Hypoxia-inducible Adrenomedullin in Colorectal Cancer

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Abstract. *Background: Recently, we determined several potential prognostic factors in vivo using hypoxic tumor cells from hepatic metastases of colorectal cancer (CRC). Among them, expression of adrenomedullin (ADM) was an interesting target because it is highly induced by hypoxia. Patients and Methods: To evaluate the prognostic impact of the expression of ADM, samples from a total of 373 CRC patients were analyzed by microarray (n=222), and quantitative reverse-transcriptase polymerase chain reaction (n=151). Results: ADM was a novel independent prognostic factor for CRC (p=0.027). ADM expression correlated with hypoxia-inducible factor-1 A (p<0.0001) and vascular endothelial growth factor (p<0.0001) in vivo. Conclusion: ADM expression is a useful marker for predicting high risk of relapse and cancer-related death in patients who have undergone curative resection for CRC.*

Hypoxia is one of the main features of cancer, and intratumoral hypoxia affects every major aspect of cancer biology including cell invasion, metastasis, and cell death (1). Recently, we detected several potential prognostic factors and therapeutic targets *in vivo* in hypoxic tumor cells from hepatic metastases of colorectal cancer (CRC) (2). Among them, adrenomedullin (*ADM*) seems to be an interesting target because it is highly induced by hypoxic conditions and is a multifunctional gene. ADM is a vasorelaxant peptide that was originally isolated from human pheochromocytoma (3), and is reported to be a multifunctional regulatory peptide. For example, under normal conditions, it regulates cellular growth and differentiation by modulating hormone secretion, and has antimicrobial effects (4). It is secreted not only in pheochromocytoma but also in multiple human tissues, including adrenal medulla, heart, lung, aorta, kidney, brain, and the gastrointestinal tract (5, 6). Several studies demonstrated ADM expression in human cancer (7-10) and confirmed the proliferative properties of ADM in a wide variety of cancer cell types (lung, breast, ovary and prostate) (11). Recently, it was reported that ADM enhances cancer cell invasion and stimulates cancer cell proliferation in pancreatic cancer (12, 13). However, to our knowledge, there are no reliable studies determining the prognostic value of ADM in cancer.

Hypoxia is a well-established feature of the tumor microenvironment that constitutes one of the driving forces of cancer growth and progression. *ADM* is up-regulated through a hypoxia-inducible factor-1 (HIF-1)-dependant pathway under hypoxic conditions (14). Several studies indicate that ADM is an angiogenic factor (15-20), and that it acts synergistically with vascular endothelial growth factor (VEGF) to induce effects related to angiogenesis (21). However, there are no clinical studies reporting the correlation between ADM and HIF-1 or VEGF in human cancer.

In the present study, we investigated the clinical significance of ADM expression in human CRC, and assessed correlations between ADM, HIF-1 and VEGF in human CRC samples.

Patients and Methods

Patients and tumor samples analyzed by cDNA microarray. Among the 2210 CRC samples available in our tumor bank (collected from 2003 to 2006), samples were selected from patients who had curative surgery, with 222 samples assayed, and who were followed up prospectively. The clinical background of the patients contributing the 222 samples is shown in Table I. The mean follow-up time of the non-recurrence group (133 patients) was 33.7±6.4 months. Eighty-nine patients developed recurrent cancer during the follow-up period; the mean time to recurrence was 10.8±5.6 months.

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Postoperative surveillance was carried out according to clinical evaluation, laboratory analysis (including serum carcinoembryonic antigen concentration, CEA), abdominal computed tomography/ultrasound (CT/US), chest radiograph, and colonoscopy in all patients using the same strategy.

Tumor samples were collected within 30 minutes from the time of resection, cut into cubes of 5 mm^3, and immediately stored in an RNAase inhibitor-containing buffer at –85˚C until RNA extraction.

Extraction and quality assessment of RNA. Samples were macrodissected from the frozen cube and homogenized by hand. Total RNA was purified from clinical samples utilizing TRIzol reagent (Invitrogen, San Diego, CA, USA) as recommended by the manufacturer. The integrity of RNA was assessed by Agilent 2100 Bioanalyzer and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNA with intact 18S and 28S ribosomal RNA was used for subsequent analyses. As a control reference, a mixture of 40 RNAs from normal colorectal mucosa was used.

Preparation of fluorescent-labeled aRNA targets and hybridization. Extracted RNA samples were amplified with T7 RNA polymerase using Amino Allyl MessageAmp™ aRNA kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The quality of each amino allyl-aRNA sample was checked using an Agilent 2100 Bioanalyzer. Five micrograms of control and experimental aRNA samples were labeled with Cy3 and Cy5, respectively, mixed, and hybridized on an oligonucleotide microarray containing 30,000 human probes (AceGene Human 30K; DNA Chip Research Inc. and Hitachi Software Engineering Co., Ltd., Yokohama Japan). The experimental protocol is available at http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf. The microarrays were then scanned using ScanArray 4000 (GSI Lumonics, Billerica, MA, USA).

Analysis of microarray data. Signal values were calculated by DNASISArray software (Hitachi Software Inc., Tokyo, Japan). Following background subtraction, data with low signal intensities were excluded from further investigation. For each sample, the Cy5/Cy3 ratio values were log-transformed and global equalization was performed to remove a deviation of the signal intensity between whole Cy3 and Cy5 fluorescence by subtracting the median of all log(Cy5/Cy3) values from each log(Cy5/Cy3) value. Genes with values missing from more than 10% of samples were excluded from further analysis.

Analysis of clinicopathological factors and disease-free survival. In this study, microarray data were analyzed following two patterns: i) analysis of all 222 patients' data, and ii), to examine the effect of *ADM* as a prognostic factor more clearly, data from patients who did not have lymph node metastasis (n=112) were analyzed.

Confirmation of microarray data. From the 222 assayed samples, 20 samples were chosen at random to confirm the microarray data. The correlation between the microarray data and the results of the RT-PCR (detail of RT-PCR is described below) was examined.

Patients and tumor samples analyzed by RT-PCR. Samples were collected from a total 148 of patients with CRC (86 males, 62 females) who had surgery from June 1998 to November 2002 at the Department of Surgery, Medical Institute of Bioregulation, Kyusyu University and its three associated institutes. These samples were collected completely independently from those samples used in the microarray. The clinical background of the patients contributing these 148 samples is shown in Table II. The mean follow-up time was 40.45±33.6 months.

Table I. *Relationship between adrenomedullin (ADM) expression and clinicopathological factors (n=222).*

	ADM expression		
	Low (n=111)	High (n=111)	*P*-value
Age (years)	66.2±9.3	66.6±10.4	0.710
Gender			
Male	71	62	0.220
Female	40	49	
Tumor location			
Colon	73	67	0.120
Rectum	35	44	
Other	3	0	
Pre-operative serum CEA			
Positive	46	58	0.100
Negative	64	52	
Depth of invasion			
≤mp	13	14	0.870
≥ss	96	97	
Lymph node metastasis			
Present	51	59	0.280
Absent	60	52	
Histological grade			
Well-differentiated	34	19	0.062
Moderately differentiated	75	87	
Poorly differentiated	2	3	
Mucinous	0	2	
TMN stage			
I:II	58	52	0.420
III:IV	53	59	
Venous invasion			
Present	65	67	0.780
Absent	46	44	
Lymphatic invasion			
Present	77	88	0.091
Absent	34	23	

CEA, Carcinoembryonic antigen; mp, muscularis propria; ss, subserosa.

Real-time quantitative RT-PCR. The sequence of the *ADM* primers were as follows: sense primer, 5'-CATTGCCAGTGGGACGTCTG-3' and antisense primer, 5'-GCAGTTCCCTCTTCCCACGA-3'. A housekeeping gene, hydroxymethylbilane synthase *(HMBS)*, was used as an internal control, and the sequences of *HMBS* primers were as follows: sense primer, 5'-AACGGCGGAAGAAAACAG-3' and antisense primer, 5'-TCCAATCTTAGAGAGTGCA-3'. Real-time monitoring of PCR reactions was performed using a LightCycler™ system (Roche Applied Science, Indianapolis, IN, USA) and SYBER-Green I dye (Roche Diagnostics).

Statistical analysis. Statistical analysis was performed using the Stat View 5.0 program (Abacus Cocepts, Inc. Berkeley. CA, USA). The Kaplan-Meier method was used to examine disease-free

Table II. *Relationship between adrenomedullin (ADM) expression and clinicopathological factors (n=148).*

	ADM expression		
	Low (n=74)	High (n=74)	*P*-value
Age (years)	68.0±10.7	66.4±11.5	0.45
Gender			
Male	41	45	0.61
Female	33	29	
Tumor location			
Colon	47	44	0.75
Rectum	27	30	
Depth of invasion			
≤mp	26	20	0.37
≥ss	48	54	
Lymph node metastasis			
Present	30	30	>0.99
Absent	44	44	
Histological grade			
Well-differentiated	26	26	>0.99
Other	48	48	
Dukes			
A:B	42	41	>0.99
C:D	32	33	
Venous invasion			
Present	14	11	0.66
Absent	60	63	
Lymphatic invasion			
Present	28	28	>0.99
Absent	46	36	

mp, Muscularis propria; ss, subserosa.

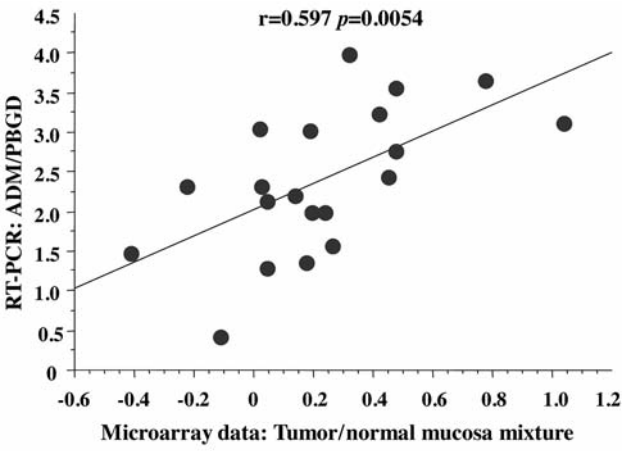

Figure 1. *Correlation coefficient for the microarray vs. the RT-PCR results for ADM expression. A significant correlation (p=0.0054) was indicated by Pearson's correlation coefficient test (correlation coefficient: r=0.597). Microarray data are shown as the fold increase compared with the expression of ADM in a mixed sample of normal colorectal mucosa. RT-PCR data are shown as the corrected values of ADM mRNA expression calculated by dividing the amount of ADM by the amount of an endogenous reference gene (HMBS) in the same samples. Data are shown on a log_2 scale.*

Table III. *Relation between liver/lung metastasis and adrenomedullin (ADM) expression.*

	ADM expression		
	Low (n=111)	High (n=111)	*P*-value
Lung metastasis			
Absent (n=194)	100	94	0.2251
Present (n=28)	11	17	
Liver metastasis			
Absent (n=171)	93	78	0.0167
Present (n=51)	18	33	

survival and the log-rank test was used to examine statistical significance. A Cox proportional hazards model was used to assess the risk ratio with simultaneous contributions from several covariates. The associations between the discrete variables were assessed using the χ^2 test of the Student's *t*-test. Correlation significance was assessed using Pearson's correlation coefficient test. Values of $p<0.05$ denoted the presence of a statistically significant difference.

Results

CRC sample microarray data

Confirmation of microarray data. When *ADM* RT-PCR expression data were plotted against *ADM* microarray expression, a correlation was noted (r=0.597, $p=0.0054$; Figure 1).

Relationship between ADM expression and clinicopathological factors. Patients were divided into two groups (high/low) according to the median value of *ADM* expression and the relationship between *ADM* expression and clinicopathological factors were examined. Table I lists the clinical and pathological characteristics of the 222 patients stratified by *ADM* expression. There were no significant differences in age, gender, tumor location, pre-operative serum CEA level, depth of tumor invasion, lymph node metastasis, histological grade, venous invasion, lymphatic invasion, or TNM stage.

The recurrence of cancer after surgery occurred in 87 patients, including 55 patients in the high *ADM* expression group and 32 patients in the low *ADM* expression group. The incidence of liver metastasis in the high *ADM* expression group was significantly higher than that of the low *ADM* expression group ($p=0.0167$; Table III). On the other hand, the incidence of lung metastasis was not related to *ADM* expression ($p=0.2251$; Table III).

Impact of ADM expression on disease-free survival. The disease-free survival curves were stratified by according to *ADM* expression level. The probability of disease-free survival was significantly higher in the low *ADM* expression group compared with the high *ADM* expression group (p=0.0028; Figure 2A).

Univariate analysis of clinicopathological factors of disease free survival. Expression of *ADM* and various clinicopathological parameters were evaluated for their impact on disease-free survival. Lymph node metastasis (p<0.0001), lymphatic invasion (p<0.0001), venous invasion (p=0.0002), pre-operative serum CEA value (p=0.0015), histological grade (p=0.0075), depth of tumor invasion (p=0.0086), gender (p=0.0368), and the expression of *ADM* (p=0.0028) were significantly associated with shorter disease-free survival (Table IV).

Multivariate analysis of clinicopathological factors of disease free survival. Multivariate Cox regression analysis demonstrated that the expression of *ADM* was a significant prognostic factor for disease-free survival (p=0.027; Table IV). As for other covariates, lymphatic invasion and venous invasion were significant prognostic factors (p=0.0480 and p=0.0366, respectively; Table IV). When several other combinations were analyzed using five, six, or eight covariates, similar results were obtained and the expression of *ADM* was a significant prognostic factor in all of the combinations.

Correlation between ADM and other genes in human colorectal tissues. As described previously, *ADM* is known as a hypoxia-induced gene and has angiogenic effects. We examined the correlation between both *HIF1A* and *VEGF* expression and *ADM* expression. When *HIF1A* expression data were plotted against *ADM* expression data (Figure 3A), a positive correlation was noted (r=0.276, p<0.0001). When *VEGF* expression data were plotted against *ADM* expression data (Figure 3B), a positive correlation was also noted (r=0.282, p<0.0001).

Analysis of 112 patients without lymph node metastasis

Relationship between ADM expression and clinicopathological factors. Lymph node metastasis is one of the most important factors that can predict a patient's prognosis. Thus we examined whether *ADM* expression is a prognostic factor in patients without lymph node metastasis (n=112). We divided the patients into two groups as in the analysis of data from the 222-patient group (high *ADM* expression: n=60; low *ADM* expression: n=52). There were no significant differences in age, gender, tumor location, pre-operative serum CEA level, depth of tumor invasion, lymph node metastasis, histological grade, venous invasion, lymphatic invasion, or TNM stage.

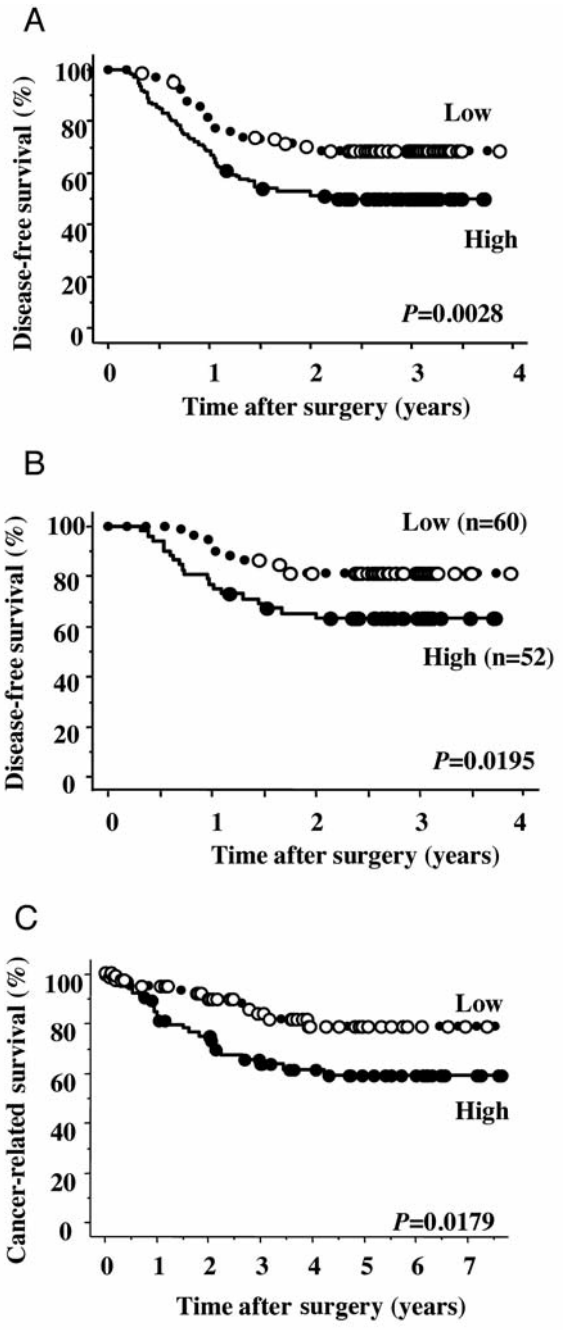

Figure 2. *Kaplan-Meier disease-free survival curves for the low and high adrenomedullin (ADM) expression groups. A: The probability of disease-free survival was significantly higher in the low ADM expression group compared with the high ADM expression group (from microarray data of all CRC sample: n=222, p=0.0028, log-rank test). B: The probability of disease-free survival was significantly higher in the low ADM expression group compared with the high ADM expression group (from microarray data of colorectal cancer patients without lymph node metastasis: n=112, p=0.00195, log-rank test). C: The probability of cancer-related survival was significantly higher in the low ADM expression group compared with the high ADM expression group (from RT-PCR data of colorectal cancer samples: n=151, p=0.0179, log-rank test).*

Table IV. *Univariate and multivariate analysis of clinicopathological factors of disease-free survival (n=222).*

	Univariate analysis	Multivariate analysis		
Variable	*P*-value	Relative risk	95% CI	*P*-value
Lymph node metastasis (+ *vs*. –)	<0.0001	1.626	1.004-2.634	0.0480
Lympatic invasion (yes *vs*. no)	<0.0001	2.057	0.910-4.648	0.0830
TNM stage (III, IV *vs*. I, II)	<0.0001			
Venous invasion (yes *vs*. no)	0.0002	1.722	1.035-2.867	0.0366
Pre-operative serum CEA (≥5 ng/ml *vs*. <5 ng/ml)	0.0015	1.533	0.984-2.389	0.0591
ADM (high *vs*. low)	0.0028	1.652	1.059-2.579	0.0270
Tumor differentiation (mod/poor/muc *vs*. well)	0.0075	1.317	0.229-1.942	0.3970
Depth of invasion (≥ss *vs*. ≤mp)	0.0086	1.499	0.515-4.369	0.4575
Tumor site (rectum *vs*. colon)	0.0691			

ADM, Adrenomedullin; CEA, carcinogenic embryonic antigen; mod, moderate; muc, mucinous; mp, muscularis propria; ss, subserosa; CI, confidence interval.

Table V. *Univariate and multivariate analysis of clinicopathological factors of disease-free survival in lymph node-negative patients (n=112).*

	Univariate analysis	Multivariate analysis		
Variable	*P*-value	Relative risk	95% CI	*P*-value
Lymph node metastasis (+ *vs*. –)	0.0046	2.724	1.139-6.514	0.0242
Lympatic invasion (yes *vs*. no)	0.1086			
Pre-operative serum CEA (≥5 ng/ml *vs*. <5 ng/ml)	0.4668			
ADM (high *vs*. low)	0.0195	2.293	1.089-4.828	0.0289
Tumor differentiation (mod/poor/muc *vs*. well)	0.0361	1.915	0.649-5.658	0.2394
Depth of invasion (≥ss *vs*. ≤mp)	0.0596			
Tumor site (rectum *vs*. colon)	0.2396			

ADM, Adrenomedullin; CEA, carcinogenic embryonic antigen; mod, moderate; muc, mucinous; mp, muscularis propria; ss, subserosa; CI, confidence interval.

Table VI. *Univariate and multivariate analysis of clinicopathological factors of cancer-related survival (from CRC sample RT-PCR data; n=148).*

	Univariate analysis	Multivariate analysis		
Variable	*P*-value	Relative risk	95% CI	*P*-value
Lymph node metastasis (+ *vs*. –)	<0.0001	4.611	1.860-11.42	0.0010
Lympatic invasion (yes *vs*. no)	<0.0001	2.688	1.130-6.395	0.0253
Duke's classification (C, D *vs*. A, B)	<0.0001			
Depth of invasion (≥ss *vs*. ≤mp)	0.0002	2.658	1.208-5.481	0.0151
Venous invasion (yes *vs*. no)	0.0099	1.936	0.841-4.456	0.1202
ADM (high *vs*. low)	0.0179	2.658	1.208-5.484	0.0151
Tumor differentiation (mod/poor/muc *vs*. well)	0.2708			
Tumor site (rectum *vs*. colon)	0.4359			

ADM, Adrenomedullin; mod, moderate; muc, mucinous; mp, muscularis propria; ss, subserosa; CI, confidence interval.

Impact of the expression of ADM on disease-free survival. The disease-free survival curves were drawn for groups stratified by the level of *ADM* expression. The probability of disease-free survival was significantly higher in the low *ADM* expression group compared with the high *ADM* expression group (p=0.00195; Figure 2B).

Univariate analysis of clinicopathological factors of disease-free survival. ADM expression and various clinicopathological parameters were evaluated for their impact on disease-free survival. Lymphatic invasion (p=0.0046), histological grade (p=0.0361), and *ADM* expression (p=0.0195) were significantly associated with shorter disease-free survival (Table V).

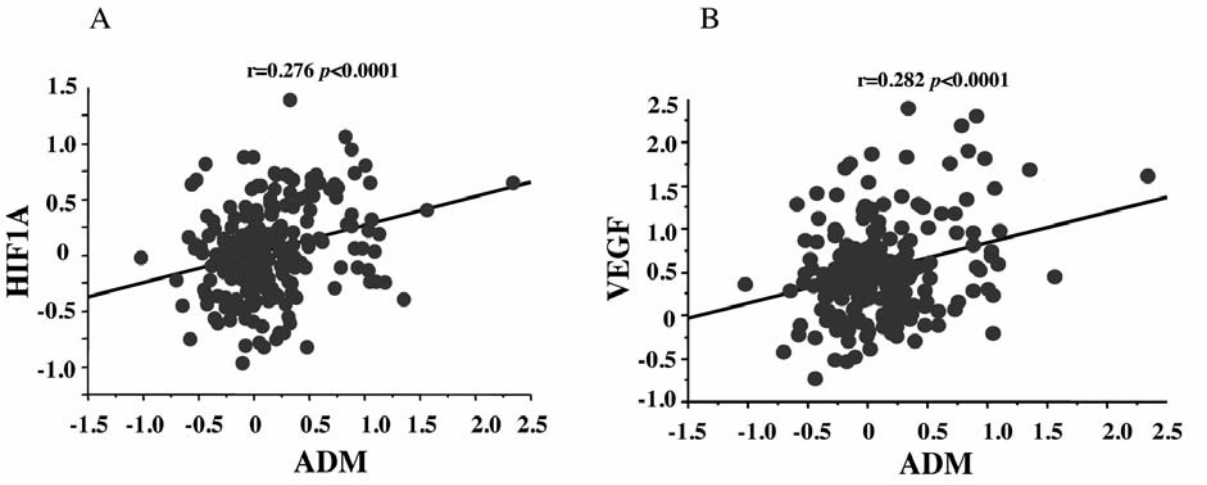

Figure 3. *Correlation of adrenomedullin (ADM) with hypoxia inducible factor-1 (HIF1A) and vascular endothelial growth factor (VEGF) mRNA expression levels. A: A significant correlation (p<0.0001) was indicated by Pearson's correlation coefficient test (correlation coefficient: r=0.276). Data are shown as the fold (log_2 scale) increase compared with the expression of ADM in a mixture of normal colorectal mucosa. B: A significant correlation (p<0.0001) was indicated by Pearson's correlation coefficient test (correlation coefficient: r=0.282).*

Multivariate analysis of clinicopathological factors of disease-free survival. Multivariate Cox regression analysis demonstrated that *ADM* expression was a significant prognostic factor for disease-free survival (p=0.0289; Table V). For other covariates, lymphatic invasion was a significant prognostic factor (p=0.0242; Table V).

RT-PCR data from CRC samples

Relationship between ADM expression and clinico-pathological factors. Patients were again divided into two groups according to the median value of *ADM* expression (high and low expression of *ADM*) and examined the relationship between *ADM* expression and clinico-pathological factors. Table II lists the clinical and pathological characteristics of the 148 patients stratified by *ADM* expression. There were no significant differences in age, gender, tumor location, depth of tumor invasion, lymph node metastasis, histological grade, venous invasion, lymphatic invasion, or Dukes' classification.

Impact of expression of ADM on cancer-related survival. The cancer-related survival curves were drawn for the two groups of patients stratified by *ADM* expression levels. The probability of cancer-related survival was significantly higher in the low *ADM* expression group compared with the high *ADM* expression group (p=0.0179; Figure 2C).

Univariate analysis of clinicopathological factors of disease-free survival. The level of *ADM* expression and various clinicopathological parameters were evaluated for their impact on disease-free survival. Lymph node metastasis (p<0.0001), lymphatic invasion (p<0.0001), venous invasion (p=0.0099), depth of tumor invasion (p=0.0002), and the expression of *ADM* (p=0.0179) were significantly associated with shorter disease-free survival (Table VI).

Multivariate analysis of clinicopathological factors of disease-free survival. Multivariate Cox regression analysis demonstrated that the *ADM* expression level was a significant prognostic factor for disease-free survival (p=0.0151; Table VI). As for other covariates, lymph node metastasis and lymphatic invasion were significant prognostic factors (p=0.0010 and 0.0253 respectively; Table VI).

Discussion

Previous reports demonstrated that ADM is a hormone involved in carcinogenesis and tumor progression from various tumor cell types (22). It is also a known antiapoptotic survival factor in tumor cells (23-26). ADM also helps tumor cells evade immune surveillance (27, 28). One of the mechanisms of the immunosuppressive effects of ADM is its ability to induce cAMP production in macrophages and significantly inhibit cytokine-induced neutrophil chemoattractant (27).

In this study, we first examined the correlation between *ADM* and clinical data from human CRC. We found that *ADM* mRNA expression in CRC tissue was a significant factor for poor prognosis. Furthermore, the same finding was obtained using two different sets of tumor samples from two different institutions.

The degree of *ADM* expression has been associated with lymph node metastasis in breast cancer (10, 29). In pancreatic adenocarcinoma, the median *ADM* mRNA expression levels

were higher in lymph node-positive compared with lymph node-negative patients (13). Lymph node metastasis is a sensitive indicator of poor prognosis in patients with CRC (30, 31) (32-34), but even in node-negative patients, approximately 10-20% patients suffer from relapse in <5 years (32).

In the present study, there were no correlations between *ADM* expression and lymph node metastasis. We also analyzed whether *ADM* expression might be a prognostic factor in node-negative patients (n=112). The probability of disease-free survival was significantly higher in the low *ADM* expression group compared with the high *ADM* expression group (p=0.00195; Figure 2B). This result strongly supports the value of *ADM* as a prognostic marker. From a clinical viewpoint, our results are important, especially in predicting disease recurrence. Moreover, there was no significant relationship between *ADM* expression and other clinicopathological factors. The apparent lack of a significant correlation between *ADM* expression and clinicopathological factors suggests that *ADM* might be a valuable marker of high prognostic significance that is independent of lymph node status.

ADM is a well-known angiogenic factor that is induced by hypoxic conditions and its role in signaling was recently demonstrated (35). VEGF and ADM act synergistically to induce angiogenic effects on endothelial cells *in vitro* (21). ADM was also reported to have the potential to act as an angiogenic factor by increasing VEGF secretion in pancreatic cancer cells (13). An *in vitro* study demonstrated that *ADM* is up-regulated through an HIF-1-dependent pathway under hypoxic conditions (14). The present study supports these findings because *ADM* expression was correlated with both *VEGF* and *HIF1A* expression (Figure 3A and 3B). These results suggest that *ADM* might potentially promote tumor progression by promoting angiogenesis.

In conclusion, our study strongly suggests that the *ADM* expression level is a useful marker for predicting a high risk for relapse and cancer-related death in CRC patients who undergo curative resection. A controlled randomized study will be necessary to ascertain whether high-risk patients identified by this method may benefit from adjuvant therapy.

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