Expression of non-coding vault RNA in human malignant cells and its importance in mitoxantrone resistance

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Short Title: vault RNA and drug resistance

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Abstract

Several non-coding RNAs perform vital cellular functions, including gene regulation and cell development. Previously, we reported that vault RNA (vRNA) has the ability to recognize chemotherapeutic compounds, such as mitoxantrone, based on biophysical and biochemical analyses. In the present study, we show that human glioblastoma, leukemia, and osteocarcinoma derived cell lines overexpress vRNA and exhibit higher resistance towards mitoxantrone. Interestingly, when vRNA expression was suppressed by RNAi in these cells, the resistance progressively decreased. In agreement with these findings, overexpression of vRNA-1 caused resistance to mitoxantrone. These results suggest a role of vRNA in mitoxantrone resistance in malignant cells and justify further studies on the importance and application of non-coding RNAs in cancer chemotherapeutics.

Introduction

Vaults are barrel-shaped particles with a mass of about 13 MDa and overall dimensions of 400 X 400 X 700 Å, as determined by scanning transmission electron microscopy (1). These particles represent the largest ribonucleoprotein complex identified thus far in eukaryotic cells. They appear to shuttle between the cytoplasm and the nucleus (2), and hence have been implicated in intracellular (3) and nucleocytoplasmic transport (2-3). They are widely distributed in eukaryotic species, including mammals, avians and amphibians. Their considerable abundance and striking evolutionary conservation argue for their important role in cell survival (4). Several lines of evidence have suggested that vaults may play an important role in intracellular detoxification processes, and thus may function in the multidrug resistance (MDR) of cancer cells (5).
In mammals, the vault particle is composed of three proteins and RNA. Among these components, the 100 kDa major vault protein (MVP) accounts for more than 75% of the particle mass (1). The other two proteins are the 290 kDa telomerase-associated protein 1 (TEP1) and the 193 kDa vault poly(ADP-ribose) polymerase (VPARP). The vault is also known to contain a non-coding RNA (vRNA) (6-11). As compared to the other components, the RNA represents <5% of the mass of the vault complex. In humans, there are three vRNA genes, the hvg-1, hvg-2 and hvg-3 RNAs, located on chromosome 5, and at least one of them is known to associate with the vault complex. Previously, relatively large amounts of hvg1 in vault particles were reported, whereas small amounts of hvg-2 and hvg-3 were found (8,11). Human hvg-1 RNA is 98 bases in length (Fig. 1a), and the other two RNAs are 88 bases long. In addition, hvg-4 is encoded on the X chromosome, but it does not appear to be expressed (8). These vRNAs share about 84% sequence identity and have similar secondary structure arrangements. In the vault particle, the TEP1 protein was shown to be important for vRNA binding and stabilization of the vault complex (9,12). The TEP1 found to locate at the terminal ends of the vault particle, based on the crystal structure of the whole vault particle, isolated from the rat, at 3.5 Å resolution (13). The vault particle consists of a dimer of half-vaults, comprising 39 identical MVP monomers. Each MVP monomer folds into 12 domains: 9 structural repeat domains, a shoulder domain, a cap helix domain and a cap-ring domain (Fig. 1b). It was proposed that
the interactions between the 42-turn-long cap-helix domains of MVP are essential for stabilizing the vault particle (13).

MDR, a major cause of failure of cancer treatment, is a phenomenon whereby cancer cells develop broad resistance to a wide variety of chemotherapeutic drugs (14). A previous study on several non-P-glycoprotein MDR cell lines with increased MVP levels demonstrated that the vault RNAs (vRNAs) and MVP were not shown to be coordinately regulated, and suggested that the entire vault particle is up-regulated in MDR and that a threshold level of vaults is required to impart MDR (6). In a clinical scenario, the elevated expression of MVP was observed in cell lines resistant to various classes of chemotherapeutic compounds, including doxorubicin and mitoxantrone (15). Support for the role of vaults in the extrusion of anthracyclines from the nuclei of resistant cells was reported by Ohno et al. (16). Cells utilize several mechanisms to prevent cytotoxic drugs from reaching their target sites, including protein modification of the drugs, up-regulation of plasma membrane pumps, and reduction of the nuclear-cytoplasmic ratio by increasing drug export from the nucleus. Multidrug resistant cell lines have been shown to utilize these routes to gain resistance to various drugs. The expected role of vaults in MDR is supported by the following evidence: 1) vaults and their components are upregulated in cells treated with cytotoxic compounds (6), 2) vaults are involved in nucleo-cytoplasmic transport (2,17), and 3) compounds that interact with the
vaults (17). Despite these findings, until recently (18), the vault components were not shown to interact directly with chemotherapeutic compounds.

RNA molecules are involved in many important metabolic processes, including peptide bond formation and replication (19,20). In recent years, non-coding RNAs have been shown to participate in complex communication pathways in gene regulation and cell differentiation (21,22). The vRNAs isolated previously from different species were predicted to fold in a similar fashion, despite their differences in length, indicating that the association of vRNAs with vaults is not fortuitous. We previously showed that vault-associating RNAs, specifically the hvg-1 and hvg-2 RNAs, have the ability to interact with chemotherapeutic compounds (18). Using a circular dichroism analysis and an in-line probing assay, we identified the mitoxantrone binding region within the vRNAs (hvg-1 and hvg-2). Moreover, we also reported that the interactions between the vRNAs and mitoxantrone take place even in the cellular milieu. These results clearly suggested that vRNAs have the ability to bind certain chemotherapeutic compounds and that these interactions may play an important role in vault functions, possibly by participating in the export of toxic compounds (18). These in vitro studies prompted us to investigate the ability of vRNA to confer mitoxantrone resistance in some cancer cell lines. We found that vRNA is overexpressed in cancer cells that display higher resistance towards mitoxantrone, and when the vRNA expression was compromised, they became sensitive. On the other hand, the mitoxantrone sensitive cancer cell line MG63,
which also expresses lower levels of vRNA-1, exhibited resistance when vRNA-1 was overexpressed endogenously. Together, our previous and present results delineate the importance of vRNA for conferring drug resistance to the cancer cells.

**Materials and Methods**

*Vault RNAi (vRNAi) design for preliminary analyses.* To prepare the various vRNAis, we chemically synthesized the following oligos: 5'-GGCUGGCUUUAGCUCAGCGUT-3' (Vault1-4RNAi-1), 5'-GCUGAGCUAAAGCCAGCCUT-3' (Vault1-4RNAi-2), 5'-GCGACUGCAUUUCGGUCCGUT-3' (Vault1-4RNAi-3), 5'-CCGACCGAAAUCGAGAGCUT-3' (Vault1-4RNAi-4), 5'-CAGUUCUUUAAUUGACUT-3' (Vault1RNAi-5); and 5'-UGUUUCAAUUAAGAAGUGUT-3' (Vault1RNAi-6). Oligofectamine (Invitrogen, CA) was used to transfect the cancer cell lines. Cells at about 90% confluence were collected for the transfection. For transient transfection, the cells were treated with 5 μl of Oligofectamine diluted 5-fold in pre-warmed, serum-free optimum medium. Two complementary vRNAi sequences, each at a 100 nM concentration, were denatured in annealing buffer (100 mM potassium acetate and 2 mM magnesium acetate in 30 mM Hepes KOH, pH 7.4) at 90°C, cooled to 37°C and kept for 1 hr, and then the mixture was further diluted to a volume equal to that of the Oligofectamine mixture. Both the Oligofectamine and vRNAi mixtures were mixed together by pipetting and incubated at room temperature for 30 min. This mixture
was then used to transfect the cell lines (10^6 cells) plated in serum-free medium in 6 well plates. After a 4 h incubation, serum was added to the cells, and growth was continued until the optimum confluence was attained for analyses.

**vRNAi design and vector construction for stable transfection.** For stable transfection, the sequences encoding all four vRNAs were cloned in-frame into the pSilencer™ puro (Ambion, USA) vector by the target sequence, according to the manufacturer’s instructions. The sequences corresponding to positions 1-19 were synthesized and used as the target to the vault transcript. Our desired vault sense and antisense strand sequences (Vault1-4RNAi-1: 5'-GGCUGGCUUUAGCUCAGCGUT-3'; Vault1-4RNAi-2: 5'-CGCUGAGCUAAAGCCAGCCUT-3'), with BamHI and HindIII restriction digestion sites and sequences for features to create a loop and a human U6 RNA pol III promoter, were inserted into the linearized pSilencer 2.1-U6 puro siRNA expression vector. The clones bearing the hairpin vRNA-coding insert were screened after ligation. The resulting vector DNA sequences were verified by sequence analysis, using an ABI PRISM™ 310 Genetic Analyzer. The correct sequences of the vRNAis were 21 nucleotides long and contained symmetric 3’ overhangs of deoxyuridine and thymidine. For negative control experiments, the reverse sequences of the vault transcripts (Vault1-4RNAi-3c: 5'-GCGACUCGAUUUCGGUCGGUT-3'; Vault1-4RNAi-4c: 5'-CCGACCGAAAUCGAGUCGCUT-3') were inserted, and in addition, vector sequences alone were also transfected.
Cell culture and Stable transfection. Human cancer cell lines (MG63, U118MG, U937 and U2OS) were obtained from ATCC and were cultured in either RPMI medium (U937) or Dulbecco’s modified Eagle’s minimal essential medium (DMEM) (for MG63, U118 and U2OS) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest) and penicillin, streptomycin and fungizone. All cells were grown at 37°C in a humidified atmosphere with 5% CO₂ supplementation. The cells were seeded at a density of 5x10⁴/cm² in 10-cm-diameter culture dishes. The U937 cell line was grown as a suspension culture, while all of the other cells were adherent. The cells were subcultured at intervals of one week after the cell density reached ~1x10⁶/ml, and they grew at the same rate.

For the vault knock-down studies, U937, U2OS and U2OS/mot-2 cells were used. The vRNAi oligos with a hairpin structure were transfected into these cells. The scrambled version of the control RNAs that form a hairpin structure with the reverse sequence, and the vector alone, which does not produce RNA with a hairpin structure, were also transfected. The cells were transfected with Fugene 6 (Boehringer Mannheim). Briefly, 4x10⁶ cells were harvested and then resuspended in 200 μl of serum-free optimum medium. Both the plasmid DNA, constructed as above, and the Fugene 6 were then diluted to a 1:3 ratio of mass (3 μg) and volume (9 μl), and were added directly to the prepared cell mix. The cells were incubated with the DNA encoding the vRNAi sequence and the
transfection reagent at room temperature for 20 min. After this incubation, the cells were further diluted with DMEM medium supplemented with 10% FBS and grown in 1 cm wells. The culture medium was removed by aspiration (or centrifugation for the suspension cultures) after one day and was replaced with fresh medium containing 1 μg/ml of puromycin for selection. After two days of drug selection, the dead cells were washed away, and the live cells were collected to monitor the efficiency of transfection. The clones transfected with the silencing vRNAi, the reverse sequence and the empty vector alone were selected for further experiments.

**Overexpression of vRNA in cancer cells confers resistance to mitoxantrone.**

We addressed whether the overexpression of vRNA-1 in cancer cells, which are sensitive to mitoxantrone and also express lower levels of vRNA-1, confers resistance to mitoxantrone. For this study, we selected the MG63 cancer cell line, as this cell line expresses vRNA-1 at relatively lower levels and is also sensitive to mitoxantrone. We prepared an expression vector (pSilencer-vRNA-1) that expressed vRNA-1 upon transfection into the MG63 cell line. The full-length vault sequence of Hvg-1 (5’–GGCUGGCUUUAGCUCAGCGGUUACUUCGACAGUUCUUUUAAAUUGAAACAAAGCAACCUGUCUGGGGUUGUUCGAGACCCGCGGCGUCUCAGUCCUUUU-3’) with BamHI and HindIII restriction digestion sites was inserted into the linearized pSilencer 2.1-U6 puro expression vector, which features a human U6 RNA pol III promoter. The clones bearing the vRNA1-coding insert were screened after ligation, and the resulting vector DNA sequences were verified by
sequence analysis, using an ABI PRISM™ 310 Genetic Analyzer. The cells were transfected as above with Fugene 6, and the cells were grown in the presence of puromycin for selection. For control experiments, vector sequences alone and a construct with the complementary sequence of Hvg1 were used.

**Detection of vRNA expression by reverse transcription-polymerase chain reaction (RT-PCR).** Total cellular RNA was isolated using a mirVana™ miRNA Isolation kit (Ambion Inc., USA), according to the manufacturer’s instructions. Using 3 μg of total RNA, reverse transcription was performed with the primer 5’ AAAAGGACTGGAGAGCGCCC 3’ and reverse transcriptase (Wako) at 42°C. PCR analyses were performed with the resultant cDNA. The primers used for vRNA amplification were 5’ GGCTGGCTTTAGCTCAGCGG 3’ and 5’ AAAAGGACTGGAGAGCGCCC 3’, which generated the correct sizes of vRNAs. The amplification conditions were 20 cycles at 95°C for 70 sec, 50°C for 50 sec, and 72°C for 70 sec. The double-stranded PCR products thus obtained were electrophoresed in a 4% agarose gel. The intensities of the PCR products on the gel were quantified using the ImageJ software [NIH (National Institutes of Health), Bethesda, MD, U.S.A.]. Similarly, the U6 promoter regions were amplified with appropriate primers (forward 5’ GGCAGCACATATACTAAAATTGGA 3’; and reverse 5’ CACCGGCCTATAAAACGTGGTCAAA 3’) as the internal control.

Similarly, RNA expression of Breast cancer resistance protein (BCRP) and Major vault protein (MVP) were checked by using the following sets of primers
Western blot analysis. The 10 cm plates with cells at 80% confluence were washed with PBS, and the cells were collected by trypsinization. The total cellular protein was extracted with 300 μl of Nonidet P-40 lysis buffer, containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 1% NP-40, supplemented with a protease inhibitor cocktail (Complete Mini; Roche Diagnostics K.K., Basel, Switzerland), at 4°C with mixing. Cell debris was removed by centrifugation at 13,000 x g for 10 min at 4°C, and the supernatants were collected. The protein concentration was quantified using a Bio-Rad protein assay kit, and bovine plasma gamma globulin (Biorad, CA) was used as the standard. An equal amount of protein (35 μg) from each sample was fractionated by 5-20% gradient gel electrophoresis (SuperSep™, Wako) and was transferred to PVDF membranes (Clear Blot membrane-P, ATTO). After immobilization of the protein on the membrane, it was blocked with 3% non-fat milk in TBS buffer at room temperature for 1 hr, and then was treated with a 1:1,000 dilution of the primary antibody (anti-mortalin monoclonal antibody) or anti-actin (Chemicon International-MAB1501R) at 4°C overnight. After removing the unbound primary antibody by washing, the membrane was incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; Santa Cruz...
Biotechnology). The immunocomplexes thus formed were visualized with an ECL kit (Amersham Pharmacia Biotech).

**Cell survival assay with the chemotherapeutic agent mitoxantrone.** Cell viability against the drug was measured using a 3-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-tetrazolium-5-carboxanilide (MTT) assay kit for cell survival and proliferation (Chemicon International, USA), according to the manufacturer’s instructions. To determine the cytotoxicity of the mitoxantrone (Sigma) treatment, the cell lines were counted with a Neubauer hemocytometer, and then were seeded into 96-well plates at a density of 1x10^4 cells/well. After the cells reached 50% confluence, different concentrations of mitoxantrone were added, and no drug was added to the control well. The treated cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cell viability was analyzed by an MTT assay on the 3rd day (72 hrs) following the mitoxantrone treatment. Cell survival with mitoxantrone treatment was calculated as the percentage (%) relative to the survival of cells in the absence of mitoxantrone.

**Results**

**Overexpression of vRNA-1 in cancer cell lines.** Previous studies revealed that vRNA-1 is present in cells, either in association with vault particles or in the free form, and on average, at any given time, only 20% of the total vRNA is associated with the vault particle in these multi-drug resistant (MDR) cancer cell lines (6). These studies also supported the hypothesis that a dynamic
relationship exists between the free vRNA and the vRNA associated with vault particles. In addition, the fraction of RNA-associated vault particles increased progressively in the drug resistant cell lines. Hu et al. (15) reported that vRNA was overexpressed in two multidrug-resistant U-937 cell lines with elevated levels of vault-associated proteins. These studies suggested that vault components are commonly overexpressed in cancer cell lines that are resistant to chemotherapeutic compounds. We showed previously that mitoxantrone has the ability to bind to vRNA-1, which potentially sequesters the drug and keeps it from reaching the target site for its function (18). In the present study, we selected four cancer cell lines (MG63, U119MG, U937 and U2OS) in order to evaluate the correlation between the expression levels of vRNA and the resistance to mitoxantrone. Among the cell lines tested, U2OS cells tend to show moderately higher expression of vRNA and resistance towards mitoxantrone (Fig. 2a,b,c). However, in MG63 and U118MG cells, the vRNA expression levels were moderately lower, and these cell lines were correspondingly more sensitive towards mitoxantrone (Fig. 2a,b,c). Similarly, the expression of U6 RNA with specific primers for 176 bases was used as internal control and the expression levels are seems to be similar in all cases. Additionally, we have evaluated the RNA expression of BCRP and MVP in these cell-lines since they are known to provide cellular resistance to mitoxantrone. Our studies show that MVP expressed at higher levels in MG63, U118MG and U2OS cells and less in U937 cells. The BCRP RNA was also expressed abundantly in U937 and U2OS cells but lesser extent in other two cell-lines (Fig. 2d).
vRNA silencing by a specific siRNA. To evaluate the importance of vRNA directly in cells, we adopted an RNAi approach to knock-down the vRNA levels. In this type of study, it is important to know the sequence of vRNA-1 overexpressed in U937 cells, even though the vRNA-1 sequence was previously reported from SW1573/2R120 and GLC4/ADR cells. Therefore, we isolated total RNA from U937 cells and performed an RT-PCR analysis using specific primers, based on the reported vRNA sequences (van Zon et al. 2001). We obtained PCR products (~100 bp) for hvg RNA-1 and hvg RNA-3 (Fig. 3a). No PCR product, however, was obtained for hvg RNA-2, even with additional PCR cycles (data not shown). To deduce the sequences of the resulting PCR products, the respective bands were isolated from the agarose gel, cloned into the TA vector (Invitrogen, USA), and fully sequenced. Among the 20 clones that were sequenced, the majority were aligned to the hvg RNA-1 sequence, even though we used two different sets of specific primers (for hvg-1 and hvg-3). Based on these results, we concluded that either the primer set may not be specific enough for the amplification of hvg RNA-3 or the hvg RNA-3 may be present in low abundance as compared to the hvg RNA-1, in U937 cells.

For the knock-down studies, at the outset, we designed two sets of duplex vRNAi oligos, which could target two different sites on the hvg RNA-1, corresponding to positions 1-19 and 30-48 (Fig. 3b). Target site I was common to hvg RNA-1, hvg RNA-2 and hvg RNA-3 (Vault1-4RNAi-1&2), and target site II
was designed to target hvg RNA-1 (Vault1RNAi-5&6) specifically. We chemically synthesized the above two vRNAi oligo sets along with a vRNAi control RNA set (Vault1-4RNAi-3c&4c), which is the reverse sequence of the site I vRNAi oligo set. The purified RNAs were transfected into U937 cells using Oligofectamine (Invitrogen, USA) as a carrier. The transfected cells were allowed to grow, and vRNA expression was analyzed by RT-PCR. As shown in Fig. 3c, the vRNA expression level was reduced by about 50% with the site I and site II vRNAi oligo sets, as compared to the control vRNAi oligos. The total RNA is also shown, as an internal loading control for RT-PCR (Fig. 3c).

**Analysis of knocked-down vRNA expression in cancer cell lines.** The above studies revealed that both sites (sites I and II) of vRNA are efficient sites to knock-down all vRNAs and vRNA-1 expression levels, respectively. In order to prepare stable cell lines that constitutively express the vRNAi oligos that target site I, we used the pSilencer vector with the U6 promoter (Ambion, USA) and embedded our target sequence (Vault1-4RNAi-1&2) (Fig. 4a). The resulting vector, pSilencer-Vault1-4RNAi-1&2, and the other control vectors, pSilencer-Vault1-4RNAi-3c&4c, were transfected into the U937 and U2OS cancer cell-lines, assuming that the vRNA sequence in these cells remains the same. The transfected cell lines were selected on puromycin-supplemented medium, and the expression levels of vRNA in these cell lines were compared by an RT-PCR analysis. Our results indicated that cells expressing Vault1-4RNAi-1&2 were compromised for the vRNA expression by about 50% in U937 cells, as compared
to the control cells transfected with pSilencer-Vault1-4RNAi-3c&4c (Fig. 4b). Similarly, the U2OS cells showed a higher level of vRNA suppression (85%) (Fig. 4b). These studies suggested that U2OS cells are suitable for further analysis, as these cells are resistant and also overexpress vRNA-1. Moreover, our previous studies revealed that the hsp70 family protein mortalin contributes to human carcinogenesis, and its overexpression increases the malignant properties of human cancer cells (23). We found that mortalin-overexpressing U2OS cells (U2OS/mot-2) tend to show moderately higher resistance to mitoxantrone (Fig. 5a). Since these cells exhibited aggressive malignant properties (data not shown) and higher resistance against mitoxantrone, we examined whether vault RNA has any role in their drug response. Interestingly, we found that U2OS/mot-2 cells have a two-fold higher level of vault RNA expression, as compared to the control cells (Fig. 5b).

**vRNA knock-down in U2OS/mot-2 cells and their response to mitoxantrone.**

In order to analyze the functional significance of vRNA overexpression in the mitoxantrone resistance of malignant cells, we knocked-down the vRNA expression in U2OS/mot-2 cells, which displayed higher resistance to mitoxantrone and also overexpressed vRNA. An RT-PCR analysis revealed that the expression levels of vRNA were reduced by about 30% when knocked-down by the RNAi approach (Fig. 6a,b). All vRNAi knocked-down and control cells were further subjected to an MTT assay, to evaluate their responses to mitoxantrone before and after knocking-down the vRNA expression. In addition,
we evaluated the levels of mortalin expression by immunoblotting with an anti-mortalin antibody. We found that in all four cases, the cells showed higher levels of mortalin expression as compared to the control cells, and there were no differences in the cells that were compromised for mortalin (Fig. 6c). On the other hand, when the vRNA expression level was reduced (30%) endogenously (Fig. 6b), due to the expression of vRNAi oligos, the resistance to mitoxantrone decreased with increasing concentrations of the drug (Fig. 6d). In contrast, the controls, such as the vector alone and the reverse sequence vRNAi-bearing vector, did not alter the resistance towards mitoxantrone. Furthermore, we have evaluated the expression levels of MCRP and MVP RNAs and found that the BCRP and MVP expressions were lower in resistance cells (U2OS/mot-2 cells) compared with wildtype (U2OS). Upon transfection with different oligos (vRNA, reverse vRNA and vector alone), no change in the expression pattern of BCRP and MVP were found (Fig. 6e).

Overexpression of vRNA in mitoxantrone-sensitive cancer cells. Next, we used an alternative approach, in which we overexpressed vRNA-1, through the U6 promoter in the mitoxantrone-sensitive cell line MG63, which is known to express endogenously lower levels of vRNA-1 (Figs. 2b & 7a), and evaluated the resulting sensitivity to the mitoxantrone drug. For these studies, we prepared two vectors, pSilencer-vRNA-1 and pSilencer-vRNA-1c, for expressing vRNA-1 and its complementary RNAs (as a negative control), respectively, and transfected them into MG63 cells. The resulting cell lines were selected in the presence of
puromycin. We analyzed the expression levels of vRNA-1 in the transfected cells, and found that vRNA-1 expression was about 1.5-fold higher, as compared to the other cell lines transfected with either the pSilencer-vRNA-1c or pSilencer vector (Fig. 7b). When these cells were tested for their resistance to mitoxantrone, the Hvg1 sequence-transfected cells exhibited higher resistance than the control cells expressing the complementary sequence of vRNA-1. The increase of the mitoxantrone resistance was more obvious at higher concentrations, as compared with that of the wild type and other control cells (Fig. 7c). Expression of BCRP and MVP RNAs were evaluated in these cell-lines and found that they expressed differently in different cell-lines and are not correlated with drug-resistance compared with wild type cells (Fig. 7d)

Discussion

Previous studies have shown that vault-associated components in cancer cells render resistant to chemotherapeutic compounds (6,15,16). In general, vault particle components including vRNA are known to be up-regulated in cancer cells that are resistant to several chemotherapeutic compounds. However, no vault components that participate or interact directly with the chemotherapeutic compound(s) have been identified. In order to understand the molecular basis for attaining resistance towards the chemotherapeutic compounds through overexpression of vault-associated components, we previously focused on the vRNA interactions with several chemotherapeutic compounds, and found that vRNA-1 has the ability to bind specifically to different chemotherapeutic
compounds (18). These studies led us to speculate that vRNA may be involved in the sequestration of chemotherapeutic compounds, thus preventing the drugs from reaching their target sites. The vRNA is known to be present in both the free form and in association with both ends of the vault particle (Fig. 1b). The purpose of this study was to evaluate the physiological relevance of the observed vRNA interactions with chemotherapeutic compounds such as mitoxantrone in malignant cells. In this study, we analyzed the expression levels of vRNA in five malignant cell lines, and found that the U2OS and U2OS/mot-2 cell lines expressed moderately higher levels of vRNA-1 (Figs. 2a,b & 5b). These cells were tends to show moderately resistant to the chemotherapeutic compound, mitoxantrone, as compared to the other cell lines (Figs. 2c & 5a). Although it remains unknown how the malignant U2OS/mot-2 cells upregulate their vault RNA-1, it appears that a higher level of vault RNA-1 expression is involved in their drug resistant phenotype, at least to mitoxantrone. The above results suggested that there is a correlation between the expression levels of vRNA-1 and the adoption of resistance to chemotherapeutics by cancer cells.

Next, to address the functional significance of vRNA-1 overexpression in cells, we used an RNAi approach to knock-down the expression levels of vRNA-1 endogenously and evaluated the resulting phenotypes for their sensitivity to mitoxantrone. This approach was very successful for elucidating the functions of many other non-coding RNAs (24). As described above, when the vRNA-1 expression in the U2OS/mot-2 cells was decreased by about 30%, the cells
became progressively sensitive to increasing mitoxantrone concentrations (Fig. 6b,d). However, the vector alone and the reverse sequence vRNAi-bearing vector did not alter the resistance towards mitoxantrone (Fig. 6d). These studies suggested that vRNA-1 expression in cancer cells leads to resistance to mitoxantrone. As a complement to these studies, we performed a rescue experiment, in which vRNA-1 was overexpressed in mitoxantrone-sensitive cell lines, such as MG63, and analyzed the resulting phenotypes. In this study, we found that when vRNA-1 was overexpressed to about 1.5 fold higher levels in MG63 cells, the resistance to mitoxantrone was also increased, as compared to the controls (Fig. 7b,c). Taken together, both the knock-down and rescue analyses described above suggest that vRNA-1 plays an important role in conferring resistance to the cancer cells against the chemotherapeutic compounds. We have also found that other known proteins such as BCRP and MVP, which are known render the cellular resistance to mitoxantrone, expressions could not be correlated for their role in mitoxantrone resistance in our studies. Recently, two non-coding RNAs (miR-221/222 and CUDR) have also been shown to play an important role in mediating drug resistance in cancer cells (25,26). In summary, this study has identified another non-coding RNA (vRNA-1) that plays an important role by mediating the drug resistant phenotype of malignant cells. Further studies on the importance and applications of non-coding RNAs in cancer chemotherapeutics are warranted.

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REFERENCES


Figure legends

**FIGURE 1. Vault RNA and vault particle components.** (a) Predicted secondary structure of vRNA-1 (RNA structure 3.71). Probable binding regions with Mitoxantrone are indicated with circles. (b) Whole vault particle. Vaults are barrel-shaped particles with a mass of about 13 MDa and overall dimensions of 400 X 400 X 700 Å. The vault particle consists of major vault protein (MVP), telomerase-associated protein 1 (TEP1) and vault poly(ADP-ribose) polymerase (VPARP). Crystal structure of the intact rat vault particle at 3.5 Å, showing details of the MVP organization in the particle.

**FIGURE 2. Analyses of vRNA expression and mitoxantrone resistance in the MG63, U118MG, U937 and U2OS cell lines.** (a) Expression of vRNA in different cell lines. Total RNA from all cell lines was isolated, and the vRNA sequences were amplified by RT, followed by PCR with specific primer sequences. The amplified sequences were fractionated on a 4% gel. U6 RNA-expression was used as the internal control. Abundance of total RNA and U6, BCRP and MVP RNAs were also shown. (b) Quantitative analysis of vRNA levels. The expressed PCR products were quantitated with the ImageJ program. Error bars are the standard deviations calculated from three independent experiments. Vault RNA and U6 RNA expression levels are evaluated statistically (the P values) in MG63 (vault, P<0.05; U6, P<0.01), U118MG (vault, P<0.01; U6, P<0.01), U937 (vault, P<0.01; U6, P<0.03), and U2OS (vault, P<0.01; U6, P<0.01) using Graphpad instat3 (Graphpad Software Inc., San Diego, USA).
Drug resistance of these cell lines by a cell viability test. An MTT assay was performed to monitor the viability in the absence and presence of mitoxantrone at 50 and 80 nM concentrations. (d) Quantitative analysis of BCRP and MVP levels. The expressed PCR products were quantitated with the ImageJ program.

**FIGURE 3. Identification of vRNA sequences, design of vRNAi oligos and analysis of vRNA expression.** (a) Identification of vRNAs and their sequences in U937 leukemia cells. Total RNA was isolated from U937 cells, and the vault RNA sequences were amplified by RT, followed by PCR with specific primer sequences. The amplified sequences were fractionated on a 4% gel, cloned into the TA vector, and sequenced. (b) Designed vRNAi oligos to target vRNAs in U937 leukemia cells. Site-1 represents the common regions from 1-19 in the vRNAs to knock-down all kinds of vRNAs. Site-II represents the region of nts 30-48 in Hvg-1, to knock-down only Hvg-1. Reverse sequences were used for a negative control. (c) Analysis of the potential of vRNAi oligos to target vRNAs in U937 leukemia cells. Transient transfections of different vRNAi oligos were performed using Oligofectamine. To evaluate the efficiency of the knocking-down ratio, total RNA from U937 cells was isolated, and the vRNA sequences were amplified by RT, followed by PCR with specific primer sequences. The amplified sequences were fractionated on a 4% gel. The expression of vRNAs was reduced by about 50% with both vRNAis (site-1 and site-II).
FIGURE 4. Construction of constitutively expressed vRNAi oligos and their ability to suppress vRNA expression in the U937 and U2OS cell lines. (a) Construction of stable cell lines expressing vRNAi oligos in the U937 and U2OS cell lines. vRNAs were suppressed using the oligos specific for site-I, which are suitable for all vRNAs. These sequences were cloned into the pSilencer 2.1-U6 puro vector. Insertion sites and sense and anti-sense sequences are indicated. (b) Levels of vRNA suppression in the U937 and U2OS cell lines. The levels of suppression were calculated with reverse sequence suppression as 100%.

FIGURE 5. Analyses of resistance to mitoxantrone and vRNA expression in U2OS and resistant cells derived from U2OS (U2OS/mot-2). (a) The drug resistance of these cell lines was assessed by a cell viability test. An MTT assay was performed to monitor the viability in the absence and presence of mitoxantrone at 50 to 100 nM concentrations. (b) Expression of vRNA in both cell lines. Total RNA was isolated from these cell lines, and the vRNA sequences were amplified by RT, followed by PCR with specific primer sequences. The amplified sequences were fractionated on a 4% gel. Abundance of total RNA and U6 RNA were also shown. The expressed PCR products were quantitated by the ImageJ program. Error bars were calculated from the standard deviations of three independent experiments. Vault RNA expression levels are evaluated statistically (the P values) in U2OS (vault, P<0.01), and U2OS/mot-2 (vault, P<0.01) as mentioned.
FIGURE 6. Analyses of vRNA and mortalin expression in U2OS/mot-2 cells constitutively expressing vRNAi oligos and their response towards mitoxantrone. (a) Levels of vRNA suppression in the U2OS/mot-2 cell line. The numbers 1, 2, 3 and 4 indicate the vRNA sequences amplified from untreated U2OS/mot-2, vRNA suppressed U2OS/mot-2, U2OS/mot-2 suppressed by reverse vRNAi sequences, and U2OS/mot-2 transfected with control vector sequences, respectively. The levels of suppression were calculated with the untreated control as 100%. Expressions of BCRP and MVP were shown with different transfection treatments. (b) Quantitative analysis of suppressed vRNA levels. The PCR products were quantitated by the ImageJ program. Error bars were calculated from the standard deviations of three independent experiments. Vault RNA expression levels are evaluated statistically (the P values) in sample 1 (vault, P<0.01), 2 (vault, P<0.01), 3 (vault, P<0.05), and 4 (vault, P<0.01). (c) Extracted proteins from the aforementioned vRNAi treated cells (1,2,3 and 4), analyzed by western blotting with an anti-mortalin monoclonal antibody. In addition to 1-4, wild-type U2OS cells were also analyzed. (d) Drug resistance of the vRNAi-treated and untreated cell lines, assessed by a cell viability test. An MTT assay was performed to monitor the viability in the absence and presence of mitoxantrone at 10 to 80 nM concentrations. (e) Quantitative analysis of BCRP and MVP levels. The expressed PCR products were quantitated with the ImageJ program.
FIGURE 7. Expression of vRNA-1 in MG63 cells, which originally express low levels of hvg1 and are sensitive to mitoxantrone. (a) Levels of vRNA suppression in the MG63 cell line. The numbers 1, 2, 3 and 4 indicate the vRNA sequences amplified from untreated MG63, vRNA-1 transfected MG63, MG63 transfected by complementary sequences, and MG63 transfected with control vector sequences, respectively. The levels of suppression were calculated with the untreated control as 100%. Expressions of BCRP and MVP were shown with different treatments. (b) Quantitative analysis of suppressed vRNA levels. The PCR products were quantitated by the ImageJ program. Error bars were calculated from the standard deviations of three independent experiments. Vault RNA expression levels are evaluated statistically (the P values) in sample 1 (vault, P<0.01), 2 (vault, P<0.01), 3 (vault, P<0.01), and 4 (vault, P<0.01). (c) Drug resistance of the vRNA-1 transfected and control cell lines, as determined by a cell viability test. An MTT assay was performed to monitor the viability in the absence and presence of mitoxantrone at 10 to 100 nM concentrations. (d) Quantitative analysis of BCRP and MVP levels. The expressed PCR products were quantitated with the ImageJ program.
Figure 3a-c

(a) 20 bp vRNA1 vRNA2 vRNA3

(b) Hvg-1:

<table>
<thead>
<tr>
<th>Site -1</th>
<th>Site -2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'</td>
<td>3'</td>
</tr>
<tr>
<td>3GCUUGCUGUUACUCAGCA</td>
<td>3AGUUCUUAAUUGAACA</td>
</tr>
<tr>
<td>50</td>
<td>80</td>
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</table>

Vault1-4 target oligos

| Vaut1-4RNAi-1: 5'-GCGACUGCUUUAGUCAGCGUT-3' |
| Vaut1-4RNAi-2: 5'-CGCUGAGCUAAAGCGCAAGCUT-3' |

Control oligos

| Vaut1-4RNAi-3c: 5'-CGACUCCUAAUUGAACAUT-3' |
| Vaut1-4RNAi-4c: 5'-CGGACUGUUAAAAGAUGUT-3' |

Vault-1 target oligos

| Vaut1RNAi-5: 5'-CAGUUCUUAAUUGAACAUT-3' |
| Vaut1RNAi-6: 5'-UGUUCCAUUAAAGAACUGUT-3' |

(c) Marker vRNA-1-3 vRNA-1-4 & Total RNA

47% 100% 52%
Figure 4a&b
Figure 5a&b

(a) Cell Viability (%)

<table>
<thead>
<tr>
<th></th>
<th>U2OS</th>
<th>U2OS/mot-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>50 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 nM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) DNA Ladder

100 bp

Total RNA

U6 Expression

Quantification of vRNA expression

U2OS  U2OS/mot-2
Figure 6d&e

**Cell Viability (%)**

- **Mitoxantrone (nM)**
  - 0, 10, 20, 30, 40, 50, 60, 70, 80
  - Lines 1 to 4

**RNA Expression (arb.)**

- **BCRP**
- **MVP**
  - Bars 1 to 4

---

*Figure 6d&e*
Figure 7a-d

(a) Gel electrophoresis of total RNA. The lanes are labeled 1 to 4.

(b) Bar graph showing vault RNA expression (arb.) levels for samples 1 to 4.

(c) Graph depicting cell viability (%) against mitoxantrone concentration (nM) for samples 1 to 4.

(d) Bar graph showing RNA expression (arb.) levels for BCRP and MVP for samples 1 to 4.
Expression of non-coding vault RNA in human malignant cells and its importance in mitoxantrone resistance

Gopinath Subash, Renu Wadhwa and Kumar K.R Penmetcha

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