

# Expression of the Tumor Suppressor Gene ARHI in Epithelial Ovarian Cancer Is Associated with Increased Expression of p21<sup>WAF1/CIP1</sup> and Prolonged Progression-Free Survival

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## ABSTRACT

**Purpose:** ARHI, an imprinted putative tumor suppressor gene, is expressed in normal ovarian epithelial cells, but its expression is down-regulated or lost in most ovarian cancer cell lines. Reexpression of ARHI in cancer cells induces p21<sup>WAF1/CIP1</sup>, down-regulates cyclin D1 promoter activity and inhibits growth in cell culture and in heterografts. To determine the relevance of these observations to clinical cancer, we have now measured ARHI expression in normal, benign and malignant ovarian tissues using immunohistochemistry and *in situ* hybridization.

**Experimental Design:** Paraffin embedded tissues from 7 normal ovaries, 22 cystadenomas and 42 borderline lesions were analyzed using standard immunoperoxidase and *in situ* hybridization techniques to assess ARHI expression. In addition, immunohistochemistry against ARHI was performed on a tissue microarray containing 441 consecutive cases of ovarian carcinoma.

**Results:** Strong ARHI expression was found in normal ovarian surface epithelial cells, cysts and follicles using immunohistochemistry and *in situ* hybridization. Reduced ARHI expression was observed in tumors of low malignant potential as well as in invasive cancers. ARHI expression was down-regulated in 63% of invasive ovarian cancer specimens and could not be detected in 47%. When immunohistochemistry and *in situ* hybridization were compared, ARHI protein expression could be down-regulated in the presence of ARHI mRNA. ARHI expression was correlated with ex-

pression of p21<sup>WAF1/CIP1</sup> ( $P = 0.0074$ ) but not with cyclin D1 and associated with prolonged disease free survival ( $P = 0.001$ ). On multivariate analysis, ARHI expression, grade and stage were independent prognostic factors. ARHI expression did not correlate with overall survival.

**Conclusions:** Persistence of ARHI expression in epithelial ovarian cancers correlated with prolonged disease free survival and expression of the cyclin dependent kinase inhibitor p21<sup>WAF1/CIP1</sup>.

## INTRODUCTION

Members of the ras family are among the most frequently activated proto-oncogenes in human cancers. Among the neoplasms that arise in the ovary, *ras* genes can be mutated in borderline tumors and in invasive cancers of mucinous and low-grade serous histotypes (1). Given the prevalence of different histotypes, no more than 20% of invasive cancers exhibit *ras* mutations. Functional activation of the ras pathway in the absence of genetic mutations has, however, been reported in a majority of ovarian cancer cell lines (2). Members of the rap family share high homology with ras proteins and have been shown to antagonize ras-mediated activation of mitogen-activated protein kinase and cellular transformation in NIH 3T3 fibroblasts (3). Thus, certain members of the ras-superfamily may act as tumor suppressor genes rather than as proto-oncogenes.

ARHI is a maternally imprinted putative human tumor suppressor gene that maps to chromosome 1p31 and that encodes a 26-kDa small G protein with 60% homology to rap and ras (4). ARHI is expressed in normal ovarian and breast epithelial cells, but its expression is lost or down-regulated in the majority of ovarian and breast cancers (4–6). As an imprinted gene, ARHI is expressed only from the paternal allele. Expression can be lost through loss of heterozygosity in up to 40% of ovarian and breast cancers and can also be down-regulated by methylation (7, 8) or by transcriptional regulation (9). Reexpression of ARHI in cancer cells induces p21<sup>WAF1/CIP1</sup>, down-regulates cyclin D1 promoter activity, and inhibits growth in cell culture and in heterografts (10). ARHI can also inhibit the motility and invasiveness of cancer cells.<sup>1</sup> Expression of ARHI can block epidermal growth factor mediated signaling through RAS/mitogen-activated protein above the level of RAF, activate c-Jun NH<sub>2</sub>-terminal kinase, and induce apoptosis through a calpain-dependent, caspase-independent mechanism (10). Growth inhibition depends upon a unique NH<sub>2</sub>-terminal extension of the molecule that sets ARHI apart from Ras (11).

Previous studies have focused on the impact of ARHI expression on ovarian cancer cell lines. The present study ex-

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amines the significance of ARHI expression using immunohistochemical analysis and *in situ* hybridization (ISH) in normal ovarian surface epithelium, benign ovarian tumors, borderline ovarian lesions, and in epithelial ovarian cancers. ARHI expression has been compared with p21<sup>WAF1/CIP1</sup> and cyclin D1 expression and correlated with histopathological data and clinical outcome.

## MATERIALS AND METHODS

**Patients and Tissues.** Subjects with primary epithelial ovarian cancer who had undergone initial surgery at The University of Texas M. D. Anderson Cancer Center between 1990 and 2001 were included in this study. A total of 441 patients was identified. Follow-up was updated through June 2003 by review of medical records and the United States Social Security Index. Demographic and survival data were entered into a comprehensive database created with Microsoft Access (version 97). Histopathologic diagnosis was based on WHO criteria; the samples were assigned a grade based on Gynecologic Oncology Group criteria (12) and staged according to the International Federation of Gynecology and Obstetrics system (13). Time to progression was defined as the interval from diagnosis until disease progression evidenced by a  $\geq 25\%$  increase in tumor size, appearance of new lesions, or an increase in serum CA 125 level to more than twice the upper limit of normal (14, 15). Mean time to progression was calculated as the average time from diagnosis until the date of relapse. The percentage of subjects who have survived the disease for a defined period of time identifies disease-specific survival. Survival was calculated from the date of diagnosis to the date of death, and only deaths from the disease were considered. The extent of cytoreduction was defined as optimal if residual disease after surgery was  $< 1$  cm or suboptimal if residual disease was  $> 1$  cm (16, 17).

The mean age of our entire population of 441 cases was 58.8 years (range, 20 to 92 years); 339 patients were white, 26 black, 53 Hispanic, 6 Asian, and 17 were of other ethnicities. With regard to surgical stage, 36 patients (8.2%) had stage I disease, 32 (7.2%), stage II disease, 291 (66%), stage III, and 82 (18.6%) stage IV. The tumor histotype was pure serous carcinoma in 243 patients (55.1%), serous carcinoma mixed with another histotype in 98 (22.2%), endometrioid in 41 (9.3%), clear cell in 18 (4%), mixed malignant Mullerian tumor in 12 (2.7%), undifferentiated carcinoma in 12 (2.7%), mucinous carcinoma in 10 (2.3%), and transitional cell carcinoma in 7 (1.8%). After primary cytoreductive surgery, 406 patients had platinum-based therapy, 10 had some other form of therapy and 25 no additional therapy. Thirty-eight patients had chemotherapy before surgery. No recurrence of disease was evident during follow-up for 120 patients, 67 patients had progressive disease during initial chemotherapy, 238 had recurrent disease, and 16 patients were lost to follow-up. The mean follow-up interval was 57.6 months (range, 1 to 120 months), and the overall survival rate at 5 years was 36.4%. As expected, the probability of death from disease was associated with primary tumor stage ( $P < 0.0001$ ), recurrence ( $P < 0.0001$ ), and tumor histotype ( $P < 0.005$ ). The mean time to progression was 17.8 months. Of all 441 cases, 407 (92%) could be scored for ARHI staining, 409 (93%) for cyclin D1 and 415 (94%) for p21<sup>WAF1/CIP1</sup>. The

remaining cases were either lost during the sectioning procedure or contained  $< 20\%$  tumor cells.

To compare ARHI expression among different ovarian lesions, 7 normal ovaries, 22 cystadenomas, and 42 borderline lesions were selected from archived files of formalin-fixed, paraffin-embedded tissues. These samples served as tissue controls. In addition, tissue microarray was designed containing five specimens of normal fallopian tube, uterus, breast, kidney, stomach, small intestine, adrenal gland, thyroid, testis, pancreas, brain, spleen, prostate, lung, heart, and liver. Studies of tissue blocks and patient information were approved by the Institutional Review Board at The University of Texas M. D. Anderson Cancer Center.

**Construction of Tissue Microarrays.** Tissue blocks were stored under ambient conditions at  $\sim 24^\circ\text{C}$ . H&E-stained sections were reviewed by a pathologist to select representative areas of tumor from which to acquire cores for microarray analysis. Core samples from morphologically representative areas of paraffin-embedded tumor tissues were assembled on a recipient paraffin block to create tissue microarrays. Arrays were constructed with a precision instrument (Beecher Instruments, Silver Spring, MD) that uses two separate core needles for punching the donor and recipient blocks and a micrometer-precise coordinate system for assembling tissue samples on a block. For each case, two replicate 1-mm core-diameter samples were collected, and each one was placed on a separate recipient block. The final ovarian tumor tissue microarray consisted of six blocks, the first two (1a and b) containing duplicates of 164 spots, the second two (2a and b) containing duplicates of 158 spots, and the third (3a and b) containing duplicates of 119 spots, with samples spaced 0.5 mm apart. Five-micron sections were obtained and stained with H&E to confirm the presence of tumor and to assess tumor histology. Tumor samples were arranged at random.

Sample tracking was based on coordinate positions for each tissue spot in the tissue microarray block. Spots were transferred onto tissue microarray slides for staining. This sample tracking system was linked to a Microsoft Access database containing demographic, clinicopathologic, and survival data, thereby allowing rapid links between histologic data and clinical features. The array was read according to the given tissue microarray map. Each core was scored individually and the results were presented as the mean of the two replicate core samples.

**Immunohistochemical Analysis.** After initial deparaffinization of tissue microarray slides, endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Deparaffinized sections were steamed in 10 mmol/L citrate buffer (pH 6.0) to restore latent epitopes. The slides were then incubated with monoclonal antibodies against ARHI as described previously (6). Cyclin D1 (Biocare, Walnut Creek, CA) and p21<sup>WAF1/CIP1</sup> (Neomarkers, Fremont, CA) were stained according to the manufacturer's specifications. Slides were washed and incubated with biotin-labeled secondary antibody for 10 min and then with streptavidin:peroxidase for 10 minutes. Tissues were then stained for 5 minutes with 0.05% 3',3'-diaminobenzidine tetrahydrochloride that had been freshly prepared. Tissues were then counterstained with hematoxylin, dehydrated, and mounted in Permount. Normal colon and colon carcinoma

were used as a positive control. Negative controls were made by replacing the primary antibody with PBS and leukocyte common antigen monoclonal antibody (IgG1). All controls gave satisfactory results.

The cytoplasmic intensity and proportion of positive cells for ARHI immunohistochemical staining were determined for the normal ovarian epithelium, benign lesions, and invasive carcinoma. On the basis of criteria used by our previous publication and similar publications (7), we prospectively chose 10% as a cutoff for positive staining cells and used a subjective scale (- to +++) to classify staining patterns: (a) -, no staining; (b) +, weak staining; (c) ++, moderate staining; and (d) +++, intense staining. If *c* or *d* was observed, the specimen was considered to be strongly positive. Cases in which <20% of the core contained tumor or no cores were available were excluded from the final data analysis.

Cyclin D1 and p21<sup>WAF1/CIP1</sup> were expressed as percentage of positive nuclear area staining by image analysis (Nikon Metavue 6.1). For cyclin D1, a positive nuclear area > 10% was considered overexpression, whereas for p21<sup>WAF1/CIP1</sup> a positive nuclear area > 2% was considered overexpression.

**Preparation of Probes for ISH.** To prepare an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, GAPDH PCR products were produced with cDNA prepared from a normal ovarian epithelial cell culture (NOE 115) derived in our laboratory. Sense (5'-GAAGGTGAAGGTCGGAGTCA-3') and antisense (5'-GAAGATGGTGTGGGATTTTC-3') primers were used. PCR products were TA cloned using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). TOPO-GAPDH clones were digested with *EcoRI* (Roche, Indianapolis, IN), releasing the GAPDH fragments, which were gel purified and subcloned into the pSPT 19 vector (Roche) at the *EcoRI* cloning site. The resulting pSPT 19 clones were sequenced to verify correct orientation with the SP6 promoter. The anti-GAPDH probe was tested on normal breast tissue known to have intact mRNA. A commercial GAPDH mRNA antisense probe (Tetra Link, Buffalo, NY) served as the control for our laboratory's GAPDH antisense construct.

**Preparation of Digoxigenin-labeled RNA Probes.** The ARHI cDNA (1012 bp), which spanned the entire open reading frame, was subcloned into plasmids in either orientation with respect to the Sp6 or T7 promoter site to transcribe antisense or sense RNA probes. The plasmids were linearized by *SmaI* digestion and purified by phenol-chloroform-isoamyl alcohol extraction. A transcription kit (Roche) was used to transcribe digoxigenin-labeled cRNA probes. Linearized DNA (1 µg/mL) was incubated for 2 hours at 37°C in a solution containing transcription buffer, nucleotide triphosphate-labeling mixture, Sp6 or T7 RNA polymerase, and RNase inhibitor. The reaction was stopped by digesting the DNA template with RNase-free DNase. The RNA probes were precipitated with LiCl and 100% ice-cold etomidate overnight at -70°C, then pelleted by centrifugation at 12,000 rpm at 4°C, and additionally washed with 70% etomidate. The probes were then dried under vacuum and dissolved in 0.1% diethyl pyrocarbonate water containing 2 mmol/L EDTA, and their concentration was adjusted to 100 ng/µL. The digoxigenin-labeled RNA probes were stored at -80°C.

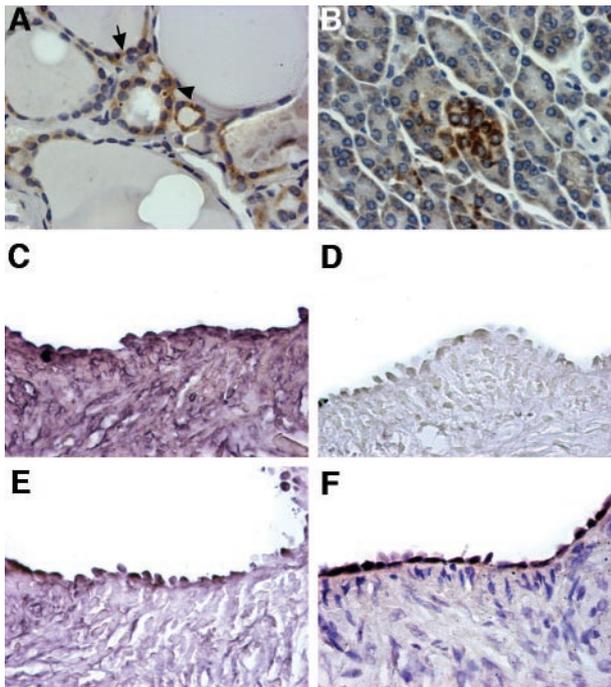
**ISH.** ISH was carried out following the procedure described by Xu Peng *et al.* (5). Briefly, the sections were deparaffinized, rehydrated, and deproteinized. The slides were then prehybridized at 42°C for 1 hour in Slide Mount Model 240000 (Boekol Scientific, Feasterville, PA) and incubated (50 µL/slide) with hybridization solution containing freshly denatured digoxigenin-labeled cRNA probe (0.625 µg/mL) at 42°C for 4 hours. The sections were washed twice in 2× SCC and additionally washed in 2× SCC containing 2% normal sheep serum and 0.05% Triton X-100 for 2 hours with mild agitation. For the immunodetection of the *in situ* hybridization signal, the slides were incubated in 0.1 mol/L maleic acid-0.15 mol/L NaCl (pH 7.5; buffer 1) containing 2% normal sheep serum and 0.3% Triton X-100 for 30 minutes at 23°C and additionally incubated overnight at 4°C with sheep anti-digoxigenin antibody (0.75 µg/mL in buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100). The slides were then washed twice in buffer 1 and then with buffer 2, which consisted of 0.1 mol/L Tris-0.1 mol/L NaCl-MgCl<sub>2</sub> (pH 9.5). The color reactions were developed by incubating the slides in a chromogen solution (45 µL of nitroblue tetrazolium and 35 µL of X-phosphate solution in 10 mL of buffer 2) in humidified light-tight containers for up to 6 hours, with observation for color development. The color reaction was stopped by washing the slides with Tris-EDTA buffer, and the slides were mounted with a cover glass in Aqua mounting medium. The specificity of the method was confirmed by the negative results obtained with corresponding sense controls, and the distinct positive staining patterns obtained with digoxigenin-labeled GAPDH mRNA antisense probes described above.

**Statistical Analysis.** Differences in proportions were evaluated by the  $\chi^2$ , Fisher's exact test of the Spearman correlation, or Mann-Whitney *U* test as appropriate. The McNemar  $\chi^2$  test was used to compare discordance between ARHI immunostaining and ISH. Disease-specific survival rates and progression-free survival were calculated using the method of Kaplan and Meier and compared by the log-rank test. The Cox proportional hazards regression model was used for multivariate analysis of survival. Statistica for windows 6.0 software (Statsoft, Inc., Tulsa, OK) was used for the statistical analysis. Results were considered statistically significant at the  $P < 0.05$  level.

## RESULTS

**ARHI Protein Is Widely Distributed in Normal Organs and Cells.** ARHI protein was expressed in epithelial cells from each of five specimens of fallopian tube, uterus, breast, kidney, stomach, small intestine, and adrenal gland judged by immunohistochemistry (IHC). Thyroid and testis showed partial expression in two of five control sections, whereas pancreas and brain expressed ARHI protein in 1 of 5. (Fig. 1, A and B). Weak or no expression was found in spleen, prostate, lung, heart, and liver. ARHI expression was more intense in the cytoplasm of epithelial cells and rarely present in the nucleus where staining exhibited a membranous reinforcement.

**ARHI Is Expressed in Normal Ovaries and Benign and Borderline Lesions.** The surface epithelium from all seven normal ovaries strongly expressed ARHI, judged both by IHC and ISH. GAPDH served as the positive control for ISH



**Fig. 1** Expression of ARHI in normal tissue and normal ovarian surface epithelial cells. *A*, expression of ARHI in normal follicular (arrow) and parafollicular cells (arrowhead) of the thyroid ( $\times 40$ ). *B*, focal expression of ARHI in pancreatic acini ( $\times 40$ ). *C*, ARHI mRNA expression by normal ovarian surface epithelial cells detected using ISH with an antisense probe ( $\times 40$ ). *D*, negative reaction with an ARHI sense probe ( $\times 40$ ). *E*, positive reaction with a GAPDH antisense probe ( $\times 40$ ). *F*, ARHI protein expression by normal ovarian epithelial cells detected using IHC and an anti-ARHI antibody ( $\times 40$ ).

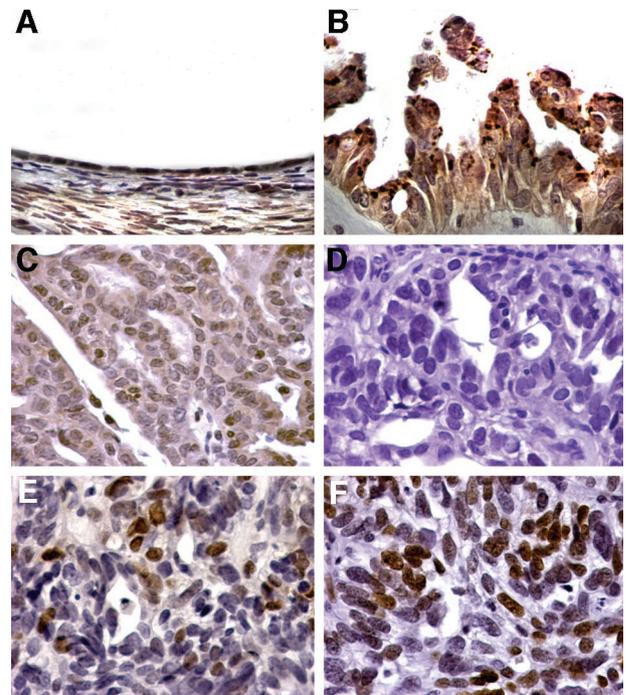
and ARHI sense serve as a negative control for ISH. Strong ARHI expression was also found with IHC and ISH in the cysts and follicles of normal ovaries, as well as in the epithelia of the fallopian tube (Fig. 1C–F). Overall, benign ovarian lesions showed greater ARHI expression than did serous or mucinous borderline tumors of low malignant potential (Fig. 2, A and B). ARHI was strongly expressed in 90 to 91% of serous and mucinous cystadenomas. ARHI protein expression was decreased in 8 of 10 mucinous tumors (80%) of low malignant potential and lost in 3 of 10 (30%). Similarly, ARHI protein expression was down-regulated, but not lost, in 12 of 22 (54%) serous borderline tumors. Consequently, loss of ARHI was characteristic of a fraction of borderline tumors that proliferate but generally do not invade or metastasize. (Fig. 3A).

**ARHI Protein Expression Varies between Histotypes of Invasive Ovarian Cancers but Does Not Correlate with Stage or Grade.** ARHI protein expression by IHC is analyzed with regard to pathological characteristic of different cancers in Table 1. ARHI expression was down-regulated in 63% (256 of 407) of ovarian cancer specimens and was not detectable in 47% (191 of 407) of these cases. Conversely, 37% (151 of 407) of ovarian cancer samples maintained levels of ARHI expression similar to those in normal ovarian

epithelial cells. The highest expression for ARHI was found among endometrioid (56%) and clear cell carcinomas (53%), whereas the lowest expression was associated with mucinous (12%), transitional cell carcinoma (14%), and serous carcinomas (32%). No correlation was found between ARHI and International Federation of Gynecology and Obstetrics stage, tumor grade, or debulking surgery status.

**ARHI Protein Expression Correlates with Prolonged Progression-free Survival.** Relapse during the course of the disease was associated with low levels of ARHI protein expression ( $P = 0.009$ ; Table 1). Association of ARHI protein expression with disease-specific survival is detailed in Fig. 3A. A trend toward longer overall survival was seen in patients with up-regulation of ARHI. Five-year survival of 39% was observed in patients whose tumors had strong expression of ARHI, 43% in those with weak expression, compared with 33% in those with negative expression. This difference, however, did not achieve statistical significance ( $P = 0.82$ ).

When time to progression (disease-free survival) was compared with ARHI protein expression (Fig. 3, B and C), results of the univariate analysis showed that patients with positive expression of ARHI lived longer without recurrence than those with negative expression. After 5 years, 82% of the patients whose tumors had lost ARHI expression had experienced dis-



**Fig. 2** ARHI protein expression in ovarian lesions. *A*, strong reactivity of a serous cystadenoma stained with anti-ARHI antibody. ( $\times 40$ ). *B*, strong reactivity of a serous tumor of low malignant potential stained with anti-ARHI antibody ( $\times 40$ ). *C*, weak reactivity of a low-grade serous carcinoma stained with anti-ARHI antibody ( $\times 40$ ). *D*, lack of reactivity in a high-grade serous carcinoma stained with anti-ARHI antibody. *E*, same case as Fig. 1D showing reactivity with antibodies against cyclin D1. *F*, same case as Fig. 1D showing reactivity with antibodies against p21<sup>WAF1/CIP1</sup>.

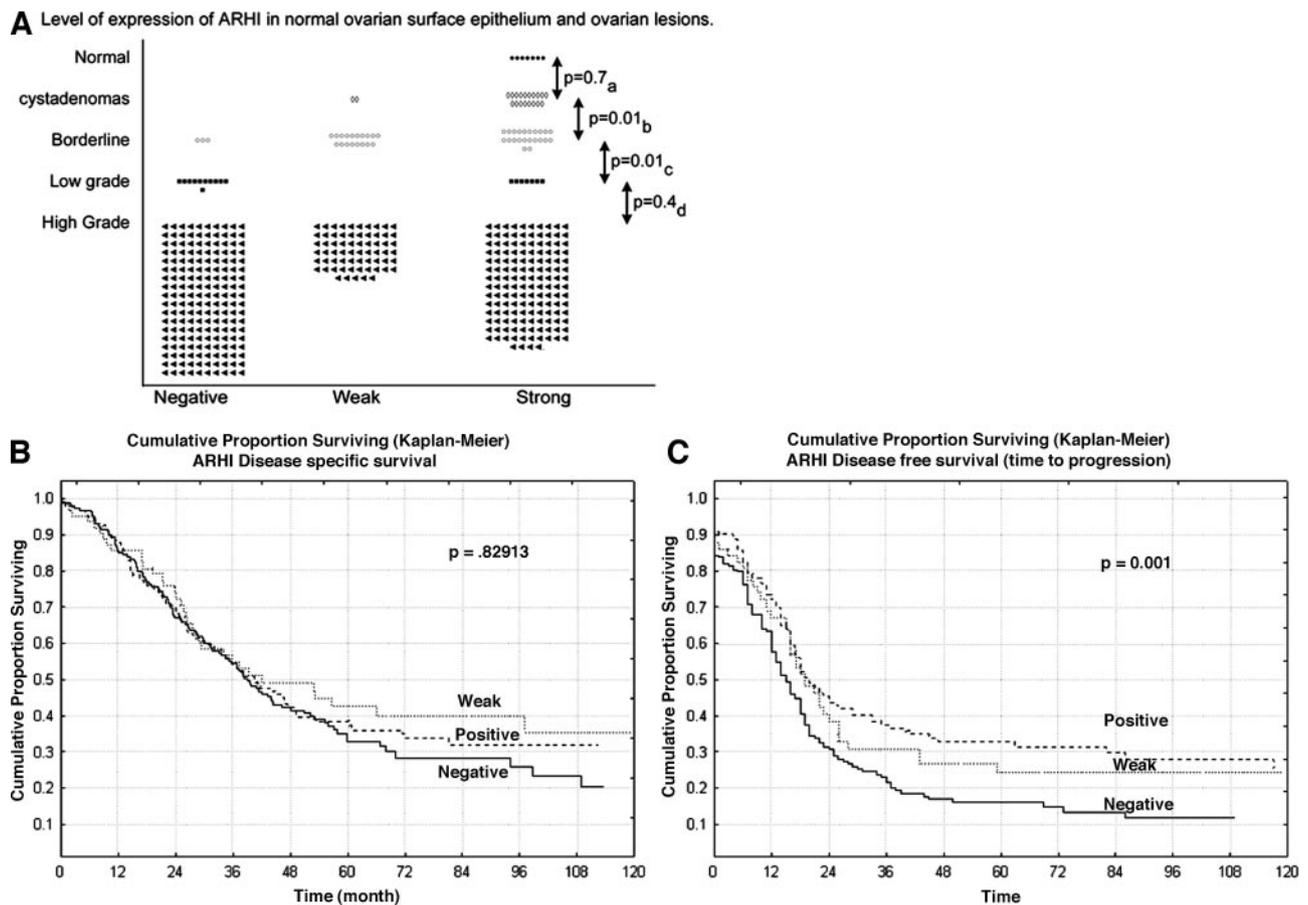


Fig. 3 A, level of expression of ARHI in normal ovarian surface epithelium and ovarian lesions. *P* values derive from Mann-Whitney *U* test to compare the differences between (a) normal ovarian surface epithelium and cystadenomas, (b) cystadenomas and borderline tumors, (c) borderline tumors and low-grade carcinomas, and (d) low-grade and high-grade carcinomas. B, ARHI overall survival using Kaplan-Meier analysis. C, ARHI disease-free survival using Kaplan-Meier analysis.

ease recurrence, compared with 66% of those with positive expression ( $P = 0.001$ ). ARHI achieved statistical significance in the multivariate analysis as a predictor of progression-free survival with tumor stage, grade, and debulking surgery status (Table 2).

**Loss of ARHI Protein Expression Can Occur in the Presence of ARHI mRNA.** To confirm the pattern of ARHI expression in ovarian cancer, we performed ISH and IHC in 322 cases of ovarian carcinoma (Table 3A). A total of 184 cases could be analyzed both for ISH and IHC. The remaining cases were either lost during the procedure, had  $<20\%$  of tumor cells, or did not bind GAPDH antisense probes, indicating that the mRNA was degraded. The ARHI sense probes did not show positive staining, indicating that the hybridization of the antisense probe was specific. ARHI mRNA was detected by ISH in lymphocytes, blood vessels, fibroblasts, and stromal cells, as well as in epithelial cells. Overall, positive ISH was found in 89% of the cancers. When ISH and IHC were compared, strong ARHI protein and RNA expression were detected in 68 of 184 cases (37%). ARHI protein was down-regulated in 96 of the 184 cases (52%), despite the presence of ARHI RNA by ISH.

**ARHI Protein Expression in Ovarian Carcinomas Correlates with p21<sup>WAF1/CIP1</sup> Expression but not with Cyclin D1 Expression.** Correlation between p21<sup>WAF1/CIP1</sup>, cyclin D1 and ARHI are summarized in Table 3B. Low expression of ARHI was found in 76% of patients with low p21<sup>WAF1/CIP1</sup> expression. Patients with high expression of ARHI show also high expression of p21<sup>WAF1/CIP1</sup> ( $P = 0.0074$ ). ARHI appeared to correlate inversely with cyclin D1 in that 70% of patients with negative expression of ARHI had high expression of cyclin D1, whereas 34% of those with increased expression of ARHI had low labeling indices for cyclin D1. However, no statistically significant correlation was found between ARHI and cyclin D1 ( $P = 0.84$ ).

## DISCUSSION

Existing data suggest that, in common to other types of tumors, ovarian oncogenesis is a multistep process during which oncogenes are activated and the function of tumor suppressor genes is lost (18). ARHI is a putative tumor suppressor gene that is expressed by normal ovarian epithelial cells. Expression of ARHI is lost in a majority of ovarian

Table 1 ARHI expression in ovarian cancers of different histotypes, stages, and grades

	ARHI Expression		
	Negative	Weak	Strong
<b>Histotype</b>			
Clear cell carcinoma	4	3	8 (53%)
Endometrioid	11	6	22 (56%)
Undifferentiated	3	4	5 (42%)
Malignant Mullerian mixed tumor	5	1	5 (45%)
Mixed	39	14	36 (40%)
Mucinous	6	1	1 (12%)
Serous	118	35	73 (32%)
Transitional cell	5	1	1 (14%)
Total	191	65	151 (37%)
	$P = 0.0001$		
<b>International Federation of Gynecology and Obstetrics stage</b>			
Stage I	13	4	18
Stage II	7	8	14
Stage III	137	41	86
Stage IV	34	12	33
Total	191	65	151
	$P = 0.1615$		
<b>Tumor grade</b>			
Low	11	0	7
Intermediate	11	4	16
High	169	61	128
Total	191	65	151
	$P = 0.2052$		
<b>Debulking surgery status</b>			
Optimal	84	34	82
Suboptimal	107	31	69
Total	191	65	151
	$P = 0.14$		
<b>Responders to primary therapy</b>			
No recurrence	40	18	51 (47%)
Recurrence	114	31	76 (34%)
Nonresponders to primary therapy	31	12	21 (32%)
Outcome unknown	6	4	3
Total	191	65	151
	$P = 0.0095$		

cancer cell lines. Reintroduction of the gene inhibits cell growth, slows motility, prevents invasion, and induces apoptosis. At a molecular level, ARHI reexpression blocks heregulin stimulation, truncates epidermal growth factor-mediated signaling through mitogen-activated protein kinase, activates c-Jun NH<sub>2</sub>-terminal kinase, induces p21<sup>WAF1/CIP1</sup>, and down-regulates cyclin D1 promoter activity (4). After

expression of ARHI, cancer cells die through caspase-independent, calpain-dependent apoptosis (10).

In the present article, widespread expression of ARHI was detected in multiple normal tissues, consistent with the possibility that this gene might have an important role in cell growth regulation in many different organs. In the female reproductive organs, ARHI message and protein were detected in endometrium, fallopian tube, and ovarian surface epithelium. ARHI expression was maintained in >90% of benign ovarian cystadenomas but decreased in 48% of tumors of low malignant potential. ARHI expression was lost, however, in only 7% of borderline cancers. In frankly malignant invasive ovarian cancers, ARHI protein was down-regulated in 63%, and expression of the gene was lost altogether in 47%. Thus, down-regulation of ARHI correlated with the malignant potential of ovarian neoplasms. Similar results have been observed with neoplasms of the breast. ARHI is expressed in normal breast epithelial cells, and loss of ARHI expression has been linked to tumor progression from *in situ* to invasive breast cancer (6).

In studies with ovarian and breast cancer cell lines, decreased expression of ARHI protein has generally corre-

Table 3 A comparison between ARHI IHC and ISH

A. IHC and ISH			
ARHI ISH	IHC		Total
	Down-regulated	Expressed	
Down-regulated	15 (8.1%)	5 (2.7%)	20
Expressed	96 (52.1%)	68 (36.9%)	164
Total	111	73	184
B. Correlation of ARHI, p21 <sup>WAF1/CIP1</sup> , and cyclin D1 expression			
	ARHI expression		
	Negative	Weak	Strong
p21 <sup>WAF1/CIP1</sup>			
<2%	142 (76.7%)	36 (61%)	90 (62.5%)
>2%	43 (23.2%)	23 (38.9%)	54 (37.5%)
	$P = 0.0074$		
Cyclin D1			
Negative	42 (31.3%)	16 (35.5%)	38 (33.9%)
Positive	92 (68.6)	29 (64.4%)	74 (66.0%)
	$P = 0.84$		

NOTE. McNemar  $\chi^2 = 32.58$ ,  $P < 0.00001$ .

Table 2 Disease-free survival: multivariate analysis using Cox proportional hazard method

	Univariate		Multivariate	
	Hazard ratio (95% CI)	$P^*$	Hazard ratio (95% CI)	$P^*$
ARHI	0.7 (0.8–0.5)	0.001	0.82 (1.0–0.6)	0.05
Tumor grade	0.7 (1.4–0.4)	0.41	0.45 (0.8–0.2)	0.01
International Federation of Gynecology and Obstetrics stage	4.7 (7.7–2.9)	<0.00001	4.7 (7.8–2.8)	<0.00001
Debulking status	1.4 (1.8–1.1)	0.004	1.1 (1.4–0.8)	0.3

\*  $P$  values were derived from the Cox proportional-hazard model, with simultaneous inclusion of all factors shown. Abbreviation: CI, confidence interval.

lated with decreased expression of ARHI mRNA. Transcriptional down-regulation can occur through multiple mechanisms. As a maternally imprinted gene, ARHI is transcribed only from the paternal allele. Loss of heterozygosity that preferentially affects the paternal allele has been observed in 40% of ovarian and breast cancers (4). Silencing of the paternal allele by methylation of CpG islands in the promoter region has been observed in breast cancers (7) but not in ovarian neoplasms. Transcriptional down-regulation of the ARHI promoter has been observed in cancers from both disease sites. In studies of breast cancers, a consistent correlation has been found between the expression of ARHI by IHC and ISH (6). A similar association was found in this study ( $P < 0.0001$ ), but a significant number of cases were found in which ARHI mRNA could be detected despite low protein expression. This raises the intriguing possibility that posttranscriptional regulation of ARHI might occur in a fraction of ovarian cancers.

Previous reports show that expression of ARHI induces p21<sup>WAF1/CIP1</sup> (4). The p21<sup>WAF1/CIP1</sup> gene is a transcriptional target of p53 and is involved in senescence (19), differentiation (20, 21) and in the coordination of DNA damage repair with cell cycle arrest (22, 23). Expression of p21<sup>WAF1/CIP1</sup> can also be induced through p53-independent pathways (24). Expression of p21<sup>WAF1/CIP1</sup> in ovarian cancers has been associated with prolonged survival, whereas lack of expression related to higher risk of recurrence disease in ovarian carcinoma (25, 26). In the present study, we show a high correlation between these two markers ( $P < 0.001$ ), consistent with the possibility that ARHI inhibits epithelial proliferation and suppresses tumor progression.

Cyclin D1 is a cell cycle-regulatory protein that plays a critical role in the growth and progression of several types of human cancer; however, its significance in ovarian carcinoma still remains controversial. In cell culture, expression of ARHI decreased cyclin D1 promoter activity (4). In this study, we compared the expression of cyclin D1 and ARHI in ovarian cancers taken directly from patients and found no correlation. The lack of correlation might relate to amplification or regulation of cyclin D1 independent of the influence of ARHI.

Using Kaplan-Meier analysis, ARHI expression correlated directly with disease-free survival (time to relapse), although correlation with overall survival did not achieve statistical significance. In predicting disease-free survival, ARHI expression retained significance with tumor grade and stage on multivariate analysis. Correlation with disease-free survival raises the interesting possibility that ARHI expression might affect the sensitivity of ovarian cancer cells to platinum-based chemotherapy. Studies of the interaction of ARHI expression with sensitivity to cytotoxic drugs are under way.

Loss of the function of several genes is thought to contribute to the progression of ovarian cancers (27). Among the tumor suppressor genes studied to date in ovarian cancer, abnormalities of p53 have been shown to occur most frequently with mutations found in at least 60% of cases (28). ARHI is down-regulated in a similar fraction of cases and lost in 43%. Expression of ARHI has been shown to inhibit growth of ovarian cancer cells independent of p53 status. Consequently, loss of

ARHI appears to be one of the most prevalent abnormalities associated with ovarian cancer progression.

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