## Original

# Involvement of Adrenomedullin Expression in Tumor Cells and Stroma in the Development of Diabetes in Pancreatic Cancer Patients

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**Abstract** : Some studies have reported that adrenomedullin（AM）is involved in diabetes mellitus（DM）associated with pancreatic cancer. Therefore, in this study we investigated the relationship between diabetes and AM expression in patients with pancreatic cancer. We examined 48 biopsies and 26 surgical resections from 74 patients with histologically diagnosed pancreatic cancer. Patients were classified into either DM or non-DM groups. The immunohistochemical expression of AM and various clinicopathological factors were compared between the two groups. Among the biopsy cases, 21 were classified as DM and 27 as non-DM. AM expression in pancreatic cancer cells was significantly lower in the DM group（$p = 0.03$）. No significant differences were noted in age, body mass index, tumor diameter or location, serum CA19-9, amylase, or C-reactive protein levels, pancreatic ductal dilatation, portal vein invasion, clinical stage, or histological differentiation between the DM and non-DM groups. The proportion of men was significantly lower in the DM group（$p = 0.04$）, as was the frequency of liver metastasis at diagnosis（$p = 0.03$）. Among the resection cases, 13 were classified as DM and 13 as non-DM. There were no significant differences in AM expression in pancreatic cancer cells between the two groups. However, marked AM expression was observed in the inflammatory cells and fibroblasts of the tumor stroma in all cases. In addition, the inflammatory response in the tumor stroma tended to be stronger in the DM group. Although the present study failed to find a positive correlation between diabetes and AM expression in pancreatic cancer cells, the results indicate that AM expression in stromal cells may be more closely related to the development of DM in pancreatic cancer patients.

**Key words** : diabetes mellitus, pancreatic cancer, adrenomedullin, inflammation, immunohistochemical study

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## Introduction

The relationship between diabetes mellitus (DM) and pancreatic cancer (PC) has long been considered. The prevalence of diabetes in PC patients is high, ranging from approximately 40% to 55%[1–3]. Long-standing diabetes is regarded as a risk factor for PC, whereas PC is thought to cause diabetes and glucose tolerance. Diabetes frequently improves after surgical resection of PC[2, 4], and Pannala *et al* reported that diabetes improved after pancreatectomy in 57% of PC patients with new-onset diabetes[2]. These facts suggest that PC itself may cause diabetes, but the underlying mechanism remains unclear.

One possible mechanism is thought to be the secretion, by tumors, of diabetogenic products that cause diabetes[5], including adrenomedullin (AM), islet amyloid polypeptide, and S-100A8 N-terminal peptide[6, 7]. AM, a 52-amino acid multifunctional hormone, was originally identified as a hypotensive peptide isolated from pheochromocytoma and subsequently shown to exert proliferative and proangiogenic effects[8]. AM inhibits insulin secretion from pancreatic islets and causes glucose tolerance *in vivo* and *in vitro*[9]. Recent studies have reported that AM expression in PC cells is a likely candidate for the induction of diabetes in patients with PC[5, 7, 10]. The main purpose of the present study was to investigate the relationship between the presence or absence of diabetes and the expression of AM in PC cells.

## Materials and methods

The study was performed on 48 patients who had undergone a pancreatic biopsy by endoscopic ultrasound-guided fine needle aspiration and 26 who had undergone pancreatic surgical resection between 2014 and 2017 at the Showa University Fujigaoka Hospital. All patients had been histopathologically diagnosed with typical pancreatic cancer (tubular adenocarcinoma). Patients were divided into two groups based on serum HbA1c levels at the time of diagnosis and their history of diabetes: (i) a DM group, with HbA1c ≥ 6.5% and no history of diabetes or HbA1c ≥ 8.0% and a history of diabetes; and (ii) a non-DM group without these abnormalities. Among the biopsied cases, 21 were classified as DM and 27 as non-DM; among the resected cases, 13 were classified as DM and 13 as non-DM.

Clinicopathological features (i.e. patient age, gender, body mass index [BMI], tumor size, location, serum concentrations of carbohydrate antigen 19-9 [CA19-9], amylase, and C-reactive protein [CRP], dilatation of the pancreatic duct, invasion to the portal vein, liver metastasis, clinical stage, and histological differentiation) were investigated in the patients who underwent pancreatic biopsy and were compared between the DM and non-DM groups. Tumor size, location, dilatation of the pancreatic duct, invasion to the portal vein, and liver metastasis were evaluated by computed tomography (CT) or magnetic resonance imaging (MRI). Clinical stage was classified according to the criteria issued by the Japan Pancreas Society[11].

For the immunohistological investigation of AM expression, 3-μm sections were obtained from each formalin-fixed, paraffin-embedded block. Immunohistochemical staining was performed using the avidin–biotin complex detection system with a BEKCHMARK automated immunostaining
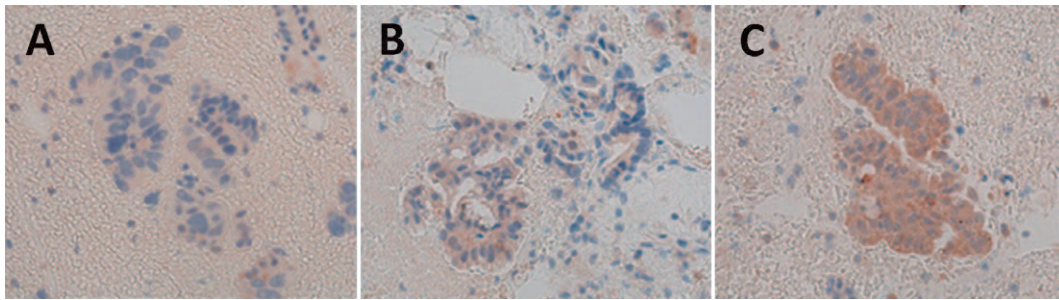
Fig. 1. Immunohistological expression of adrenomedullin in pancreatic cancer cells was categorized into three grades based on staining intensity : (A) mild (0), (B) moderate (1+), and severe (2+). Representative images of each grade are shown (original magnification×400).
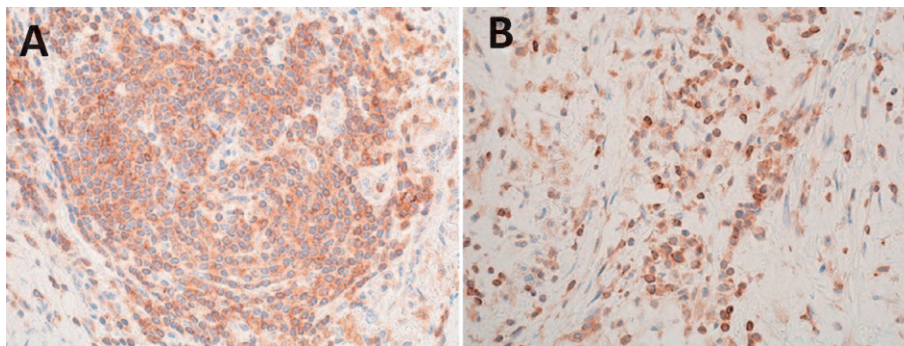


Fig. 2. Immunohistological expression of adrenomedullin (AM) in (A) inflammatory cells and (B) fibroblasts in the tumor stroma (original magnification × 400). Marked AM expression was seen in both inflammatory cells (A) and fibroblasts (B), and it was particularly prominent in lymph follicles.
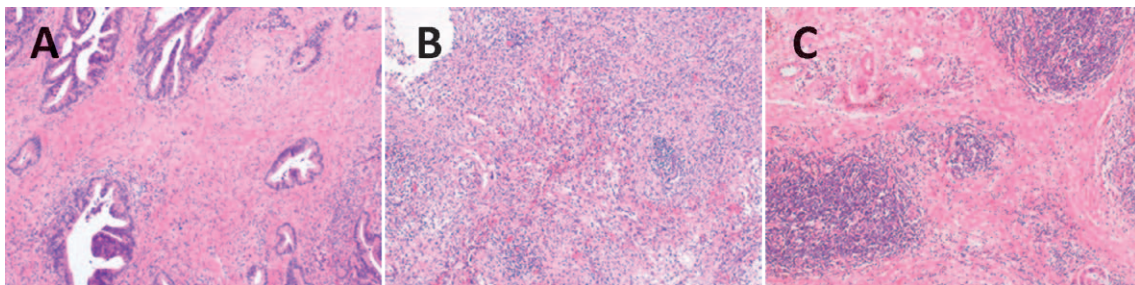


Fig. 3. The degree of inflammation of the tumor stroma was categorized into three grades : (A) mild (0), (B) moderate (1+), and severe (2+). Representative images of each grade are shown (original magnification×100 ; hematoxylin and eosin staining). There was scant inflammatory cell infiltration in the mild group (A), whereas prominent infiltration forming many lymph follicles was seen in the severe group (C). Intermediate infiltration was seen in the moderate group (B).

device (Ventana Medical Systems, Tucson, AZ, USA). A rabbit anti-human AM polyclonal antibody (200-fold dilution ; abcam, Cambridge, UK) was used. The staining intensity of AM expression in tumor cells was classified as mild (0), moderate (1+), or severe (2+), as shown in Fig. 1, and was compared between the DM and non-DM groups for both biopsied and resected cases.

In surgical specimens, AM expression was observed in the tumor stroma (Fig. 2) and the degree of inflammation was investigated and classified as follows: mild (0), scant infiltration of inflammatory cells; moderate (1+), intermediate infiltration; and severe (2+), prominent infiltration of inflammatory cells containing many lymph follicles (Fig. 3). The degree of inflammation was compared between the DM and non-DM groups. In addition, we investigated whether there were significant relationships between serum HbA1c, diabetic status (i.e. DM or non-DM group), the degree of AM expression in tumor cells, and the degree of inflammation. Pathological scoring was performed by two pathologists (H.I. and N.O.) in a blinded manner. Statistical analyses were performed using the $\chi^2$ test, Fisher's exact test, or Wilcoxon's rank sum test. Two-tailed $p < 0.05$ was considered significant.

The present study was a retrospective study, and consent was obtained by opt-out. Information regarding the study was posted on the Showa University Fujigaoka Hospital homepage. Subjects were guaranteed the opportunity to opt-out of the study. Approval for this study was obtained from the Ethics Committee at Showa University Fujigaoka Hospital (Permission no. F2017C62).

## Results

*Clinicopathological comparisons between the DM and non-DM groups (pancreatic biopsies)*

Clinicopathological factors in the DM (n = 21) and non-DM (n = 27) groups were compared for biopsy cases (Table 1). The non-DM group included three patients who had a history of diabetes but did not meet the criteria of the DM group. In the DM and non-DM groups,

Table 1.  Clinicopathological characteristics of the diabetes mellitus (DM) and non-DM groups of patients who underwent pancreatic biopsy by endoscopic ultrasound-guided fine needle aspiration

|  | DM group (n=21) | Non-DM group (n=27) | *p*-value |
|---|---|---|---|
| Age (years) | 71 [65.5–75] | 72 [68–79] | 0.3 |
| Male | 7 (33) | 17 (63) | 0.04 |
| BMI (kg/m²) | 21.2 [18.6–21.9] | 20.6 [17.4–22.1] | 0.63 |
| Tumor size (mm) | 33 [20–40] | 34 [29–47] | 0.65 |
| Location pancreatic head | 12 (57) | 10 (37) | 0.17 |
| CA19-9 (U/ml) | 724 [107–2,840] | 1,144 [292–9,640] | 0.29 |
| Amylase (IU/l) | 58 [45.5–107] | 72 [50–118] | 0.48 |
| CRP (mg/dl) | 0.14 [0.04–0.50] | 0.12 [0.05–0.77] | 0.61 |
| Dilatation of pancreatic duct ≥ 5 mm | 11 (52) | 9 (33) | 0.18 |
| Invasion to portal vein | 4 (19) | 9 (33) | 0.27 |
| Liver metastasis | 5 (24) | 15 (56) | 0.03 |
| Clinical Stage Ⅳ | 14 (67) | 21 (78) | 0.39 |
| Poorly differentiated histologically | 3 (14) | 2 (7) | 0.64 |

Data are expressed as the median [25th–75th percentile] or as the number of patients (%).
BMI, body mass index; CA19-9, carbohydrate antigen 19-9; CRP, C-reactive protein.

A   Intensity of AM
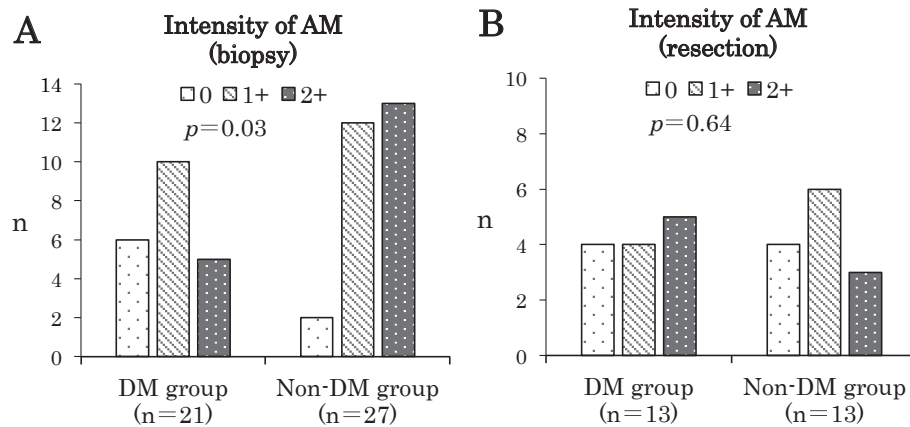(biopsy)

B   Intensity of AM
(resection)



Fig. 4. Comparison of adrenomedullin (AM) expression in tumor cells from specimens obtained by (A) pancreatic biopsy or (B) surgical resection and separated into diabetes mellitus (DM) and non-DM groups. AM expression was classified as follows: 0, mild; 1+, moderate; and 2+, severe. AM expression in tumor cells of specimens obtained by pancreatic biopsy was significantly lower in the DM than in the non-DM group ($p=0.03$), but there was no significant difference between these two groups for samples obtained by surgical resection.

respectively, the median age was 71 and 72 years, 33% and 63% of patients were male, median BMI was 21.2 and 20.6 kg/m$^2$, and median tumor diameter was 33 and 34 mm. When the tumor location was classified into pancreatic head versus body and tail, 57% and 37% of tumors were located in the head in the DM and non-DM groups, respectively. In the DM and non-DM groups, respectively, median serum CA19-9 was 724 and 1,144 U/ml, median amylase was 58 and 72 IU/l, and median CRP levels were 0.14 and 0.12 mg/dl. The number of significant dilatations ($\geq 5$ mm in diameter) of the main pancreatic duct was 11 (52%) and 9 (33%) in the DM and non-DM groups, respectively, whereas the number of patients in the DM and non-DM groups with invasion to the portal vein was 4 (19%) and 9 (33%), respectively, and 5 (24%) and 15 (56%) patients, respectively, had liver metastases. The number of clinical Stage IV cases in the DM and non-DM groups was 14 (67%) and 21 (78%), respectively, and there were 3 (14%) and 2 (7%) cases with poorly differentiated adenocarcinoma in these two groups, respectively. Significant differences were found with regard to gender ($p=0.04$) and liver metastasis ($p=0.03$). The DM group tended to be more frequently diagnosed with PC due to new-onset diabetes or elevated blood glucose before developing liver metastasis.

*AM expression in tumor cells from pancreatic biopsy or surgically resected specimens*

Immunohistochemical analysis revealed uniform AM staining throughout the lesions in all cases, but of varying degree. In biopsy cases, mild (0), moderate (1+), and severe (2+) AM expression was found in tumor cells in 6 (28%), 10 (48%), and 5 (24%) cases in the DM group, respectively, compared with 2 (7%), 12 (45%), and 13 (48%) cases in the non-DM group (Fig. 4A). AM expression was significantly lower in the DM than in the non-DM group ($p=0.03$). In surgically
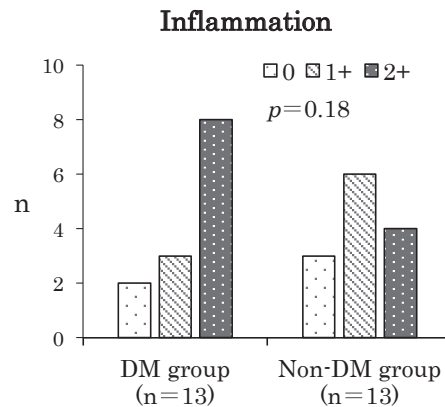
**Inflammation**



Fig. 5.  Comparison of inflammation of the tumor stroma
in specimens obtained by surgical resection between
the diabetes mellitus (DM) and non-DM groups.
Inflammation of the tumor stroma was categorized
as follows : 0, mild ; 1+, moderate ; and 2+, severe.
Although there was no significant difference between
the two groups, the degree of inflammation tended to
be greater in the DM than in the non-DM group.

resected specimens, there were 4 (31%), 4 (31%), and 5 (38%) cases of mild (0), moderate (1+),
and severe (2+) AM expression in tumor cells, respectively, in the DM group, compared with
4 (31%), 6 (46%), and 3 (23%) cases in the non-DM group (Fig. 4B).  There were no signifi-
cant differences in AM staining intensity between the two groups ($p = 0.64$).

*Inflammation of the tumor stroma in surgically resected specimens*

 Significant AM expression was observed in inflammatory cells and fibroblasts in the tumor
stroma.  In particular, AM expression was prominent in lymph follicles.  There were 2 (15%), 3
(23%), and 8 (62%) cases of mild (0), moderate (1+), and severe (2+) inflammation of the
tumor stroma, respectively, in the DM group, compared with 3 (23%), 6 (46%), and 4 (31%)
in the non-DM group (Fig. 5).  These observations suggest that the inflammatory response of
the tumor stroma tended to be stronger in the DM than in the non-DM group, although the
difference was not statistically significant ($p = 0.18$).

*Relationships between serum HbA1c, group, the degree of AM expression in tumor cells, and the*
*degree of inflammation in surgically resected specimens*

 As indicated in Table 2, there were no significant differences in serum HbA1c, DM group, and
inflammation of the tumor stroma between different grades of AM expression in tumor cells.  In
addition, there were no significant differences in serum HbA1c, DM group, and AM expression
in tumor cells between different grades of inflammation of the tumor stroma (Table 3).  How-
ever, there was a tendency for a greater number of cases in the DM group to exhibit stronger
inflammation of the tumor stroma ($p = 0.12$).

Table 2. Relationship between adrenomedullin expression in tumor cells and HbA1c, diabetes status, and stromal inflammation in surgically resected cases

| | AM expression in tumor cells | | | p-value[A] |
|---|---|---|---|---|
| | 0 (n=8) | 1+ (n=10) | 2+ (n=8) | |
| HbA1c (%) | 6.4 [6.0–9.8] | 6.9 [5.8–11.8] | 7.4 [6.2–9.3] | 0.66 |
| DM group | 4 (50) | 4 (40) | 5 (63) | 1.00 |
| Inflammation of tumor stroma | | | | 0.53 |
| 0 | 2 (25) | 1 (10) | 2 (25) | |
| 1+ | 1 (12.5) | 6 (60) | 2 (25) | |
| 2+ | 5 (62.5) | 3 (30) | 4 (50) | |

Data are expressed as the median [25th–75th percentile] or as the number of patients (%).
Adrenomedullin (AM) expression and inflammation of the tumor stroma were categorized as follows : 0, mild ; 1+, moderate ; and 2+, severe.
[A]P-values are for comparisons of Grade 0 expression vs Grades 1+ and 2+.
DM, diabetes mellitus.

Table 3. Relationship between stromal inflammation and HbA1c, diabetes status, and adrenomedullin expression in tumor cells in surgically resected cases

| | Inflammation of tumor stroma | | | p-value[A] |
|---|---|---|---|---|
| | 0 (n=5) | 1+ (n=9) | 2+ (n=12) | |
| HbA1c (%) | 6.2 [5.8–6.6] | 7.0 [6.2–7.7] | 9.1 [6.0–11.0] | 0.18 |
| DM group | 2 (40) | 3 (33) | 8 (67) | 0.12 |
| AM expression in tumor cells | | | | 0.64 |
| 0 | 2 (40) | 1 (11) | 5 (42) | |
| 1+ | 1 (20) | 6 (67) | 3 (25) | |
| 2+ | 2 (40) | 2 (22) | 4 (33) | |

Data are expressed as the median [25th–75th percentile] or as the number of patients (%).
Adrenomedullin (AM) expression and inflammation of the tumor stroma were categorized as follows : 0, mild ; 1+, moderate ; and 2+, severe.
[A]P-values are for comparisons of Grade 0 expression vs Grades 1+ and 2+.
DM, diabetes mellitus.

## Discussion

The underlying mechanism and pathogenesis of PC-induced diabetes remain unclear. Previous reports have suggested direct destruction of islet cells or a decrease in the number of islet cells as a result of PC invasion and/or PC-associated obstructive pancreatitis and fibrosis[12, 13]. However, diabetes frequently improves after surgical resection of PC, and even small tumors cause glucose tolerance ; therefore, it seems difficult to attribute the pathogenesis of DM only to the destruction of or a decrease in the number of islet cells[2, 7]. The biopsy case study reported herein also showed no significant differences in factors related to tumor development, such as tumor size and dilatation of the pancreatic duct, between the DM and non-DM groups.

Several diabetogenic tumor-secreted products are hypothesized to be involved in PC-associated diabetes[6,7], including AM[5,7,10,14]. AM is a multifunctional hormone expressed in various tissues of the human body. In the pancreas, AM receptors are found on $\beta$-cells, and AM expression is found in pancreatic polypeptide cells of the islets[15]. Furthermore, AM inhibits insulin secretion from islets and causes glucose tolerance in *in vivo* and *in vitro* in rat models[9].

Ramachandran *et al*[16] showed that AM is expressed in PC and it stimulates cell proliferation and invasion in an autocrine manner via the AM receptor[16]. Aggarwal *et al*[10], using both *in vivo* and *in vitro* experiments, showed that AM is upregulated in patients with PC and it mediates PC-induced inhibition of insulin section in $\beta$-cells. AM levels are higher in patients with PC who developed diabetes compared to those who did not[10]. In contrast to our expectations, in the present study we failed to find a significantly higher AM expression in the DM group, in either the biopsy or resection cases ; rather, we found that the AM expression was significantly lower in the DM than in the non-DM group.

To determine the cause of this unexpected result, we focused on AM expression in surgically resected specimens ; in these specimens, in almost all cases, AM expression was more prominent in the inflammatory cells (especially lymph follicles) and fibroblasts of the tumor stroma than in PC cells. Although the differences were not significant, we did find a stronger inflammatory response in the DM than in the non-DM group, suggesting that AM expression in response to inflammation may be more closely related to the development of diabetes in PC patients, as reported previously[6,7,17]. Regarding fibroblasts, Bhowmick *et al*[18] showed that stromal fibroblasts in tumors are biologically distinct from normal fibroblasts, and Benyahia *et al*[19] reported that stromal fibroblasts in breast cancer promote tumor growth and angiogenesis through AM secretion. We expect that the AM expression of stromal fibroblasts in PC will be found to have multiple functions and to be associated with the onset of diabetes.

In conclusion, the present study failed to find a positive correlation between diabetes and AM expression in PC cells. Conversely, the inflammatory response in the tumor stroma tended to be stronger in the DM group, although the difference was not statistically significant. Therefore, the findings of the present study indicate that AM expression in inflammatory tissues may be one of the potential mechanisms by which PC induces diabetes.

**Conflict of interest statement**

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