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ORIGINAL ARTICLE

Clinical significance of altering epithelial–mesenchymal transition in metastatic lymph nodes of gastric cancer

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Abstract

Background The E-cadherin, N-cadherin, and Snail genes are epithelial–mesenchymal transition (EMT)-inducible genes. Previous studies demonstrated that the expression of EMT markers in the primary tumor sites of gastric cancer correlates with tumor progression and prognosis. However, the clinical significance of the expression of these EMT markers in metastatic lymph nodes remains unclear. In the present study, we investigated the expression of these EMT markers in the primary tumor sites and metastatic lymph nodes.

Methods Immunohistochemistry was used to investigate the expression of E-cadherin, N-cadherin, and Snail in 89 primary tumors and 511 metastatic lymph nodes obtained from patients with gastric cancer.

Results The weak expression of E-cadherin in tumors and lymph nodes increased with more lymph node metastasis and in more undifferentiated tumors. The strong expression of N-cadherin in lymph nodes correlated with more lymph nodes metastasis, an advanced stage, and poor prognosis. The weak expression of Snail in tumors correlated with lymphatic invasion. The strong expression of Snail in lymph nodes correlated with more lymph node metastasis and an advanced stage. The strong expression of Snail in tumors and its weak expression in lymph nodes correlated with more lymph node metastasis, an advanced stage, and poor prognosis.

Conclusions The expression of N-cadherin in metastatic lymph nodes is useful for predicting the prognosis of patients with gastric cancer. The Snail switch—namely, the positive-to-negative conversion of the Snail status—between primary tumors and lymph node metastasis may be important for confirming EMT and mesenchymal–epithelial transition.

Keywords Epithelial–mesenchymal transition · Gastric cancer · Metastatic lymph nodes · Snail switch

Introduction

Gastric cancer is one of the commonest malignancies, and patients with advanced gastric cancer have a poor prognosis [1]. Recent studies clearly demonstrate that epithelial–mesenchymal transition (EMT), a developmental process in which epithelial cells lose intercellular adhesion and myofibroblastic features, plays an important role in tumor progression and metastasis [2–5]. Significant changes occur during EMT, including the downregulation of epithelial markers such as E-cadherin and upregulation of mesenchymal markers such as N-cadherin [6–9]. A switch in cadherin from the loss of E-cadherin to gain of N-cadherin is part of the EMT process.

Snail, Slug, and Twist are some of the transcription factors that govern EMT [3]. Snail was previously reported to be important during EMT in several carcinomas, including non-small-cell lung carcinomas, ovarian carcinomas, urothelial carcinomas, esophageal squamous cell carcinomas, and gastric adenocarcinomas [10–14]. Natsugoe et al. [13] and Shin et al. [14] reported that overexpression of Snail in the main tumors of esophageal

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squamous cell carcinoma and gastric cancer was associated with a poor prognosis. A recent meta-analysis by Chen et al. [15] revealed Snail expression in gastric cancer. Their findings indicated that overexpression of Snail is associated with more lymph node metastasis (LNM) and an advanced stage. Snail family proteins are core EMT regulatory factors that play essential roles in developmental and disease processes and have been associated with metastasis in carcinomas [16–23]. The overexpression of Snail in different epithelial cells has been shown to strongly induce conversion to a fibroblastic phenotype at the same time as E-cadherin expression is lost, and invasive and migratory properties are acquired [16]. E-cadherin, N-cadherin, and Snail family proteins play a role in tumor progression in primary gastric cancer [14, 24–27]. However, the expression of these markers in LNM during EMT remains to be clarified. The aim of this study was to examine the clinical significance of E-cadherin, N-cadherin, and Snail expression in the primary tumors and LNM of gastric cancer.

Materials and methods

Patients and specimens

The study participants comprised 89 patients with gastric cancer who underwent gastrectomy with lymph node dissection between 2005 and 2012 at Kagoshima University Hospital, Kagoshima, Japan. All 511 metastatic lymph nodes were examined in this study. There were 60 men and 29 women, with a median age of 67.1 years (range 33–89 years). None of the patients received preoperative chemotherapy. Clinicopathological findings were based on the criteria of the tumor–node–metastasis (TNM) classification of the Union for International Cancer Control. The number of patients in each pT category was as follows: 20 in pT1, 5 in pT2, 33 in pT3, and 31 in pT4. All patients had LNM: 28 in pN1, 26 in pN2, and 35 in pN3. Postoperative follow-up data were obtained from all patients, with a median follow-up period of 49.6 months (range 3–157 months).

The Ethics Committee of Kagoshima University approved this study, and all patients provided written informed consent for the use of their information.

Immunohistochemical staining and evaluation

All resected specimens were fixed in 10% formaldehyde and routinely embedded in paraffin, and 3-μm-thick sections were prepared for immunohistochemistry. Sections were soaked in methanol with 3% H_2O_2 for 30 min to block endogenous peroxidase activity. Sections were incubated with an anti-E-cadherin monoclonal antibody (1:100; NCH-38; Dako, Tokyo, Japan), an anti-N-cadherin monoclonal antibody (1:50; 6G11; Dako, Tokyo, Japan), or an anti-Snail polyclonal antibody (1:500; ab85936; Abcam, Tokyo, Japan) at 4 °C overnight. E-cadherin, N-cadherin, and Snail expression in cancer tissue was visualized by the avidin–biotinylated peroxidase method.

Immunohistochemical evaluations were performed by two independent investigators (K.O. and Y.U.). To assess the expression of E-cadherin, N-cadherin, and Snail, ten fields (within the tumor and at the invasive front) were selected, and expression in 1000 tumor cells (100 cells per field) was examined by high-power (×200) microscopy. For E-cadherin, more than 60% of tumor cell staining was considered to reflect the preserved expression of E-cadherin, whereas 60% or less indicated reduced expression. The positive expression of N-cadherin and Snail was defined as detectable immunoreactivity in more than 5% and 75% of cancer cells respectively. These cutoff values for immunohistochemical evaluations of E-cadherin [28], N-cadherin [25], and Snail [14] expression were set on the basis of previously published data.

In LNM, all 511 metastatic lymph nodes were evaluated by the same methods as described for the primary tumors. E-cadherin-positive cases were defined by the positive expression in all LNM. N-cadherin-positive and Snail-positive cases were defined by more than one lymph node showing positive expression because the expression of N-cadherin and Snail was detected in only a few LNM.

Statistical analysis

Statistical analyses of group differences were performed by the χ^2 test and the t test. The Kaplan–Meier method was used for survival analysis, and differences in survival were examined by the log-rank test. Prognostic factors were assessed by univariate and multivariate analyses (Cox's proportional hazards regression model). All statistical calculations were performed with SAS (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered significant.

Results

Expression of E-cadherin, N-cadherin, and Snail

The expression of E-cadherin was observed on the cell membranes of cancer cells, indicating preserved expression, in 50.5% of primary tumors (45 of 89) and 51.6% of LNM (46 of 89) (Fig. 1a, b). The expression of N-cadherin was observed on the cell membranes of cancer cells in 31.4% of primary tumors (28 of 89) and 31.4% of LNM (28 of 89) (Fig. 1c, d). The expression of Snail was observed in the nuclei of cancer cells in 48.3% of primary tumors (43 of 89) and 51.7% of LNM (46 of 89) (Fig. 1e, f).

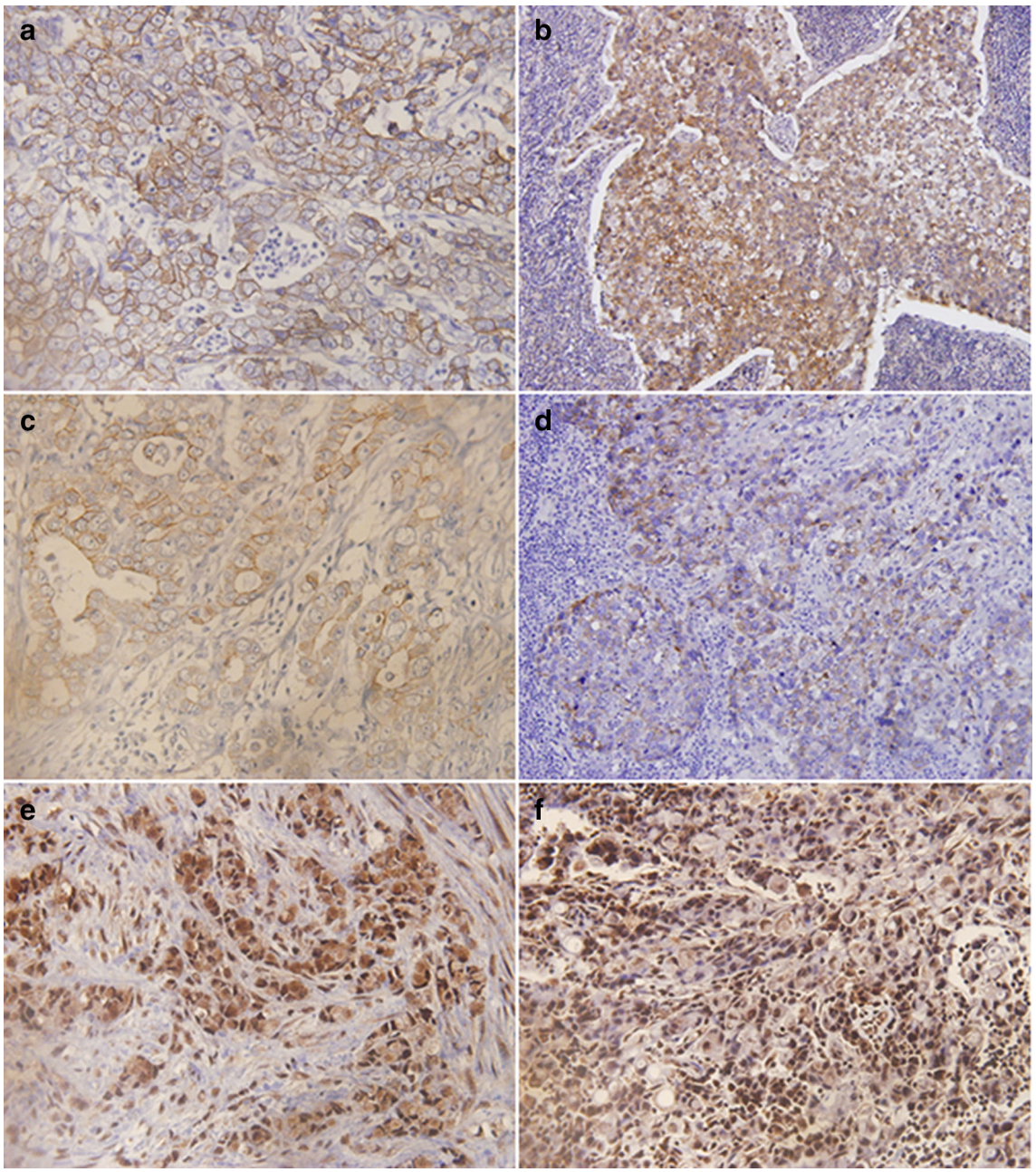

Fig. 1 Expression of E-cadherin, N-cadherin, and Snail in gastric cancer. E-cadherin expression was detected in the cell membranes of cancer cells: **a** primary tumor; **b** lymph node. N-cadherin expression was detected in the cell membranes of cancer cells: **c** primary tumor; **d** lymph node. Snail expression was detected in the nuclei of cancer cells: **e** primary tumor; **f** lymph node

Relationships between primary tumors and metastatic lymph nodes for E-cadherin, N-cadherin, and Snail expression

Table S1 shows E-cadherin, N-cadherin, and Snail expression in 511 metastatic lymph nodes, including primary tumors. Preserved E-cadherin expression in primary tumors was identified in 142 (41.6%) of 341 metastatic lymph nodes by its strong expression. On the other hand, reduced N-cadherin expression in primary tumors was identified in 272 (66.6%) of 408 metastatic lymph nodes by its weak expression.

Relationships between E-cadherin, N-cadherin, and Snail expression and clinicopathological factors

The weak expression of E-cadherin in primary tumors and lymph nodes increased with more LNM (primary tumor

$P = 0.027$; lymph nodes $P = 0.003$) and in more undifferentiated tumors (primary tumor $P < 0.0001$; lymph nodes $P = 0.015$) (Tables 1, 2). The strong expression of N-cadherin in primary tumors was associated with more LNM ($P = 0.061$) (Table 1). The strong expression of N-cadherin in lymph nodes correlated with more LNM and lymphatic invasion and an advanced stage ($P = 0.004$, $P = 0.004$, and $P = 0.015$ respectively) (Table 2). The strong expression of Snail in primary tumors correlated with lymphatic invasion ($P = 0.001$) (Table 1). The weak expression of Snail in lymph nodes correlated with more LNM ($P = 0.002$) and an advanced stage ($P = 0.048$) (Table 2).

In lymph nodes, a correlation was observed between the expression of E-cadherin and N-cadherin. The weak expression of E-cadherin correlated with the strong expression of N-cadherin in lymph nodes ($P = 0.012$) (Table 3). In most LNM cases, the expression of E-cadherin was weak, whereas that of N-cadherin was strong in primary tumors and lymph nodes. These expression patterns of E-cadherin and N-cadherin have emerged as one of the commonest indicators of the onset of EMT. On the other hand, in most LNM cases, the expression of Snail was strong in primary tumors and weak in lymph nodes. These expression patterns in primary tumors corresponded to the onset of EMT, whereas those in lymph nodes did not.

Relationships between E-cadherin, N-cadherin, and Snail expression and prognosis

No significant differences were observed in 5-year overall survival among patients with primary tumors and lymph nodes expressing E-cadherin (Fig. S1). Furthermore, the expression of N-cadherin in primary tumors did not correlate with 5-year overall survival (Fig. S2). However, a correlation was found between the expression of N-cadherin in lymph nodes and 5-year overall survival ($P = 0.0029$) (Fig. 2). No correlation was observed between the expression of Snail in primary tumors and lymph nodes and 5-year overall survival (Fig. S3). In LNM, the reduced expression of E-cadherin and preserved expression of N-cadherin, reflecting the EMT status, correlated with a poor prognosis ($P = 0.041$) (Fig. S4).

Relationship between the Snail switch and clinicopathological factors

We defined the positive-to-negative conversion of the Snail status in primary tumors and lymph nodes as the Snail switch and evaluated its clinicopathological and prognostic significance. Patients with the Snail switch showed positive Snail expression in primary tumors and negative Snail

Table 1 Relationships between E-cadherin, N-cadherin, and Snail expression and clinicopathological factors in primary tumors

	E-cadherin		*P*	N-cadherin		*P*	Snail		*P*
	Preserved ($n = 45$)	Reduced ($n = 44$)		Preserved ($n = 28$)	Reduced ($n = 61$)		Preserved ($n = 43$)	Reduced ($n = 46$)	
Sex									
Male	33	27	0.228	18	42	0.669	25	35	0.070
Female	12	17		10	19		18	11	
T category									
T1/T2	15	10	0.265	9	16	0.564	14	11	0.364
T3/T4	30	34		19	45		29	35	
N category									
N1	19	9	0.027	5	23	0.061	10	18	0.107
N2/N3	26	35		23	38		33	28	
Pathological stage									
I/II	20	12	0.091	11	21	0.657	15	17	0.838
III/IV	25	32		17	40		28	29	
Lymphatic invasion									
0/1	21	21	0.902	14	28	0.719	13	29	0.001
2/3	24	23		14	33		30	17	
Venous invasion									
0/1	24	28	0.324	18	34	0.447	22	30	0.178
2/3	21	16		10	27		21	16	
Histopathological type									
Differentiated	33	14	<0.0001	12	35	0.202	23	24	0.901
Undifferentiated	12	30		15	26		20	22	

Table 2 Relationship between E-cadherin, N-cadherin, and Snail expression and clinicopathological factors in lymph node metastasis

	E-cadherin		*P*	N-cadherin		*P*	Snail		*P*
	Preserved ($n = 46$)	Reduced ($n = 43$)		Preserved ($n = 28$)	Reduced ($n = 61$)		Preserved ($n = 46$)	Reduced ($n = 43$)	
Sex									
Male	33	27	0.368	20	40	0.584	29	31	0.362
Female	13	16		8	21		17	12	
T category									
T1/T2	16	9	0.146	5	20	0.145	16	9	0.146
T3/T4	30	34		23	41		30	34	
N category									
N1	21	7	0.003	3	25	0.004	21	7	0.002
N2/N3	25	36		25	36		25	36	
Pathological stage									
I/II	20	12	0.126	5	27	0.015	21	11	0.048
III/IV	26	31		23	34		25	32	
Lymphatic invasion									
0/1	25	17	0.161	7	35	0.004	26	16	0.068
2/3	21	26		21	26		20	27	
Venous invasion									
0/1	25	27	0.419	11	41	0.013	27	25	0.957
2/3	21	16		17	20		19	18	
Histopathological type									
Differentiated	30	17	0.015	13	34	0.414	23	24	0.583
Undifferentiated	16	26		15	27		23	19	

Table 3 Relationship between E-cadherin and N-cadherin expression

N-cadherin expression	E-cadherin expression		*P*
	Preserved	Reduced	
Primary tumor			
Preserved	14	31	0.942
Reduced	14	30	
Lymph nodes			
Preserved	9	37	0.012
Reduced	19	24	

expression in lymph nodes. The Snail switch was detected in 87 (17.0%) of 511 metastatic lymph nodes (Table S1).

Patients with the Snail switch accounted for 21.3% of all patients (19 of 89) (Table 4). Patients with the Snail switch had more LNM ($P = 0.0009$) and lymphatic invasion ($P = 0.002$) and an advanced stage ($P = 0.038$). N-cadherin expression levels in patients with the Snail switch were significantly high in primary tumors and LNM (Table 5). Furthermore, the Snail switch correlated with poor overall survival ($P = 0.0002$) (Fig. 3).

Discussion

EMT is a process through which epithelial cells are converted into mesenchymal cells and are changed such as the loss of cell–cell adhesion, loss of cell polarity, and gain of migration and invasion. The EMT process has been correlated with the presence of LNM, distant metastases, and a poor prognosis. Although previous studies examined only primary tumors, we have shown here EMT in primary tumors and metastatic lymph nodes. Significant changes generally occur during EMT, including the downregulation of epithelial markers such as E-cadherin and upregulation of mesenchymal markers such as N-cadherin. We previously examined the relationship between E-cadherin and Slug and N-cadherin in patients with gastric cancer: Uchikado et al. [24] reported that patients with weaker E-cadherin expression or positive Slug expression had poor clinical outcomes; Kamikihara et al. [25] found that neoexpression of N-cadherin may be a useful prognostic marker independent of E-cadherin expression.

In the present study, the expression of E-cadherin and N-cadherin in primary tumors was consistent with previous findings. However, their expression in primary tumors did

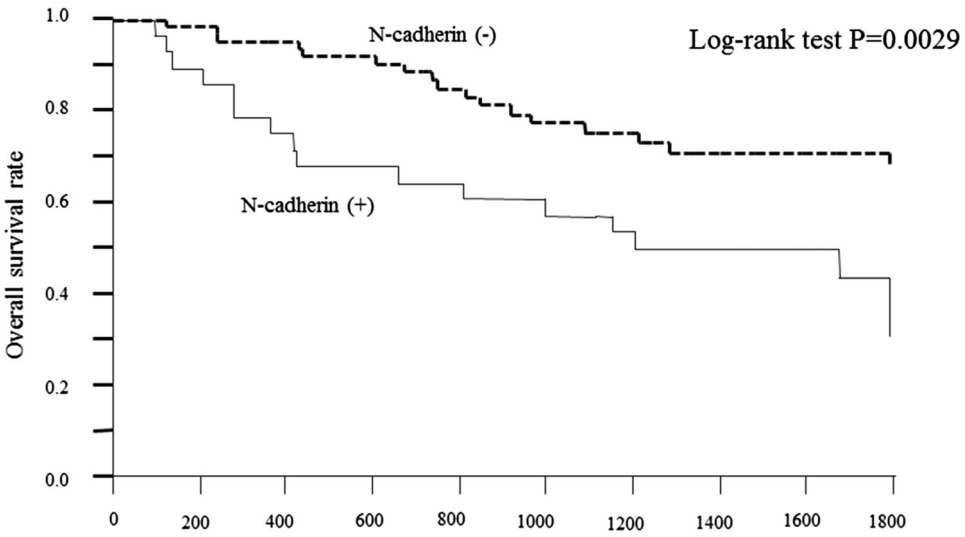

Fig. 2 Postoperative 5-year survival curves of patients according to the expression of N-cadherin in lymph nodes. The preserved expression of N-cadherin in lymph nodes correlated with a poor prognosis ($P = 0.0029$)

Table 4 Relationships between the Snail switch and clinicopathological factors

	Snail switch		*P*
	Positive ($n = 19$)	Negative ($n = 70$)	
Sex			
Male	13	47	0.916
Female	6	23	
T category			
T1/T2	3	22	0.178
T3/T4	16	48	
N category			
N1	0	28	0.0009
N2/N3	19	42	
Pathological stage			
I/II	3	29	0.038
III/IV	16	41	
Lymphatic invasion			
0/1	3	29	0.002
2/3	16	41	
Venous invasion			
0/1	9	43	0.270
2/3	10	27	
Histopathological type			
Differentiated	9	38	0.592
Undifferentiated	10	32	

not correlate with prognosis. The reason for this may be that all patients in this series had LNM; no non-LNM patients were included. In LNM, the weaker expression of E-cadherin and the preserved expression of N-cadherin correlated with a poor prognosis. Therefore, EMT—namely, the weaker expression of E-cadherin and preserved expression of N-cadherin—may have been induced in LNM. The evaluation of EMT-related markers in LNM may be more useful than in primary tumors. To our knowledge, the clinical significance of EMT in metastatic lymph nodes of gastric cancer has not been studied before; therefore, this is the first investigation to further understanding of the EMT process in metastatic sites.

Markiewicz et al. [29] reported that the expression levels of *TWIST1* (which encodes Twist), *SNAIL* (which encodes Snail), and *SLUG* (which encodes Slug) were significantly higher in LNM than in primary tumors. Furthermore, the negative-to-positive conversion of the Snail status correlated with worse survival in breast cancer. However, this is in contrast to our results for the conversion of the Snail status. The weaker expression of Snail in the lymph nodes was associated with LNM and stage, but did not correlate with overall survival, although the Snail switch, which is the positive-to-negative conversion of the Snail status, is associated with LNM, stage, and lymphatic invasion. The Snail switch correlated with a poor prognosis. These results indicate that patients with the Snail switch have more aggressive disease.

Snail switch is a new concept to understand the EMT phenomenon. When tumor cells transfer from metastatic lymph nodes to another lymph node, Snail expression in metastatic lymph nodes seems to be generally overexpressed. The main reason for this hypothesis is that Snail operates to reduce E-cadherin expression and to increase N-cadherin expression in metastatic lymph nodes. However, this study demonstrated that metastatic lymph nodes had reduced expression of Snail. Consequently, these findings may be caused by mesenchymal–epithelial transition (MET) rather than EMT in metastatic lymph nodes. Moreover, patients with the Snail switch had high levels of N-cadherin expression in both primary tumors and LNM

Table 5 Relationship between the Snail switch and E-cadherin and N-cadherin expression

	Snail switch		*P*
	Positive ($n = 19$)	Negative ($n = 70$)	
E-cadherin expression in primary tumors			
Preserved ($n = 45$)	10	35	0.838
Reduced ($n = 44$)	9	35	
E-cadherin expression in lymph node metastasis			
Preserved ($n = 46$)	8	38	0.346
Reduced ($n = 43$)	11	32	
N-cadherin expression in primary tumors			
Preserved ($n = 28$)	11	17	0.005
Reduced ($n = 61$)	8	53	
N-cadherin expression in lymph node metastasis			
Preserved ($n = 28$)	13	15	<0.001
Reduced ($n = 61$)	6	55	

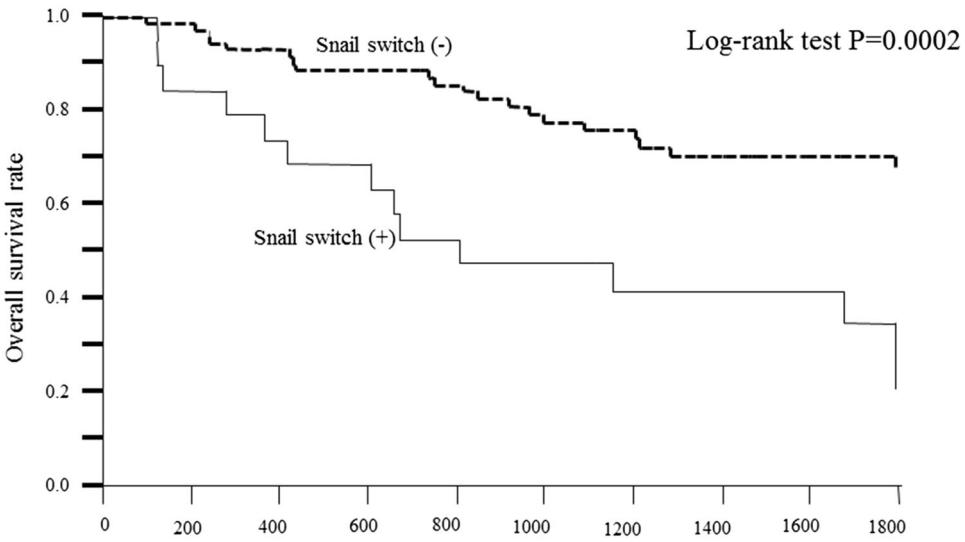

Fig. 3 Postoperative 5-year survival curves of patients according to the Snail switch. The Snail switch correlated with poor overall survival ($P = 0.0002$)

(Table 5). However, the Snail switch was not significantly correlated with E-cadherin expression levels in both primary tumors and LNM (Table 5). These findings may indicate that E-cadherin expression levels in LNM are reduced by EMT after MET caused by the function of Snail. Therefore, Snail may reduce the expression of E-cadherin during development and tumor progression in the gastrointestinal tract. Snail may also be downregulated in lymph nodes so as to adhere to metastatic sites.

Hur et al. [30] analyzed the expression and methylation status of miR-200 family members in primary colorectal cancer and liver metastasis. The expression of the *ZEB1* was significantly weaker in liver metastasis than in the corresponding primary tumors. Metastasized liver cells become hypomethylated at the miR-200c locus, which initiates the MET process. Kurashige et al. [31] indicated that miR-200 inhibits the expression of *ZEB2* and enhances that of E-cadherin in gastric cancer. Their findings suggest that miR-200 negatively regulates EMT, and thus may reduce the risk of metastasis in gastric cancer. Saito et al. [32] reported a relationship between long noncoding RNA activated by transforming growth factor β and the expression of *ZEB1* and miR-200 in gastric cancer. Comijn et al. [33] showed that ZEB family members, similarly to Snail gene family members, also bind to the E-box in the E-cadherin gene promoter through their two zinc finger domains. Thus, the expression of Snail may be the same as that of *ZEB1* of the zinc finger family. Our results indicate that the expression of Snail was reduced by the hypomethylation of miR-200 in LNM.

In the present study, most LNM cases were associated with the reduced expression of E-cadherin and preserved expression of N-cadherin. Therefore, EMT appears to be induced in tumors for metastasis and MET appears to be induced in lymph nodes for adherence to metastatic sites through the downregulation of Snail. EMT is then induced

in lymph nodes so that metastasis to the surrounding lymph nodes can occur. However, EMT in lymph nodes may be associated with a factor other than Snail.

A recent study reported that EMT may be associated with "cancer stem cells" and this is sufficient to induce stemness and tumorigenicity. Ryu et al. [34] performed immunohistochemistry for EMT-related proteins including Snail, ZEB-1, E-cadherin, vimentin, and β-catenin as well as the cancer stem cell marker CD44 in 276 consecutive primary gastric cancers and 54 matched LNM. They showed that the gastric cancer stem cell marker CD44 correlated with the expression of EMT-activating transcription factors. Moreover, in the gastric epithelium, stem cells at the base of the pyloric gastric glands were found to be reliant on an active and dynamically regulated Wnt pathway [35, 36]. Further studies are needed to elucidate the relationship between the Wnt pathway, Notch pathway, and cancer stem cells.

In the present study, we performed only immunohistochemistry to examine protein expression. Therefore, we were unable to identify biological processes occurring in lymph nodes similar to those involving miR-200. However, the Snail switch between the primary tumor and LNM may be important for confirming EMT and MET.

The present study had several limitations. The median follow-up period was 49.6 months. Furthermore, this study was based on a retrospective analysis of a small patient sample in a single institution. Accordingly, larger validation studies are needed to strengthen the results from this study.

Conclusions

The reduced expression of E-cadherin and the preserved expression of N-cadherin play key roles in EMT. The expression of N-cadherin in LNM is useful for predicting prognoses of patients with gastric cancer. The positive-to-negative conversion of the Snail status correlated with LNM and a poor prognosis. The Snail switch may be important for confirming EMT and MET. Further studies are needed to elucidate the biological processes occurring in LNM.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. The Ethics Committee of Kagoshima University approved the study, and all patients provided written informed consent for the use of their information.

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