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RARRES1 expression is significantly related to tumour differentiation and staging in colorectal adenocarcinoma

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ABSTRACT

Retinoic acid receptor responder 1 (RARRES1) is a retinoid regulated gene. Its expression is frequently down-regulated through DNA hypermethylation in several types of malignant tissues. This study investigated the clinical significance of RARRES1 protein and its association with RARRES3 protein expression in 161 (26 adenoma, 13 distal normal mucosa and 122 primary colorectal adenocarcinoma) paraffin-embedded colorectal tissues by immunohistochemistry. RARRES1 protein was detected at the highest levels in terminally differentiated cells of normal mucosal tissues and all 26 adenoma tissues. Among 122 colorectal adenocarcinomas, the poorly differentiated adenocarcinomas and Dukes' stage D tumours showed a significant decrease in RARRES1 expression ($P < 0.001$ and $P < 0.01$, respectively). RARRES1 expression was significantly ($P < 0.001$) correlated with RARRES3 expression, which was positively associated with tumour differentiation ($P < 0.001$). Difference in expression of RARRES1 among 119 patients had no apparent effect on patient survival. Our results suggest the role of RARRES1 in colorectal epithelial differentiation, and the down-regulation of RARRES1 is related to stage D progression.

1. Introduction

Retinoids, which are natural and synthetic analogues of vitamin A, display a wide range of biological activities on cellular growth, differentiation, vision, immune function, and malignant transformation. Retinoids also exhibit a wide spectrum of antitumour activities through induction of cell cycle arrest, cellular differentiation or apoptosis,[1] and are clinically active for cancer treatment and prevention.[1,2] The mechanism by which retinoic acid (RA) regulates such diverse biological processes is mediated through direct or indirect modulation of transcription of numerous target genes after RA binds with nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs).[1] Among a number

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of RA regulated genes, retinoic acid receptor responder 1 (*RARRES1*) and retinoic acid receptor responder 3 (*RARRES3*) have been found to have tumour suppressor effects in human cancer.

The *RARRES1* gene, also known as *TIG1* (tazarotene-induced gene 1), was first isolated from retinoid-treated human skin keratinocytes using subtractive hybridization.[3] Sequence analysis predicted that the 228-amino acid RARRES1 protein is a transmembrane protein with a small-N-terminal intracellular region, a single membrane-spanning hydrophobic region and a long C-terminal extracellular region containing a glycosylation signal and a hyaluronic acid-binding motif. The C-terminal region of RARRES1 shares 30% sequence similarity with the carboxypeptidase inhibitor latexin, which suggests that RARRES1 may act to inhibit extracellular proteolysis.[4] Expression of RARRES1 is induced in psoriatic lesions induced to differentiation by the synthetic retinoids AGN190168[3] or in well-differentiated Caco-2 colon cancer cells treated with vitamin D.[5] The endogenous function of RARRES1 remains unclear. Results from a recent study suggest that RARRES1 may play a regulatory role in tumour growth and metastasis. This hypothesis is supported by the finding that restoration of RARRES1 expression in prostate cancer cells resulted in a decrease of in vitro invasiveness and in vivo tumourigenicity.[6]

RARRES1 is expressed in several normal tissues with high RARRES1 levels detected in prostate, heart, lung, liver, colon and small intestine.[6] However, a loss or decrease in RARRES1 expression is observed in several types of cancer.[6–9] In cancerous tissues, RARRES1 expression is progressively lost from benign prostate tissues to malignant lesions.[6] This decrease in RARRES1 transcription was also reported in cancerous prostate, breast, colon, nasopharynx and leukemia tissues,[7–9] suggesting significance of the aberrant RARRES1 expression during carcinogenesis. A CpG island identified in the *RARRES1* promoter is frequently hypermethylated in cancer cell lines and tissues that had loss of RARRES1 expression.[7–9] Furthermore, the induction of *RARRES1* hypomethylation restored RARRES1 expression.[7–9] These studies suggest promoter hypermethylation may play an important role in the loss of RARRES1 expression in cancer cells.

RARRES3,[10] also referred to as *TIG3*[11] or *RIG1*,[12] encodes a growth regulatory protein that belongs to the HREV107 protein family.[13] Proteins of this family exhibit activity in suppressing H-Ras-mediated transformation or transactivation.[14,15] Ectopic RARRES3 expression leads to cellular apoptosis, growth suppression, or differentiation of cancer cells and human keratinocytes.[11,15,16] RARRES3 is expressed ubiquitously in various normal tissues, and the expression is decreased in cancer cells in vitro and in vivo.[11,17,18] Although the molecular mechanism of RARRES3 action remains unknown, it is believed that RARRES3 plays a role in the regulation of cell differentiation, growth, and apoptosis.

Both *RARRES1* and *RARRES3* are retinoid inducible genes. Expression of these genes are upregulated in vivo in precancer or cancer lesions treated with the synthetic retinoid tazarotene,[3,17,19] indicating the potential use of these two genes as markers of retinoid sensitivity. Results from our previous study have shown that RARRES3 expression is positively associated with differentiation of normal and malignant tissues of the colorectum and biliary tract.[18,20] Currently, studies of RARRES1 expression in tumour tissues are limited to the mRNA level. Clinical significance of RARRES1 has not been investigated at the protein level. To understand the significance of RARRES1 in colorectal tumourigenesis, we have investigated the expression of RARRES1 protein in normal, precancer and malignant tissues of the colorectum. Significance of RARRES1 expression in tumour differentiation and staging was investigated. Finally, the association between RARRES1 and RARRES3 expression and role of RARRES1 in patient survival were analysed.

2. Materials and methods

2.1. *RARRES1 antiserum preparation*

A peptide corresponding to amino acids 169–187 of the RARRES1 protein,[3,6] was synthesized and conjugated to keyhole limpet hemocyanin (Genosys Biotechnologies Inc., Woodlands, TX, USA). The conjugated peptide antigen was mixed thoroughly with Freund's complete adjuvant and was injected subcutaneously into a New Zealand white rabbit. Followed by four injections of 100 μg RARRES1 peptide, mixed with incomplete Freund's adjuvant at 2 week intervals, titers of the antiserum were determined using an enzyme immunoassay. Results of Western blot analysis showed that RARRES1 antiserum specifically detects the 29.2 kDa RARRES1 fusion protein containing myc and His epitopes from cytosol extracts of HtTA cervical cancer cells (data not shown). The specificity of RARRES1 staining on tissues was determined by a competition test. Briefly, RARRES1 antisera was preincubated with 10–100 μg of immunized peptide at 4 °C overnight. Samples were spun at 14,000*g* at 4 °C for 20 min before adding the absorbed antisera to the tissue section.

2.2. *Specimen collection and preparation*

A total of 161 colorectal specimens (26 adenoma, 13 distal normal colon mucosa and 122 primary adenocarcinoma) were obtained from the Tri-Service General Hospital and collection conformed the requests of hospital institutional review board. Adenoma tissues (one mild, 21 moderate and 4 severe dysplasia) were obtained from 18 male and 8 female patients with a mean age of 67.1 years. Primary tumours were obtained from 71 male and 51 female patients with a mean age of 63.0 years. Seventy adjacent normal tissues were observed within the same tissue sections among 122 primary tumour tissues. The distribution of tumours according to the level of differentiation and Dukes' stages are listed in Table 1. In addition, 13 distal normal tissues were taken from regions >10 cm away from the bulk of those tumour tissues that had clearly defined margins. Tissue sections (4 μm thick) were prepared from paraffin-embedded blocks. Specimens were evaluated by the same pathologist to define the differentiation status of carcinoma tissues and the degree of dysplasia of adenoma tissues. Assessment of tumour differentiation was based on the architectural and glandular differentiation as well as nuclear features of tumours.[21] Primary tumours were

Table 1 – Expression of RARRES1 in 161 colorectal tissues

Tissues	Number of cases	RARRES1 protein levels (no. (%) of cases)			IRS[a] mean ± SE
		Negative	Weak	Strong	
Distal normal	13	8 (61.5)	3 (23.1)	2 (15.4)	2.00 ± 0.67
Adenoma	26	0 (0)	3 (11.5)	23 (88.5)	8.08 ± 0.35
Adjacent normal	70	10 (14.3)	43 (61.4)	17 (24.3)	4.14 ± 0.31
Carcinoma differentiation					
Well	20	5 (25.0)	3 (15.0)	12 (60.0)	5.55 ± 0.84[b]
Moderate	79	24 (30.4)	36 (45.6)	19 (24.0)	3.27 ± 0.30[c]
Poor	23	17 (73.9)	5 (21.7)	1 (4.4)	0.91 ± 0.35[b,c]
Dukes' staging					
A + B	51	13 (25.5)	22 (43.1)	16 (31.4)	3.86 ± 0.42[d]
C	49	18 (36.7)	18 (36.7)	13 (26.6)	3.31 ± 0.46[e]
D	22	15 (68.2)	4 (18.2)	3 (13.6)	1.41 ± 0.45[d,e]
RARRES3 protein levels					
Negative	35	27 (77.1)	8 (22.9)	0 (0)	0.71 ± 0.23[f,g]
Weak	36	18 (50.0)	10 (27.8)	8 (22.2)	2.61 ± 0.48[f,h]
Strong	51	1 (2.0)	26 (51.0)	24 (47.0)	5.31 ± 0.36[g,h]

a IRS: immunoreactive score.
b $P < 0.001$.
c $P < 0.001$.
d $P < 0.01$.
e $P < 0.05$.
f $P < 0.01$.
g $P < 0.001$.
h $P < 0.001$.

staged according to a modification of Dukes' classification system.[22]

2.3. Immunohistochemical analysis

Tissue sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. To retrieve antigens, the sections were boiled for 30 min in 10% DAKO ChemMate™ solution (DAKO Co., Carpinteria, CA, USA) containing 0.05% Nonidet P-40. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min. The sections were then incubated at room temperature for 1 h in RARRES1 or RARRES3[18] antiserum diluted at 1:1200 or 1:1000 respectively in DAKO antibody diluent. Tissue sections were also incubated with 50-fold diluted anti-p53 antibody (DO-7, DAKO) at room temperature for 30 min. The DAKO LSAB® 2 Peroxidase kit was used to stain protein expression in tissue sections. This was followed by incubation with 3-amino-9-ethylcarbazole solution (DAKO) for 10 min to visualize the peroxidase complex. Finally, sections were lightly counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and mounted with DAKO Faramount. Control sections were incubated with pre-immune rabbit serum.

2.4. Reviewing and scoring of sections

To minimize subjectivity, stained sections were reviewed and scored by two researchers, including a pathologist. Staining was repeated in equivocal cases, and consensus between the two researchers was achieved in all cases. Patterns, cellular localization, staining intensity and percentage of RARRES1, RARRES3 or TP53 expressed cells were recorded. The evaluation of RARRES1 and RARRES3 expression was performed using an immunoreactive score (IRS) system, in which IRS = SI (staining intensity) × PP (percentage of positive cells).[18] SI was determined as 0, negative; 1, weak; 2, moderate; 3, strong. PP was defined as 1, <10% positive cells; 2, 10–50% positive cells; and 3, >50% positive cells. A total of 10 high-power visual fields, with 100 cells per field counted from different area of each section were chosen at random for IRS evaluation, and the average of the IRS was calculated. The final intensity of RARRES1 or RARRES3 staining was defined as 'negative', 'weak', and 'strong', corresponding to IRS values of 0–1, 2–4, and 6–9, respectively. Sections stained for TP53 protein were assigned as positive when 10% or more cells expressed the protein in the nucleus.

2.5. Statistical analysis

The non-parametric Kruskal–Wallis tests were applied to compare IRS of RARRES1 and RARRES3 associated with different clinicopathologic parameters including various levels of differentiation as well as modified Dukes' staging. Further, Dunn's procedure was applied to compare the IRS between groups. Logistic regression analyses were used to assess the association and trend between tumour differentiation and the probability of positive RARRES1 staining while controlling for potential confounding factors, which were subjects' gender and age. The Spearman's test was used to evaluate the correlation among RARRES1, TP53 and RARRES3. Survival rates were calculated using the Kaplan–Meier method and its significance was calculated by the log-rank

test. To further validate the effect of RARRES1 staining on survival, a multivariate Cox proportional hazard method was used to adjust for age, cancer tissue differentiation and stage.

3. Results

3.1. *Validation of RARRES1 antiserum*

To analyse the specificity of RARRES1 antiserum, tissue sections of well-differentiated colon adenocarcinoma were stained with RARRES1 antiserum, RARRES1 antiserum pre-treated with immunization peptide and pre-immune rabbit serum. Strong RARRES1 staining with granular pattern localized at the supranuclear regions of cancer cells is shown in a section incubated with RARRES1 antiserum (Fig. 1A). Pre-incubation of RARRES1 antiserum with 30 μg of immunized peptide resulted in suppression of staining, which supports the specificity of RARRES1 staining (Fig. 1B). No staining was observed in section incubated with pre-immune rabbit serum (Fig. 1C).

3.2. *Expression of RARRES1 in normal and adenoma tissues*

A total of 13 distal normal tissues, 70 adjacent normal tissues of carcinomas and 26 adenoma tissues were analysed for RARRES1 expression (Table 1). Positive RARRES1 staining was detected in 5 (38.5%) distal normal tissues and 60 (85.7%) adjacent normal tissues with a mean IRS of 2.00 and 4.14, respectively. In contrast, all 26 adenoma tissues expressed RARRES1 protein and 23 (88.5%) tissues showed strong RARRES1 expression regardless of the variation in the degree of dysplasia. In normal mucosal cells, levels of RARRES1 protein varied with difference in cellular differentiation, and the terminally-differentiated mucosal cells expressed the highest level of RARRES1 protein. Representative staining of RARRES1 in adenoma, adjacent normal and distal normal tissues is shown in Fig. 2.

3.3. *Expression of RARRES1 in colorectal adenocarcinoma tissues*

Levels of RARRES1 protein varied among 122 tissues of colorectal adenocarcinoma (Table 1). When analysed with respect to differences in tumour differentiation, 12 (60.0%) out of 20 well-differentiated tissues had strong RARRES1 expression. Among 79 moderately differentiated tumour tissues, 24 (30.4%) tumours did not express RARRES1 protein and 19 (24.0%) tumours had strong RARRES1 expression. Furthermore, 17 (73.9%) out of 23 poorly differentiated colorectal carcinoma tissues did not express the RARRES1 protein. Representative staining of RARRES1 protein expression in well, moderately and poorly differentiated tumour tissues is shown in Fig. 3. RARRES1 expression levels in terms of IRS of poorly differentiated tumours were significantly lower than that of tumours with moderate or well differentiation ($P < 0.001$) (Table 1).

When analysed with respect to difference in patient's Dukes' staging, RARRES1 expression was not detected in 13

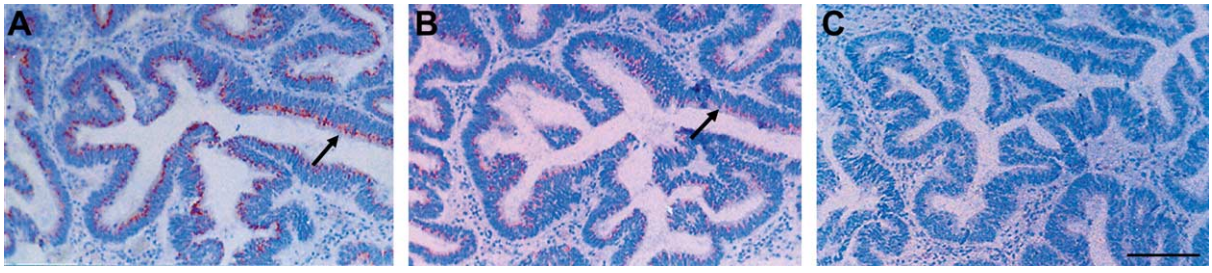

Fig. 1 – Specificity of RARRES1 antiserum. Sections of well-differentiated colon adenocarcinoma were incubated with (A) RARRES1 antiserum, (B) RARRES1 antiserum pre-treated with 30 μg of immunization peptide or (C) pre-immune rabbit serum. Arrows indicate positive RARRES1 staining. Scale bar: 100 μm.

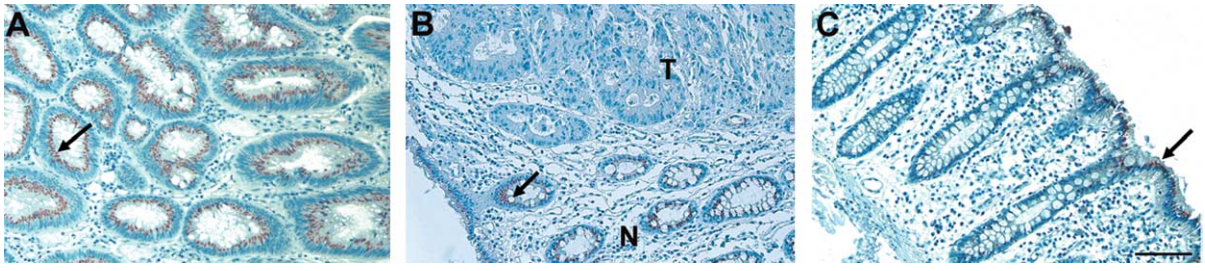

Fig. 2 – Expression of RARRES1 in colorectal adenoma and normal mucosal tissues. Sections of (A) colonic adenoma, (B) adjacent normal tissues or (C) distal normal mucosal tissues were analysed. Adenoma cells and terminally-differentiated normal colonic mucosa expressed high levels of RARRES1 protein. Arrows indicate positive RARRES1 staining. N: adjacent normal tissues; T: tumour tissues. Scale bar: 100 μm.

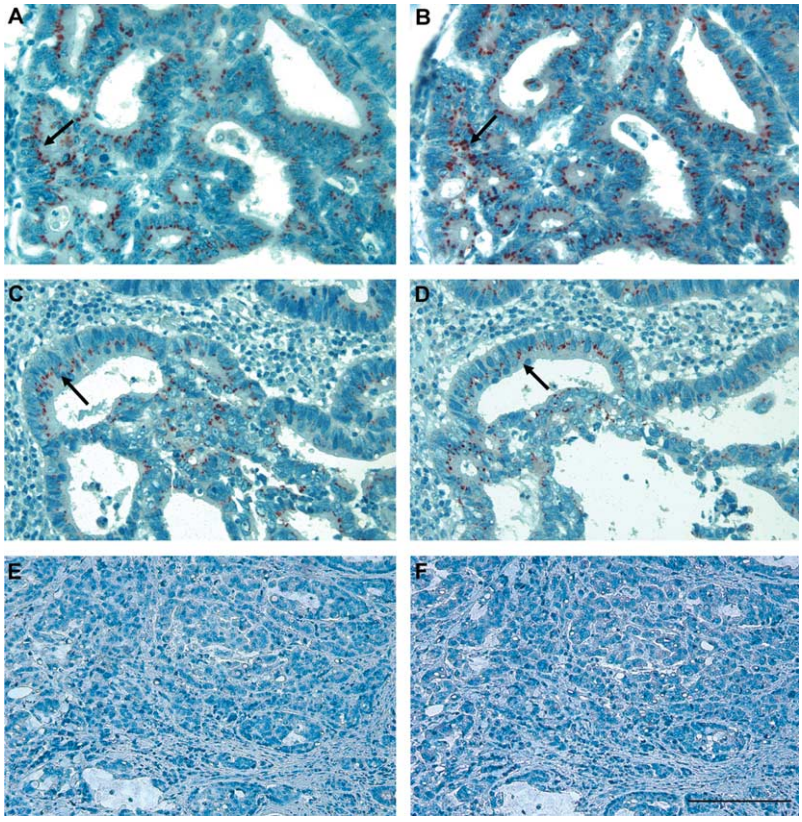

Fig. 3 – Expression of RARRES1 and RARRES3 in colorectal adenocarcinoma tissue. Sections of (A–B) well-, (C–D) moderately-, and (E–F) poorly-differentiated colorectal adenocarcinoma were stained for: (A, C, E) RARRES1; and (B, D, F) RARRES3 protein expression. Arrows indicate positive staining of RARRES1 or RARRES3. Scale bar: 100 μm.

(25.5%), 18 (36.7%) or 15 (68.2%) primary tumours obtained from patients with Dukes' stages of A and B, C or D colorectal adenocarcinoma, respectively. The IRS of RARRES1 expression in Dukes' stage D primary tumours was significantly lower than that of tumours from Dukes' stage C ($P < 0.05$) or Dukes' stages A and B ($P < 0.01$) (Table 1).

Levels of RARRES1 protein between adjacent normal and tumour tissues within the same tissue slide among 70 carcinoma tissues were also analysed (Table 2). Seven out of 12 (58.3%) of well-differentiated tumours expressed higher levels of RARRES1 than that of adjacent normal tissues, in contrast to higher levels of RARRES1 protein in adjacent normal tissues among nine (75.0%) of 12 poorly differentiated tumours.

Table 2 – Comparison of RARRES1 expression between adjacent normal and adenocarcinoma tissues

Differentiation	Number of cases	Number of cases (% of total)		
		[1]N > T	[2]N = T	[3]N < T
Well	12	2 (16.7)	3 (25.0)	7 (58.3)[a]
Moderate	46	13 (28.3)	22 (47.8)	11 (23.9)[b]
Poor	12	9 (75.0)	2 (16.7)	1 (8.3)[a,b]
Total cases	70	24 (34.3)	27 (38.6)	19 (27.1)

Staining between adjacent normal and adenocarcinoma tissues: [1]higher, [2]similar, [3]lower. [a]$P < 0.01$, [b]$P < 0.05$ and test for trend: $P < 0.05$.

Levels of RARRES1 protein in 46 moderately differentiated tumours were relatively similar between tumour and adjacent normal tissues. Compared to poorly differentiated tumours, moderately and well-differentiated carcinomas had a significantly increased chance of having higher RARRES1 protein levels in the adjacent normal tissues than in tumour tissues ($P < 0.05$).

3.4. *Expression of RARRES3 in colorectal adenocarcinoma tissues*

Consistent with our previous report,[18] strong RARRES3 expression was detected in 15 (75.0%), 36 (45.6%) and no tissues with well, moderate or poor differentiation, respectively (Table 3). The IRS of RARRES3 expression in tumours with well or moderate differentiation was significantly higher than that of the poorly differentiated tumours ($P < 0.001$) (Table 3). A significant positive linear trend was found between tumour differentiation and RARRES3 expression ($P < 0.001$). Representative results of RARRES3 expression in tumours with well, moderate or poor differentiation are shown in Fig. 3.

When analysed with respect to difference in Dukes' staging, 27 (52.9%) out of 51 primary tumours obtained from patients in Dukes' stage A or B had strong RARRES3 expression, in contrast to 19 (38.8%) or 5 (22.7%) primary tumours obtained from patients with Dukes' stage C or D, respectively. Further analysis of the IRS of RARRES3 expression showed that RARRES3 expression in Dukes' stage A or

Table 3 – Expression of RARRES3 in 122 tissues of colorectal adenocarcinoma

Categories	Number of cases	RARRES3 protein levels (no. (%) of cases)			IRS[a] mean ± SE
		Negative	Weak	Strong	
Differentiation					
Well	20	4 (20.0)	1 (5.0)	15 (75.0)	5.80 ± 0.72[b]
Moderate	79	14 (17.7)	29 (36.7)	36 (45.6)	4.68 ± 0.34[c]
Poor	23	17 (73.9)	6 (26.1)	0 (0)	0.87 ± 0.26[b,c]
Dukes' staging					
A + B	51	11 (21.6)	13 (25.5)	27 (52.9)	4.92 ± 0.45[d]
C	49	14 (28.6)	16 (32.6)	19 (38.8)	3.98 ± 0.45
D	22	10 (45.5)	7 (31.8)	5 (22.7)	2.73 ± 0.68[d]

a IRS: immunoreactive score.
b $P < 0.001$.
c $P < 0.001$.
d $P < 0.05$.

B tumours was significantly higher than that of Dukes' stage D tumours ($P < 0.05$) (Table 3).

3.5. *Comparison of RARRES1 and RARRES3 expression in colorectal adenocarcinoma tissues*

Expression of RARRES1 was analysed for its correlation with the expression of RARRES3 in the same specimen of primary colorectal adenocarcinomas. (Table 1, Fig. 3). Twenty-seven (77.1%) out of 35 RARRES3 negative tumours were also stained negative for RARRES1 protein. Fifty (98.0%) out of 51 tumours with strong RARRES3 expression stained positively for RARRES1 expression. RARRES1 protein levels in tumours with variation in RARRES3 expression levels were significantly different ($P < 0.01$ or $P < 0.001$). Significant positive correlations between RARRES1 and RARRES3 expression were found ($P < 0.001$). A total of 112 tumours were also stained for expression of TP53 protein, and 63 (56.3%) tumours showed positive TP53 expression. No correlation between TP53 expression and RARRES1 or RARRES3 expression was noted (data not shown).

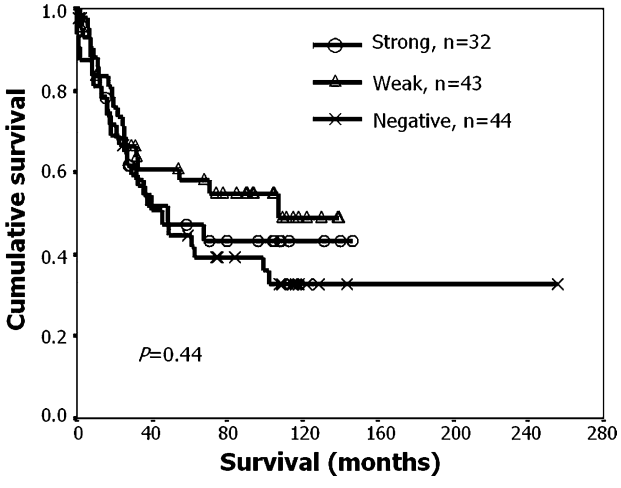

Fig. 4 – Expression levels of RARRES1 protein and overall survival in colorectal adenocarcinoma patients calculated by the Kaplan–Meier method. The significance was calculated by the log-rank test.

3.6. *Prognostic impact of RARRES1 and RERRES3 proteins*

A total of 119 patients with colorectal adenocarcinoma with different staging and tissue differentiation were analysed for prognosis related to different protein expression. Kaplan–Meier survival curves for the different RARRES1 expression levels are presented in Fig. 4. No difference in survival was found comparing patients with negative, weak and strong RARRES1 staining in tumours ($P = 0.44$). Consistent with our previous results, levels of RARRES3 staining in colorectal adenocarcinoma tissues were not associated with patient survival.[18] No significant difference in patient survival was found when the effect of RARRES1 on patient survival in conjunction with RARRES3 and TP53 was analysed. In addition, there was no difference between the survival rates of RARRES1-positive and RARRES1-negative patients among the 62 patients with TP53-positive tumours.

4. Discussion

Retinoids regulate a broad spectrum of biological processes including cell growth and differentiation. In this study, tissues of normal, premalignant and malignant colorectum were analysed for expression of the RARRES1 protein, whose expression may be directly regulated by RA due to presence of the RA response element in the RARRES1 promoter.[8] RARRES1 protein is expressed at the highest level in terminally-differentiated epithelial cells of normal mucosal tissues. All adenoma tissues expressed RARRES1 protein. In malignant tissues, expression of RARRES1 is significantly associated with the differentiation, staging and RARRES3 expression of colorectal adenocarcinoma. However, RARRES1 expression does not influence patient prognosis.

A decrease in RARRES1 expression has been demonstrated in several cancer tissues. Jing and coworkers first used RNA in situ hybridization to show progressive loss of RARRES1 expression in precancerous lesions and prostate carcinomas.[6] Subsequent analysis of cDNA also indicated a decrease in RARRES1 expression in malignant tumours derived from

prostate,[8] colon,[7] and nasopharynx.[9] Similarly, this study showed that 46 (37.7%) among 122 tumours of colorectal adenocarcinoma had lost RARRES1 protein expression. Down-regulation of the RARRES1 expression in tumours may be caused by hypermethylation of the CpG island located in the *RARRES1* promoter. This hypothesis is supported first by the finding of frequent hypermethylation of the *RARRES1* promoter in several cancer cell lines and tissues.[7–9] Secondly, restoration of RARRES1 expression and unmethylated alleles are observed in cancer cells after 5-aza-deoxycytidine treatment.[7,9] The *RARRES1* is located at chromosome 3q25.32, where loss of heterozygosity is frequently found in nasopharyngeal cancer.[23] Therefore, significance of allelic loss of chromosome 3q25.32 and promoter hypermethylation on the aberrant RARRES1 expression in tissues of colorectal adenocarcinoma merits further investigation. Frequent loss or decrease of RARRES1 expression in several types of cancer tissues shown in this and previous studies, as well as the suppression of tumourigenicity in prostate cancer cells stably expressed the RARRES1,[6] support the potential of RARRES1 as a tumour suppressor.

RA induces cellular differentiation through activation of nuclear retinoid receptors, which subsequently induces expression of various target genes that participate actively during process of cellular differentiation. A study by Sterniolo and coworkers has demonstrated the induction of keratinocyte terminal differentiation following RARRES3 expression in vitro.[16] This is in accordance with high levels of RARRES3 expression in terminally-differentiated epithelium of the skin[17,19] and colorectum,[18] as well as in well-differentiated adenocarcinoma derived from the colorectum and biliary tract.[18,20] Similarly, this study shows that RARRES1 is expressed at the highest levels in both terminally-differentiated epithelial cells of colorectum and well-differentiated colorectal adenocarcinomas. The increase in *RARRES1* expression was also observed in psoriatic lesions induced to differentiation following treatment with the synthetic retinoid tazarotene[3] or in Caco-2 colorectal adenocarcinoma cells incubated with vitamin D, an agent known to exhibit prodifferentiating and antiproliferative actions on intestinal enterocytes.[5] Whether the enhanced RARRES1 expression in differentiated cells of normal and malignant colorectum represents the active role of RARRES1 in cellular differentiation or as a marker of differentiation remains unclear. Further analysis of cellular differentiation in cells induced to express RARRES1 may be helpful for understanding the role of RARRES1 in cellular differentiation.

When it was first isolated from skin tissues, the RARRES1 protein was initially predicted to be a transmembrane protein with a long extracellular region;[3] however, this has never been confirmed. This study is the first report of expression of RARRES1 at the protein level. Normal colorectal mucosa, adenoma and adenocarcinoma cells showed granular staining localized to the supranuclear region, which is consistent with the localization of secretory proteins in the epithelia of the intestinal tract, such as S100A6.[24] The possibility that RARRES1 is a secreted protein without an N-terminal signal peptide is supported by analysis using the pTARGET,[25] SecretomeP[26] and WoLF PSORT (http://wolfpsort.seq.cbrc.jp) programs. In addition, RARRES1 is predicted to localize in various types of membranes, including the plasma membrane, Golgi apparatus and endoplasmic reticulum. We observed weak positive staining in the secreted mucin on the mucosal surfaces of some tissues. Whether or not this staining represents secreted RARRES1 remains to be verified. The RARRES3 protein contains a hydrophobic domain at the C-terminus. Intracellular localization of the protein is in both the soluble and membrane fractions.[16,27] The fact that RARRES1 and RARRES3 proteins in colorectal tissues have the same staining patterns suggests a relationship between the proteins at the functional levels or common protein trafficking.

The C-terminal domain of RARRES1 is related to latexin, the only endogenous protein inhibitor known for metallocarboxypeptidase.[28] Both proteins share a similar sequence and crystal structure,[4] suggesting that RARRES1 may have protease inhibitor activity, although to our knowledge this has not been tested. Human latexin consists of two topologically equivalent subdomains that share systatin fold architecture found in proteins that inhibit cysteine proteases.[4,28] If sequence identity between latexin and the C-terminal domain of RARRES1 translates to functional similarity, RARRES1 may function to inhibit the degradation of extracellular matrix. This hypothesis is supported first by the suppression of cellular invasion and tumourigenicity of human PC-3 prostate cancer cells stably expressing RARRES1,[6] and second by the significant loss of RARRES1 expression in late stages of colorectal adenocarcinomas observed in our study. Future analysis of the substrate specificity of purified RARRES1 will be needed to reveal the functional basis for the tumour suppressive activity of RARRES1.

RARRES1 was detected only in terminally-differentiated cells obtained from distal normal tissues. Due to the low percentage of RARRES1 positive cells, epithelial cells of distal normal tissues had the lowest IRS of RARRES1 among tested non-malignant tissues (Table 1). Expression of RARRES1 was enhanced in normal tissues adjacent to tumours, and all adenomas expressed the protein with most (88.5%) tissues having strong expression. Well-differentiated carcinomas expressed RARRES1 at high levels, and the levels of expression decreased with a decrease in tumour differentiation and disease progression. The enhanced RARRES1 expression in adenomas and well-differentiated tumours is in agreement with expression of the vitamin D receptor in colonic tissues,[29,30] whose signalling leads to enhanced RARRES1 expression.[5] Similar data were also observed for expression of RARRES3 in colorectal tissues.[18] During evolution of colorectal carcinogenesis, precancerous and cancerous lesions are shown to harbour mutations at genes like *APC* and *K-Ras* at high frequency, which leads to enhanced cellular proliferation. The up-regulation of RARRES1 and RARRES3 expression that occurs during early stages of adenoma and tumour progression, suggests that adenoma and differentiated colorectal cancer cells may respond to stimulation of growth by increasing expression of genes like *RARRES1* and *RARRES3*. These genes are shown to exhibit activity to induce differentiation and apoptosis or suppression of cellular proliferation. The enhanced RARRES1 and RARRES3 expression therefore likely plays a role in the "yin/yan feedback homeostasis" proposed by Weinstein during multistage carcinogenesis.[31] The fact that poorly-differentiated or advanced-stage

tumours have profound loss of RARRES1 and RARRES3 expression suggests an aberration in feedback regulation, similar to the frequently lost of p27/Kip1 expression in poorly differentiated tumours.[31]

In conclusion, this study has demonstrated profound RARRES1 expression in terminally-differentiated mucosa cells, adenoma, and well-differentiated colorectal adenocarcinomas. Expression of RARRES1 progressively decreased with a decrease in tumour differentiation and increase in disease progression. Although *RARRES1* and *RARRES3* are both RA inducible genes that encode proteins with distinct structures, their expression in tissues is closely related. Analysis of the expression of nuclear retinoid receptors in colorectal tissues and molecular mechanisms of RARRES1 and RARRES3 may provide further insight into their role and the significance of retinoid signalling in colorectal tumourigenesis.

Conflict of interest statement

None declared.

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