Title Page **Original Contribution**

Clinical significance of β2-adrenergic receptor expression in patients with surgically resected gastric adenocarcinoma

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Abstract

BACKGROUND: The β2-adrenergic receptor (β2AR) is highly expressed in various human neoplasms and has been considered a novel therapeutic target of cancer treatment. However, the clinicopathological significance of β2AR expression in patients with gastric cancer (GC) remains unclear. The aim of this study was to explore β2AR expression and its prognostic significance.

METHODS: A total of 331 patients with surgically resected GC were evaluated. Tumor sections were stained immunohistochemically for β2AR. And we confirmed β2AR expression in GC cell lines by Western blot.

RESULTS: β2AR was highly expressed in 30.5% of GC patients. Expression was significantly associated with age, T factor, tumor differentiation, histology of non-signet cells, lymphatic permeation, and vascular invasion. All GC cell lines expressed β2AR. On univariate analysis, age, disease stage, T factor, N factor, lymphatic permeation, vascular invasion, and β2AR expression were significantly associated with

overall survival. Although multivariate analysis did not indicate that β2AR expression was independently prognostic of survival, high-level β2AR expression was associated with significantly poorer survival of GC patients with well- or moderately differentiated tumors.

CONCLUSIONS: β2AR expression was a significant predictor of tumor aggressiveness in, and poorer survival of, patients with GC.

Key words: β2-adrenergic receptor, gastric cancer, prognosis, immunohistochemistry, β2-blocker

Introduction

Gastric cancer (GC) is the fourth most common malignant disease and the second most common cause of cancer-related death worldwide, especially in East Asian countries such as Japan, China and South Korea [1]. Curative gastrectomy with regional lymphadenectomy remains the standard treatment for patients with stages I-III GC, but approximately 20-30% of patients develop local or distant recurrences and die of progressive disease, despite a seemingly favorable prognosis after surgery [1]. Disease staging is the most important consideration in predicting the prognosis of patients with GC. Although novel drugs targeting HER2 and VEGFR-2 have been developed and administered clinically, no established biomarker has yet been associated with the prognosis or therapeutic response of patients with GC and the discovery of some promising biomarkers is expected to improve the survival of GC. Thus, more research is required to discover the molecular mechanisms associated with the identification of more promising biomarkers for patients with GC.

Beta-blockers are commonly used for the treatment of heart disease [2]. The β2-adrenergic receptor (β2-AR), a member of the transmembrane G protein-coupled receptor (GPCR) family,

triggers multiple signaling cascades and regulates cell proliferation through a classical cyclic-adenosine-monophosphate (cAMP)/protein kinase A (PKA) pathway [3]. Chemokines and neurotransmitters (ligands) bind to GPCRs and play essential roles in regulating cancer recurrence [4]. In a recent *in vitro* study, beta-adrenergic activation of the cAMP-PKA signaling pathway via β2-AR was shown to affect angiogenesis, followed by tumor proliferation and cell growth [5].

β2-AR is highly expressed in various human neoplasms, such as breast cancer, oral cancer, melanoma, prostate cancer and hepatocellular carcinoma, and it is closely associated with poorer survival, metastasis and tumor recurrence [6-10]. Interestingly, beta-blockers, especially propranolol, have been reported to prolong the survival of patients with breast cancer [11,12]. Recently, Shi *et al.* found that β2-AR was highly expressed in some gastric lesions, particularly metastatic tissues, suggesting that activation of β2-AR plays an important role in the metastasis of, and invasion by, GC [13]. However, little is known about the clinicopathological significance of β2-AR expression in patients with GC. The cited studies suggest that β2-AR might be an attractive therapeutic target in various human cancers including GC. Based on this background,

we conducted a clinical and pathological investigation of β2-AR expression in GC patients.

Materials and Methods

Patients and cell lines

Between January 2000 and December 2009, a total of 331 consecutive patients who underwent surgery for GC at our institution were selected for inclusion in this study. We obtained clinicopathological data (age, gender, histology, lymphatic permeation status, vascular invasion status, presence/absence of lymph node metastasis, and disease stage) from our database. Our study was retrospectively analyzed. This study was approved by the institutional review board of Gunma University Hospital (the Ethical Committee for Clinical Studies-Gunma University Faculty of Medicine).

Patient ages ranged from 28 to 90 years, with a median age of 69 years. No patient had received neo-adjuvant chemotherapy. Ninety-eight patients received adjuvant chemotherapy, of whom single-agent 5-fluorouracil (5-FU) was administered to 80 patients, a combination of 5-FU and taxane to 17 patients, and single-agent taxane to 1 patient.

All surgical specimens were reviewed and classified according to the WHO classification (seventh edition) by an experienced pathologist blinded to the clinical and imaging findings. Pathological tumor-node metastasis stages were defined using the International System for Staging adopted by the American Joint Committee on Cancer and the Union Internationale Centre le Cancer. Histologically, all patients had adenocarcinoma, and 181, 66, 64, and 20 patients had stage I, II, III, and IV tumors, respectively. The day of surgery was Day 1 in terms of the measurement of postoperative survival. The follow-up duration ranged from 72 to 5,430 days (median, 2,047 days). In terms of comorbid disease, 125 patients were taking anti-hypertensive drugs, of whom only 8 patients were taking β-blockers.

Human gastric cancer cell lines KATO-III, MKN7, MKN45 and MKN74 were maintained in RPMI 1640 containing 10% FBS and supplemented with 100 units/ml penicillin and streptomycin sulfate, and cultured in a humidified 5% CO_2 incubator at 37°C.

Immunohistochemical staining

β2AR expression was assessed by immunohistochemical staining using a rabbit anti-human

β2AR monoclonal antibody (Abcam, Inc., Cambridge, UK; 1:100 dilution) raised against a C-terminal peptide of human β2AR. Immunohistochemical staining was performed on paraffin sections using a polymer peroxidase method (Histofine Simple Stain MAX PO [MULTI] kit; Nichirei Corporation, Tokyo, Japan). Briefly, deparaffinized rehydrated sections were treated with 0.3% hydrogen peroxidase in methanol for 30 min to block endogenous peroxidase activity. To expose the antigen, sections were autoclaved in 10 mmol/L sodium citrate buffer (pH 6.0) for 5 min and cooled for 30 min. After rinsing in phosphate-buffered saline, sections were incubated with anti-β2AR antibody (1:100) overnight. Thereafter, sections were incubated with the Histofine Simple Stain of the MAX PO (MULTI) kit (Nichirei Corporation). The peroxidase reaction was performed using 0.02% 3,3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxidase in 0.05 M Tris-HCl buffer, pH 7.6. Negative control tissue sections featured omission of the primary antibody. β2AR expression was considered positive only if distinct cytoplasmic and plasma membrane staining was present. β2AR expression was scored as follows: I, ≤10% of tumor area stained; II, 11-25% stained; III, 26-50% stained; and IV, ≥51% stained. The tumors in which stained tumor cells scored ≥ IV were defined as "high-expression" tumors.

Five fields (x400) were analyzed to determine the frequency of the positive β2AR staining cells. Sections were evaluated by two investigators separately and in case of discrepancies both would evaluate the slide simultaneously and would agree in their final assessment. Neither investigators had knowledge of patient outcome.

Western blot

Total proteins were extracted from gastric cancer cells using the PRO-PREP (iNtRON Biotechnology, Kyungki-Do, Korea), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Bis-Tris gels, and transferred to membranes. The membranes were incubated overnight at 4°C with the antibodies against β2AR (1:1000; abcam), and β-actin (1:2000; Sigma, St. Louis, MO, USA), and then treated with horseradish peroxidase-conjugated secondary antibodies. Specific signals were detected using the ECL Prime Western Blotting Detection System (GE Healthcare, Tokyo, Japan) and quantified using an Image Quant LAS 4000 instrument (GE Healthcare).

Statistical analysis

A probability value <0.05 indicated a statistically significant difference. The significance of among-group differences was determined using Fisher's exact test. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. Overall survival (OS) was the time from tumor resection to death from any cause. Progression-free survival (PFS) was the time between tumor resection and the first episode of disease progression or death. Multivariate analyses were performed using stepwise Cox's proportional hazards model to identify factors independently prognostic of survival. Statistical analysis was performed using GraphPad Prism 4 software (Graph Pad Software, San Diego, CA, USA) and JMP 8 (SAS, Institute Inc., Cary, NC, USA) for Windows.

Results

Immunohistochemical analysis

Immunohistochemical analyses were performed on all 331 primary GC lesions. Figure 1 shows the immunohistochemical staining of β2AR in GC tissue. β2AR immunostaining was evident in

GC cells and was localized predominantly on the cytoplasmic and plasma membranes (Fig. 1). Of 331 GC sections, β2AR was highly expressed in 30.5% (101/331). On the other hand, we evaluated the expression level of β2AR in the normal gastric tissue of consecutive 40 patients with GC between January 2000 and December 2001. The high expression of β2AR was not observed in the normal gastric tissue (0%; 0/40). A statistically significant difference in the expression of β2AR was recognized between GC and normal gastric tissue. Next, we evaluated the expression of β2AR in the different grades of gastric cancer cell lines. The expression of β2AR was observed in the gastric cancer cell lines (MKN7, MKN74, MKN45 and KATO III) (Fig. 2). The expression of β2AR in KATO III was slightly low compared to the other cell lines.

Patient demographics by β2AR expression status are listed in Table 1. High β2AR expression was significantly associated with age, T factor, degree of tumor differentiation, histology of non-signet cells, lymphatic permeation, and vascular invasion.

Survival analysis by β2AR expression

The 5-year overall survival (OS) and progression-free survival (PFS) rates for all patients were

75% and 74%, respectively. Of the 331 patients, 102(30.8%) died after the initial surgery. On univariate analysis, age, disease stage, T factor, N factor, lymphatic permeation, vascular invasion, and β2AR expression were significantly related to poor OS. Also, disease stage, T factor, N factor, lymphatic permeation, and vascular invasion were significantly related to poor PFS (Table 2). Multivariable analysis confirmed that disease stage, age and lymphatic permeation were independent prognostic factor of poor OS, and disease stage and vascular invasion were significant predictors of poor PFS in GC patients. Figure 3 shows the Kaplan-Meier survival curves of patients with high- and low-level β2AR expression.

Next, we analyzed the prognostic significance of β2AR expression with respect to different variables (Table 3). High-level β2AR expression was associated with significantly poorer survival of patients with well- or moderately differentiated tumors, early-stage (I or II) disease, T factor classification (T1 or T2), and lymphatic permeation. Figure 3C and 3D shows the survival curve according to tumor differentiation.

Discussion

This is the first study to examine the prognostic significance of β2AR expression in a large sample of GC patients. We found that β2AR was highly expressed in approximately 30% of GC patients, and such high expression was closely associated with advanced age, well or moderate tumor differentiation, tumor aggressiveness, and the histology of non-signet cells. Moreover, the present study showed that the expression level of β2AR was significantly higher in gastric cancer specimens than in normal gastric tissue. In our *in vitro* study, the expression of β2AR was recognized in gastric cancer cells with different grades. High-level expression of β2AR was significantly prognostic of a poor outcome, but β2AR was not shown to be an independent prognostic predictor. Our study suggests that β2AR plays a crucial role as a predictor of poor outcomes in GC patients with well- or moderately differentiated tumors. However, it remains unclear why β2AR overexpression is significantly associated with tumor aggressiveness and shorter survival after surgery in patients with carcinomas that are not poorly differentiated. We found in the present study that β2AR expression may predict tumor aggressiveness and GC metastasis. Especially, it was suggested that the expression level of β2AR yields a close

relationship with lymph node metastases.

Recently, Ming *et al.* reported that β2AR expression was potentially associated with the HER2 signaling pathway, and inhibition of HER2 may not be effective in GC patients expressing β2AR [14]. As shown previously, the expression level of HER2 is higher in GC patients with well- or moderately differentiated tumors than in those with poorly differentiated tumors [15], in line with the results of our present study. However, the HER2 expression level was significantly higher in male than female GC patients and did not change with age [15], unlike our findings.

Several authors have described the clinical and pathological features associated with increased β2AR expression in different cancers, including oral squamous cell carcinoma, hepatocellular carcinoma and pancreatic cancer [10,16,17]. These studies showed that the prognostic significance of β2AR expression seemed to differ by cancer histological type. Higher-level expression of β2AR was shown to be associated with better survival in patients with squamous cell carcinoma, but a significant relationship was evident between enhanced β2AR expression and shorter survival in patients with non-squamous cell carcinoma [10,16,17]. High-level expression of β2AR may thus be a useful predictor of negative outcomes in patients

with adenocarcinoma. However, the differences in the β2AR prognostic role by histology and cancer type remain poorly understood. Further study is required to confirm the clinicopathological significance of β2AR expression as a prognostic predictor in patients with various neoplasms.

Shan *et al.* showed that norepinephrine mediated epithelial-mesenchymal transition (EMT) in GC patients, through the β2AR-hypoxia-inducible factor-1α regulatory axis (18). EMT is essential for cancer cell invasion and metastasis and is a key event during cancer progression and development [19]. Especially, EMT facilitates tumor cell migration from a primary site to a metastatic site. The cited study suggested that β2AR expression might be a very significant risk factor for cancer progression and development in patients with GC. In our present study, the β2AR expression level was closely associated with aspects of tumor progression such as lymphatic permeation and vascular invasion, although we did not explore the clinicopathological relationships between β2AR expression and EMT in our GC tumor specimens.

It has been shown experimentally that the β2AR antagonist propranolol significantly suppresses norepinephrine-induced stimulation of cell proliferation in cancer cell lines [20]. *In*

vivo, propranolol has been shown to inhibit prostate cancer cell growth [21]. Moreover, it has been suggested that inhibition of β2-adrenergic signaling slows tumor progression, metastasis, recurrence, and mortality in patients with breast cancer [11,12]. Several researchers have that inhibition of β2AR using propranolol could have clinical potential in the treatment of malignant melanoma [22,23]. Therefore, β2AR has received attention as an attractive therapeutic target for the treatment of cancer. In fact, we have some preliminary data on the associations between β2AR expression levels and tumor cell aggressiveness in several cancer cell lines; our preclinical results indicate that β2-adrenergic signaling plays a crucial role in carcinogenesis and the further development of cancers. However, only a few reports have explored the clinicopathological significance of β2AR expression immunohistochemically. Further work is required to investigate whether the β2AR expression level has clinical implications, perhaps as a predictive and/or prognostic marker in patients with various neoplasms.

There are several limitations to the present study. First, only eight patients were prescribed β-blockers to treat hypertension. Therefore, the relationship between the β2AR expression level and patient outcomes after administration of oral β-blockers remains unknown. Earlier reports

found that β-blockers might prolong the survival of patients with neoplasms [11,12]. Further work is warranted to evaluate whether β-blockers can suppress tumor growth in GC patients. Second, the optimal predictive β2AR cut-off levels for various neoplasms remain obscure. The immunohistochemical techniques used to evaluate β2AR expression levels have varied among studies. Thus, no recognized immunohistochemical technique has yet been generally accepted. This means that the β2AR expression levels reported may vary depending on the methodology used. Finally, this was a clinicopathological study on a heterogeneous population such with diseases of different stages and including patients with lymph node metastasis. This may have biased our survival analysis. Although we sought to perform such analysis on a maximally homogenous population, several limitations may have been at play when we sought to predict outcomes after surgery correctly.

In conclusion, β2AR was highly expressed in GC patients, and β2AR overexpression significantly predicted tumor aggressiveness in, and poor survival of, patients with GC. Further work is needed to explore whether β2AR inhibition is a useful treatment for patients with advanced GC.

Conflict of interest statement:

No author has any financial or personal relationship with any other person or organization that could inappropriately influence our work.

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Ethical statement

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Gunma University Hospital) and with the Helsinki Declaration of 1964 and later versions.

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Figure legends

Figure 1:

Immunohistochemical staining of β2AR in gastric cancer. Positive staining is evident on the cytoplasmic and plasma membranes of cancer cells. The β2AR immunostaining grades were Ⅳ(A), Ⅲ(B) and Ⅱ(C).

Figure 2:

Western Blotting of β2AR expression in gastric cancer cell lines.

The expression of β2AR was observed in all of the gastric cancer cell lines (MKN7, MKN74, MKN45 and KATO III). β-actin was used as a loading control.

Figure 3:

Outcomes after surgical resection as shown by Kaplan-Meier analysis of overall survival (OS) and progression-free survival (PFS) according to the β2AR expression level. Statistically significant differences in both OS (A) and PFS (B) were evident between patients with high- and low-level β2AR expression. A statistically significant difference in OS was observed between high- and low-level β2AR expression in patients with well- or moderately differentiated tumors

(C), but not in those with poorly differentiated tumors (D).