Alterations of the Tumor Suppressor Gene ARLTS1 in Ovarian Cancer

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Abstract

ARLTS1 is a tumor suppressor gene initially described as a low-penetrance cancer gene: a truncated Trp149Stop (MUT) polymorphism is associated with general familial cancer aggregation and, particularly, high-risk familial breast cancer. DNA hypermethylation has been identified as a mechanism of ARLTS1 expression down-regulation in lung carcinomas and ARLTS1 DNA hypermethylation has been identified as a mechanism of ARLTS1 expression down-regulation in lung carcinomas and ARLTS1 expression inhibition in ovarian cancer. After the initial description, an independent group linked ARLTS1 Trp149Stop to both familial risk of breast cancer in BRCA1-negative and BRCA2-negative families (7) and risk of bilateral breast cancer (8). Moreover, they showed that another variant, the Cys148Arg, revealed a significant association with high-risk familial breast cancer (8) and melanomas (9).

Ovarian cancer is the most lethal of gynecologic malignancies in the United States with ~23,000 new cases and >15,000 deaths estimated for 2006 (10), and hereditary breast and ovarian cancers are among the most commonly encountered adult genetic diseases (11). Therefore, we decided to investigate the roles of ARLTS1 in ovarian cancer. Herein, we showed that ARLTS1 is frequently down-regulated in ovarian primary tumors and cell lines and restoration of its expression by adenoviral ARLTS1 or by the demethylation agent 5-AZA-2-deoxycytidine (5-AZA) effectively induced apoptosis in vitro and suppressed ovarian cancer tumorigenicity in nude mice. No similar effects on ovarian cancer cell lines constitutively expressing the ARLTS1 protein have been observed. In addition, we showed that ARLTS1 Trp149Stop polymorphism greatly reduced the protein apoptotic function in ovarian and breast cancer cell lines.

Introduction

ARLTS1 [ADP ribosylation factor-like 1 (ARL11)] is a recently identified tumor suppressor gene described in association with familial cancers: a Trp149Stop (G446A) variant (MUT) leading to premature termination of translation was identified in cancer kindreds displaying various combinations of breast, ovarian, prostate, gastric, and lung carcinomas, melanomas, and B-cell chronic lymphocytic leukemias (B-CLL; ref. 1). However, the Trp149Stop variant frequency is similar in control cases and patients with sporadic cancers (1), and in fact, it was shown that no association with general risk of CLL (2) or various types of sporadic cancers (3) could be identified. ARLTS1 is located at chromosome 13q14.3, a genomic region frequently homozygously or heterozygously deleted in various types of solid and hematologic malignancies, including CLL and prostate, breast, and colon carcinomas (4–6). Furthermore, down-regulation by promoter hypermethylation of ARLTS1 was reported in sporadic cases of B-CLL and lung cancers (1). After the initial description, an independent group linked ARLTS1 Trp149Stop to both familial risk of breast cancer in BRCA1-negative and BRCA2-negative families (7) and risk of bilateral breast cancer (8). Moreover, they showed that another variant, the Cys148Arg, revealed a significant association with high-risk familial breast cancer (8) and melanomas (9).

Materials and Methods

Tumor samples and cell lines. Sixteen ovarian tumor samples, including 13 malignant, 2 borderline, and 1 benign tumor, were obtained from Fox Chase Cancer Center (Philadelphia, PA) according to institutional guidelines. The clinical data are presented in Supplementary Table S1. A portion of the tissue specimens were assessed for tumor content by histology, and only tissues with >60% tumor cells were used. A set of 11 ovarian and breast cell lines, including TOV-112, OV-90, OV-90, MB-453, DU-4475, MB-436, SkBr3, MCF7, BT-474, BT-549, and MB-361 cancer cell lines, was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained according to ATCC protocols. As controls, multiple normal total RNAs from ovary (three samples) and breast (three samples) were used (Ambion, Austin, TX and Stratagene, La Jolla, CA).

ARLTS1 expression analysis. Total RNA from ovarian and breast cancer samples was processed as described previously (1). The relative expression of ARLTS1 normalized with β-actin in each sample was quantified using the GelDoc software (Bio-Rad, Hercules, CA).
Recombinant adeno- or lentiviral vectors and in vitro transduction. Wild-type ARLTS1 and mutant (G446A) ARLTS1 full-length cDNAs were cloned into Adenovaptor-CMV3(CuO)-IRES-GFP transfer vector (Qiogene, Irvine, CA). This vector allows transgene expression driven by the cumate-inducible CMV3(CuO) promoter. An internal ribosome entry site sequence ensures coexpression of GFP. Cells were transduced with recombinant adeno- or lentiviral vectors at multiplicity of infection (MOI) 75, and transduction efficiency was determined by visualization of GFP-expressing cells.

Cell growth and cell cycle kinetics. Cells (2 × 10^5) were infected at MOIs of 75 and they were harvested at 48-hour intervals for 6 days, stained with trypan blue, and counted (ViCyte count, Beckman Coulter, Fullerton, CA). Number of viable cells was assayed using CCK-8 test (Dojindo, Gaithersburg, MD) as per the manufacturer’s protocol. For flow cytometry by EPICS XL scan (Beckman Coulter), cells were harvested 2, 4, and 6 days after infection, fixed in cold methanol, RNase treated, and stained with propidium iodide (50 µg/mL). All analyses were done in duplicate.

Western blot analysis. Protein extraction and immunoblot analysis were done as described in ref. 12. The following primary antisera were used: rabbit polyclonal anti-caspase-3 (1:1,000; Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-caspase-9 (1200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-caspase-8 (1200; Chemicon, Temecula, CA), rabbit monoclonal anti-Bcl-2 (1500; Santa Cruz Biotechnology), and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000; Santa Cruz Biotechnology).

DNA methylation studies. TOV-112 cells were treated with 5 µmol/L 5-aza-dC (Sigma, St. Louis, MO) and 1 µmol/L trichostatin A (TSA). Maximal ARLTS1 reexpression was observed by treating 1 × 10^5 cells with 5 µmol/L 5-aza on days 2 and 5. After the 5th day, medium was replaced with medium containing 1 µmol/L TSA and incubation was continued for 24 hours and cells were collected for use. RNA was extracted and reverse transcription-PCR (RT-PCR) was done as described (1).

Caspase inhibition assay. The caspase inhibitors z-VAD-FMK (general), z-IETD-FMK (casepsase-8), and z-LEHD-FMK (casepsase-9) were purchased from BD Biosciences (San Jose, CA). TOV cells were seeded (10^4 per well) in 96-well culture plates and the infection was done using MOI 75. The cells were incubated for 2, 4, and 6 days with different caspase inhibitors (50 µmol/L), and the media with the inhibitors were replaced daily. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay (CCK8) was done as recommended by the manufacturer (Dojindo).

RNA interference. TOV-112 cells were transfected with ARL11 SMART-pool (Dharmacon, Lafayette, CO) at different concentrations (10, 50, and 100 nM). We used siPORT NeoFX Transfection Reagent (Ambion) as per the manufacturer’s protocol. Twenty-four hours after transfection, cells were treated with 5-aza only as described above, and ARLTS1 reexpression was assayed at 2, 4, and 6 days by RT-PCR.

In vivo studies. Animal studies were done according to institutional guidelines. TOV-112 cells were infected in vitro with Ad-ARLTS1 or Ad-ARLTS1-MUT or Ad-GFP or mock infected (MOI 75). Alternatively, they were treated in vitro with 5 µmol/L 5-aza. At 24 hours after infection/treatment, 10^5 viable cells were injected s.c. into left flanks of 6-week-old female nude mice (Charles River Breeding Laboratories, Wilmington, MA), five mice per infected/treated or control group. Tumorigenic controls were untreated TOV-112. Tumor diameters were measured every 5 days, and tumors were weighed after necropsy. Tumor volumes were calculated by using the equation

\[ V = \frac{a \times b^2}{2} \]

where a is the largest diameter and b is the perpendicular diameter.

Statistical analysis. Results of in vitro and in vivo experiments were expressed as mean ± SD. Student’s two-sided t test was used to compare values of test and control samples. *P < 0.05 indicated significant difference.

Results

ARLTS1 is down-regulated in ovarian cancers. To identify a potential role of ARLTS1 in ovarian cancer, we analyzed its expression by semiquantitative RT-PCR in a set of 19 ovarian cancer samples, consisting of 13 malignant primary tumors, 2 borderline primary tumors, 1 benign tumor, and 3 normal ovarian tissues (Supplementary Table S2). Eight malignant tumors (8 of 13, 61.5%), but none of the normal tissues, displayed a reduction to <33%, with four (25%) ovarian cancer showing a reduction to <10% of the normal expression, respectively (Fig. 1A). ARLTS1 was highly down-regulated in the only benign ovarian tumor that we analyzed (#89, Fig. 1A) suggesting that, at least in this case, down-regulation of ARLTS1 level can be an early event in ovarian tumorigenesis. Because the majority of primary malignant tumors showed consistent reduction in ARLTS1 expression, we analyzed a second panel of 11 ovarian and breast cancer cell lines. In five cell lines (TOV-112, BT-549, BT-474, MB-436, and MB-453), a reduction to <10% of normal expression was found (Fig. 1B). Globally, 14 of 27 (52%) tumor samples (primary tumors and cell lines combined) and none of 6 normal samples showed reduction in ARLTS1 expression (*P < 0.05, Fisher’s exact test). Therefore, ARLTS1 is highly down-regulated in about half of primary ovarian cancer and in a similar proportion of ovarian and breast cancer cell lines.

ARLTS1 inhibits proliferation in ovarian and breast cancer cells. To assess the effect of ARLTS1 restoration in ovary and breast cancer cell lines, we selected an ARLTS1-negative ovarian cell line (TOV-112), an ARLTS1-positive ovarian cell line (OV-90), and the MCF7 breast cancer cell line expressing only the truncated form (MUT) of ARLTS1 (1). Of note, both ovarian cell lines harbor mutations in TP53, although MCF7 is TP53 wild-type.

Gene reexpression was achieved through adenoviral transduction of either the wild-type (Ad-ARLTS1-WT) or mutated G446A (Ad-ARLTS1-MUT) forms of ARLTS1. The expression was confirmed by RT-PCR (Supplementary Fig. S1). Infection with MOI 75 was sufficient to restore gene expression in >90% of infected cells as estimated by the percentage of fluorescent cells. Infection with control vector (Ad-GFP) showed no significant toxic effect at MOI 75.

Ninety-six hours after Ad-ARLTS1-WT infection, both TOV-112 and MCF7 cell lines showed markedly decreased proliferative activity and higher cell death as determined by cell count and viability (CCK8) assay (Supplementary Fig. S2A and B). Specifically, at 96 hours after Ad-ARLTS1-WT infection, a 76% cell growth inhibition in TOV-112 cells compared with the untreated cells (*P < 0.001) was found, whereas at 144 hours the cell growth inhibition increased to 86% (*P < 0.001). MCF7 cells, which express only the truncated form of ARLTS1, showed a 63% cell growth inhibition.
inhibition ($P < 0.001$) after 144 hours of treatment with wild-type ARLTS1. There was no statistically significant difference in the growth of OV-90 cells after ARLTS1 gene restoration, suggesting that exogenous ARLTS1 has no effect in cells where its expression is normal, whereas it has significant effects in cells either lacking ARLTS1 expression or expressing only the truncated form.

On the other hand, infection with Ad-ARLTS1-MUT had a minimal effect on cell proliferation and viability (Fig. 2), showing that the truncated ARLTS1 protein has no significant activity in vitro. Specifically, restoration of mutant ARLTS1 form in TOV-112 cells did not affect the cell growth significantly ($P = 0.12$). Moreover, the strong antiproliferative effect (65% cell growth inhibition; $P < 0.001$) of Ad-ARLTS1-WT in the MCF7 cell line confirms that the endogenous mutated form expressed in this cell line has no significant biological activity.

**Restoration of ARLTS1 induces apoptosis in ovarian and breast cancer cells.** To fully understand the nature of the antiproliferative response to ARLTS1 restoration, we studied cell cycle kinetics and observed an evident apoptotic peak 96 hours after infection in TOV-112 (50.75% in sub-G1 phase) and MCF7 (36.21% in sub-G1 phase) cell lines infected with Ad-ARLTS1-WT (Fig. 2A and B; Supplementary Fig. S3). Accordingly, with growth curves and CCK-8 assay, the apoptotic program was not activated in OV-90 cell line (Fig. 2C) or in any cell line infected with Ad-ARLTS1-MUT, confirming its lack of significant function in vitro.

Western blot analysis revealed that Ad-ARLTS1-WT activates the intrinsic apoptotic pathway as shown by the cleavage of the initiator caspase-9 and the effector caspase-3. In addition, down-regulation of the antiapoptotic protein Bcl-2 was detected (Fig. 2D). To validate that ARLTS1-induced apoptosis is caspase dependent, we infected TOV cells with ARLTS1 and treated them with different caspase inhibitors (Fig. 2E). Treatment with the pan-caspase and caspase-9 inhibitors blocked the ARLTS1-induced apoptosis, confirming that ARLTS1 induced apoptosis through the intrinsic (mitochondrial) caspase pathway.

**Treatment with 5-AZA restores ARLTS1 expression in ovarian cancer cells and inhibits their proliferation.** Because hypermethylation had already been described as a potential mechanism of ARLTS1 down-regulation (1), we decided to study if ARLTS1 expression could be restored in TOV-112 cells using either DNA methyltransferase inhibitor 5-AZA or the histone deacetylase inhibitor TSA or both. RT-PCR on total RNA extracted from treated cells showed that only 5-AZA was able to restore ARLTS1 expression in TOV-112 (Fig. 2F), confirming that DNA hypermethylation, but not histone modifications, regulates ARLTS1 expression in this cell line.

To assess whether an antiproliferative effect of demethylation on TOV-112 was associated with ARLTS1 restoration, we transsected cells with small interfering RNA (siRNA) targeting ARLTS1, and after 24 hours, we treated them with 5-AZA. Treatment with 100 nmol/L siRNA against ARLTS1 was able to dramatically down-regulate its expression (Fig. 3B). TOV-112 showed a 4-fold decreased proliferation rate ($P < 0.001$) after treatment with 5-AZA in vitro (Fig. 3C). Notably, cells in which ARLTS1 expression was silenced by siRNA showed decreased sensibility to 5-AZA treatment, suggesting that ARLTS1 has an effective role in response to demethylating agents (Fig. 3D).

**Ex vivo ARLTS1 gene restoration suppresses tumor growth in vivo.** To verify the in vivo effect of ARLTS1 restoration in ovarian cancer cells, we did xenografts of ex vivo–treated TOV-112 cells in nude mice. At 30 days after injection, tumor growth was completely suppressed in mice inoculated with TOV-112 cells infected with Ad-ARLTS1-WT (Fig. 4A). The average tumor weights for controls (Ad-GFP and untreated TOV-112 cells) at day 30 were 1.39 ± 0.85 g and 1.53 ± 0.29 g, respectively. At 30 days, four of five mice inoculated with Ad-ARLTS1-infected TOV-112 cells showed no tumors, and tumor weight of the only mouse who developed tumor was 0.15 g, significantly lower ($P < 0.001$) than tumors of Ad-GFP-infected TOV-112 cells (1.53 ± 0.29 g) and mock-treated TOV-112 cells (1.39 ± 0.85 g; Fig. 4B). In the five mice injected with 5-AZA-treated TOV-112 cells, only one mouse developed a small tumor (weight of 0.21 g).

Notably, ARLTS1-MUT retained partial activity in vivo because two of five mice showed no tumor after 30 days and the average tumor weight in mice that developed tumors was 0.68 ± 0.35 g (Fig. 4). This is at least four times higher as the only Ad-ARLTS1-WT developed tumor and thrice higher as the 5-AZA-treated cells induced tumors but also about twice as smaller as in the two
control groups. These in vivo data support the idea that the Trp149Stop is retained in the general population and predisposes to cancer because of a reduction, but not full loss, of normal ARLTS1 function.

Discussion

In the present study, we showed the tumor suppressor function of ARLTS1 in ovarian cancer and we investigated the biological importance of the Trp149Stop polymorphism in both ovarian and breast cancer models. We previously described the methylation of a predicted promoter as the cause of reduced or absent ARLTS1 expression (1). Here, we proved that the same mechanism is involved in the silencing of ARLTS1 in TOV-112 cells: treatment with demethylating agent 5-AZA, but not with the histone deacetylase inhibitor TSA, induced its reexpression. We adopted two different methods to restore gene expression: adenoviral transduction (in cancer models with ARLTS1 deletion and/or mutation) and use of demethylating agents (in cancer models with ARLTS1 hypermethylation). In both cases, the apoptotic effects are evident in ARLTS1-negative ovarian cancer cell line TOV-112. On the other hand, overexpression of ARLTS1 in positive ovarian cancer cell lines OV-90 has no effect, suggesting that ARLTS1 selectively restores apoptotic programs only in negative cancer cells, where lack of expression is selected during tumor evolution. Restoration of wild-type ARLTS1 expression in MCF7 breast cancer cell line, which expresses only the truncated ARLTS1, determines a strong apoptotic response. Exogenous expression of the variant Trp149Stop has no significant in vitro antiproliferative effect in any of the cell lines we analyzed; however, ARLTS1 variant Trp149Stop retained some antiproliferative function in vivo. The same partial effect was proved also in A549, a lung carcinoma cell line with very low expression of ARLTS1 (1). Another polymorphism in position Cys148Arg has been described to be associated with increased risk of familial breast cancers (8), and therefore, it is very likely that a functional domain important in cancer predisposition resides in this region of the protein. Of note, a mutation in ARL1 (Asp151Gly), a close member of the same ADP ribosylation-like family of genes as ARLTS1 that corresponds to Asp146 in human ARLTS1, has been proven to inhibit the autophagic cell death in yeast consequent to a defect in
However, we showed that silencing multiple genes whose expression is restored on demethylation. As ARLTS1 is a member of the ADP ribosylation factor/ADP ribosylation-like subfamily of GTP-binding proteins (15) involved in membrane trafficking, it is possible that reexpression of ARLTS1 in negative cancer cells induces a type of cell death resembling the autophagic cell death. Our experiments showed that a single treatment of TOV-112 cells with either Ad-ARLTS1 or 5-AZA ex vivo is sufficient to restore ARLTS1 expression and to inhibit tumor growth. The tumor suppressor effect in cells treated with 5-AZA might be due to multiple genes whose expression is restored on demethylation. However, we showed that silencing ARLTS1 by siRNA in vitro partially reduced response of TOV-112 cells to 5-AZA, suggesting that ARLTS1 plays an effective role in response to this drug. In addition, it would be valuable to study an eventual association between ARLTS1 down-regulation and resistance to specific therapeutic agents like decitabine.

In conclusion, we have shown that ARLTS1 is a tumor suppressor gene down-regulated in a large proportion of ovarian cancers. Restoration of expression by adenoviral infection in negative cells induced high levels of apoptosis, whereas introduction of the truncated form of ARLTS1 associated with familial predisposition to breast cancer has no effect. Breast cancer cells endogenously expressing the Trp149Stop variant are sensitive to apoptosis induced by the wild-type protein, supporting the idea that the truncated variant is maintained in the general population and is predisposing to cancer because of a reduction in normal function.

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