Expression of Stathmin1 in Gastric Adenocarcinoma

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Abstract. *Background: Over expression of Stathmin1 (STMN1), activation-induced cytidine deaminase (AID) and protein kinase C iota (PKCi) proteins participate in the regulation of carcinogenesis. In the present study, we investigated the expression of STMN1 in patients with gastric adenocarcinoma and also determined the correlation of STMN1 with AID and PKCi proteins. Materials and Methods: This study was conducted in the Tokushima University Hospital between September 2009 and September 2010 on a total of 59 patients with gastric adenocarcinoma. Stathmin1, AID and PKCi protein expressions were evaluated by immuno-histochemistry in gastric adenocarcinoma. Results: A strong expression of STMN1 was significantly associated with gender- and poorly differentiated gastric adenocarcinoma (p<0.05). A high mRNA level of STMN1 was found in the tumor tissue of gastric adenocarcinoma compared to non-tumor tissue (p<0.05). In addition, STMN1 expression was significantly correlated with AID and PKCi protein expressions in gastric adenocarcinoma (p<0.05). Conclusion: High mRNA level of the Stathmin1 gene was significantly expressed in gastric tumor tissue than non-tumor and strong expression of STMN1 protein is correlated with poorly-differentiated gastric adenocarcinoma.*

Gastric cancer is the fourth most common cancer and the second most common cause of cancer-related deaths worldwide. The five-year survival rate for patients with gastric cancer is under 30% in most countries (1), however that rate is 60% in Japan (2). Tumor metastasis is a pivotal reason for death, and failure of effective therapy for cancer patients. Stathmin1 (*STMN1*) is one of the genes associated with cancer, its expression is up-regulated in tumor cells and lightly detected in normal cells.

STMN1 is an oncoprotein 18- and an important cytosolic micro tubule-destabilizing protein, which plays the pivotal role of mitosis *via* regulation of micro-tubule dynamics (3). Studies showed that overexpression of STMN1 protein is found in several cancer types such as breast (4), prostate (5), lung (6) and cervical carcinoma (7), and is associated with disease progression and poor prognosis outcomes (8, 9). Previous studies have shown that activation-induced cytidine deaminase (AID) inhibits the cyclin-dependent kinase (CDK) inhibitor that leads to activation of STMN1 (10, 11).

AID is a member of the cytidine deaminase family and is closely related to apolipoprotein B RNA editing cytidine deaminase (12), which breaks down the DNA through deamination cytosines into uracils (13). A previous *in vitro* study examined the expression of AID protein by enhancing the susceptibility to mutagenesis in a variety of epithelial organs (14). Reports referred to previously mentioned work that protein kinase C iota (PKCi) regulates CDK, which activates STMN1 protein (15). The PKCi protein plays different roles in epithelial cell functions, such as cell polarity, cell survival and cell growth (16, 17).

In general, STMN1 could be activated by the different upstream genes. In this study we investigated the correlation between STMN1 expression and AID and PKCi at the protein level by immuno-histochemistry in gastric adenocarcinoma. In addition we checked the relationship of STMN1 with clinico-pathological factors and disease-free survival in patients with gastric adenocarcinoma.

Patients and Methods

Patients. We enrolled a total of 59 patients consisting of 13 males and 46 females, surgically-resected for gastric adenocarcinoma at the Tokushima University Hospital between September 2009 and September 2010. The age of the study participants ranged from 40 years to 87 years with a mean age of 62 years. The clinico-pathological parameters were obtained from the pathological reports, including tumor differentiation;- lymph node metastasis- and Tumor Node Metastasis staging system. All of these data were reviewed and confirmed by experienced pathologists in our department.

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Immunohistochemistry. The sample processing and immuno-histochemistry procedures were performed following standard immuno-histochemical staining protocols. In brief, sections were cut with a thickness of 4 μm, de-paraffinized in xylene- and dehydrated in descending ethanol concentration. Endogenous peroxidase activity was blocked by 10 minutes of incubation with 0.3% hydrogen peroxidase in 50% of methanol. Antigen retrieval was performed by using a T/T Mega multi-functional microwave histoprocessor (HACKER Instruments & Industries Inc, Winnsboro, SC, USA) at 100oC and by using a microwave heating in citrate buffer (pH 6.0) for 24 minutes. Sections were incubated for 60 minutes at ambient temperature with primary antibody and then washed with phosphate buffered saline (PBS) 3 times for 5 minutes.

Immuno-histochemical staining was completed by applying a polyclonal rabbit antibody to STMN1 (Cell Signaling Technology, no. 3352; dilution 1:50; Chiyoda-ku, Tokyo); a monoclonal mouse antibody to AID (ZA001, catalog no.39-250, dilution 1:100; Invitrogen, Zymed Laboratories, San Francisco, CA, USA)- and PKCi (Mat.Number 610176, dilution 1:100; BD Biosciences, Qume Drive San Jose, CA, USA). After washing with PBS, sections were incubated for 60 minutes at ambient temperature with the second antibody of the detection reagent (code K5027, Glostrup, Denmark). Diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd) was used as the chromogen. Finally, sections were counter stained with Mayer's hematoxylin.

Quantitative real-time reverse transcription polymerase chain reaction. Total ribonucleic acid (RNA) was extracted from cancer tissue and non-cancer tissue of the gastric adenocarcinoma patients and then reverse transcribed into cDNA using the high capacity cDNA Reverse transcription Kit (Applied Bio systems, California, USA). Quantitative real time RT-PCR was performed with SYBR Green master mix real-time core reagents on an ABI 7500 (Applied Bio systems) according to the manufacturer's instructions. Primers for quantitative real time RT-PCR were as follows: Taqman® stemness genes: Stathmin1 Hs00606370_m1 (Life Technologies).

Immuno-histochemistry evaluation. For a positive control of STMN1, we used a human breast carcinoma, while negative controls were performed by omitting the primary antibody. The mean percentage of positive tumor cells was determined using an Image System (Nikon Digital Camera, DXM1200F, Gotenba, Shizuoka, Japan).

The cytoplasmic staining of STMN1 expression was assessed by combining a proportion score and an intensity score. The proportion score was according to the proportion of tumor cells with positive cytoplasmic staining (0, none; 1, <=10%; 2, 10 to <=25%; 3, >25 to 50%; 4, >50%). The intensity score was assigned for the average intensity of positive tumor cells (0, none; 1, weak; 2, intermediate; 3, strong). The cytoplasmic score of STMN1 was the product of proportion and intensity scores, ranging from 0 to 12. The cytoplasmic expression was categorized into negative (score 0); 1+(score 1 to 3); 2+ (score 4-6); and 3+(score 7-12) (20). Scores of 0 and 1+ were defined as negative, scores of 2 + and 3 were defined as positive. Figure 1 shows representative images of immuno-histochemistry.

Cytoplasmic AID expression was evaluated by summing the staining percentage and intensity scores. The staining percentage was assigned to one of the following categories: 0=no staining; 1=up to 5% of stained cells; 2=5%-10% of cells stained; 3=10%-25% of cells stained; 4=25%-50% of cells stained; and 5=over 50% of cells stained. The staining intensity was assigned as: 0=no expression; 1+=weak expression; 2+=strong expression. The cytoplasmic expression levels of AID were classified as low (score ≤4) and high (score ≥5) (21). PKCi was scored according to a previous method as follows: 0, no staining; 1+, weak; 2+, moderate intensity; and 3+, strong intensity (22).

Table I. *Correlation between stathmin 1 (STMN1) expression and clinic-pathological findings in gastric adenocarcinoma.*

Factor	Stathmin 1 weak expression group (n=29)	Stathmin 1 wtrong expression group (n=30)	*p*-Value
Age (years)	61.7±13.6	66.7±10.2	0.8
Gender M/F	14/15	24/6	0.01
Invasion T 1,2/ T 3,4	22/7	26/4	0.29
Lymph node metastasis –/+	20/9	16/14	0.22
Peritoneal metastasis –/+	25/4	28/2	0.37
Stage I,II/III,IV	20/9	22/8	0.71
Venous invasion –/+	17/12	14/16	0.36
Lymphatic invasion –/+	17/12	14/16	0.36
Well Differentiated/poor differentiated	19/10	10/20	0.01
AID –/+	10/19	2/28	0.01
PKCi –/+	15/14	5/25	0.005

M/F: Male/Female; AID: activation-induced cytidine deaminase; PKCi: protein kinase C iota.

Statistical analysis. The *p*-value of the disease-free survival of the patients was defined by using the Kaplan Meier analysis with the JMP10 software (SAS Campus Drive, Cary, NC, USA). Information from the laboratory analysis and patients were entered into Stat. View 5.0 software (SAS Campus Drive, Cary, NC) and statistical analyses were performed using Student's *t*-test- and the chi-square test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Tumor tissues express high mRNA levels of STMN1. The *STMN1* mRNA level was checked by q real time RT-PCR in non-tumor and tumor tissue of 27 patients with gastric adenocarcinoma. The mRNA level of *STMN1* significantly expressed in tumor tissue compared to non-tumor tissue of gastric adenocarcinoma ($p<0.05$) (Figure 2).

Relationship between STMN1 protein and clinico-pathalogical findings in gastric adenocarcinoma. The relationship between STMN1 protein expression and the clinico-pathological characteristics, such as tumor differentiation; age; gender; tumor stages; and venous or lymphatic invasion were evaluated as shown in Table I. STMN1 expression was significantly associated with poorly-differentiated gastric adenocarcinoma ($p< 0.05$), and gender

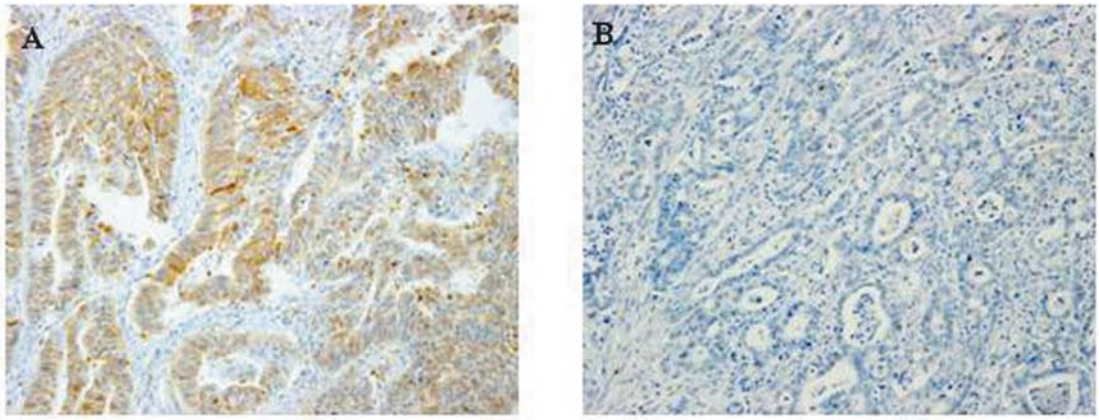

Figure 1. *Representative histological features of gastric adenocarcinoma by stathmin 1 (STMN1) immunostaining. A: STMN1 strong expression. B: STMN1 weak expression.*

($p<0.05$). However there was no significant correlation between STMN1 protein expression and age, stage, TNM classification and venous or lymphatic invasion ($p>0.05$).

Correlation between STMN1, AID and PKCi protein expression in gastric adenocarcinoma. In this study, 30 (50.8%) cases of STMN1 strong expression and 29 (49.2%) cases of STMN1-weak expression of gastric adenocarcinoma were examined. There were 47 (80%) high-cases and 12 (20%) low-cases of AID protein expression, a ratio implying a significant positive correlation with the STMN1 protein in gastric adenocarcinoma ($p<0.05$) (Table I). In addition 39 (66%) PKCi-positive cases and 20 (34%) PKCi-negative cases were observed, which correlated significantly with STMN1 protein in gastric adenocarcinoma ($p<0.05$) (Table I).

Disease-free survival rate of patients according to STMN1 expression in gastric adenocarcinoma. Disease-free survival was evaluated in 51 patients who underwent resection. Patients with stage IV of the disease were excluded from the disease-free survival evaluation. Median follow-up time was 36 months, with a range of 4 to 46 months. There was no significant difference, but disease-free survival of STMN1 expression was 80% in the strong and 100% in the low STMN1 expression groups at 4 years ($p=0.14$) (Figure 3).

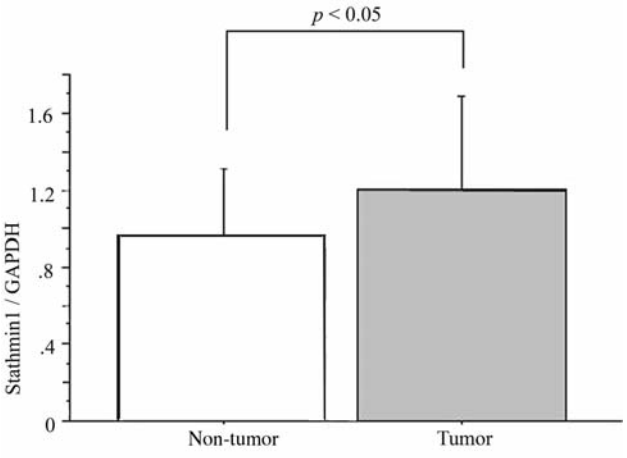

Figure 2. *mRNA expression level of STMN1 in the tumor and non-tumor tissue of gastric adenocarcinoma.*

Discussion

Recently, STMN1 has been proposed to function as an oncogene based on some relevant studies in multiple types of human cancers such as breast cancer (4), prostate cancer (5), lung cancer (6) and cervical carcinoma (7). Chen *et al*. reported that STMN1 protein was overexpressed in lung adenocarcinomas compared to normal lung tissue and in poorly-differentiated compared with well- or moderately-differentiated lung adenocarcinoma (23). However, the role of STMN1 in gastric cancer carcinogenesis in relation with AID and PKCi proteins in gastric adenocarcinoma patients has not been well-elucidated. In the present study the expression of STMN1 was detected by real-time quantitative reverse transcription polymerase chain reaction in gastric cancer and adjacent non-tumor tissues. In addition, the STMN1 expression was analyzed by immunohistochemistry in gastric cancer patients. The expression levels of *STMN1* mRNA and protein in gastric cancer tissues were both higher than those in adjacent non tumor tissues.

Clinicopathological studies have suggested that over-expression of STMN1 was significantly correlated with

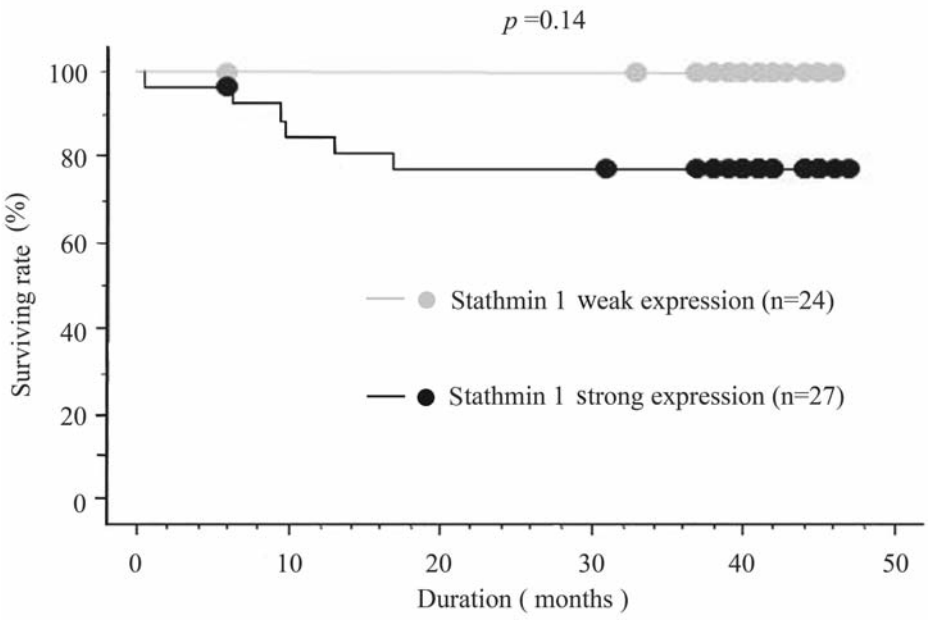

Figure 3. *Disease-free survival curve according to STMN1 protein expression in gastric adenocarcinoma.*

invasion, recurrence and poor prognosis in hepatoma and hepatocellular carcinoma (9) and that the expression of STMN1 was significantly related with lymph node metastasis in gastric cancer (8). However, in the present study, STMN1 was more highly expressed in poorly differentiated types of gastric adenocarcinoma than in well-differentiated tissues. The Kaplan-Maier analysis revealed that STMN1 strong expression has a tendency of recurrence for patients with gastric adenocarcinoma. This result showed that the STMN1 over expression might play an important role in the pathogenesis and subsequent progression of gastric adenocarcinoma. STMN1 could also be a potential therapeutic target in gastric cancer, especially for poorly-differentiated types of gastric adenocarcinoma.

Previous reports have shown that AID (10) and PKCi (15) proteins activate STMN1 expression through cyclin-dependent kinase (CDK) (11). Moreover, our study showed a significant correlation between STMN1 and AID, PKCi proteins in gastric adenocarcinoma patients, indicating that these proteins may influence each other and promote carcinogenesis.

Our study demonstrated that AID could be one of the activators of STMN1 protein expression in gastric adenocarcinoma. *P27* and STMN1 correlation was related either directly or by some other pathways. These include the pathway by which cyclin-dependent kinases activate STMN1 expression. Lancu-Rubin *et al*. reported that inhibition of CDK1 *via* p27 induces the STMN1 inactivation, which leads to de-stabilization of micro tubules (11). This finding indicates that activation of CDK induces the STMN1 activation. Matsumoto *et al*. reported that aberrant AID reduces the copy number of cyclin-dependent kinase inhibitors such as *CDKN2A* and *CDKN2B*, which are tumor suppressive genes and negatively controlled CDK (10). Thus, AID could be an activator of the STMN1 protein *via* the AID/CDKN2A/CDK/STMN1 pathway, although our result shows significant positive correlation between AID and STMN1 expression in gastric adenocarcinoma. Over expression of the AID protein reduces the cyclin-dependent kinase inhibitor, which leads to activation of CDK; its activation or up-regulation produces an activation of the STMN1 protein.

Although, the PKCi protein may play a role as a stimulator of STMN1 expression in gastric adenocarcinoma, its up-regulation is related to recurrence. There is a similar mechanism, where the PKCi protein induces activation of cyclin-dependent kinase, which induces up-regulation of STMN1. Desai *et al*. reported that PKCi induces glioma cell-cycle progression and proliferation *via* regulating Cdk7/Cdk2 activity in a PI (3)-kinase-dependent manner (15).

Previous studies reported STMN1 up-regulation to be correlated with *p53* mutations in hepatocellular cancer (HCC) (24, 25). Moreover microR-223 has a potential suppressive effect on STMN1 in hepatocellular carcinoma (HCC) (26) and in gastric cancer (20), as its effect is a down-regulation of microR-223 which leads to up-regulation of STMN1.

Conclusion

High mRNA level of the *STMN1* gene was significantly expressed in gastric tumor tissues compared to non-tumor and strong expression of STMN1 protein is correlated with poorly-differentiated gastric adenocarcinoma.

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