Clinical significance of NOTCH1 intracellular cytoplasmic domain translocation into the nucleus in gastric cancer

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Abstract. Recent studies have shown constitutive activation of the Notch signaling pathway in various types of malignancies. However, it remains unclear whether this signaling pathway is activated in gastric cancer. In the present study, the aim was to investigate the role of Notch signaling in gastric cancer by investigating the subcellular localization of Notch-associated proteins in tissue samples from gastric cancer patients. Samples were obtained from 115 gastric cancer patients who had undergone surgery at the Department of Gastroenterological Surgery, Nagoya City University Graduate School of Medical Science without pre-operative chemotherapy or radiation. Subsequently the correlation between translocation of NOTCH1 intracellular cytoplasmic domain (NICD) into the nucleus (as measured by immunostaining) and survival in gastric cancer patients after surgery was investigated. The results were analyzed in reference to the patients' clinicopathological characteristics and the effects of these results on patient prognosis were determined. Significant correlations were observed between NICD nuclear localization and clinicopathological characteristics, such as tumor status (T factor), lymph node status (N factor), pathological stage and differentiation status. No significant correlations were observed between NICD nuclear localization and age, gender, tumor location, vein invasion or lymphatic invasion. Patients with >30% of cancer cell nuclei positively stained for NICD (as revealed by immunostaining) were associated with a significantly shorter survival following surgery than patients with <30% NICD-positive cancer cell nuclei (log-rank test, P=0.0194). Univariate analysis revealed that among the clinicopathological factors examined, T factor [risk rate (RR)=10.870; P=0.0016], N factor (RR=41.667; P=0.0003), lymphatic invasion (RR=13.158; P=0.0125), vein invasion (RR=25.000; P= 0.0019) and translocation of NICD to the nucleus (RR=3.937; P=0.0312) were all identified to be statistically significant prognostic factors. However, multivariate analysis revealed that translocation of NICD to the nucleus was not independently associated with an unfavourable prognosis in patients with gastric cancer. The present results suggest that NOTCH1 acts as an oncogene in gastric cancer. It is hypothesized that translocation of NICD into the nucleus may be used as a therapeutic target in gastric cancer.

Introduction

Gastric cancer is one of the most prevalent types of human cancer. In Japan, ~50,000 people succumb due to gastric cancer every year (1). Survival prognoses for patients with gastric cancer remain poor, prompting researchers to continue searching for novel treatment strategies. To develop novel therapeutic options, it is important to understand the molecular mechanisms of gastric cancer.

Previous studies have shown constitutive activation of the Notch signaling pathway in various types of malignancies (2). However, it remains unclear whether this signaling pathway is activated in gastric cancer. The Notch signaling pathway is an evolutionary conserved pathway that is critical in various cellular processes, including cell fate decisions, proliferation, development, adult homeostasis and stem cell maintenance (3-7). NOTCH1 functions as a receptor in this signaling pathway.

The NOTCH1 receptor binds with one of its ligands, which include jagged 1 (JAG1), JAG2, delta-like 1 (*Drosophila*) (DLL1), DLL3 or DLL4 (2). The notch intracellular cytoplasmic domain (NICD) of the NOTCH receptor is then subjected to processing by proteases [a disintegrin and metalloproteinase (ADAM) protease or γ-secretase] and subsequently translocates into the nucleus (2). NICD then binds with target proteins to activate downstream targets and promote Notch signaling.

In the present study, immunostaining for NICD was performed in 115 gastric cancer tissue samples collected during gastric surgery to determine the correlation between localization of NICD, clinicopathological characteristics and survival prognoses in gastric cancer patients.

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Materials and methods

Tissue samples. Tumor tissue samples were obtained from 115 gastric cancer patients (comprising 74 males and 41 females; mean age, 63.1 years) who had undergone surgery at the Department of Gastroenterological Surgery, Nagoya City University Graduate School of Medical Science (Nagoya, Japan) between 1996 and 2007 without pre-operative chemotherapy or radiation. All samples were snap-frozen in liquid nitrogen and stored at -80˚C. The tumors were classified according to 6 th UICC guidelines for clinical and pathological studies on gastric cancer (8). Written, informed consent was obtained from each patient and approval was obtained from the ethical committee on human research of Nagoya City University (code, no. 71).

Immunohistochemistry. Immunohistochemical staining was performed on gastric cancer tissue samples that were fixed with 10% formalin for 1 day at room temperature, and then embedded in paraffin. Paraffin-embedded 3-μm tumor sections were deparaffinized using xylene (Wako Laboratory Chemicals, Osaka, Japan), rehydrated, and heat-treated by microwaving in 10 mM citrate buffer (Cell Signaling Technology, Tokyo, Japan) for 15 min for antigen retrieval. The sections were cooled to room temperature and washed three times with phosphate-buffered saline (PBS; Wako Laboratory Chemicals), for 5 min each time. Sections were then treated with 0.3% H_2O_2 in methanol for 30 min to neutralize endogenous peroxidases, blocked with Block-Ace (Dainihon Sumitomo Seiyaku, Osaka, Japan) for 10 min, and incubated with primary monoclonal antibodies targeting human NOTCH1 (1:50; cat. no. ST1028; Calbiochem; EMD Millipore, Billerica, MA, USA) overnight at 4˚C. The sections were then washed with PBS three times. The sections were incubated with a secondary antibody (1:10; EnVision+ kit; cat. no. K400211: Dako North America, Inc., Carpinteria, CA, USA) for 30 min and washed with PBS three times. Detection of immunoreactive proteins was facilitated with 3,3'-diaminobenzidine buffer tablets (EMD Millipore) and the sections were counterstained with hematoxylin. For the evaluation of NOTCH1 expression, immunostaining was considered positive only when unequivocally strong nuclear staining was present in >30% of the tumor cells, as analyzed using a light microscope. Cases with faint staining only were considered negative.

Statistical analysis. Statistical analysis was performed using the StatView 5.0 software package (Abacus Concepts, Berkeley, CA, USA). χ^2 tests were used to analyse the associations between NICD immunostaining and the clinical histopathological parameters of the patients. The survival rate of gastric cancer patients after surgery was examined using the Kaplan-Meier method, and survival times were compared using the log-rank test. Cox regression analysis of factors potentially associated with survival was conducted to identify independent factors that may exert a significant joint effect on survival. All tests were two-tailed and P<0.05 was considered to indicate a statistically significant difference.

Results

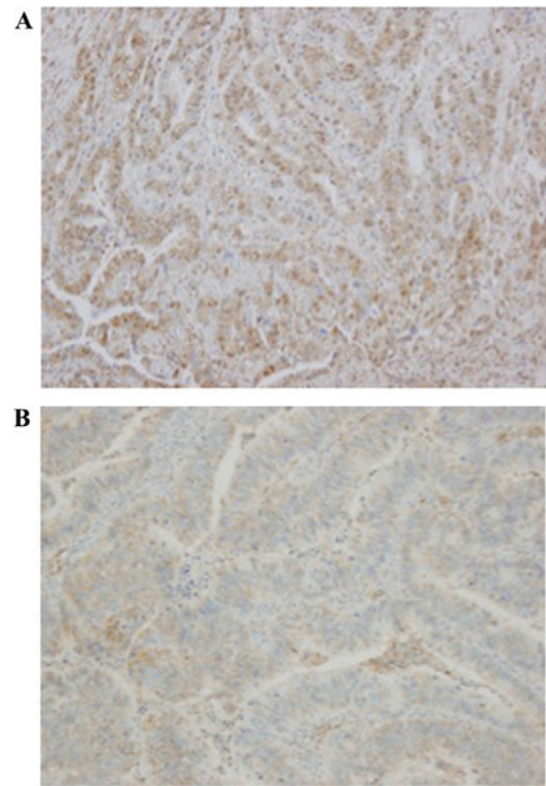*Expression of NICD in gastric cancer.* First, NOTCH1 localization was examined by immunostaining. Representative

Figure 1. Representative images of NICD immunostaining in gastric cancer tissues (magnification, x100). (A) More than 30% of cancer cell nuclei were stained positive for NICD. (B) Less than 30% of cancer cell nuclei were stained for NICD. NICD, NOTCH1 intracellular cytoplasmic domain.

images are presented in Fig. 1. The 115 gastric cancer patients were divided into two groups according to NICD expression: One group of patients exhibited NICD-positive staining in >30% of all cancer cell nuclei (n=61; positive group), and the other group had NICD-positive staining in <30% of nuclei (n=54; negative group).

Correlation between clinicopathological factors and NICD in gastric cancer. Correlations between NICD nuclear localization and the clinicopathological characteristics of the patients are presented in Table I. No significant correlation was observed between the NICD-positive group and age, gender, location, lymphatic invasion or blood vessel invasion (Table I). Significant correlations were observed between NICD nuclear localization and clinicopathological characteristics, such as tumor status (T factor; P=0.0069), lymph node status (N factor; P=0.0118), pathological stage (P=0.0160) and histological differentiation (P=0.0232).

Survival curves and expression of NICD. The correlation between nuclear localization of NICD and survival time in gastric cancer patients after surgery was investigated, and the mean follow-up was 32.53 months. The NICD-positive group (n=61) had a significantly shorter survival time following surgery when compared with the NICD-negative group [n=54; Fig. 2 (P=0.0194, log-rank test)].

Among the clinicopathological factors that were evaluated, univariate analysis indicated that local invasiveness [risk rate (RR)=10.870; P=0.0016], lymph node metastasis

Table I. Correlation of nuclear NICD in gastric cancer with clinicopathological factors, including patient and tumor characteristics (n=115).

Characteristics	NICD-positive patients/total patients	P-value
Age at surgery (years)		0.1315
≤65	39/66	
>65	22/49	
Gender		0.4953
Male	41/74	
Female	20/41	
Location		0.9236
Upper	15/27	
Middle	21/39	
Lower	25/49	
Tumor status		0.0069
T1	23/57	
T2	9/15	
T3	9/17	
T4	20/26	
T1 vs. T2-4	23/57 vs. 38/58	
Lymph node status		0.0118
N0	30/69	
N-positive	31/46	
Pathological stage		0.0160
I	27/63	
II	13/21	
III	21/31	
I vs. II-IV	27/63 vs. 34/52	
Histological differentiation		0.0232
Differentiated	38/82	
Undifferentiated	23/33	
Lymphatic invasion		0.1385
Negative	20/45	
Positive	41/70	
Blood vessel invasion		0.0786
Negative	25/56	
Positive	36/59	

NICD, NOTCH1 intracellular cytoplasmic domain.

Table II. Univariate analysis.

| Parameters	Risk ratio	95% confidence interval	P-value
Age at surgery (years)			
≤65	1		
>65	1.958	0.754-5.085	0.1677
Gender			
Male	1		
Female	1.379	0.532-3.577	0.5085
Primary tumor			
T1	1		
T2-4	10.870	2.481-47.619	0.0016
Lymph node metastasis			
N0	1		
N-positive	41.667	5.435-41.667	0.0003
Lymphatic invasion			
Negative	1		
Positive	13.158	1.739-100.00	0.0125
Vein invasion			
Negative	1		
Positive	25.000	3.289-200.00	0.0019
Differentiation status			
Differentiated	1		
Undifferentiated	3.039	1.172-7.874	0.0223
Immunostaining for NICD			
Negative	1		
Positive	3.937	1.131-13.70	0.0312

NICD, NOTCH1 intracellular cytoplasmic domain.
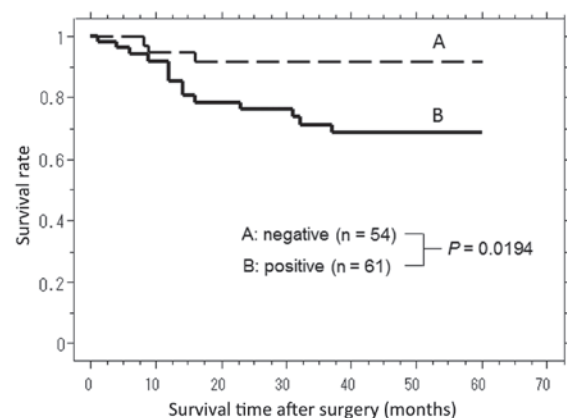
Figure 2. Overall survival rate of patients with gastric cancer according to NICD immunostaining. Patients with >30% of cancer cell nuclei positively stained for NICD had a significantly shorter survival time following surgery than patients with <30% of nuclei positively stained for NICD (P=0.0194).

(RR=41.667; P=0.0003), lymphatic invasion (RR=13.158; P=0.0125), vein invasion (RR=25.00; P=0.0019) and NICD nuclear localization (RR=3.937; P=0.0312) were statistically significant prognostic factors (Table II). Multivariate analysis revealed that only lymph node metastasis was an independent prognostic factor (Table III).

Discussion

NOTCH proteins are single-pass transmembrane receptors that regulate cell-fate decisions during development (9). The

NOTCH family includes four receptors, NOTCH1, NOTCH2, NOTCH3 and NOTCH4, whose ligands include JAG1, JAG2,

Table III. Multivariate analysis including NICD.

Parameters	Risk ratio	95% confidence interval	P-value
Primary tumor			
T1	1		
T2-4	0.371	0.0528-2.618	0.3204
Lymph node metastasis			
N0	1		
N-positive	27.778	2.632-333.33	0.0056
Lymphatic invasion			
Negative	1		
Positive	0.924	0.104-8.197	0.9435
Vein invasion			
Negative	1		
Positive	8.403	0.827-83.333	0.0719
Differentiation status			
Differentiated	1		
Undifferentiated	2.933	0.946-9.091	0.0623
Immunostaining for NICD			
Positive	1		
Negative	2.0747	0.451-9.524	0.3486

NICD, NOTCH1 intracellular cytoplasmic domain.

DLL1, DLL3, and DLL4 (2). The mature NOTCH1 receptor is a heterodimeric class I transmembrane glycoprotein, generated by proteolytic processing of a precursor polypeptide (proNOTCH1) in the trans-Golgi network (10). Receptors bind to their ligand NOTCH receptor, which is then subjected to processing by proteases (ADAM protease and γ-secretase) and translocate into the nucleus (11). NICD activates its targets, promoting protein-protein interactions (12). Data from the present study revealed that the translocation of NICD into the nucleus occurred in approximately half of the examined gastric cancer cases (53%; Table I). Therefore, it was hypothesized that the Notch signaling pathway is activated in approximately half of all gastric cancer cases.

Oncogenic roles for Notch signaling have also been discovered in Hodgkin's lymphoma (HL), anaplastic large-cell non-HL, certain types of acute myeloid leukemia and B-cell chronic lymphoid leukemia, gliomas, medulloblastomas, sarcomas, and various epithelial malignancies of the breast, cervix, lung, colon, prostate, head and neck, kidney, and pancreas (13-19). However, while many of the mechanisms underlying the deregulation in these malignancies remain unclear, the altered expression of Notch receptors or other Notch signaling pathway components is often associated with poor prognosis or tumor metastasis (20). Together, these facts indicate that Notch signaling is oncogenic in a variety of types of human tumor. Consistent with this, the present data indicates that NOTCH1 is oncogenic in gastric cancer, as nuclear translocation of NOTCH1 was correlated with the T and N factors, and a poor prognosis.

Conversely, Notch signaling is anti-oncogenic in squamous cell carcinoma (SCC) of the skin and cervical uterus, and for basal cell carcinoma (BCC) of the skin (21-23), partially due to its interference with canonical WNT signaling. Thus, it remains unclear whether NOTCH1 acts as a tumor suppressor or oncogene. However, according to the present data, activation of the Notch signaling pathway in gastric cancer is indicated to promote cancer, since NICD expression correlated with tumor status and lymph node metastasis (Table I). Therefore, the present study hypothesizes that NOTCH1 acts as an oncogene in gastric cancer.

The role of the Notch signaling pathway in gastric cancer is particularly complicated, and it is not clear what mechanisms regulate the NOTCH1 signaling pathway. However, a previous study suggested that NOTCH1 signaling contributes to the progression of human gastric cancer through induction of prostaglandin-endoperoxide synthase 2 (PTGS2) expression (24).

The *NOTCH1* gene is located on chromosome 9q34. A previous study reported that the most frequent chromosomal changes in gastric adenoma, as analyzed by comparative genomic hybridization, were gains on 9q (25). Thus, amplification of NOTCH1 signaling via chromosomal alterations may be involved in gastric cancer carcinogenesis.

In gastric cancer patients, prognostic markers, including erb-b2 receptor tyrosine kinase 2 (*ERBB2*) (26,27), *CD44* (28,29) and matrix metallopeptidase 12 (30) have been reported. Additionally, a previous study by the present team demonstrated that the expression of the microRNAs *mir-20b* and *mir-150* may be a prognostic marker for undifferentiated gastric cancer (1). Thus, NOTCH1 may be added to this list of prognostic markers. However, further investigations into the role of NOTCH1 in gastric cancer progression are required. Although the precise molecular mechanisms involved in the activation of the NOTCH1 signaling pathway remain to be clarified, the present data suggest that NOTCH1 may be a molecular target for the development of an effective therapeutic intervention for patients with gastric cancer.

Acknowledgements

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