effects of vRNA on cell growth and colony formation, indicates the competitive binding of RBD with vRNA to endogenous PSF in vivo.

The role of vtRNA1-1 in acquisition of chemoresistance in a PSF-dependent way

The upregulation of vtRNAs in drug resistant cell line suggests that vtRNAs may function as both suppressor of drug sensitivity and promoter of tumor malignancies. For exploring the
possibility that vtRNA1-1 influence the chemosensitivity of breast cancer, we used MCF-7 cells overexpressing either empty vector or construct coding for vtRNA1-1 to create the responder phenotype in vitro. CCK-8 assay was performed to detect the Doxorubicin (Sigma-Aldrich, St. Louis, MO, USA) sensitivity. As shown in Fig 4A, overexpression of vtRNA1-1 desensitized MCF-7 to Doxorubicin treatment, while co-introduction of RBD abolished the effect of over-expressed vtRNA1-1 on chemoresistance. In IC_{30}-Doxorubicin treated MCF-7 cells, GAGE6 was transcriptional downregulated (Fig 4B & 4C), and the introduction of vtRNA1-1 significantly upregulation GAGE6 expressing levels. For confirming the transcriptional change of GAGE6 after vtRNA1-1 introduction, we performed ChIP for detecting the effects of vtRNA1-1 introduction on interaction between PSF and GAGE6 promoter region. As expected, Doxorubicin treatment significantly increased the binding of PSF to GAGE6 promoter region, which is reversed by vtRNA1-1 introduction (Fig 4D). By detecting cell viability and cell death rate, it is illustrated that vtRNA1-1 expression remarkably exerted cellular protective effects by decreasing the death rate caused by DOX treatment (Fig 4E & 4F). For further determine whether.

VtRNA1-1 contributes to doxorubicin resistance independent with MVP

MVP, which was also known as LRP (lung resistance-related protein), is correlated with the drug resistance potentially via modulating the nucleocytoplasmic transport, such as hormones, ribosomes, mRNA and drug [32–34]. This raised the question that whether the contribution of vtRNA1-1 to chemoresistance is dependent on the presence of MVP or not. For identifying whether the resistance to chemoagent acquired by vtRNA1-1 presence is dependent on the expression of MVP, Doxorubicin resistant MCF-7 sub-cell line (MCF-7/DOX) was employed for detect the drug sensitivity after MVP or vtRNA1-1 knockdown, respectively. The results showed that, without disturbing the DOX sensitivity in MCF-7 (data not shown), both MVP and vtRNA1-1 knockdown sensitized MCF-7/DOX to Doxorubicin, demonstrating that both MVP and vtRNA1-1 are important in causing Doxorubicin resistance (Fig 5A & 5B). It is reported that MVP contributes to doxorubicin resistance in MCF-7/DOX by altering intracellular distribution of doxorubicin [35]. For confirming whether knockdown of vtRNA1-1 contributes to doxorubicin sensitivity by affecting the distribution of doxorubicin, doxorubicin distribution was observed in MCF-7/DOX with or without vtRNA1-1 knockdown. As shown in Fig 5C, as expected, MVP knockdown increased nuclear accumulation of doxorubicin which is not disturbed by knockdown of vtRNA1-1. Taken together, vtRNA1-1 potentially exerted as a promoter of chemoresistance in MCF-7/DOX independent with MVP.

Discussion

Previous studies have been reported that vault-associated components in cancer cells is responsible for causing resistance to chemotherapeutic compounds [7, 21]. In cancer cells, the upregulation of the components of vault particle, including vtRNAs and protein parts, is tightly associated with the raising of chemoresistance. Interestingly, the upregulation of the protein parts failed to affect the chemoresistant capacity significantly, indicating the critical role of vtRNAs in raising chemoresistance. For understanding the molecular basis for causing chemoresistant capacity toward the chemotherapeutic compounds through overexpression of vault components, Gopinath et al. found that, vtRNAs presented the ability to recognize chemotherapy compounds, such as mitoxantrone, based on biophysical and biochemical analyses [13]. However, the molecular mechanism of the causing of chemoresistance involving vtRNAs is still largely unknown.
With the accumulating evidence showing that non-coding RNA significantly regulates the physiological processes, including the attaining resistance in cancer cells, it leads us to...

https://doi.org/10.1371/journal.pone.0191325.g004
speculate that vtRNAs may be involved in the regulatory processes through interaction with the other proteins, not only the vault components, and functions as an epigenetic regulator. Thus, the purpose of this study was to evaluate the interaction of vtRNAs with protein. Through EMSA assay, it was found that vtRNAs binds specifically with PSF’s RBD, but not with its DBD. The binding dramatically decreases the interaction between PSF’s DBD with its target gene, such as GAGE6. This interaction was further confirmed in vivo by RNA-IP. Consequently, the expressing level of GAGE6 was increased significantly after the epigenetic expression of vtRNA1-1 in MCF7 cells and led to the competitively release of PSF from GAGE6’s promoter region. Although it remains unknown whether there is a conserved sequence in vtRNA1-1 recognized by PSF, it seems that the interaction of PSF’s RBD with vtRNA1-1 is involved in their transcriptional regulation to its target genes, at least to GAGE6. The above results suggested that there is a correlation between the expression level of vtRNA1-1 and the chemoresistance in cancer cells. PSF was reported exerts a central role in the reversible regulation of cell proliferation and tumorigenesis [36]. Our results showed that inhibition of PSF activity by vtRNA1-1 interaction is consistent with the previous finding logically.

Next, to address the functional significance of vtRNA1-1 overexpression in cells, we used the shRNA targeting to vtRNA1-1 to knockdown the expression levels of vtRNA1-1.
endogenously [21]. These strategies were proved successful for evaluating the functions of many noncoding RNAs [37]. As it was shown in Figs 4 and 5, vtRNA1-1 knockdown by shRNA transfection in MCF-7 chemoresistant-strain significantly sensitize them to chemoagent.

Taken together, both the knockdown and rescue analyses in this study reveal that vtRNA1-1 plays a critical role in conferring resistance to several kinds of cancer cells against the chemotherapeutic compounds. As a supplement of its role involving the induction of chemoresistance by binding to chemoagent directly, for the first time, we revealed the direct binding of vtRNA1-1 to PSF protein, and leading to the competitive release of PSF’s target gene from the DBD, such as GAGE6. The transcriptional regulation of GAGE6 by vtRNA1-1 binding tightly regulates the physiological processes in cancer cells. This novel epigenetic regulatory mechanism identified in this study may contribute a new therapeutic target for against chemoresistance.

Author Contributions

Data curation: Xuemei An.
Formal analysis: Xuemei An.
Investigation: Jianjun Chen, Hui OuYang, Shixi Liu.
Methodology: Jianjun Chen, Hui OuYang.
Software: Xuemei An.
Supervision: Shixi Liu.
Writing – original draft: Jianjun Chen.
Writing – review & editing: Shixi Liu.

References

1. Kedersha NL, Heuser JE, Chugani DC, Rome LH. Vaults. III. Vault ribonucleoprotein particles oper into flower-like structures with octagonal symmetry. J Cell Biol. 1991; 122: 225–235. PMID: 1988458.
2. Abbondanza C, Rossi V, Roscigno A, Gallo L, Belsito A, Piluso G, et al. Interaction of vault particles with estrogen receptor in the MCF-7 breast cancer cells. J Cell Biol. 1998; 141: 1301–1310. PMID: 9628887.
3. Christine H, Elaheh G, Elisabeth I, Leonard R, Walter V. Recombinatn major vault protein is targeted to neuritic tips of PC12 cells. J Cell Biol. 1999; 144: 1163–1172. PMID: 10087261.
4. Kitazono M, Sumizawa Y, Takebayashi Y, Chen ZS, Furukawa T, Nagayama S, et al. Multidrug resistance and the lung resistance-related protein in human colon carcinoma SW-620 cells. J Natl Cancer Inst. 1999; 91: 1647–1653. PMID: 10511592.
5. Kedersha NL, Rome LH. Isolation and characteriz ation of a novel ribonucleoprotein particle: large structures contain a single species of small RNA. J Cell Biol. 1986; 103: 699–709. PMID: 2943744.
6. Kedersha NL, Rome LH. Preparative agarose gel electrophoresis for purification of small organelles and particles. Anal Biochem. 1986; 156: 161–170. PMID: 2429868.
7. Kickhoefer VA, Rajavel KS, Scheffer GL, Dalton WS, Scheper RJ, Rome LH. vaults are up-regulated in multidrug-resistant cancer cells. J Biol Chem. 1998; 273: 8971–8974. PMID: 9535882.
8. Siva AC, Raval-Fernandes S, Stephen AG, LaFemina MJ, Scheper RJ, Kickhoefer VA et al. Up-regulation of vaults may be necessary but not sufficient for multidrug resistance. Int J Cancer. 2001; 92: 195–202. PMID: 11291045.
9. van Zon A, Mossink MH, Schoester M, Scheffer GL, Scheper RJ, Sonneveld P et al. Multiple human vault RNAs. Expression and association with the vault complex. J Biol Chem. 2001; 276: 37715–37721. https://doi.org/10.1074/jbc.M106055200 PMID: 11479319.
10. Poderycki MJ, Rome LH, Harrington L, Kickhoefer VA. The p80 homology region of TEP1 is sufficient for its association with the telomerase and vault RNAs. Nuclei Acids Res. 2005; 33: 893–902. https://doi.org/10.1093/nar/gki234 PMID: 15701761.
11. Huffman KE, Corey DR. Major vault protein does not play a role in chemoresistance or drug localization in a non-small cell lung cancer cell line. Biochemistry. 2005; 44: 2253–2261. https://doi.org/10.1021/bi047948g PMID: 15709737.

12. Steiner E, Holzmann K, Elbling L, Micksche M, Berger W. Cellular functions of vaults and their involvement in multidrug resistance. Curr Drug Targets. 2006; 7: 923–934. PMID: 16918321.

13. Gopinath SC, Wadhwa R, Kumar PK. Expression of noncoding vault RNA in human malignant cells and its importance in mitoxantrone resistance. Mol Cancer Res. 2010; 8: 1536–1546. https://doi.org/10.1158/1541-7786.MCR-10-0242 PMID: 20881010.

14. Patton JG, Porro EB, Galceran J, Tempst P, Nadal-Ginard B. Cloning and characterization of PSF, a novel pre-mRNA splicing factor. Genes Dev. 1993; 7: 393–406. PMID: 8449401.

15. Shoham T, Parameswaran R, Shay-Tal Y, Bara-Saad M, Zipori D. The mesenchymal stroma negatively regulates B cell lymphopoiesis through the expression of activating A. Ann N Y Acad Sci. 2003; 996: 245–260. PMID: 12799303.

16. Song X, Sun Y, Garen A. Roles of PSF protein and VL30 RNA in reversible gene regulation. Proc Natl Acad Sci U S A. 2005; 102: 12189–12193. https://doi.org/10.1073/pnas.0505179102 PMID: 16079199.

17. Duan Z, Duan Y, Lamendola DE, Yusuf RZ, Naeem R, Penson RT, et al. Overexpression of MAGE/GAGE genes in paclitaxel/doxorubicin-resistant human cancer cell lines. Proc Natl Acad Sci U S A. 2003; 9: 2778–2785. PMID: 12855658.

18. Cheryl SB, Kevin GB, William HW, Zhang YQ, Patrice JM. Gene expression and pathway analysis of ovarian cancer cells selected for resistance to cisplatin, paclitaxel, or doxorubicin. J Ovarian Res. 2011; 4: 21–31. https://doi.org/10.1186/1757-2215-4-21 PMID: 22141344.

19. Li L, Feng T, Lian Y, Zhang G, Garen A, Song X. Role of human noncoding RNAs in the control of tumorigenesis. Proc Natl Acad Sci U S A. 2009; 106: 12956–12961. https://doi.org/10.1073/pnas.0906051106 PMID: 19625619.

20. Ji Q, Zhang L, Liu X, Zhou L, Wang W, Han Z, et al. Long non-coding RNA MALAT1 promotes tumour growth and metastasis in colorectal cancer through binding to SFPQ and releasing oncogene PTBP2 from SFPQ/PTBP2 complex. Br J Cancer. 2014; 111: 736–748. https://doi.org/10.1038/bjc.2014.383 PMID: 25025966.

21. Hu Y, Stephen AG, Cao J, Tanzer LR, Slapak CA, Harrison SD, et al. Very early induction of major vault protein accompanied by increased drug resistance in U-937 cells. Int J Cancer. 2002; 97: 149–156. PMID: 11774257.

22. Ohno N, Tani A, Uozumi K, Hanada S, Furukawa T, Akiba S, et al. Expression of function lung resistance-related protein predicts poor outcome in adult T-cell leukemia. Blood. 2001; 98: 1160–1165. PMID: 11493465.

23. Taylor PD, Oon BB, Thomas CR, Poston L. Prevention by insulin treatment of endothelial dysfunction but not enhanced noradrenaline-induced contractility in mesenteric resistance arteries from streptozotocin-induced diabetic rats. Br J Pharmacol. 1994; 111: 35–41. PMID: 8012717.

24. Zijlstra JG, de Vries EGE, Mulder NH. Multifactorial drug resistance in an Adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res. 1987; 47: 1780–1784. PMID: 3028613.

25. Versantvoort CH, Withoff S, Broxterman HJ, Kuiper CM, Scheper MR JNH, de Vries EG. Resistance-associated factors in human small-cell lung carcinoma GLC4 sub-lines with increasing Adriamycin resistance. Int J Cancer. 1995; 61: 375–380. PMID: 7729950.

26. Taylor CW, Dalton WS, Parrish PR, Gleason MC, Bellamy WT, Thompson FH, et al. Different mechanisms of decreased drug accumulation in doxorubicin and mitoxantrone resistant variants of the MCF7 human breast cancer cell line. Br J Cancer. 1991; 63: 923–929. PMID: 1676902.

27. Janke C, Magiera MM, Rathfelder N, Taxis C, Reber S, Maekawa H, et al. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast. 2004; 21: 947–962. https://doi.org/10.1002/yea.1142 PMID: 15334558.

28. Bochud-Allemann N, Schneider A. Mitochondrial substrate level phosphorylation is essential for growth of procyclic Trypanosoma brucei. J Biol Chem. 2002; 277: 32849–32854. https://doi.org/10.1074/jbc.M205776200 PMID: 12095995.

29. Cui R, Widlund HR, Feige E, Lin JY, Wilensky DL, Igras VE, et al. Central role of p53 in the suntan response and pathologic hyperpigmentation. Cell. 2007; 128: 853–864. https://doi.org/10.1016/j.cell.2006.12.045 PMID: 17350573.

30. D’Orazio JA, Nobuhisa T, Cui R, Araya M, Spry M, Wakamatsu K, et al. Topical drugrescue strategy and skin protection based on the role of Mc1r in UV-induced tanning. Nature. 2006; 443: 340–344. https://doi.org/10.1038/nature05098 PMID: 16988713.

31. Kickhoefer VA, Rajavel KS, Scheffer GL, Dalton WS, Scheper RJ, Rome LH. Vaults are up-regulated in multidrug-resistant cancer cell lines. J Biol Chem. 1998; 273: 8971–8974. PMID: 9535882.
32. Izquierdo MA, Scheffer GL, Flens MJ, Giaccone G, Broxterman HJ, Meijer CJ, et al. Broad distribution of the multidrug resistance-related vault lung resistance protein in normal human tissues and tumors. Scheper RJ Am J Pathol. 1996; 148: 877–887. PMID: 8774142.

33. Kickhoefer VA, Rajavel KS, Scheffer GL, Dalton WS, Scheper RJ, Rome LH. Vaults are up-regulated in multidrug-resistant cancer cell lines. J Biol Chem. 1998; 273: 8971–8974. PMID: 9535882.

34. Izquierdo MA, Shoemaker RH, Flens MJ, Scheffer GL, Wu L, Prather TR, et al. Overlapping phenotypes of multidrug resistance among panels of human cancer-cell lines. Int J Cancer. 1996; 65: 230–237. https://doi.org/10.1002/(SICI)1097-0215(19960117)65:2<230::AID-IJC17>3.0.CO;2-H PMID: 867122.

35. Han M, Lv Q, Tang XJ, Hu YL, Xu DH, Li FZ, et al. Overcoming drug resistance of MCF-7/ADR cells by altering intracellular distribution of doxorubicin via MVP knockdown with a novel siRNA polyamidoamine-hyaluronic acid complex. J Control Release. 2012; 163: 136–144. https://doi.org/10.1016/j.jconrel.2012.08.020 PMID: 22940126.

36. Wang G, Cui Y, Zhang GF, Garen A, Song X. Regulation of proto-oncogen transcription, cell proliferation, and tumorigenesis in mice by PSF protein and a VL30 noncoding RNA. Proc Natl Acad Sci U S A. 2009; 106: 16794–16798. https://doi.org/10.1073/pnas.0909022106 PMID: 19805375.

37. Li LC, Okino ST, Zhao H, Pookot D, Place RF, Urakami S, et al. Small dsRNA induce transcriptional activation in human cells. Proc Natl Acad Sci U S A. 2006; 103: 17337–17342. https://doi.org/10.1073/pnas.0607015103 PMID: 17085592.