Abstract. Background: Ghrelin, an orexigenic peptide, is primarily produced and secreted by the gastrointestinal tract. As far as we are aware of, there is no evidence of ghrelin expression in esophageal squamous cell carcinoma (ESCC).

Materials and Methods: Two hundred and ten patients with ESCC who underwent surgical resection were enrolled in this study. We immunohistochemically investigated ghrelin expression in primary ESCC specimens and analyzed the relationship with clinicopathological factors.

Results: High ghrelin expression was observed in 61 patients (29.0%). Depth of tumor invasion and histological differentiation were statistically associated with ghrelin expression. As for depth of tumor invasion, the deeper it was, the higher was the expression of ghrelin. Well-differentiated tumors had a significantly higher proportion of ghrelin-expressing cells than other types.

Conclusion: Ghrelin expression correlated with tumor depth and tumor differentiation, suggesting an important role of ghrelin in tumor growth in ESCC.

Malignant tumors are characterized by extensive invasion, metastasis, and marked cachexia. Several reports have referred to the role of ghrelin which stimulates the release of growth hormone (GH), in malignant cell invasion and proliferation. Murata et al. first suggested the possibility of proliferative role of ghrelin in a hepatoma cell line (1). Ghrelin is a 28-amino acid peptide hormone, produced mainly in the stomach, by neuroendocrine cells (X/A-like cells in rodents and P/D1 cells in humans) in the fundus, and secreted into the circulation (2-4). Since its discovery, it has been implicated in a wide range of physiological activities, including the control of food intake and metabolism (5-8). Obese people have been shown to have low fasting ghrelin levels compared to those with normal body-mass index (BMI) in an examination to limit uptake of fat (5, 6, 9, 10). In contrast, cachectic patients exhibit higher fasting ghrelin levels (5, 6, 11, 12).

Ghrelin is produced mainly in a non-acylated form. Orexigenic and growth hormone-stimulating effects of ghrelin depend on its acylation with an octanoyl fatty acid residue at the serine-3 position (4, 6). Lower levels of ghrelin are found in the small and large intestine, cells of the pancreatic islet, kidney, testis, placenta, and immune cells (13-19). Locally produced ghrelin possibly affects cells with which can act as receptors for ghrelin. Receptors of ghrelin are known as growth hormone secretagogue receptors (GHS-Rs) (20-23). GHS-Rs have two sub-types: type Ia (GHS-RIa) transduces the GH-releasing effect of GHS, whereas type 1b GHS-RIb is a non-spliced, non-functional receptor mRNA variant. GHS-RIa is mainly expressed in the pituitary, hypothalamus and at low levels in other brain regions such as ventral tegmental area, substantia nigra, nucleus tractus solitaries, and hippocampus as well as peripheral tissue such as the heart, lungs, pancreas, intestine, kidneys, and adipose tissue (19, 24-27).

Some groups have reported the proliferative effect of ghrelin in neuronal, adrenal, prostatic, adipose, mammary, chondroblastic and pancreatic cells (7, 20, 21, 28-34). In contrast there are reports of the anti-proliferative role of ghrelin in few cells (35). Furthermore, some authors reported that ghrelin expression is suppressed in malignant tumors (36, 37), while others emphasized on ghrelin expression in tumors (38, 39). Recently, much effort has been put into research regarding the role of ghrelin in cancer, which is still unclear.
Esophageal cancer is the most aggressive type of cancer among gastrointestinal cancers because of the existence of metastases even in the early stages (40). Mottershead et al. reported the negligible expression of ghrelin in esophageal adenocarcinoma (37). Nevertheless, as far as we are aware of, there have been no reports on the expression of ghrelin in esophageal squamous cell carcinoma (ESCC). Herein, we examined ghrelin expression in resected specimens of ESCC and its relationship with clinicopathological features.

Materials and Methods

Patients. We enrolled two hundred and ten patients with ESCC (189 males and 21 females) who underwent curative esophagectomy with lymph node dissection between 1990 and 2004 at the Kagoshima University Hospital, Kagoshima, Japan (Table I). The age of the patients ranged from 38 to 86 years (mean=64.8±9.3 years). None of them underwent endoscopic mucosal resection, palliative resection, preoperative chemotherapy, or radiotherapy. Patients with synchronous or metachronous multiple cancers in other organs were excluded. Specimens of cancer tissues and noncancerous adjacent tissues were collected from the patients after informed consent had been obtained in accordance with the institutional guidelines of our hospital.

Clinicopathological findings were based on the criteria of the TNM classification for esophageal carcinoma of the International Union Against Cancer (41). We classified 67 of the ESCCs as well differentiated, 106 as moderately-differentiated, and 37 as poorly-differentiated. Forty of the tumors were located in the upper third of the esophagus, 102 in the middle third, and 68 in the lower third. Regarding tumor depth, 63 patients had pT1 tumors, 32 had pT2 tumors and 115 had pT3 tumors. Lymph node metastasis was found in 138 of the 210 patients (65.7%). Lymphatic and venous invasion were found in 74.3% (156/210) and 56.7% (119/210) of the patients, respectively (Table I).

Immunohistochemistry. After primary lesions were fixed in 10% formaldehyde and routinely embedded in paraffin, 3-μm thick sections were prepared for immunohistochemistry. Sections were de-paraffinized in xylene, rehydrated in graded ethanol, and incubated in 0.3% H2O2 solution in methanol for 30 min to block endogeneous peroxidases. All sections were autoclaved in 10 mM sodium citrate (pH 6.0) for 10 min and allowed to cool at room temperature. After washing three times with Phosphate buffered saline (PBS) for 5 min each, the sections were treated with 1% bovine serum albumin for 30 min at room temperature. The sections were incubated overnight at 4°C with the mouse monoclonal antibody to human ghrelin (1: 200; Abcam, Tokyo, Japan). These reactions were developed with an avidin-biotin immunoperoxidase technique (ABC method). The reaction was visualized using the Vectastain Elite ABC kit and a 3,3’diaminobenzidine solution (Vector Laboratories, Inc., Burlingame, CA, USA). Sections were then slightly counterstained with hematoxylin. Normal gastric mucosa of corpus was utilized as a positive control.

The expression of ghrelin was evaluated in 10 fields each containing 100 tumor cells using high-power microscopy (×200) expressing the proportion of positively stained cells as a percentage of total cells examined. All immunostained slides were evaluated by two independent observers (IO and MM). According to the proportion of ghrelin expression, patients were divided into two groups (≥20%: high expression, <20%: low expression), because the mean proportion of ghrelin-expressing cells in the patients’ tumor was 17.19±16.66%.

Statistical analysis. Statistical analysis consisting of Student’s t-test, the Chi-square test, the Kaplan-Meier method, and the log-rank test was performed using the JMP IN version 5.0.1 software system (SAS institute Inc., Cary, NC, USA). A p-value of less than 0.05 was considered to indicate statistical significance.

Results

Expression of ghrelin in esophageal carcinoma tissue. The expression of ghrelin was expressed throughout the cytoplasm of cancer cells, but was not found in the nucleus of cancer cells (Figure 1). Sixty-one patients had high expression of ghrelin. Ghrelin was expressed diffusely throughout the tumor without heterogeneity. No expression was observed in intratumoral stromal or inflammatory cells.

Correlation between clinicopathological factors and expression of ghrelin. Table II shows the correlation between ghrelin expression and pathological findings. High ghrelin expression was significantly correlated with depth of tumor invasion, pathological stage, tumor differentiation and venous invasion (p<0.0001, p<0.005, p<0.0005 and p<0.05, respectively). As for the proportion of lymph node metastasis and lymphatic invasion, patients with high expression of ghrelin had a higher incidence than those with lower expression, although the difference was not significant (p=0.21 vs. 0.10). Tumor location and recurrence rate were...
Correlation between prognosis and expression of ghrelin.

The five-year survival rate was analyzed according to the expression of ghrelin. No significant differences were found in the expression of ghrelin (Figure 4). According to Figure 4, in the short term, the difference in the one-year survival rate between the high and low expression groups approached borderline statistical significance ($p=0.072$, log rank test); hence there is a possibility that ghrelin expression in ESCC is of relevance regarding short-term survival.

Discussion

As far as we are aware of this is the first study to show a correlation between ghrelin expression and clinicopathological findings in ESCC. This study revealed the existence of ghrelin-expressing cells in ESCC.

Previous studies have shown that immunoreactivity for ghrelin was found in gastric endocrine tumors, pancreatic endocrine tumors, intestine endocrine tumors, lung carcinoids (42), prostate cancer (33, 43, 44), testicular
tumors (45), gastrointestinal carcinoids (46), pituitary adenomas (47), gastric adenocarcinoma (36, 37), salivary gland tumors (36), colorectal cancer (48), esophageal adenocarcinomas (37), breast cancers (31) (49), renal cancers (50), and oral squamous cell carcinomas (51). Some tumors have ghrelin-expressing cells, while others do not. The discrepancies among the results may be related to tissue differences, suggesting that the different genetic and epigenetic backgrounds of tissues as well as their embryological origins might contribute to differences in ghrelin expression in different cancer types.

Our results showed that ghrelin expression in ESCC was significantly correlated with depth of tumor invasion, pathological stage, tumor differentiation and venous invasion. As for tumor differentiation, the results were compatible with those of Rindi et al. (42) and Waseem et al. (48). They reported that ghrelin expression decreases with poorer differentiation. Rindi et al. suggested that the presence of protoendocrine cells void of hormonal storage was the main reason for the absence of ghrelin in poorly-differentiated endocrine tumors (42). Waseem et al. suggested that ghrelin was not required for enhanced malignant cell behavior in colorectal malignancy, (48) and also suggested that the ghrelin system contributes to the spread of malignancy rather than increasing the grade of the tumor as many other growth factors do, including insulin and insulin like growth factors (52). Our results also suggest that well-differentiated tumors of ESCC with high expression of ghrelin may have deeper invasion and be of more advanced stage. Furthermore, as we have indicated, ghrelin has a proliferative effect on malignant cells (1, 7, 20, 21, 28-34).

Our results also suggest that ghrelin has an important role in carcinogenesis and cancer promotion.

Regarding the possibility of ghrelin being used as a tumor marker, in tumors expression ghrelin, Gronberg et al. examined breast cancer samples immunohistochemically and suggested that ghrelin expression correlated with lower risk for recurrence and cancer-related death (49). In contrast, another report by Jeffery et al. revealed that ghrelin treatment significantly increased the proliferation of cancer cells, and that low grade carcinomas strongly expressed ghrelin and its receptor. They suggested that members of the ghrelin signaling axis may become novel markers for breast cancer and potential therapeutic targets (31). Alnema et al. also immunohistochemically investigated oral squamous cell carcinoma, and suggest that ghrelin expression in cancer was lower than in benign tissue, and so it might be helpful to distinguish malignant from benign tumors (51). Our results suggest that if we examine ghrelin expression in ESCC biopsy tissues, it may be useful in making a diagnosis of depth and differentiation. In this study, the five-year survival rate had no statistical significance, but there was a trend that the one-year survival rate for those with positive ghrelin expression was worse than for those without expression. Thus, it is still unclear whether ghrelin expression is truly a marker of malignancy.

Murphy et al. reported that lower serum level of ghrelin was associated with an increase of the risk of ESCC (53).
Waseem et al. examined correlation between serum ghrelin level and clinicopathological factors of patients with colorectal cancer, resulting in close correlation only with BMI change over six months (48). Other investigators also have tried to find if there is any relationship between serum ghrelin levels and malignancies, however, none have yet reported such correlation (54-59). The relationship between serum ghrelin levels and ghrelin expression in tumors remains unclear.

In treatment of cancer cachexia, several trials have been carried-out on ghrelin administration. The rodent model of cancer cachexia revealed that ghrelin led to significant increase in weight and appetite (11, 60, 61). Another report by Nearry et al. showed that ghrelin infusion increased food intake of cachexic patients (62). In contrast, Strasser et al. reported opposite findings (63). As we have indicated, however, in many investigations on cancer cells treated with ghrelin, a proliferative effect was demonstrated and ghrelin possibly stimulated tumor growth. We also showed a correlation between ghrelin expression and depth of tumor invasion and advanced staging. A long-term clinical trial is required to determine if ghrelin treatment indeed stimulates tumor growth.

In conclusion we showed for the first time that some ESCCs express ghrelin and this was correlated with different clinicopathological findings. In the present study we suggest an important role of ghrelin for the tumor growth in ESCC. However, further investigation is required to establish the clinical significance of ghrelin as a biomarker or a therapeutic target in ESCC.

References


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