Clinicopathological significance of N-cadherin and VEGF in advanced gastric cancer brain metastasis and the effects of metformin in preclinical models

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Abstract. Gastric cancer is the second most common cause of cancer-related death worldwide. Although brain metastasis is a rare complication of gastric cancer, no standard therapy for gastric cancer brain metastasis has been established. We attempted to identify biological markers that predict brain metastasis, and investigated how to modulate such markers. A case-control study of patients newly diagnosed with gastric cancer who had developed brain metastasis during follow-up, was conducted. These patients were compared with patients who had advanced gastric cancer but no evidence of brain metastasis. Immunohistochemistry was used to analyze the expression of E-cadherin, N-cadherin, MSS1, claudin-3, claudin-4, Glut1, clusterin, ITGB4, vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) and p53. The expression of VEGF tended to be higher in the case group (33.3 vs. 0%, p=0.055). Median survival was significantly correlated with vascular invasion (12 vs. 33 months, p=0.008) and N-cadherin expression (36 vs. 12 months, p=0.027). We also investigated the effects of metformin in tumor-bearing mouse models. VEGF expression was decreased and E-cadherin increased in the metformin-treated group when compared with the control group. The expression of the mesenchymal marker MMP9 was decreased in the metformin-treated group. Brain metastasis of advanced gastric cancer was associated with the expression of VEGF. Metformin treatment may be useful for modulating the metastatic capacity by reducing VEGF expression and blocking epithelial-to-mesenchymal transition.

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Introduction

Gastric cancer is the fourth most common malignancy and the second most common cause of cancer-related death worldwide (1). As a result of early detection, developments in surgical techniques and the development of anticancer agents, the survival of patients with gastric cancer has improved. However, the prognosis of advanced gastric cancer is still poor. While a 5-year survival rate of patients with stage I is more than 96%, that of patients with stage IV is less than 20% (2). Central nervous system (CNS) metastasis is a rare complication of gastric cancer, occurring in 0.16-0.69% of gastric cancer patients, and its clinical features, treatment outcomes and prognostic factors remain unclear (3).

Numerous molecular events are involved in metastatic progression, such as loss of cellular adhesion, detachment of tumor cells from the original sites, degradation of the extracellular matrix, migration of tumor cells, angiogenesis, and implantation of tumor cells at distant sites (4). The transmembrane glycoproteins E- and N-cadherin play a role in calcium-dependent cell-to-cell adhesion, and reduced E-cadherin expression is associated with gastric and colon cancer progression and metastasis (5,6). MSS1 is a nuclear-encoded mitochondrial GTPase, and silencing of MSS1 gene expression is associated with neuroinflammation (7). Claudin-3 and -4 are major structural molecules of the tight junctions that link epithelial cells (8). They are associated with worse malignancy grades, not only in terms of size and invasiveness, yet also in potential metastatic ability and patient outcome. Malignant cells show increased glucose uptake *in vitro* and *in vivo*, and this is facilitated by glucose transporters (Gluts) (9). The expression of Glut1 has been described in several malignancies, including those of the esophagus, colon and brain, and Glut1 expression is associated

with tumor aggressiveness and survival in gastric cancer (10). Clusterin expression is upregulated in many malignancies, including head and neck, breast and prostate cancers (11). The function of clusterin in cell reconstruction contributes to the occurrence and progression of cancer (12). ITGB4 is an adhesion receptor for lamins and plays a key role in the progression of various carcinomas via its ability to orchestrate key signal transduction events and promote cell motility (13). Vascular endothelial growth factor (VEGF) is one of the most important factors promoting vascularization, and it plays a role in both physiological and malignant conditions (14). Several studies have reported that VEGF is associated with lymph node metastasis in gastric cancer. Epidermal growth factor receptor (EGFR) is a transmembrane receptor that contributes to many processes involved in cell survival and proliferation and also inhibits apoptosis, which leads to cancer development (15). Preusser *et al* evaluated the expression of EGFR immunohistochemically in tissue samples of gastroesophageal cancer brain metastasis, which they proposed could be treated by agents targeting EGFR (16). p53 is a nuclear phosphoprotein involved in regulating cell proliferation by inhibiting the G_1 to S phase progression of the cell cycle, and it is frequently mutated in a variety of human cancers (17). The present study evaluated the expression of E- and N-cadherin, MSS1, claudin-3 and -4, Glut1, clusterin, ITGB4, VEGF, EGFR and p53 in surgical specimens of gastric cancer with and without brain metastasis and analyzed their correlations with clinicopathological features and survival.

Metformin is an oral biguanide introduced in the 1950 for the treatment of type 2 diabetes (18). A recent epidemiologic survey found that metformin had also significant effects on tumorigenesis (19). It was also reported that metformin inhibits human gastric cancer cell proliferation and tumor growth, possibly by suppressing cell cycle-related molecules (20). Candidate markers associated with brain metastasis were investigated in tumor-bearing mouse models treated with metformin to evaluate its use in preventing or targeting brain metastasis therapeutically.

Materials and methods

Patients. Nine samples of gastric adenocarcinoma with brain metastasis were acquired from St. Vincent's Hospital, The Catholic University of Korea from June 1997 to September 2009. Two of the nine patients underwent craniotomies to resect metastatic brain lesions. Seven patients were diagnosed with brain metastasis using brain magnetic resonance imaging (MRI), four of whom showed positive cytology in the cerebrospinal fluid (CSF). Thirteen samples of advanced gastric adenocarcinoma without brain metastasis were also included.

The study protocol was approved by the Institutional Review Board of St. Vincent's Hospital, The Catholic University of Korea. The stages of all patients were evaluated in accordance with the guidelines of the Japanese Classification of Gastric Carcinoma (21). The surgical treatment comprised gastric resection, bypass procedures or laparotomy alone. All patients were followed for a median of 12.5 (range 3-80) months. Patient data were obtained from our own and the Korea Central Cancer Registry database. Survival time was measured from the date of the initial surgery to that of death or last follow-up. Patients who died as a result of surgery or from other causes were excluded from the present study.

Construction of the tissue microarray block. Formalin-fixed paraffin-embedded tissues were obtained from the subjects. Using hematoxylin and eosin (H&E)-stained slides, a representative tumor site was chosen, and the site corresponding to the confirmed tumor site in the paraffin block was marked. Areas with necrosis, hemorrhage or artifacts were excluded. Single-core biopsy specimens 2 mm in diameter were taken from the representative regions (Seongkohn Trader's Co, Seoul, Korea), placed on a trimethylamine (TMA) mold with 60-pores, and re-embedded with paraffin. The TMA blocks were prepared as 4-μm-thick sections and stained with H&E. The tissues were then examined to determine whether an appropriate tumor site had been selected.

Immunohistochemistry. We performed immunohistochemical staining using tissue microarray sections and a BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA), according to the manufacturer's protocol. The respective antibodies used for immunohistochemistry of E- and N-cadherin, MSS1, claudin-3 and -4, Glut1, clusterin, ITGB4, VEGF, EGFR and p53 were 36/E-cadherin (1:50; BD Biosciences, Rockville, MD, USA), N-cadherin rabbit monoclonal antibody (1:100; Enzo Life Sciences, Plymouth Meeting, PA, USA), ab78161 (1:25), ab15102 (1:50) (both from Abcam, Cambridge, MA, USA), mouse monoclonal antibody to claudin-4 (1:100; Young in Frontier, Seoul, Korea), ab14683 (1:200; Abcam), mouse monoclonal antibody to clusterin (1:50; Young in Frontier), ITGB4 rabbit monoclonal antibody (1:15; Abgent, San Diego, CA, USA), mouse monoclonal antibodies to VEGF (1:100), EGFR (1:100) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ab61256 (1:100; Abcam). Ki-67 p53, bcl-2 and Bax were detected using clone PP-67 (1:100; Abcam). Clone images were acquired using an Olympus BX41 microscope with a DP72 digital camera (both from Olympus, Tokyo, Japan).

The immunostained slides were examined under light microscopy by one of the authors (S.H. Kim). The immunohistochemical results were scored semi-quantitatively using a four-point scale: 0, no immunoreaction; 1+, faint or equivocal immunoreaction in <10% of cells; 2+, unequivocal, strong immunoreaction in <30% of cells; and 3+, unequivocal, strong immunoreaction in >30% of cells. Tumors with 1+, 2+ or 3+ expression were interpreted as positive, while tumors with no expression were interpreted as negative.

Inoculation of intracranial cancer cells and experimental design. The human gastric adenocarcinoma MK28 cell line was provided by one of the authors (S.H. Kim) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) under routine tissue culture conditions. Four-weeks-old male athymic nude mice (Central Laboratory Animals, Seoul, Republic of Korea) were used. The nude mice were anesthetized using an intraperitoneal (i.p.) injection of 12 mg/kg xylazine (Rompun; Cutter Laboratories, Shawnee, KS, USA) and 30 mg/kg ketamine (Ketalar; Parke-Davis, Morris Plains, NJ, USA). The mice were then stereotactically inoculated with $1x10^6$ MK28 cells into the right frontal lobe

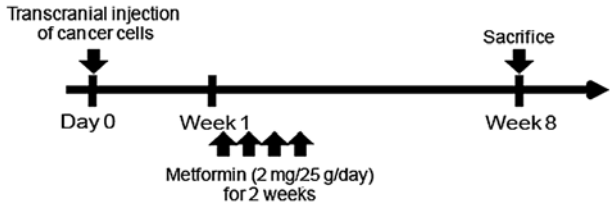

Figure 1. Therapeutic schedule.

(2 mm lateral and 1 mm anterior to the bregma at a depth of 2.5 mm from the skull) using a sterile Hamilton syringe fitted with a 26-gauge needle (Hamilton, Reno, NV, USA) and a microinfusion pump (Harvard Apparatus, Holliston, MA, USA).

The experimental design is shown in Fig. 1. Each group contained five mice initially. Mice in the treatment group were treated with metformin (2 mg/25 g/day) via i.p. injection for 14 days, 1 week after being inoculated intracranially with MK28 cells. Mice in the control and treatment groups were euthanized 8 weeks after the intracranial inoculation, and tumor specimens were obtained for reverse transcription-polymerase chain reaction (RT-PCR) and western blotting. All experiments were approved by the Ethics Committee of the St. Vincent's Hospital, The Catholic University of Korea.

Reverse transcription-PCR. Total RNA from all specimens was extracted using an RNeasy Mini kit (Qiagen), and 1 μg was reverse-transcribed using RT-premix (M-Biotech, Seoul, Korea). RT-PCR was performed on cDNA samples using a DNA Thermal Cycler (Bio-Rad) with Go *Taq* Green Master Mix (Promega, Madison, WI, USA), RNase-free water and the following primers: E-cadherin 5'-AAGTGACCGATGATGA TGCC-3' (forward), and 5'-CTTCTCTGTCCATCTCAGCG-3' (reverse); and 18S rRNA 5'-CGCGGTTCTATTTTGTTGGT-3' (forward), and 5'-AGTCGGCATCGTTTATGGTC-3' (reverse). Expression of MMP9, N-cadherin and VEGF was assessed by RT-PCR using the following primers: MMP9 5'-AGTTTGGTG TCGCGGAGCAC-3' (forward), and 5'-TACATGAGCGCTT CCGGCAC-3' (reverse); N-cadherin 5'-GCCACCATATGACT CCCTCTTAGT-3' (forward), and 5'-CAGAAAACTAATTCC AATCTGAAA-3' (reverse); and VEGF 5'-CCAGCGAAGCTA CTGCCGTCCA-3' (forward), and 5'-ACAGCGCATCAGCGG CACAC-3' (reverse). The RT-PCR products were separated on 1.2% agarose gels containing ethidium bromide and were visualized with ultraviolet light.

Western blot analysis. Total proteins in the tumor specimens were extracted using PRO-PREP Protein Extraction Solution (Intron Biotechnology, Gyeonggi-Do, Korea) according to the manufacturer's instructions. Western blot analysis was performed to confirm expression using antibodies recognizing the specific epitopes. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and detected with antibodies to E-cadherin (BD Biosciences, Franklin Lakes, NJ, USA), MMP9 (Abcam), VEGF (Santa Cruz Biotechnology), N-cadherin (Abcam) and β-actin (Sigma-Aldrich, St. Louis, MO, USA). After washing, the membrane was incubated with anti-mouse IgG or anti-rabbit IgG, horseradish peroxidase (HRP)-linked secondary antibody (Cell Signaling Technology, Danvers, MA, USA) or anti-goat IgG HRP-antibody derived from rabbit (Sigma-Aldrich). Immunoreactivity was detected using the ECL chemiluminescence system (Thermo, Rockford, IL, USA) and quantified using an imaging densitometer (Model LAS 4000 mini; GE Healthcare Bio-Sciences AB Hercules, Japan). The density of each band was quantified using Quantity One software (Bio-Rad Laboratory, Hercules, CA, USA).

Table I. Characteristics of the patients.

Parameters	Case group	Control group	P-value
Gender			>0.05
Men	7	10	
Women	2	3	
Age (years)			>0.05
Median (range)	74 (45-82)	71 (43-81)	
Differentiation			>0.05
Well	0	1	
Moderate	4	4	
Poor	5	7	
Mucinous	0	1	
Size			>0.05
Mean ± SD	5.0±1.8	6.7±2.5	
Stage			>0.05
I	1	0	
II	0	2	
III	7	4	
IV	1	7	
Lymphatic invasion	44.40%	92.30%	0.023
Vascular invasion	44.40%	38.50%	>0.05
Neural invasion	33.30%	69.20%	0.192
Follow-up (months)			>0.05
Median (range)	12 (4-80)	18 (3-36)	

Statistical analysis. The distributions of patient characteristics were compared between the study and control groups using the Wilcoxon rank-sum and Fisher's exact tests for continuous and discrete variables, respectively. Differences in the immunohistochemical results between groups were analyzed using the Chi-square test. Overall survival was analyzed using the Kaplan-Meier method, and survival data were compared using a log-rank test. A p-value of <0.05 was considered to indicate a statistically significant result.

Results

Patient characteristics. The characteristics of the present study population are listed in Table I. There were no significant differences in age, gender, histology type, tumor size,

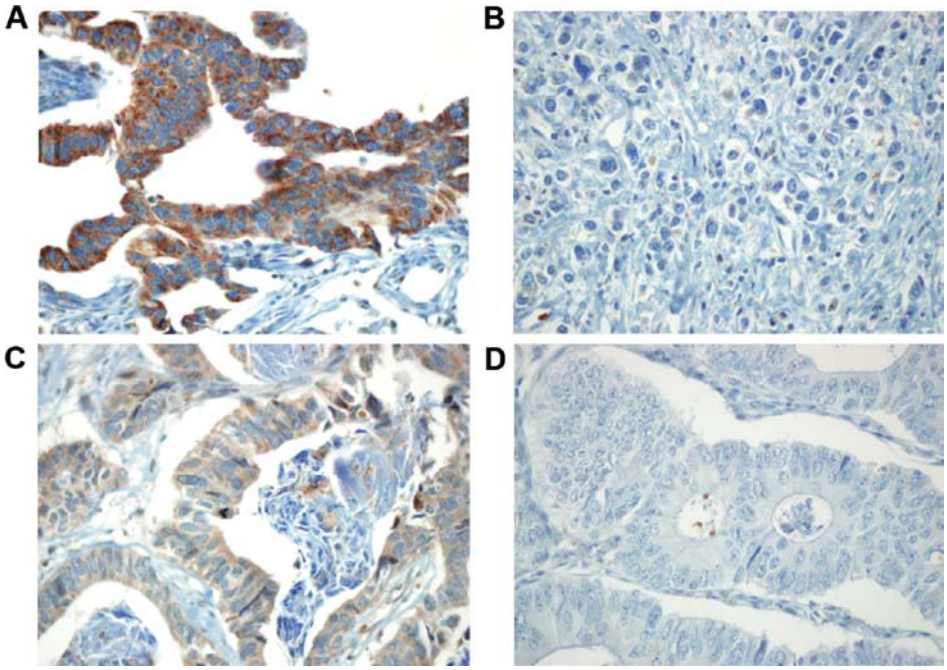

Figure 2. Immunohistochemical staining for N-cadherin and VEGF. A tissue core of gastric adenocarcinoma showed strong N-cadherin expression (A, 3+, x400), whereas another tissue core showed negative N-cadherin expression (B, negative, x400). Weak (C, 1+, x400) and negative (D, negative, x400) VEGF expression are also shown. VEGF, vascular endothelial growth factor.

Table II. Immunostaining.

	Positive		
Biomarker	Case (%)	Control (%)	P-value
E-cadherin	100	69.2	0.115
N-cadherin	66.7	46.2	>0.05
MSS1	66.7	38.5	>0.05
Claudin 3	44.4	23.1	>0.05
Claudin 4	55.6	23.1	>0.05
Glut 1	33.3	30.8	>0.05
Clusterin	44.4	38.5	>0.05
ITGB4	22.2	0	>0.05
VEGF	33.3	0	0.055
EGFR	0	7.7	>0.05
p53	66.7	53.9	>0.05

Data are expressed as a percentage of cases. VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor.

Table III. Analysis of immunostaining for overall survival.

	Median survival (months) Positivity vs. negativity	P-value
E-cadherin	20 vs. 8	>0.05
N-cadherin	36 vs. 12	0.027
MSS1	20 vs. 16	>0.05
Claudin 3	32 vs. 16	>0.05
Claudin 4	32 vs. 16	>0.05
Glut 1	20 vs. 33	>0.05
Clusterin	16 vs. 32	>0.05
VEGF	16 vs. 20	>0.05
EGFR	20 vs. 32	>0.05
p53	16 vs. 36	>0.05

VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor.

stage, vascular invasion or perineural invasion between the case and control groups. Lymphatic invasion was significantly higher in the control group than the case group (92.3 vs. 44.4%, p=0.023). The median interval between the diagnosis of stomach cancer and the detection of brain metastasis was 9 (range 1-77) months.

Immunohistochemistry. The immunohistochemical staining results for each protein are presented in Table II. E-cadherin had the strongest expression in both groups (100 vs. 69.2%). EGFR expression was lowest in the case group (0%), while the expression levels of ITGB4 and VEGF were lowest in the control group (0%). No significant differences in biomarker expression were detected between cases and controls. However, VEGF expression tended to be higher in the case group (33.3 vs. 0%, p=0.055). The immunoreactivity of N-cadherin and VEGF is shown in Fig. 2.

Survival analysis. Survival analyses with respect to the clinicopathological variables and biomarker expression profiles using the Kaplan-Meier method are summarized in Table III and IV. N-cadherin expression (36 vs. 12 months, p=0.027) and vascular invasion (12 vs. 33 months, p=0.008) were significantly correlated with the median survival.

Table IV. Analysis of clinical factors for overall survival.

	Median survival (months)	P-value
Age (<70 vs. ≥70)	18 vs. 32	>0.05
Tumor size (mm) (<50 vs. ≥50)	16 vs. 33	>0.05
M stage (0 vs. 1)	32 vs. 12	>0.05
Lymphatic invasion (yes vs. no)	20 vs. NR	>0.05
Vascular invasion (yes vs. no)	12 vs. 33	0.008
Neural invasion (yes vs. no)	16 vs. 33	0.061
Brain metastasis (yes vs. no)	16 vs. 20	>0.05

VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; NR, not reported.

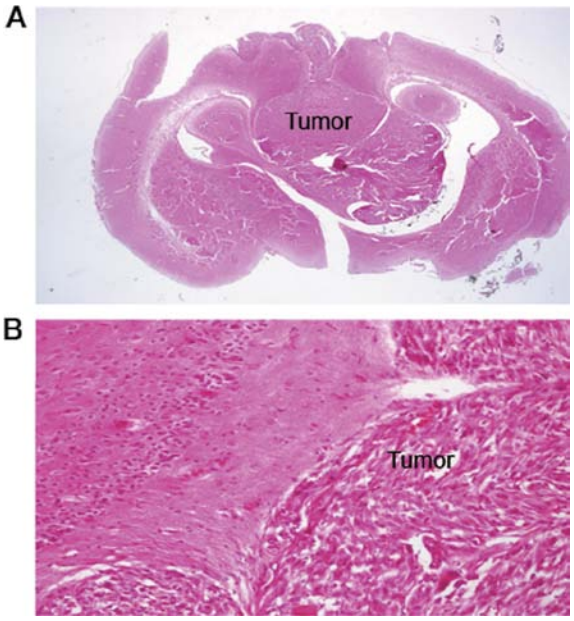

Figure 3. H&E staining of xenograft mouse model (sacrificed 8 weeks after intracranial inoculation) (A, x12; B, x200). H&E, hematoxylin and eosin.

Modulation of VEGF and N-cadherin by metformin in tumor-bearing mice. As shown in Fig. 3, the inoculation of MK28 cells was found in the mouse brain. The level of VEGF mRNA was decreased in the treatment group compared with the controls. E-cadherin was increased in the treatment group, while N-cadherin did not alter after administration of metformin. To investigate changes in epithelial-to-mesenchymal transition (EMT), expression of the mesenchymal marker MMP9 was assessed. The MMP9 expression was decreased in the metformin-treated group compared with the controls. These findings are compatible with the western blotting results (Fig. 4).

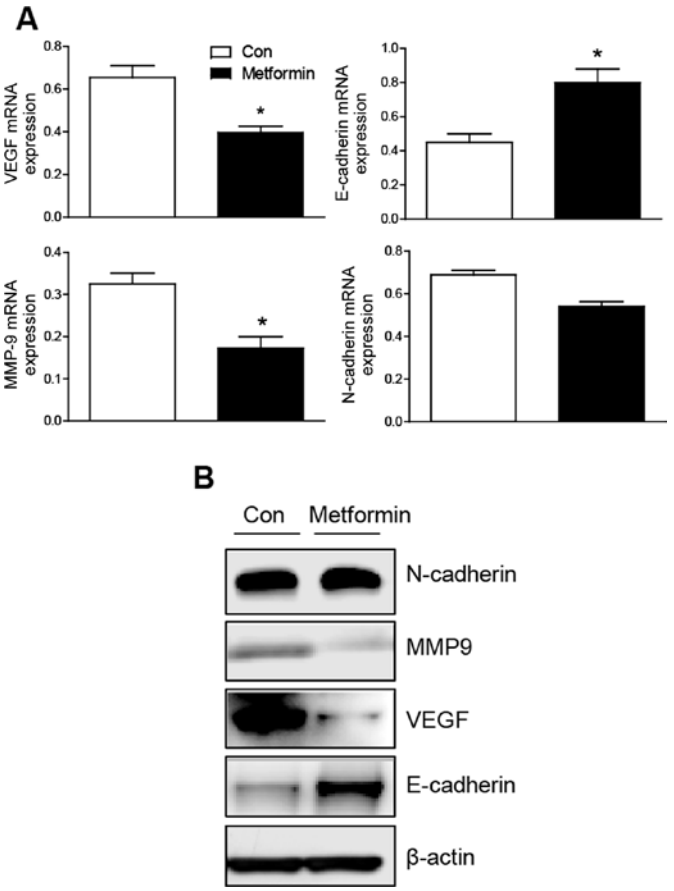

Figure 4. (A) The results of reverse-transcription polymerase chain reaction (RT-PCR) and (B) western blotting of VEGF, N-cadherin, E-cadherin and MMP9. The expression of VEGF and MMP9 were decreased in the metformin-treated group when compared with the controls. E-cadherin expression was increased in the metformin-treated group, while N-cadherin expression was not changed following metformin treatment. Data are presented as the means ± SEM (n=5 each). *p<0.05 vs. control. VEGF, vascular endothelial growth factor.

Discussion

The present study evaluated the correlations between clinicopathological findings and survival in brain metastasis of advanced gastric cancers. The overall survival of all 22 patients with advanced gastric cancer was influenced by vascular invasion and expression of N-cadherin. Our results suggested a role for VEGF expression in gastric cancer brain metastasis. It has been reported that elevated VEGF expression contributes to the ability of breast and prostate cancer cells to metastasize to the brain in nude mice (22,23). Angiogenic factors expressed by metastatic cancers are potent mediators of angiogenesis and vascular permeability in brain metastasis. High VEGF levels in CSF have a very high specificity for leptomeningeal metastasis (24). The role of angiogenesis in brain metastasis is not clear. Bevacizumab monoclonal antibody to VEGF and sunitinib, a multi-kinase inhibitor that affects the VEGF receptor are in clinical trials for treating brain metastasis of breast and lung cancer (25).

Generally, the loss of E-cadherin and the gain of N-cadherin indicate the conversion of tumor cells into a metastatic phenotype; nevertheless, we could not find any correlation

between brain metastasis and E- or N-cadherin expression. In 146 gastric cancer patients, including 84 patients with stage I, the 5-year survival rate of the N-cadherin-positive group was significantly worse than that of the N-cadherin-negative group (58 vs. 78%) (26). By contrast, in our series of advanced gastric cancers, the survival of patients with positive N-cadherin immunoreactivity was longer than that of those with negative immunoreactivity, while E-cadherin expression did not correlate with survival. This discrepancy could have arisen since the number of patients we enrolled was very small, and early-stage gastric cancers were excluded from our series.

Of the nine patients with brain metastasis, resection and radiation therapy were performed in two, radiation only in two, intrathecal methotrexate chemotherapy in one and four were conservatively treated. In 19 cases of cytologically confirmed leptomeningeal carcinomatosis, the median survival was 4-8 weeks (27,28). Other authors have described a longer median survival of ~13 months for patients who underwent surgical resection of brain metastasis followed by radiotherapy (29,30). Since the systemic disease was already advanced and multiple lesions were not accessible surgically, it is not difficult to choose aggressive multidisciplinary treatment. The overall survival of patients with and without brain metastasis did not differ in our series of advanced gastric cancers. However, since no standard therapy for gastric cancer brain metastasis has been established, it is important to investigate the predictors of brain metastasis and develop a preventive strategy for gastric cancer brain metastasis. We investigated modulation of the expression of VEGF, N- and E-cadherin, and MMP9 in mouse models of brain metastasis that had been inoculated with gastric cancer cells transcranially.

The invasion and metastasis of solid tumors require EMT for tumor cells to invade and metastasize (31). During EMT, non-motile, polarized epithelial cells embedded via cell-cell junctions in a cell collective dissolve their cell-cell junctions and transform into individual, non-polarized, motile and invasive mesenchymal cells. Previously, we reported that the reduced expression of molecules involved in EMT, such as E-cadherin, could be used to identify patients at high risk for developing brain metastasis of non-small cell lung cancer (32). In the present study, we suggest that VEGF expression is associated with brain metastasis of advanced gastric cancer. Metformin inhibited VEGF expression in MCF-7 breast cancer cells via the AMP-activated protein kinase pathway (33). Metformin inhibits both angiogenesis and the metastatic spread of various cancer cells by preventing EMT (34,35). In tumor-bearing mouse models, metformin administration decreased the expression of VEGF and MMP9. In addition, the expression of E-cadherin was increased, although that of N-cadherin did not significantly change. In mouse models, the dose of metformin administered to the mice (2 mg/25 g/day) is in the same range as that administered to diabetic patients (3 g/75 kg/day) (36). These findings suggest that metformin may prevent or attenuate the EMT process and cell invasiveness.

In conclusion, brain metastasis in advanced gastric cancer was associated with the expression of VEGF. Metformin treatment affects the metastatic capacity of gastric cancers by reducing VEGF expression and blocking EMT.

Acknowledgements

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