Tumor Suppressor Functions of ARLTS1 in Lung Cancers

Sai Yendamuri,1 Francesco Trapasso,1,2 Manuela Ferracin,4 Rossano Cesari,1 Cinzia Sevignani,1 Masayoshi Shimizu,4 Shashi Rattan,1 Tamotsu Kuroki,1 Kristoffel R. Dumon,2 Florencia Bullrich,1 Chang-gong Liu,1 Massimo Negrini,4 Noel N. Williams,2 Larry R. Kaiser,2 Carlo M. Croce,1 and George A. Calin1

1Kimmel Cancer Center, Thomas Jefferson University; Department of Surgery, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania; 2Department of Experimental and Diagnostic Medicine, Interdepartment Center for Catanzaro, Catanzaro, Italy; and 4Department of Experimental and Diagnostic Medicine, Interdepartment Center for Cancer Research, University of Ferrara, Ferrara, Italy

Abstract

ARLTS1 is a newly characterized tumor suppressor gene located at chromosome 13q14.3 and involved in the pathogenesis of various types of tumors: two single-nucleotide polymorphisms, one of them responsible for protein truncation, were found statistically associated with familial malignancies, whereas DNA hypermethylation and genomic deletions have been identified as a mechanism of ARLTS1 down-regulation in sporadic cancers. We found that in a large portion of lung carcinomas (37%) and in all analyzed lung cancer cell lines, ARLTS1 is strongly down-regulated due to DNA methylation in its promoter region. After its restoration by adenoviral transduction, ARLTS1-negative A549 and H1299 cells underwent apoptosis and inhibition of cell growth. Furthermore, ARLTS1 reexpression significantly reduced the ability of A549 and H1299 to form tumors in nude mice. Finally, we identified ∼650 transcripts differentially expressed after restoration of ARLTS1 expression in A549 cells, suggesting that various pathways involved in cell survival, proliferation, signaling, and development mediate the effects of wild-type ARLTS1 in a lung cancer system. [Cancer Res 2007; 67(16):7738–45]

Introduction

ARLTS1 (ARL11, OMIM 609351) is a newly characterized tumor suppressor gene located at chromosome 13q14.3 and involved in the pathogenesis of various types of tumors (1). It encodes for a small guanine nucleotide binding protein and is a member of the ADP-ribosylation factor (ARF)-ARF-like (ARL) family of the Ras protein superfamily (2, 3). Several lines of evidence support its proposed suppressor function. First, the G446A (W149X) and T442C (C148R) polymorphisms in ARLTS1 gene have been shown to influence familial cancer risk for B-cell chronic lymphocytic leukemia (B-CLL) and BRCA-1/BRCA-2-negative breast cancers (1, 4, 5). Second, down-regulation of ARLTS1 was consistently reported after analyzing a small number of CLL and lung cancers (1) and a larger panel of ovarian primary tumors or cell lines (6). The mechanisms responsible for these low levels of expressions are varied and include promoter hypermethylation or loss of heterozygosity. The chromosomal region 13q14.3 where the gene is located was reported to be deleted in a variety of hematopoietic and solid tumors, including B-CLL, mantle cell lymphomas, multiple myeloma, and myeloid malignancies, and in prostate cancer as well as low-malignant and benign lipomatous tumors (1). Furthermore, after ARLTS1 restoration by adenoviral transduction, only the negative ovarian TOV-112 cells, but not the OV-90 cells expressing a normal ARLTS1 product, underwent apoptosis and inhibition of cell growth (6). In addition, ARLTS1 reexpression significantly reduced the tumorigenic potential of TOV-112 cells in nude mice (6). Here, we reported an expression study on a large panel of lung cancers and investigate by using an adenoviral system the effects of restoration of ARLTS1 expression in lung cancers cells. We found that ARLTS1 has a suppressor function also in this histotype, supporting for a large spectrum of malignancies in which its down-regulation has pathogenetic effects.

Materials and Methods

Northern blot analysis. The analysis was done on filters with human poly(A)+ from several tissues purchased from Clontech. Filters were probed with a cDNA corresponding to the entire coding sequence of ARLTS1 and β-actin hybridization was used to normalize the sample loading.

Homology tree and multiple protein sequence alignments. These were done using the CLUSTALW (text only) program. Twenty-six human members of ARF/ARL family were uploaded and used for the analyses. The homologous genes were found in Mus masculus, Rattus norvegicus, Denio rerro, Drosophila melanogaster, and Arabidopsis thaliana and aligned using the same software.

Cell lines and tissues. Human lung cancer cell lines A549, NCI-H1299, NCI-H460, NCI-H522, Calu-3, NCI-H23, NCI-H650, NCI-H1573, and SKMES-1 were obtained from the American Type Culture Collection; HEK293 cells were purchased from Qbiogene. Lung cancer cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS); HEK293 cells were cultured in DMEM supplemented with 10% FBS. Tumors and corresponding noncancerous tissues were obtained from patients who underwent surgery at the Hospital of the University of Pennsylvania. Tissue samples were excised and immediately stored at −80°C. DNA was extracted from each of the 9 cell lines and from the 27 paired lung tissues according to methods previously described (1).

Real-time PCR. RNA was extracted with Trizol (Invitrogen Life Technologies, Inc.) following the manufacturer’s recommendations.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: George A. Calin, University of Texas, M. D. Anderson Cancer Center, Experimental Therapeutics Department, Houston, TX 77030.

5 http://align.genome.jp/
Real-time PCR was done using in-house designed primers (sequences are available on request from the authors). The probe sequence was labeled with the reporter dye FAM. TAMRA was used as the quencher. β-Actin was used as an internal control. Thirty-five cycles were run with an annealing temperature of 62°C and an elongation time of 30 s. All reactions were done in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Each sample was run in triplicate. After normalization of the samples with the respective β-actin control, a t test was used to compare the quantitation of ARLTS1 expression. All analyses were done with the Statistical Package for the Social Sciences for Windows (SPSS, Inc., release 10.0.7, 2000). The t tests were considered to be statistically significant if P < 0.05.

Methylation studies. Methylation studies were done by bisulfite sequencing as described in ref. 1 in three cancer cell lines, A549, H1299, and H460, and in four normal tissues including two lung samples and two lymphoblastoid cell lines.

Western blot analysis and antibodies. Proteins were extracted by lysing cells in a Triton extraction buffer containing 10 mmol/L Tris- HCl (pH 7.4), 5 mmol/L NaCl, 5 mmol/L EDTA, 10% glycerol, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL, and 1 mmol/L sodium orthovanadate. Concentrations of proteins in the lysates were measured with the Bio-Rad protein assay reagent (Bio-Rad, In c.) according to the manufacturer’s protocol. Equal amounts of protein (20 μg) were boiled in Laemmli sample buffer, separated in a 4% to 20% SDS-PAGE gel (Ready Gels, Bio-Rad), and transferred to nitrocellulose membranes (Bio-Rad). Filters were blocked in TBS with 5% skim milk for 2 h. Primary antibodies used in this study were a monoclonal antibody raised against ARLTS1 (1) and antibodies against poly(ADP-ribose) polymerase (PARP), caspase-9, and β-actin (Amersham Biosciences).

Adenovirus preparation. A recombinant adenovirus carrying the ARLTS1 coding sequence under the transcriptional control of a constitutively expressed cytomegalovirus promoter (Ad ARLTS1) was generated using the Adenovator kit according to the manufacturer’s procedures (Qbiogene) as previously described (6). A recombinant adenovirus carrying the green fluorescent protein (GFP) reporter was purchased from Qbiogene and used as a control. Adenoviruses were amplified in HEK293, purified and used as an internal control. Thirty-five cycles were run with an annealing temperature of 62°C and an elongation time of 30 s. All reactions were done in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function. We have analyzed the expression of human ARLTS1 in different normal tissues. The analysis of the expression pattern of a gene during adulthood can provide valuable insights about its function.
**ARLTS1 is highly conserved during evolution.** We used the alignments of *ARLTS1* cDNA sequence with other 25 human ARF/ARL homologues to generate a dendogram with sequence similarities: the resulted diagram indicates that the closest homologues of *ARLTS1* are *ARL14*, followed by *ARL4D*, *ARL4C*, and *ARL4A* (Fig. 2A and B). Furthermore, we found homologues of *ARLTS1* in *M. musculus*, *R. norvegicus*, *D. rerio*, *D. melanogaster*, and *A. thaliana* (Fig. 2C). This finding indicates that this gene is highly conserved among various species during evolution, suggesting an important role for *ARLTS1* in eukaryotic cells.

**ARLTS1 expression is strongly reduced or absent in lung tumor specimens and lung cancer cell lines.** To evaluate *ARLTS1* expression levels in human lung cancer, we carried out a real-time PCR on 27 non–small-cell lung cancer tumors (including 12 adenocarcinomas, 9 squamous cell cancers, and 6 with other histology) and their normal paired lung tissues. Among the analyzed tumors, a statistically significant reduction in *ARLTS1* expression was observed in 10 (37%) tumor samples [including 50% (6 of 12) of analyzed adenocarcinomas] compared with their normal counterparts, but in none of two nonpithelial analyzed tumors (Fig. 3A and Supplementary Table S1). Furthermore, *ARLTS1* expression was also assessed with the same approach in a panel of nine lung cancer cell lines. As shown in Fig. 3B, *ARLTS1* messenger was slightly reduced in H552 and H1573 cells, strongly reduced in H460 and

![Figure 2. ARLTS1 is a highly conserved gene. A, dendogram of 26 members of ARF/ARL family.](cancerres.aacrjournals.org)
SK-MES-1 cells, and, finally, virtually absent or extremely reduced in the remaining five cell lines (A549, H1299, Calu-3, H23, and H1650). Taken together, these data indicate that \textit{ARLTS1} was expressed at low levels or not expressed at all in more than half of the analyzed cancer samples (19 of 39, 52.7%), meaning that this is an extensive phenomenon in the lung type of cancers. No association with a particular histotype was found, but this study was not designed for this purpose, and a much larger set of samples has to be analyzed to achieve definite conclusions. In two samples, higher levels of expression were present in cancers as in corresponding normal tissues; unfortunately, no other material was available to test if these cases were harboring any of the dysfunctional polymorphisms described in \textit{ARLTS1}, making the protein less functional in comparison with the normal wild-type. This is the case of the breast cancer MCF7 cells, which express \textit{ARLTS1} but only the truncated version of the protein is present (1, 6).

We recently described hypermethylation of a putative promoter region as a possible mechanism for \textit{ARLTS1} down-regulation (1). Therefore, we carried out methylation analyses by bisulfite treatment and correlation analysis with expression levels in three lung cancer cell lines (A549, H1299, and H460), two normal lung samples, and two lymphoblastoid cells. Whereas in all normal samples the putative \textit{ARLTS1} promoter was not hypermethylated and the expression of \textit{ARLTS1} was present, in all three analyzed cell lines the lack/reduced levels of expression were concordant.

Figure 2. Continued. B, sequence alignments for the closest four human homologues; C, sequence alignments for eukaryotic homologues. In white on black background are presented the most conserved amino acids.
with the levels of methylation of the promoter (Supplementary Table S2).

Reconstitution of ARLTS1 through a recombinant adenovirus blocks proliferation of human lung cancer cells. To assess the role of ARLTS1 as a therapeutic gene in lung cancer cells, we infected A549 and H1299 cells with a recombinant adenovirus carrying ARLTS1 cDNA (Ad ARLTS1; ref. 6). A recombinant adenovirus for the GFP reporter was used as a control. Cells were infected at MOI 50 and ARLTS1 overexpression in Ad ARLTS1–transduced A549 and H1299 cells was confirmed by Western blot analysis 48 h after infection (Fig. 4A). ARLTS1 transgene dramatically suppressed the growth rate of both Ad ARLTS1–infected cell lines; conversely, no effects were observed in A549 and H1299 cells infected with Ad GFP at the same MOI after comparison with their parental mock-infected cells (Fig. 4B).

ARLTS1 overexpression in lung cancer cells induces apoptosis. To evaluate cell cycle perturbations in A549 and H1299 cells virally transduced with Ad ARLTS1, we did a flow cytometric analysis of these cells harvested 5 days after infection at MOI 50. Both A549 and H1299 Ad ARLTS1–infected cells displayed a significant appearance of a sub-G1 fraction (22.9% and 31.7% in A549-Ad ARLTS1 and H1299-Ad ARLTS1 cells, respectively). No significant alterations in the cell cycle profile were observed in both cell lines infected with Ad GFP compared with noninfected controls (Fig. 5A). A TUNEL assay on H1299 and A549 ARLTS1-overexpressing cells confirmed that the sub-G1 cell fraction was indeed primarily composed of apoptotic cells (Fig. 5B and data not shown).

We previously showed that A549 cells transfected with an expression vector carrying ARLTS1 cDNA resulted in apoptosis mediated by the activation of the apoptotic intrinsic pathway (1). To evaluate if adenovirally mediated ARLTS1 reconstitution in A549 cells could itself be responsible for triggering the same apoptotic pathway, we did a Western blot with protein lysates from A549 cells infected with Ad ARLTS1 at MOI 50, 96 h postinfection; filters were probed with caspase-9 and PARP antibodies. Both markers are clearly down-regulated in A549 Ad ARLTS1–infected cells compared with controls (Fig. 4A), confirming the role of the apoptotic intrinsic pathway in the ARLTS1-mediated apoptosis.

Ad ARLTS1 leads to decreased tumorigenicity of human lung cancer cell lines. We finally assessed ARLTS1 tumor suppressor activity also in vivo. Both A549 and H1299 cells were infected with Ad ARLTS1 at MOI 50; 24 h after infection, cells were injected in the right flank of 6-week-old nude mice. As a control, we injected mock-infected cells and cells infected with Ad GFP at MOI 50; three groups of six mice each were used in this study. Mice were
monitored for tumor appearance for 1 month; mice were then sacrificed and tumors excised and weighed. Statistically significantly smaller tumors (4-fold reduction, \(P < 0.05\)) were obtained from cells preinfected in vitro with Ad ARLTS1 compared with their controls in both cell lines, thus showing the ability of ARLTS1 to block tumor formation in preclinical models (Fig. 6).

The molecular bases of ARLTS1 tumor suppressor effect in A549 lung cancer cell line. To analyze the global molecular changes following A549 cell line stable transfection with ARLTS1, we did a microarray assay with an 18K EST microarray (described in Supplementary Methods). Three clones transfected with full-length ARLTS1 were hybridized on microarray and compared with A549 normal gene expression pattern. When compared with untransfected cells, the ARLTS1-transfected cells showed a different expression pattern; in fact, 453 genes were found up-regulated whereas 198 were down-regulated after ARLTS1 transfection (Supplementary Table S3). A Gene Ontology (GO) analysis of deregulated genes revealed the functions altered after ARLTS1 transfection (Supplementary Table S4). The most frequent GO function among the up-regulated genes was “Developmental process” (56 times), whereas among the down-regulated genes it was “Transport” (26 times), which is in agreement with the intracellular functions of the ARF/ARL proteins in vesicular transport and membrane trafficking (7).

The highest level of induction was found for neuronal pentraxin 1 (NPTXI; 166 times), a gene exclusively expressed in neurons and

Figure 5. ARLTS1 overexpression triggers apoptosis of lung cancer cells. A, flow cytometric analysis of A549 (top) and H1299 (bottom) lung cancer cells after infection with Ad ARLTS1. The percentage of sub-G1 cell population is representative of three different experiments. B, TUNEL assay of H1299 lung cancer cells after infection with Ad ARLTS1. Columns, mean of three different experiments; black blocks, SD.
mediating probably the uptake of synaptic material (8). The second most up-regulated gene (~74 times) is DDX3X (also known as DDX3), a DEAD box RNA helicase with tumor growth-suppressive property and transcriptional regulation activity of the p21waf1/cip1 promoter, which was recently shown to be a candidate tumor suppressor (9) and to be deregulated in hepatitis virus–associated hepatocellular carcinomas (10). Ras-induced senescence and is also up-regulated by the transcription candidate tumor suppressor specifically activated during Ras-induced senescence 1 (RIS-1) gene, which was ~55 times more expressed in ARLTS1 stably transfected than wild-type A549 cells, is a candidate tumor suppressor specifically activated during Ras-induced senescence and is also up-regulated by the transcription factor Ets2 (11). None of the ras genes as well as Ets2 showed significant expression differences in comparison with the wild-type A549 levels, suggesting that ARLTS1 can be involved in this pathway. The gene expressed at lowest levels (reduced ~30 times) in transfected clones was Fc fragment of immunoglobulin G binding protein (FCGBP), a gene that may play roles in immune protection and inflammation in the intestine (Supplementary Table S3).

Several apoptosis-related mRNAs with correspondent proteins found by Western blotting or by caspase-3 assay to be activated (caspase-9, caspase-3, APAF-1, and PARP) in the present study or in ref. 1 show normal levels of mRNA expression. Moreover, other important genes for apoptosis regulation are deregulated in transfected A549 cells. The list includes the up-regulated proapoptotic genes such as tumor necrosis factor receptor superfamily, member 12A (TNFRSF12A; ref. 12), microtubule-associated protein τ (MAPT; ref. 13), receptor-interacting serine-threonine kinase 2 (RIPK2; ref. 14), and caspase 4, apoptosis-related cytostatic protein (CASP4; ref. 15; Supplementary Table S3).

A further confirmation of microarray results was the observation that ARLTS1 reexpression after 5-aza-2-deoxycytidine treatment of A549 cells has the same effects on the expression of SNAI2 and NPTXI genes as ARLTS1 transfection (Supplementary Fig. S1).

Discussion

In the present study, we proved the tumor suppressor effect of ARLTS1 in lung cancers by showing a significant reduction of ARLTS1 levels in about half of the analyzed samples and by proving in vitro the block of proliferation and apoptosis and in vivo the reduced tumorigenic effect after restoration of ARLTS1 expression in defective cells. Furthermore, we investigated at the genome-wide level by microarray the mechanisms associated with these effects and found that significant variation in ~650 transcripts is associated with the restoration of ARLTS1 expression. These data expand the spectrum of tissues where ARLTS1 exerts suppressor roles by the addition of lung cancers. It was shown before that the gene is acting in the same way in B-CLL cells and in breast and ovarian cancer cells (1, 6).

An uncommon single nucleotide polymorphism in ARLTS1 (i.e., G446A that causes a premature stop codon) has been suggested to be predisposing for familial cancer because it was shown to be more frequent among cancer patients with a family history of cancers, including CLL, pancreatic, melanoma, prostate, and breast, compared with a control population (1, 16). Furthermore, association of the ARLTS1 Cys148Arg variant with familial breast cancer risk has been also described (5). Cys148Arg revealed also a statistically significant association with an increased risk of melanoma for heterozygous carriers (17), whereas G446A was associated with an increased cancer familial aggregation of melanomas (18). Although significantly associated with familial risk of cancer, ARLTS1 G446A was not found to be more frequently present in sporadic cancers in respect with normal population by several studies, including our initial one (1, 18, 19).

These data suggest that ARLTS1 could be involved by a distinct pathogenetic mechanism in sporadic cancers. In fact, homozygous or heterozygous deletions at chromosome 13q14.3 are found in a variety of hematopoietic and solid tumors, including B-CLL (20), mantle cell lymphomas, multiple myeloma, and myeloid malignancies, and in prostate cancer (21) as well as low-malignant and benign lipomatous tumors (21–23). Hypermethylation of protective promoter region located in the immediate 5’ genomic region of the gene was proved to be also associated with low levels of expression in B-CLL, ovarian, breast, and lung cancers (1, 6).

The exogenous restoration of ARLTS1 gene expression in cancer cell lines with very low levels of expression changes the expression of several hundreds of genes, mainly by overexpression (455 of 653, 70%). Interestingly, among these are 30 transcription factors up-regulated (and only 4 down-regulated), and the most frequently
found GO function is represented by “Developmental processes” (56 up-regulated and 18 down-regulated genes) followed by “Transport” (34 up-regulated and 26 down-regulated genes). Furthermore, the expression of several oncogenes including the FOS gene involved in cell growth and/or maintenance, the small GTPase RAB31, or the antiapoptotic BIRC5 gene is induced by ARLTS1 transfection (Supplementary Table S4). All these data together suggest that various pathways involved in cell survival, proliferation, signaling, and development mediate the effects of wild-type ARLTS1 in a lung cancer system.

References

Tumor Suppressor Functions of \textit{ARLTS1} in Lung Cancers
Sai Yendamuri, Francesco Trapasso, Manuela Ferracin, et al.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/16/7738

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2007/08/15/67.16.7738.DC1

Cited articles
This article cites 23 articles, 6 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/16/7738.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/16/7738.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.