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Low Expression of *ARHI* Is Associated with Shorter Progression-Free Survival in Pancreatic Endocrine Tumors[1*]

Irene Dalai, Edoardo Missiaglia†, Stefano Barbi†, Giovanni Butturini*, Claudio Doglioni‡, Massimo Falconi* and Aldo Scarpa†*

Departments of *Surgical and Gastroenterological Sciences and †Pathology, University of Verona, Verona, Italy; ‡Department of Pathology, San Raffaele Hospital, Milan, Italy

Abstract

Little is known about the molecular anomalies involved in the development and progression of malignancy of pancreatic endocrine tumors (PETs). A recently identified member of the Ras family, Ras homologue member I (*ARHI*), has been shown to be involved in breast, ovary, and thyroid carcinogenesis. Unlike other members, it acts as a tumor suppressor gene that inhibits cell growth. Here we analyzed the mRNA expression of *ARHI* in 52 primary PETs and 16 normal pancreata using quantitative reverse transcription-polymerase chain reaction. *ARHI* expression showed a statistically significant difference between either normal pancreas or well-differentiated endocrine tumors (WDET) and poorly differentiated endocrine carcinomas (PDECs) (*P* < .001 and *P* < .001, respectively). Moreover, *ARHI* expression among WDEC samples was more heterogeneous than in WDET, with several tumors showing level of expression analogous to that observed in PDECs. A significant correlation between lower *ARHI* expression and shorter survival (*P* = .020) was identified, and a low *ARHI* expression was associated to a shorter time to progression (*P* < .001), even considering the proliferation index Ki67 in the multivariate analysis. *ARHI* is involved in PET progression. Its mRNA expression seemed to be a prognostic factor for disease outcome and, in association with the proliferative index Ki67, a predictor for a rapid tumor relapse.

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Keywords: ARHI, pancreatic endocrine tumor, tumor differentiation, survival, time to progression.

Introduction

Pancreatic endocrine tumors (PETs) are a heterogeneous group of neoplasms that are clinically classified as functioning or nonfunctioning according to the presence of symptoms due to hormone hypersecretion. According to the World Health Organization classification [1], PETs, whether functioning or not, are classified into three categories: 1) well-differentiated endocrine tumors (WDETs), 2) well-differentiated endocrine carcinomas (WDECs), and 3) poorly differentiated endocrine carcinomas (PDECs). The WDET category is further distinguished into two subgroups with either benign or uncertain clinical behavior [1]. Carcinomas are characterized by the presence of invasion or metastases, and among them PDECs are highly aggressive and show a poorer outcome compared with WDECs [1]. To date, little is known about the molecular pathways and anomalies involved in PET development and progression [2].

Ras homologue member I (*ARHI*) is one of the first identified tumor suppressors belonging to the Ras superfamily. It encodes a 26-kDa GTPase with 50% to 60% amino acid homology to Ras but exerts opposite functions that inhibit cell growth, motility, and invasion [3–5]. Notably, it has been shown that *ARHI* underexpression correlated with breast tumor progression as well as reduced progression-free survival in ovarian cancer [6,7].

In the present work, we analyzed the expression of *ARHI* in 52 PETs and 16 normal pancreata to assess possible clinical-pathological correlations with tumor differentiation, proliferation rate, and clinical behavior including time to progression (TTP) and survival.

Materials and Methods

Materials

The study involved 52 primary PETs (22 WDETs, 26 WDECs, and 4 PDECs), obtained from patients that underwent either explorative or radical surgery with their informed consent

Abbreviations: PET, pancreatic endocrine tumor; WDET, well-differentiated endocrine tumor; WDEC, well-differentiated endocrine carcinoma; PDEC, poorly differentiated endocrine carcinoma; TTP, time to progression

Address all correspondence to: Aldo Scarpa, Department of Pathology, University of Verona, Strada Le Grazie 8, 37134 Verona, Italy. E-mail: aldo.scarpa@univr.it

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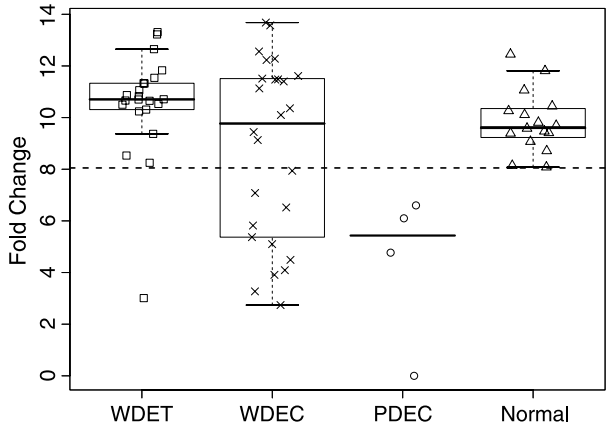

Figure 1. *Box and whiskers plot of* ARHI *mRNA expression in 52 PETs and 16 normal pancreatic samples. Dotted line indicate the cutoff level used to distinguish samples with low (below the line) and normal (above the line)* ARHI *mRNA expression. WDET, well-differentiated tumor; WDEC, well-differentiated carcinoma; PDEC, poorly differentiated carcinoma.*

(Table W1), and 16 normal samples (Table W2). These included 12 pancreatic bulk tissues and four human pancreatic islets of Langerhans cell preparations [8]. PETs were diagnosed by histopathological and cell marker analysis and classified according to World Health Organization criteria [1].

Total RNA was prepared from 10 to 20 cryostat sections (40 μm thick) of snap-frozen tissue, checking the cellularity every four sections. Tissue sections were placed in 4 M guanidine thiocyanate containing 0.1 M 2-mercaptoethanol and centrifuged through a $CsCl_2$ gradient. RNA quality was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). cDNA was synthesized from 1 μg of total RNA using random primers and the Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Quantitative Reverse Transcription Polymerase Chain Reaction

Quantitative Reverse Transcription polymerase chain reaction (PCR) mRNA expression analysis was performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Oligonucleotide primers used were the following: ARHI-F, AGAAAGGGGTCTCCTGCTG, and ARHI-R, GCAGCTTCTGTTCCTTGGAG; β-actin-F, GGAGTCCTGTGGCATCCACG, and β-actin-R, CTAGAAGCATTTGCGGTGGA. β-Actin transcript level (RefSeq ID NM_001101) was used to normalize the samples.

Calibration curves of each couple of primers were obtained by serial dilution of cDNA. Expression data were analyzed by the comparative threshold cycle (Ct) method accordingly to User Bulletin No. 2 (Applied Biosystems). Results were expressed in terms of the ΔΔCt value and obtained as follows: $\Delta Ct_{ARHI} = Ct_{ARHI} - Ct_{\beta\text{-actin}}$ and $\Delta\Delta Ct_{ARHI} = ||\Delta Ct_{ARHI} - \max(\Delta Ct_{ARHI})||$, where Ct_{ARHI} and $Ct_{\beta\text{-actin}}$ represent the comparative threshold cycles for *ARHI* and β-actin, respectively. All experiments were performed in duplicate.

Time to Progression and Survival Evaluation

Patients were monitored every 3- months with contrast-enhanced CT scan, clinical evaluation of symptoms and body weight, and measurement of blood parameters and tumor markers. Time to progression was defined as the interval from the surgery until disease progression, as previously described [9]. Survival was calculated from the date of diagnosis to the date of death, and only deaths from the disease were considered. *ARHI* expression was categorized by applying a cutoff level defined as the lowest ΔΔCt measured in the normal control samples. Therefore, tumors with ΔΔCt below this cutoff were considered to have a low *ARHI* expression, whereas those with ΔΔCt above the cutoff were considered as having a normal level of expression.

Statistical Analysis

The statistical significance of the differences between subgroups was investigated by either logistic regression analysis or the Mann-Whitney test. Survival and TTP data were analyzed using Kaplan Meier function and the log-rank test for univariate analyses or Cox proportional hazard regression. All *P* values were two sided and considered significant when less than .05. All the analyses were performed using R software v. 2.1.1 and Survival package (http://www.R-project.org).

Results and Discussion

The Ras homologue member I (*ARHI*) is a tumor suppressor gene and a member of the Ras family that is able to negatively regulate the Ras/mitogen-activated protein kinases (MAPK) signaling pathway inhibiting cancer cell growth [3]. Wang et al. have shown that *ARHI* was frequently downregulated in breast carcinoma and was negatively associated with tumor progression [7]. More interestingly, *ARHI* expression was associated with expression of $p21^{WAF/CIP1}$ and prolonged disease-free survival in epithelial ovarian cancer [6]. In addition, it has been shown that *ARHI* silencing could contribute to the carcinogenesis of follicular thyroid carcinomas, which are the most common endocrine cancers [10].

We analyzed the mRNA level of *ARHI* by quantitative real-time PCR in 52 PETs to identify possible correlations with clinical-pathological parameters (Tables W1 and W3). Logistic regression analysis identified a significant correlation between *ARHI* expression and tumor differentiation (odds ratio, 0.916; 95% confidence interval, 0.894–0.938; $P < .001$). In fact, PDEC samples showed a significant lower expression

Table 1. Multivariate Analysis of TTP in PETs.

	Hazard Ratio (95% Confidence Interval)	*P*
Low *ARHI* expression	4.0 (1.5–11.1)	.007
Ki67 ≥5%	8.3 (2.8–24.8)	.0002

Hazard ratio represents an estimate of the ratio of mortality rate in patients with low *ARHI* expression versus patients with normal *ARHI* expression, or patients with proliferation index Ki67 <5% versus patients with Ki67 ≥5%. Cox proportional hazard model was used with simultaneous inclusion of all factors shown.

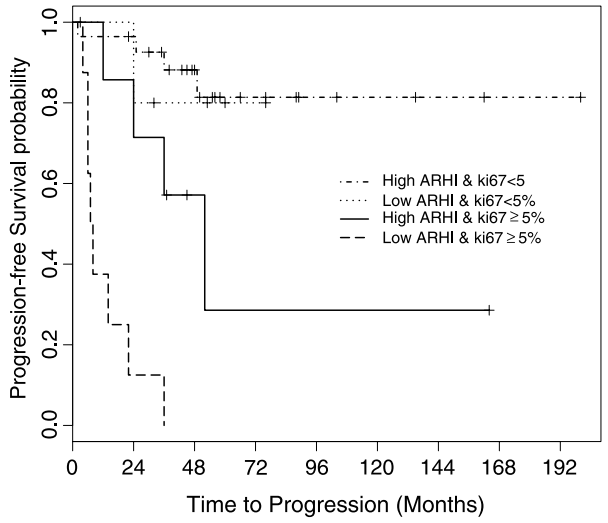

Figure 2. *Progression free-survival curve; 49 patients with high or low* ARHI *mRNA expression and Ki-67 proliferation index less than or greater than 5% were considered.*

of *ARHI* compared with normal pancreas and WDET (Mann-Whitney test, $P < .001$ and $P < .001$, respectively), whereas no significant difference was observed between WDET and WDEC. However, the expression of *ARHI* among the WDEC samples was heterogeneous, with several tumors showing levels of expression analogous to those observed in PDEC samples (Figure 1). Notably, patients with WDEC with low *ARHI* expression showed a median TTP of 30 months versus 49 months observed in WDEC patients with normal *ARHI* expression, although this difference was not statistically significant.

The correlation between *ARHI* expression and survival or TTP was tested in 47 and 49 patients, respectively, for which these data were available. A shorter survival was associated with tumor differentiation ($P < .001$), low *ARHI* expression ($P = .020$; Figure W1) and high proliferative index (Ki67 · 5%; $P < .001$). The association between survival and Ki67 remained significant when PDECs were excluded from the analysis ($P = .016$), confirming the reported prognostic value of proliferative index [1,11]. Similarly, a shorter TTP was associated with tumor differentiation ($P < .001$), reduced *ARHI* expression ($P < .001$; Figure W2), and high proliferative index ($P < .001$). In addition, shorter TTP was found to be associated with low *ARHI* expression ($P = .036$) and high proliferation index ($P = .001$) when only WDET and WDEC were included in the analysis.

Notably, multivariate analysis showed that *ARHI* expression was an independent prognostic factor for TTP in association with the proliferative index Ki67 (Cox proportional hazard; Table 1, Figure 2), which is the most informative molecular marker of PET outcome [11]. In particular, patients with low *ARHI* expression showed a significantly higher hazard ratio for disease progression compared with those with normal *ARHI* expression. Moreover, all the seven patients with low *ARHI* expression and high proliferation index showed tumor progression after a shorter time.

These findings suggest that *ARHI* is involved in PET progression. In addition, *ARHI* mRNA level seems to be a prognostic factor for disease outcome and, in association with the proliferative index Ki67, a predictor of a rapid tumor relapse.

References

[1] Kloppel G, Perren A, and Heitz PU (2004). The gastroenteropancreatic neuroendocrine cell system and its tumors: the WHO classification. *Ann N Y Acad Sci* **1014**, 13–27.

[2] Perren A, Komminoth P, and Heitz PU (2004). Molecular genetics of gastroenteropancreatic endocrine tumors. *Ann N Y Acad Sci* **1014**, 199–208.

[3] Luo RZ, Fang X, Marquez R, Liu SY, Mills GB, Liao WS, Yu Y, and Bast RC (2003). ARHI is a Ras-related small G-protein with a novel N-terminal extension that inhibits growth of ovarian and breast cancers. *Oncogene* **22**, 2897–2909.

[4] Yu Y, Luo R, Lu Z, Wei Feng W, Badgwell D, Issa JP, Rosen DG, Liu J, and Bast RC Jr (2005). Biochemistry and biology of ARHI (DIRAS3), an imprinted tumor suppressor gene whose expression is lost in ovarian and breast cancers. *Methods Enzymol* **407**, 455–468.

[5] Yu Y, Xu F, Peng H, Fang X, Zhao S, Li Y, Cuevas B, Kuo WL, Gray JW, Siciliano M, et al. (1999). NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. *Proc Natl Acad Sci USA* **96**, 214–219.

[6] Rosen DG, Wang L, Jain AN, Lu KH, Luo RZ, Yu Y, Liu J, and Bast RC Jr (2004). Expression of the tumor suppressor gene ARHI in epithelial ovarian cancer is associated with increased expression of p21WAF1/CIP1 and prolonged progression-free survival. *Clin Cancer Res* **10**, 6559–6566.

[7] Wang L, Hoque A, Luo RZ, Yuan J, Lu Z, Nishimoto A, Liu J, Sahin AA, Lippman SM, Bast RC Jr, et al. (2003). Loss of the expression of the tumor suppressor gene ARHI is associated with progression of breast cancer. *Clin Cancer Res* **9**, 3660–3666.

[8] Piemonti L, Leone BE, Nano R, Saccani A, Monti P, Maffi P, Bianchi G, Sica A, Peri G, Melzi R, et al. (2002). Human pancreatic islets produce and secrete MCP-1/CCL2: relevance in human islet transplantation. *Diabetes* **51**, 55–65.

[9] Butturini G, Bettini R, Missiaglia E, Mantovani W, Dalai I, Capelli P, Ferdeghini M, Pederzoli P, Scarpa A, and Falconi M (2006). Predictive factors of efficacy of the somatostatin analogue octreotide as first line therapy for advanced pancreatic endocrine carcinoma. *Endocr Relat Cancer* **13**, 1213–1221.

[10] Weber F, Aldred MA, Morrison CD, Plass C, Frilling A, Broelsch CE, Waite KA, and Eng C (2005). Silencing of the maternally imprinted tumor suppressor ARHI contributes to follicular thyroid carcinogenesis. *J Clin Endocrinol Metab* **90**, 1149–1155.

[11] Pelosi G, Bresaola E, Bogina G, Pasini F, Rodella S, Castelli P, Iacono C, Serio G, and Zamboni G (1996). Endocrine tumors of the pancreas: Ki-67 immunoreactivity on paraffin sections is an independent predictor for malignancy: a comparative study with proliferating-cell nuclear antigen and progesterone receptor protein immunostaining, mitotic index, and other clinicopathologic variables. *Hum Pathol* **27**, 1124–1134.

Table W1. Supplementary PET Clinical-Pathological Data and *ARHI* mRNA Expression.

Sample	Tumor Type	Diagnosis	Follow-up (months)	Progression*	TTP* (months)	Outcome	Ki67 (%)	*ARHI* $\Delta\Delta Ct^{\dagger}$
1	NF	WDEC	58	Yes	52	DOD	5	12.24
2	NF	PDEC	12	Yes	6	DOD	30	6.61
3	NF	WDEC	73	Yes	36	AWD	10	7.94
4	NF	WDET	88	No		AW	2	10.87
5	NF	WDEC	49	Yes	22	AWD	8	4.49
6	F	WDET	50	No		AW	2	10.31
7	F	WDEC	25	Yes	12	DOD	17	10.10
8	NF	WDET	89	No		AW	2	11.33
9	NF	WDEC	71	Yes	14	DOD	7	4.09
10	NF	WDET	48	No		AW	2	10.53
11	NF	WDEC	31	Yes	8	AWD	5	2.74
12	NF	WDEC	58	No		AW	3	13.57
13	F	WDET	48	No		AW	1	10.65
14	NF	WDEC	32	Yes	25	AWD	3	11.40
15	NF	PDEC	6	Yes	6	DOD	40	6.10
16	NF	WDEC	38	No		AW	2	10.36
17	NF	WDEC	91	Yes	36	DOD	2	11.47
18	NF	WDEC	37	No		AW	5	11.51
19	F	WDET	47	No		AW	1	10.50
20	NF	WDET	76	No		AW	1	10.71
21	NF	WDET	66	No		DOC	2	10.83
22	F	WDEC	45	No		AW	3	9.13
23	NF	WDET	25	No		AW	2	9.37
24	NF	WDET	22	No		AW	2	8.53
25	NF	WDET	30	No		AW	1	10.24
26	NF	WDEC	38	No		AW	2	11.48
27	NF	WDET	43	No		AW	2	13.22
28	NF	WDEC	na	na	na	na	2	7.09
29	NF	WDEC	45	No		AW	5	12.56
30	NF	WDET	162	No		AW	1	11.32
31	NF	WDEC	32	No		AW	2	3.91
32	NF	WDEC	68	Yes	24	DOD	17	11.13
33	NF	WDEC	76	No		AW	2	5.82
34	NF	WDET	56	No		AW	2	12.65
35	NF	PDEC	13	Yes	4	DOD	25	4.77
36	NF	WDEC	4	Yes	2	DOD	1	11.61
37	F	WDET	55	No		AW	2	10.84
38	F	WDET	200	No		AW	2	11.83
39	NF	WDEC	55	Yes	36	DOD	5	9.45
40	NF	WDET	60	No		AW	1	3.01
41	NF	WDEC	na	na	na	na	2	3.27
42	NF	PDEC	13	Yes	7	DOD	40	0.00
43	NF	WDEC	55	Yes	24	DOD	2	5.38
44	NF	WDET	164	No		AW	5	13.32
45	NF	WDEC	3	No		DOC	3	5.10
46	NF	WDET	135	No		AW	1	11.06
47	F	WDET	35	No		AW	2	11.54
48	NF	WDET	22	No		AW	2	10.66
49	NF	WDEC	53	No		AW	2	6.52
50	NF	WDEC	104	Yes	104	AW	3	12.28
51	NF	WDEC	91	Yes	49	AWD	2	13.68
52	NF	WDET	na	na	na	na	1	8.29

NF, nonfunctioning; F, functioning; WDET, well-differentiated endocrine tumor; WDEC, well-differentiated endocrine carcinoma; PDEC: poorly differentiated endocrine carcinoma; na, not available; AW, alive and well; AWD, alive with disease; DOD, dead of disease; DOC, dead of other cause.

*Time to progression calculated as the interval from surgery to disease progression.

$^{\dagger}\Delta\Delta Ct_{ARHI} = \left|\left|\Delta Ct_{ARHI} - \max(\Delta Ct_{ARHI})\right|\right|$, where Ct_{ARHI} and $Ct_{\beta\text{-actin}}$ represent the comparative threshold cycle for *ARHI* and β-actin, respectively.

Table W2. *ARHI* mRNA Expression in Normal Pancreatic Bulk Tissues and Islets.

Sample*	*ARHI* ΔΔCt†
N1	11.06
N2	9.07
N3	9.39
N4	9.41
N5	9.47
N6	9.58
N7	9.69
N8	9.81
N9	10.11
N10	10.26
N11	10.44
N12	11.81
I1	8.09
I2	12.01
I3	8.14
I4	8.71

*N1 to N12: normal pancreatic bulk tissues; I1 to I4: normal pancreatic islets.
†$\Delta\Delta Ct_{ARHI} = ||\Delta Ct_{ARHI} - \max(\Delta Ct_{ARHI})||$, where Ct_{ARHI} and $Ct_{\beta\text{-actin}}$ represent the comparative threshold cycle for *ARHI* and β-actin, respectively.

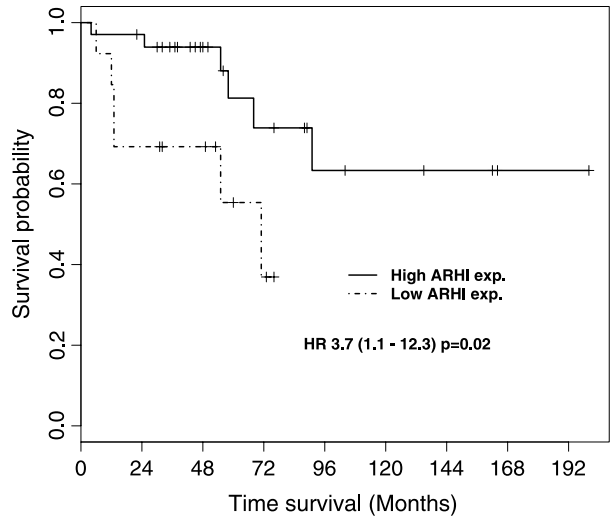

Figure W1.

Table W3. *ARHI* and Ki67 Expression in Relation to Tumor Category and Outcome.

	No. of patients*	Mean Ki67 (%)	Mean *ARHI* ΔΔCt†	Outcome (DOD)‡
WDET	20	2	10.62	0/20
WDEC	23	5	9.25	8/23
PDEC	4	34	4.37	4/4

*Only patients with survival data available are included.
†$\Delta\Delta Ct_{ARHI} = ||\Delta Ct_{ARHI} - \max(\Delta Ct_{ARHI})||$, where Ct_{ARHI} and $Ct_{\beta\text{-actin}}$ represent the comparative threshold cycle for *ARHI* and β-actin, respectively.
‡Outcome: number of patients dead of disease (DOD).

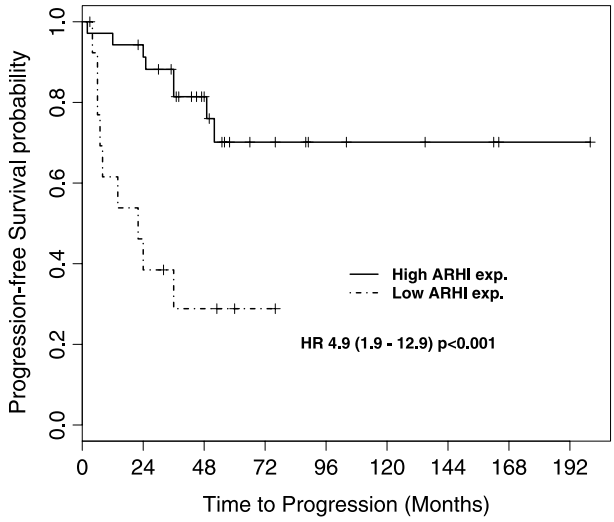

Figure W2.