

ORIGINAL ARTICLE

Familial Cancer Associated with a Polymorphism in *ARLTS1*

George Adrian Calin, M.D., Ph.D., Francesco Trapasso, M.D., Ph.D., Masayoshi Shimizu, B.S., Calin Dan Dumitru, M.D., Ph.D., Sai Yendamuri, M.D., Andrew K. Godwin, Ph.D., Manuela Ferracin, Ph.D., Guido Bernardi, Ph.D., Devjani Chatterjee, Ph.D., Gustavo Baldassarre, M.D., Shashi Rattan, B.S., Hansjuerg Alder, Ph.D., Hideaki Mabuchi, M.D., Takeshi Shiraishi, M.D., Lise Lotte Hansen, Ph.D., Jens Overgaard, M.D., D.M.Sc., Vlad Herlea, M.D., Francesca Romana Mauro, M.D., Guillaume Dighiero, M.D., Benjamin Movsas, M.D., Laura Rassenti, Ph.D., Thomas Kipps, M.D., Ph.D., Raffaele Baffa, M.D., Ph.D., Alfredo Fusco, M.D., Masaki Mori, M.D., Ph.D., Giandomenico Russo, Ph.D., Chang-Gong Liu, Ph.D., Donna Neuberg, Sc.D., Florencia Bullrich, Ph.D., Massimo Negrini, Ph.D., and Carlo M. Croce, M.D.

ABSTRACT

BACKGROUND

The finding of hemizygous or homozygous deletions at band 14 on chromosome 13 in a variety of neoplasms suggests the presence of a tumor-suppressor locus telomeric to the *RB1* gene.

METHODS

We studied samples from 216 patients with various types of sporadic tumors or idiopathic pancytopenia, peripheral-blood samples from 109 patients with familial cancer or multiple cancers, and control blood samples from 475 healthy people or patients with diseases other than cancer. We performed functional studies of cell lines lacking *ARLTS1* expression with the use of both the full-length *ARLTS1* gene and a truncated variant.

RESULTS

We found a gene at 13q14, *ARLTS1*, a member of the ADP-ribosylation factor family, with properties of a tumor-suppressor gene. We analyzed 800 DNA samples from tumors and blood cells from patients with sporadic or familial cancer and controls and found that the frequency of a nonsense polymorphism, G446A (Trp149Stop), was similar in controls and patients with sporadic tumors but was significantly more common among patients with familial cancer than among those in the other two groups ($P=0.02$; odds ratio, 5.7; 95 percent confidence interval, 1.3 to 24.8). *ARLTS1* was down-regulated by promoter methylation in 25 percent of the primary tumors we analyzed. Transfection of wild-type *ARLTS1* into A549 lung-cancer cells suppressed tumor formation in immunodeficient mice and induced apoptosis, whereas transfection of truncated *ARLTS1* had a limited effect on apoptosis and tumor suppression. Microarray analysis revealed that the wild-type and Trp149Stop-transfected clones had different expression profiles.

CONCLUSIONS

A genetic variant of *ARLTS1* predisposes patients to familial cancer.

From Thomas Jefferson University, Philadelphia (G.A.C., F.T., M.S., C.D.D., S.Y., D.C., S.R., H.A., H.M., T.S., R.B., C.-G.L., F.B., M.N., C.M.C.); Fox Chase Cancer Center, Philadelphia (A.K.G., B.M.); University of Ferrara, Ferrara, Italy (M.F., G. Bernardi, M.N.); the National Cancer Institute, Aviano, Italy (G. Baldassarre); Aarhus University, Aarhus, Denmark (L.L.H., J.O.); Fundeni Hospital, Bucharest, Romania (V.H.); University La Sapienza, Rome (F.R.M.); the Pasteur Institute, Paris (G.D.); the University of California San Diego, La Jolla (L.R., T.K.); the University of Catanzaro, Catanzaro, Italy (A.F.); Kyushu University, Beppu, Japan (M.M.); Istituto di Ricovero e Cura a Carattere Scientifico, Rome (G.R.); and the Dana-Farber Cancer Institute, Boston (D.N.). Address reprint requests to Dr. Croce at Ohio State University Comprehensive Cancer Center, 385K Wiseman Hall, 400 W. 12th Ave., Columbus, OH 43210, or at carlo.croce@osumc.edu.

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HOMOZYGOUS OR HETEROZYGOUS deletions at chromosome 13q14.3 occur in a variety of hematopoietic and solid tumors.¹⁻⁵ In some cases of chronic lymphocytic leukemia (CLL), these deletions are the only detectable cytogenetic abnormality.^{6,7} The 13 known genes in this region are expressed in hematopoietic cells and solid tissues, but none have been found to be inactivated in tumors.^{2,8-10} Because of the absence of any detectable pathogenic mutation and the active transcription of all retained genes at 13q14.3 (except the microRNA genes *miR-15a* and *miR-16-1*), it is possible that haploinsufficiency, in which one allele is deleted and the remaining normal allele is insufficient to support normal function, contributes to CLL.^{1,8,10}

To identify putative tumor-suppressor genes at 13q14.3, we sequenced and characterized a 790-kb segment spanning the minimal region of loss¹ and performed a detailed mutational study of most of the known genes in this region (*DLEU1*, *DLEU2*, *miR-15a*, *miR-16-1*, *RFP2*, *KCNRG*, *DLEU6*, *DLEU7*, and *DLEU8*).^{1,11,12} Using computational and experimental approaches, we identified a gene encoding a member of the Ras superfamily, *ARLTS1* (also referred to as *ARL11* by the Human Genome Organisation Gene Nomenclature Committee). Here, we describe the results of studies to determine whether *ARLTS1* is a tumor-suppressor gene.

METHODS

STUDY SAMPLES

We studied 215 samples of sporadic tumors or blood (from 65 patients with a thyroid tumor, 58 with colorectal adenocarcinoma, 48 with breast carcinoma, 39 with CLL, and 5 with lung carcinoma) and 1 sample from a patient with idiopathic pancytopenia. The specimens of colorectal cancer and idiopathic pancytopenia were from patients in Bucharest, Romania; the breast-cancer specimens were from patients in Ferrara, Italy (38 specimens), and Aarhus, Denmark (10 specimens); the CLL samples were from patients overseen at the CLL Consortium in the United States; the lung-cancer specimens were also from patients in Ferrara; and the thyroid-tumor specimens were from patients in Naples, Italy.

We obtained 109 peripheral-blood samples from patients with familial or multiple cancers: they included 69 women with *BRCA1*- and *BRCA2*-negative familial breast cancer, 17 men with both prostate cancer and malignant melanoma (nega-

tive for mutations in the *p16* gene), 17 patients with familial CLL (at least two first-degree relatives affected), and 6 patients with pancreatic cancer or melanoma (with no mutations in the *p16* or *p14* gene and a family history of at least one case of pancreatic cancer or melanoma). A sample from only one affected member per family was analyzed. The familial CLL specimens were from patients in Paris (11 specimens) and the CLL consortium in the United States (6 specimens). All other specimens from patients with familial or multiple cancer were from Philadelphia.

We also obtained 475 control blood samples from healthy people or patients with diseases other than cancer. The control samples were from 156 renal-transplantation donors in Bucharest, 203 blood donors in Ferrara, and 116 blood donors in Philadelphia.

Written informed consent was obtained for the use of all specimens in accordance with the guidelines for the protection of human subjects at each participating institution. All subjects were white, as indicated by medical records in the case of the patients and information obtained during interviews with the controls. Of the patients with cancer, 58 percent were European and 42 percent were American. Of the controls, 76 percent were European and 24 percent were American.

MOLECULAR STUDIES

Molecular studies, examination for the loss of heterozygosity, cloning of *ARLTS1*, and methylation studies were performed as described previously.¹³⁻¹⁶ We searched a computer database using Exofish (www.genoscope.cns.fr) and found a 182-bp, evolutionarily conserved region and obtained the full-length complementary DNA (cDNA) with the use of expressed sequence tags and rapid amplification of cDNA ends.

DETECTION OF *ARLTS1* MUTATIONS

We directly sequenced DNA on both strands from 597 samples using a DNA-sequencing system (model 377, Applied Biosystems). DNA from the 203 Italian control subjects was analyzed by denaturing high-performance liquid chromatography (Transgenomics), and all the samples with abnormal patterns were directly sequenced.

STABLE TRANSFECTION OF A549 CELLS

A549 is a highly tumorigenic, non-small-cell lung-carcinoma cell line that has wild-type *TP53* and *RB1* genes but does not express the *p16^{INK4a}* gene. We

constructed *ARLTS1* expression vectors, one containing the full-length gene (pMV7-*ARLTS1*-sense) and the other containing a truncated gene encoding a protein product lacking a C-terminal and identical to the polymorphic variant implicated by the genetic data (pMV7-*ARLTS1*-ΔC-terminal) by ligating the relevant open reading frame in a sense orientation into a mammalian expression vector (pMV7). All sequenced constructs were transfected with the use of FuGENE6, according to the manufacturer's instructions (Boehringer Mannheim). Stably transfected cells were selected with the use of G418 and examined for the transformed phenotype by establishing *in vivo* tumorigenicity in Nu/Nu nude mice and selected for apoptosis with the use of the Active Caspase-3 phycoerythrin monoclonal antibody apoptosis kit (Pharmingen, BD Biosciences). Cell-cycle profiles were identified with the use of flow cytometry of cells stained with propidium iodide, and gene-expression profiles were determined with the use of a Kimmel Cancer Center/Thomas Jefferson University human 18.5K expression bioarray (Compugen Human Oligo Set 1.0), as described previously.¹⁷

STATISTICAL ANALYSIS

Statistical analysis of categorical results was performed with the use of Fisher's exact test. A logistic-regression model was used to determine the odds ratio for cancer in association with specific mutations. Tumor weights in immunodeficient mice were examined in an analysis-of-variance model, which included the treatment group and the time at which the animal was killed; two-sided P values for specific comparisons between groups were calculated. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

DELETION OF *ARLTS1* IN VARIOUS TYPES OF CANCER

Using Exofish¹⁸ on 1.4 Mb of the assembled genomic sequence at chromosome 13q14^{1,11,19,20} and rapid amplification of cDNA ends, we cloned cDNA from bone marrow and spleen that encodes a conceptual protein of 196 amino acids with a predicted molecular mass of 21 kD. Analysis of protein databases with the basic local alignment search tool (BLAST) revealed significant homology with the ADP-ribosylation factor (ARF) and ARF-like (ARL) protein family of the Ras superfamily^{21,22}; we there-

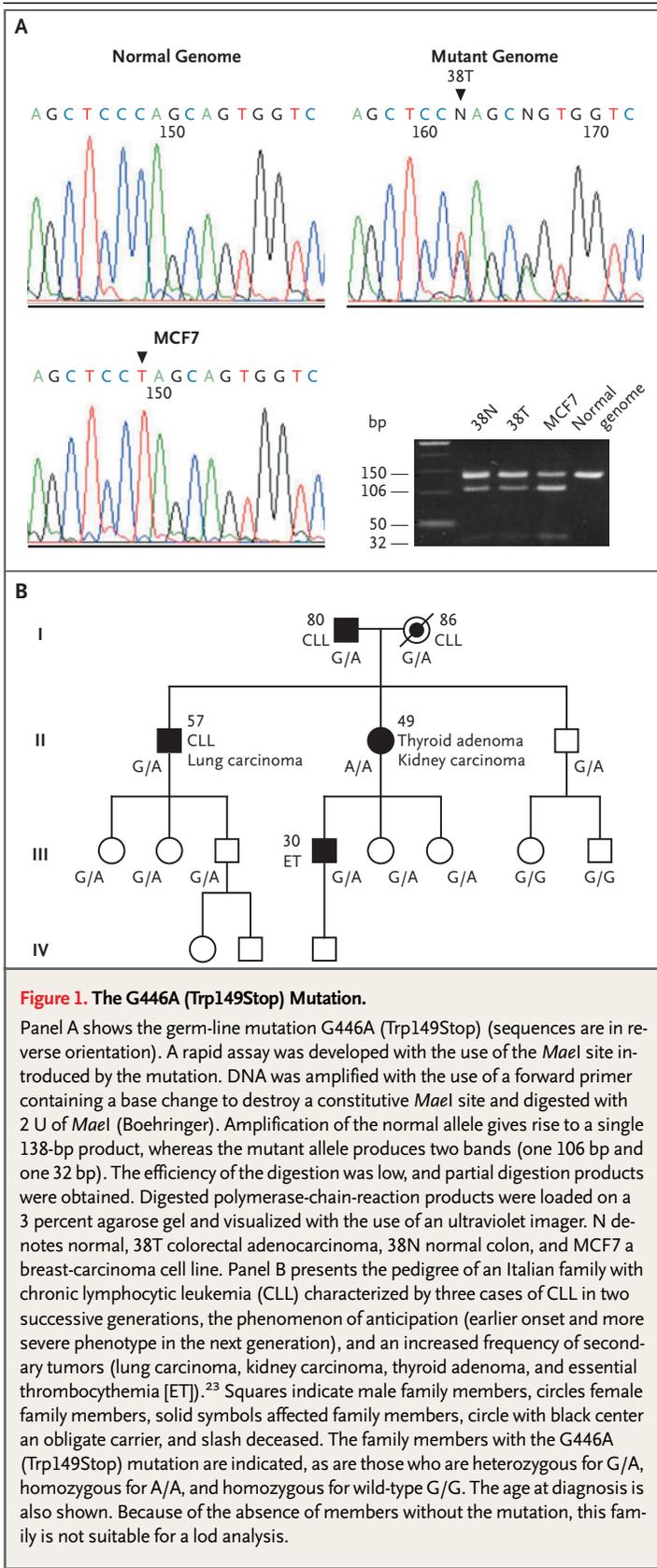
fore named the gene *ARLTS1* (for ADP-ribosylation factor-like tumor-suppressor gene 1; GenBank accession number, AF441378; European Molecular Biology Laboratory-European Bioinformatics Institute Minimum Information about a Microarray Experiment accession number, E-MEXP-274). The genomic structure of *ARLTS1* is very similar to that of class III ARF (ARF6): it has two exons, and the second contains the entire open reading frame. We found highly conserved protein homologues in mouse and rat and similar proteins in zebrafish, *Drosophila melanogaster*, and *Arabidopsis thaliana* (data not shown), indicating that *ARLTS1* has been evolutionarily conserved over time.

Previously, we reported that the genomic region at 13q14.3 is hemizygotously deleted in approximately 20 percent of the CLL samples we analyzed.¹⁴ We confirmed that *ARLTS1* is within the region targeted by deletions by using loss-of-heterozygosity analysis on DNA samples from 20 colorectal carcinomas; 10 percent of the specimens (2 of 20) had the hemizygous deletion (data not shown). We therefore hypothesized that monoallelic loss of *ARLTS1* occurs in a fraction of both hematopoietic and solid tumors. To test this idea, we sought to identify secondary events, such as a mutation or an epigenetic alteration, that could inactivate the remaining allele.

ASSOCIATION OF A GERM-LINE TRUNCATING POLYMORPHISM IN *ARLTS1* WITH FAMILIAL CANCER

We identified a germ-line polymorphism—the substitution of adenine for guanine at position 446 (G446A), resulting in a stop codon at position 149 (Trp149Stop) (Fig. 1A)—in samples from both patients with cancer and controls. The position of the stop codon predicts premature termination of translation, leading to the synthesis of a 148-amino-acid protein. Trp149 is a conserved amino acid in 12 other ARF or ARF-related proteins, including all six ARF members, whereas the stretch of 25 amino acids in the C-terminal (which is lost in the truncated form) is conserved in both mouse- and rat-homologue genes.

The polymorphism was detected in 2.1 percent of the control subjects (Table 1), with the prevalence ranging from 0.9 percent in the U.S. population (1 in 116) to 3.4 percent in the Italian population (7 in 203). Overall, DNA from the blood of 10 of the 475 control subjects and from 8 of the 216 patients with sporadic cancer (3 of 48 with breast cancer, 2 of 58 with colorectal carcinoma, 1 of



5 with lung carcinoma, and 1 of 65 with a thyroid tumor) or idiopathic pancytopenia (1) carried the stop mutation. This difference was not significant ($P=0.09$). The Trp149Stop mutation was, however, significantly more frequent among patients with a family history of cancer or with multiple cancers than among patients with sporadic cancer ($P=0.02$; odds ratio, 5.7; 95 percent confidence interval, 1.3 to 24.8). It was found in 2 of 17 blood samples from patients with familial CLL, in 1 of 69 with familial breast cancer, in 2 of 17 patients with both malignant melanoma and prostate carcinoma, and in 1 of 6 patients with both pancreatic cancer and melanoma (Table 2). All tumor samples had both the wild-type and polymorphic alleles except one breast cancer, which lacked the wild-type *ARLTS1* allele (one allele was mutated and the second was deleted). Sequence analysis of *ARLTS1* in paired samples of normal tissue, which were available from two patients with a colorectal tumor and one patient with breast carcinoma, suggested that the status of the gene was the same in the normal cells and the tumor cells.

In one kindred with familial CLL, all five members with cancer harbored the truncating polymorphism, whereas two unaffected members who were analyzed did not (Fig. 1B). The only member of this kindred with a homozygous mutation was found to have kidney carcinoma and thyroid adenoma when she was less than 50 years old. In the third generation, six members, one of whom had received a diagnosis of essential thrombocythemia (a pre-malignant state), had the polymorphism; the other five members were less than 40 years old.

In addition to the G446A (Trp149Stop) variant, we identified four other variations in *ARLTS1*: C65T (Ser22Leu) and G490A (Glu164Lys), both of which were found in thyroid adenomas, and C392T (Pro131Leu) and T442C (Cys148Arg), which were present in heterozygous form in 6.2 percent and 66.9 percent, respectively, of the controls (Table 1). Glu164 is well conserved in homologues of *ARLTS1* protein, suggesting that it is critical to protein function. Interestingly, we found two C65T missense mutations, one G446A nonsense mutation, and one G490A missense mutation among 23 thyroid adenomas of follicular origin, whereas wild-type *ARLTS1* was present in all 42 samples of the nonfollicular type. It is unlikely that this allelic distribution is random ($P=0.005$ by Fisher's exact test). A member of a family with CLL who was homozygous for the G446A polymorphism also had thyroid adenoma (Table 2).

DOWN-REGULATION OF ARLTS1 BY PROMOTER HYPERMETHYLATION

Analysis of RNA from normal hematopoietic and solid tissues with the use of an *ARLTS1* probe revealed ubiquitous expression of a 2.2-kb transcript and additional 1.3- and 5.5-kb transcripts resulting from the use of different polyadenylation sites. Northern blotting, a semiquantitative reverse-transcriptase–polymerase-chain-reaction assay, or both showed a reduction or absence of *ARLTS1* expression in 4 of 16 fresh tumor samples (2 of 7 lung carcinomas and 2 of 9 samples of CLL cells) for which cDNA, RNA, or both were available, as compared with the levels of expression in their normal counterparts (Fig. 2).

We examined tumors to determine whether *ARLTS1* is down-regulated through hypermethylation of the putative promoter, which was located by a computer search of the first exon (bases 10 to 59 of the cDNA). On Southern blotting, fresh

Table 1. Results of *ARLTS1* Sequence Analysis in Specimens of Sporadic Tumors, Blood Samples from Patients with Familial Cancer, and Blood Specimens from Control Subjects.

Variant*	Amino Acid Change	Sporadic Tumors	Familial Cancers	Controls
				no./total no. (%)
C65T	Ser22Leu	2/216 (0.9)	0/109	1/272 (0.4)†
C392T	Pro131Leu	14/216 (6.5)	4/109 (3.7)	17/272 (6.2)†
T442C	Cys148Arg	127/216 (58.8)	80/109 (73.4)	182/272 (66.9)†
G446A	Trp149Stop	8/216 (3.7)	6/109 (5.5)‡	10/475 (2.1)
G490A	Glu164Lys	1/216 (0.5)	0/109	0/272†

* Several synonymous polymorphisms were also identified, such as C175T (Leu59), G297A (Ser99), C345T (Val115), G396C (Leu132), and G546A (Gln182).

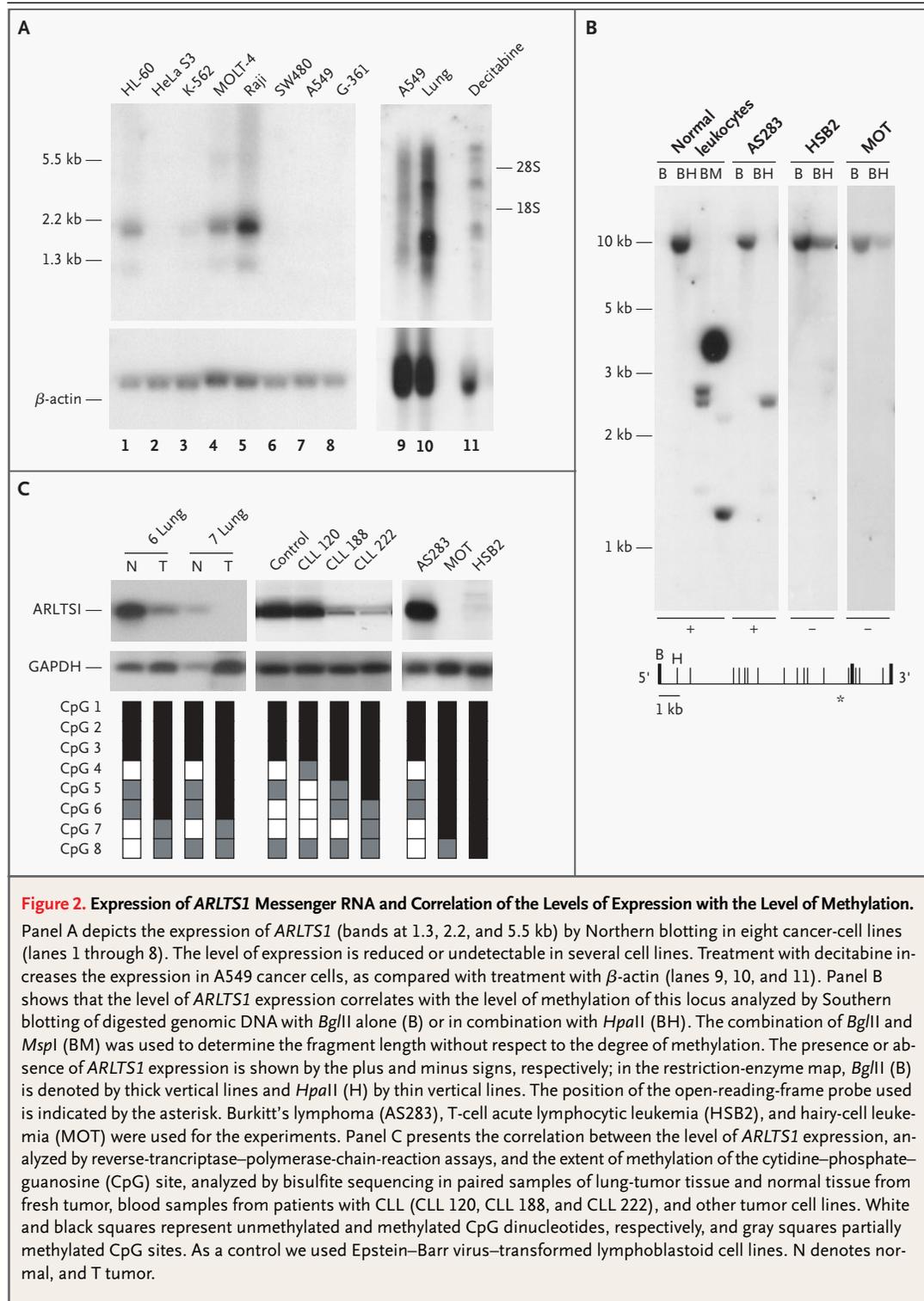
† The analysis included only samples that were directly sequenced.

‡ P=0.02 for the comparison with sporadic tumors.

Table 2. Clinical Characteristics of Persons with the G446A (Trp149Stop) Mutation.*

Sex of Proband	Type of Cancer and Age at Diagnosis	Family History
Female	B-cell CLL, 46 yr	Twin sister: G446A mutation and B-cell CLL
Male	B-cell CLL and lung cancer, 57 yr	Sister: 49 yr old; homozygous for G446A; thyroid adenoma and kidney carcinoma Nephew: 30 yr old; heterozygous for G446A; essential thrombocythemia Brother: heterozygous for G446A; healthy Mother: deceased; obligate carrier of G446A; B-cell CLL at 86 yr of age Father: 80 yr old; heterozygous for G446A; B-cell CLL
Male	Gastric carcinoma, 72 yr Melanoma, 72 yr Prostate cancer, 73 yr	None; personal history of multiple cancers
Male	Prostate cancer, 66 yr Melanoma, 67 yr	Mother: cancer at unknown location and age Brother: prostate cancer at 73 yr of age Sister: diagnosis of “black moles” at unknown age Daughter: breast cancer at unknown age
Male	Melanoma, 50 yr Lung metastasis, 55 yr (died)	Paternal uncle: melanoma at unknown age Paternal aunt: pancreatic cancer at unknown age Paternal cousin: pancreatic cancer at unknown age Paternal cousin: head and neck cancer at unknown age
Female	Bilateral breast cancers, 32 and 35 yr Ovarian cancer, 50 yr	Daughter: 48 yr old; G446A carrier; unaffected Family history of breast, ovarian, and other solid-tissue cancers 2 of 5 Relatives with cancer and 2 of 14 unaffected relatives had the C392T but not the G446A variant

* All probands tested negative for *BRCA1*, *BRCA2*, *p16^{INK4a}*, and *p14ARF* germ-line mutations.



tumor samples with low levels or no expression of *ARLTS1* showed higher methylation levels than normal tissues or tumors with normal levels of expression. The most 3' cytidine-phosphate-guanosine repeats (CpGs) were methylated in both normal and tumor tissues, with no correlation between the degree of methylation and the level of expression of *ARLTS1*, whereas the 5' CpGs located near the promoter were differentially methylated (Fig. 2C). One thyroid adenoma with a heterozygous C65T (Ser22Leu) mutation also exhibited hypermethylation. Treatment of A549 cells (Fig. 2A) and H1299 (data not shown) lung cancer cells with decitabine increased the levels of expression of *ARLTS1* to levels similar to those in normal lung (Fig. 2A).

tumors weighed 50 percent less than those of wild-type A549 clones), and we found a significant difference between the size of *ARLTS1-FL*-induced tumors and *ARLTS1-Stop*-induced tumors ($P=0.04$) (Fig. 3C and 3D). Thus, *ARLTS1* by itself has tumor-suppress-

INDUCTION OF APOPTOSIS IN VIVO BY FULL-LENGTH ARLTS1

ARLTS1 expression was dramatically decreased in the A549 cell line. This line was transfected with the use of the pMV7 vector containing the full-length *ARLTS1* coding sequence (*ARLTS1-FL*), the C-terminal-deleted cDNA (*ARLTS1-Stop*), or the control (empty) vector. The transfectants were selected according to the level of expression of the transfected *ARLTS1* minigene (Fig. 3A). We evaluated the ability of these transfected cells to form tumors in Nu/Nu mice, which lack an immune system. During eight weeks of observation, all *ARLTS1-FL*-transfected cells consistently formed smaller tumors (i.e., tumors that weighed 80 percent less) than did cells transfected with empty vector or wild-type A549 cells ($P<0.001$). Furthermore, tumor size was intermediate in the group of mice injected with A549 clones expressing the Δ C-terminal protein (i.e.,

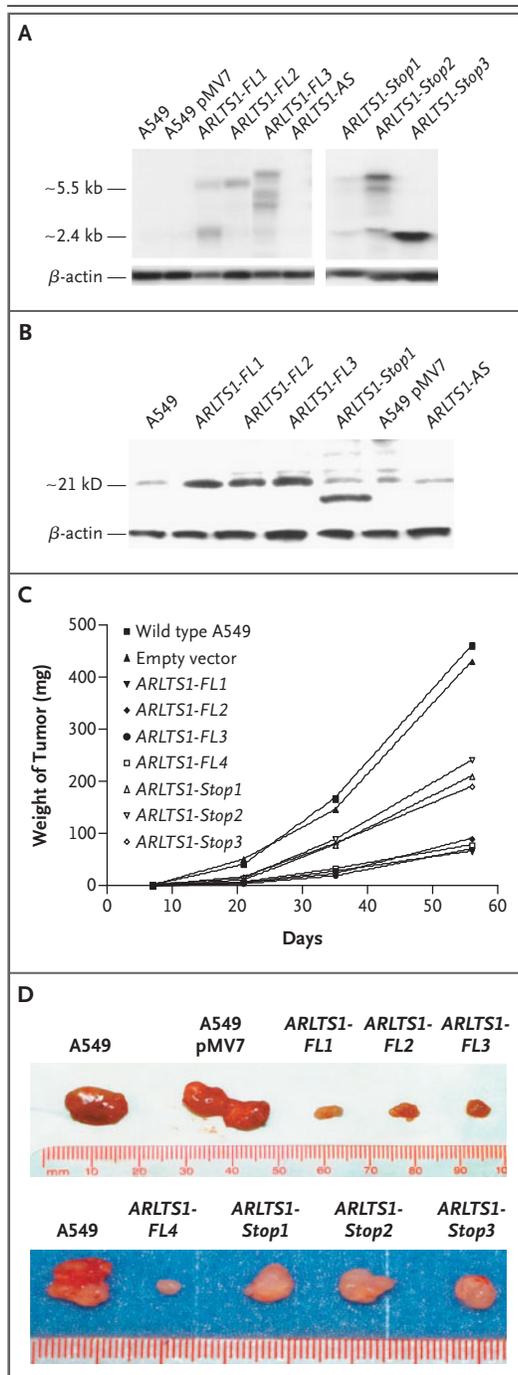
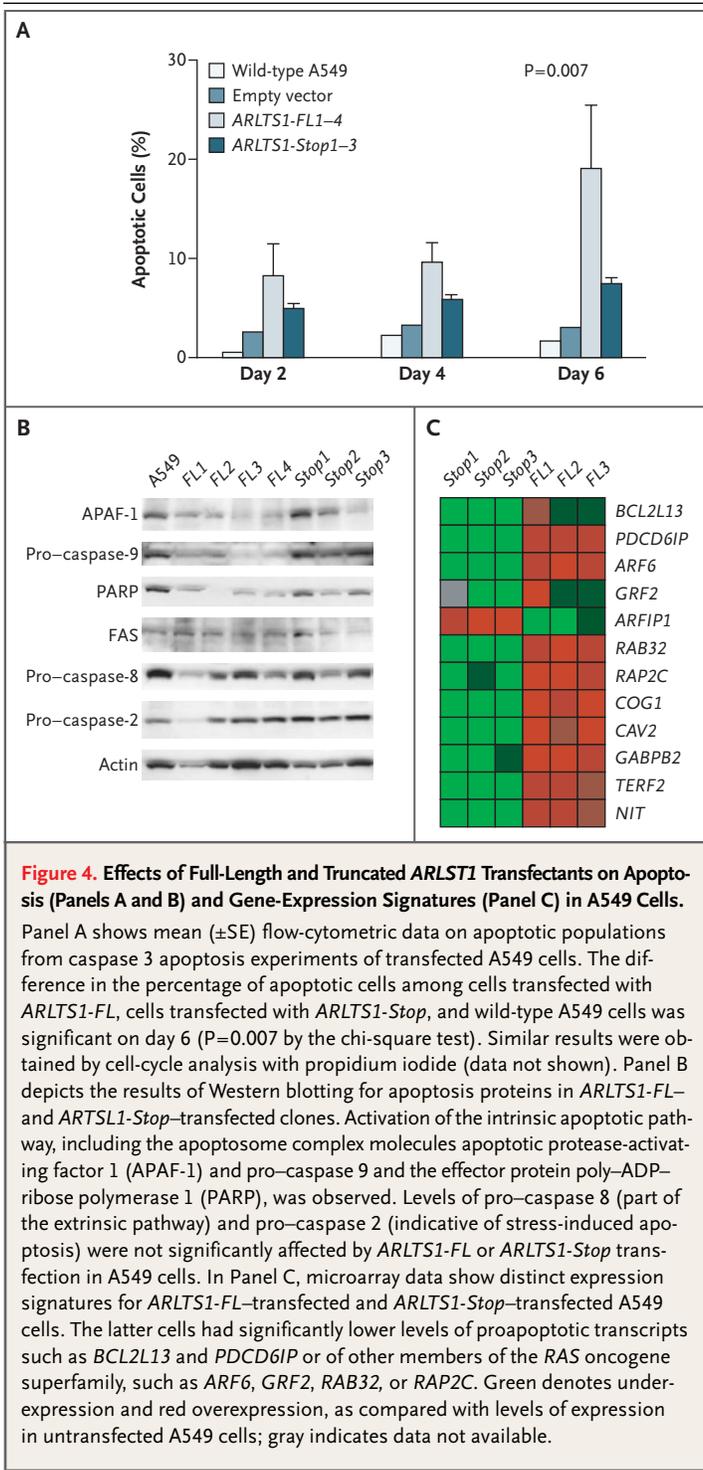


Figure 3. Effect of *ARLTS1* on the Tumorigenicity of A549 Cells.

Panels A and B show the restoration of expression of *ARLTS1*, identified by Northern blotting and Western blotting, respectively, by transfection of the minigene into A549. Panel C presents an example of tumorigenesis in nude mice. A total of 10^6 cells from A549 wild-type cells, A549 cells transfected with pMV7 empty vector, and several transfectant clones expressing full-length (FL) and stop (Stop) cDNA were injected subcutaneously in triplicate experiments. Panel D shows tumors from nude mice. The weight of tumors for the nine analyzed clones at the indicated times are shown. Similar results were obtained by measurement of tumor volumes. *ARLTS1-AS* denotes the antisense controls.



sor activity in A549 cells, and this activity is partially lost in the presence of the truncated protein.

A higher percentage of transfected *ARLTS1-FL* cells than of parental cells underwent apoptosis,

whereas the populations in the G₀ or G₁ phase or S phase did not differ significantly between the two types of cells. By contrast, cells expressing the truncated protein were less susceptible to the induction of apoptosis than cells expressing the full-length protein (P=0.007) (Fig. 4A). Western blotting showed different levels of the apoptosome complex molecules apoptotic protease-activating factor 1 and pro-caspase 9 and of the effector protein poly-ADP-ribose polymerase 1 in full-length and truncated clones (Fig. 4B), with higher levels of activation in the former, in concordance with the findings in the caspase 3 assay.

We also found that the gene-expression profiles of A549 cells transfected with full-length *ARLTS1* minigenes differed from those of A549 cells transfected with truncated *ARLTS1* minigenes (Fig. 4C). The truncated transfectants had significantly lower levels of transcripts promoting apoptosis (such as *BCL2L13*) than did the full-length clones (P=0.003). These data are consistent with the comparative ease with which *ARLTS1-FL*-transfected A549 cells and *ARLTS1-ΔC*-transfected A549 cells could be induced to undergo apoptosis. Furthermore, several members of the small GTPase family (such as *ARF6*) were expressed at significantly lower levels in the truncated transfectants (P=0.005). Together these data suggest that the full-length product increases the propensity of the cell to undergo apoptosis.

DISCUSSION

We identified *ARLTS1*, a widely expressed member of the ARF-ARL family that functions as a tumor-suppressor gene in cancers in humans. ARFs are 20-kD guanine nucleotide-binding proteins, members of the Ras GTPase superfamily involved in various cellular functions, including vesicular transport and membrane transport.²¹ ARLs are structurally very similar to ARFs, and there is a continuum of ARF-ARL functions. Of the 18 known members of this family, only *ARL5* has been found to be overexpressed in hepatocellular carcinoma,²⁴ and levels of *ARF6* protein correlate with the invasiveness of breast-cancer cells.²⁵ The most common mechanism of *ARLTS1* inactivation in cancers in humans seems to be biallelic down-regulation by hypermethylation of the promoter. In data consistent with the properties of a classic tumor-suppressor gene, *ARLTS1* alterations were found to consist of combinations of a hypomorphic polymorphism plus loss of heterozygosity in a case of breast cancer and the

polymorphism plus hypermethylation in a case of thyroid adenoma.

Since the *ARLTS1* G446A mutation was nearly three times as frequent among patients with familial cancers and nearly twice as high among patients with sporadic cancers as among persons in the general population, we propose that *ARLTS1* is a low-penetrance tumor-suppressor gene that accounts for a small percentage of familial melanoma or familial CLL. Some kindreds may carry the polymorphism but not have cancer. A similar situation was described for other tumor-suppressor genes, such as the *BRCA2* germ-line mutations in pancreatic cancer.²⁶ An apparently neutral polymorphic stop codon has been identified in a *BRCA2* gene,²⁷ but wild-type and truncated *ARLTS1* proteins were distinguishable because the truncated protein induced lower levels of apoptosis than the full-length protein when expressed in A549 cells. This observation suggests that *ARLTS1* is a dose-sensitive gene, a hypothesis in accord with the variations in the levels of expression that we found in tumor samples and cell lines.

The G446A (Trp149Stop) polymorphism is probably maintained in the general population, because the *ARLTS1*-ΔC protein retains some functions of the full-length protein and is in the same intracellular location (unpublished data); it retains an antiapoptotic function but at a significantly lower level than does the normal product. We propose that

ARLTS1-ΔC predisposes patients to cancer in several ways. First, transfected cells harboring the truncated gene up-regulate fewer proapoptotic genes than cells with the normal gene. Second, because *ARLTS1* transfection influences the levels of expression of several other members of the ARF-ARL family, the *ARLTS1* product is probably involved in some common functions with other members of this family. The *ARLTS1*-ΔC protein induces these genes at significantly lower levels, suggesting a partial loss of common functions from the ARF-ARL spectrum. Supporting this hypothesis is the fact that the truncated protein lacks the C-terminal motif involved in nucleotide binding and hydrolysis, which are characteristic of Ras-related GTPases.²⁸

The participation of *ARLTS1* in an apoptosis pathway is in accord with data showing that the yeast homologue of *ARL1* has a role in programmed cell death.²⁹ Furthermore, a substitution in *ARL1* near the position corresponding to the stop mutation that we have described inhibits the promotion of programmed cell death induced by Bax in yeast. Therefore, human *ARLTS1* and yeast *ARL1* may be involved in a conserved apoptosis pathway.

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