**Research Article**

**Clinicopathological and Prognostic Significance of MUC-2, MUC-4 and MUC-5AC Expression in Japanese Gastric Carcinomas**

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**Abstract**

**Background:** The mucin components of the gastric gel layer function as a protective and lubricating factor against luminal acid and proteolytic enzymes. Alteration of mucin expression in gastric preneoplastic and neoplastic lesions has suggested potential roles in neoplastic processes. This study aimed to assess the clinicopathological and prognostic significance of MUC-2, MUC-4 and MUC-5AC in Japanese gastric cancer. **Methods:** Expression of MUC-2, -4 and -5AC was evaluated on tissue microarrays of gastric carcinomas and adjacent non-cancerous mucosa specimens by immunohistochemistry and compared with clinicopathological parameters and survival time of the patients. **Results:** The three mucins were found to be expressed to a lesser extent in gastric carcinomas in comparison with non-cancerous mucosa (p<0.05). MUC-2 expression was negatively correlated with tumor size, depth of invasion, and TNM staging of gastric cancer (p<0.05), while that of MUC-5AC was negatively associated with the depth of invasion, venous invasion, lymph node metastasis and TNM staging (p<0.05), but positively with MUC-4 and MUC-2 expression (p<0.05). There was higher MUC-2 expression in intestinal- than diffuse-type carcinomas (p<0.05). Kaplan-Meier analysis indicated no relationship between expression of the three mucins and the cumulative survival rate of patients, even stratified according to the depth of invasion (p>0.05). **Conclusion:** Down-regulated expression of MUC-2, -4 and -5AC may be involved in pathogenesis, invasion, metastasis or differentiation of gastric carcinoma. Their altered expression might therefore be employed as an indicator of pathobiological behavior.

**Keywords:** Gastric carcinoma - mucins - clinicopathological behaviors - prognosis - tumorigenesis

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**Introduction**

Despite a worldwide decline in incidence and mortality in the last 60 years, gastric cancer is still ranked as the fourth most common and the second most frequent cause of death from cancer. It continues to be a major health concern because of the slow decrease in incidence in Asia and high mortality from diagnosed gastric carcinomas in the West, even though advanced diagnostic and operative techniques are widely applied in clinical practice (Kelley et al., 2003; Rivera et al., 2007). Increased understanding of the changes that occur in gene expression in gastric cancer, particularly identification of novel biomarkers for cancer diagnosis and novel targets for treatment, may result in the improvement of diagnosis, treatment and prevention.

Mucins are a family of high molecular weight, heavily glycosylated proteins (glycoconjugates) with many oligosaccharide side chains linked to a protein backbone called apomucin. Their key function is their ability to form gels and act as a chemical barrier. Additionally, some mucins are associated with controlling mineralization, including nacre formation in molluscs, calcification in echinoderms and bone formation in vertebrates (McGuckin et al., 2011). They bind to pathogens as part of the immune system. Thus far, at least 19 mucins have been identified and divided into two distinct classes according to their structure and function: (i) secreted types: MUC-2, -5AC, -5B, -6, -7, -8, -9 and -19; (ii) membrane-associated types: MUC-1, -3A, -3B, -4, -12, -13, -15, -16, -17 and -20 (Zheng et al., 2006; Li et al., 2008). Secreted mucins are glycoproteins constituting the major macromolecular component of mucus, while membrane-associated mucins contribute to epithelial cell-cell interactions. Their patterns...
of expression, especially of secreted mucins, appear to be relatively cell-, tissue-, or organ- specific (Zheng et al., 2006). Qualitative and quantitative alteration of mucin expression in preneoplastic and neoplastic lesions has suggested potential roles in neoplastic processes, as reviewed by Cozzi et al. (2005). Furthermore, numerous pieces of evidence indicate a close association between aberrant mucin expression and aggressive behaviors of malignancies (Akyürek et al., 2002; Huang et al., 2002; Wang et al., 2003; Wang et al., 2003; Levi et al., 2004; Cozzi et al., 2005; Zheng et al., 2006).

In the stomach, the mucin component of gastric gel layer functions as a protective and lubricating factor against luminal acid and proteolytic enzymes, which also hinders access of carcinogens causing DNA damage. When the stomach suffers from infection with Helicobacter Pylori (HP), HP lipopolysaccharides decrease mucin synthesis by the phosphatidylinositol 3-kinase/ERK pathway and via inhibition of galactosyltransferase (Slomiany et al., 2003; Slomiany et al., 2005). A large body of in vitro evidences indicate that treatment of gastric epithelial or adenocarcinoma cell lines with HP will cause the loss or reduced synthesis of mucin (Wang, et al., 2003; Durai Babu et al., 2006). In vivo studies have also suggested that HP infection is positively correlated with low expression of some mucins during the pathogenesis and development of gastric carcinomas (Byrd et al. 2000; Kim et al. 2003). Proinflammatory and inflammatory cytokines (IL-1, IL-6 and TNFα) trigger MUC-2, MUC-4 and MUC-5AC expression in gastrointestinal cancers (Enss et al., 2000; Mejías-Luque et al., 2008; Mejías-Luque et al., 2010). Therefore, altered mucin expression might be a key molecular event in gastric carcinogenesis. In previous work, we found that down-regulated MUC-6 expression was linked to gastric carcinogenesis, and subsequent progression of Japanese gastric cancer, while the converse was true for MUC-1 (Zheng et al., 2006; Li et al., 2008). Japan is within the high-risk area for gastric carcinoma worldwide and the observed gastric carcinomas are characterized as follows: (i) predominance in the distal stomach; (ii) frequently detected at an early stage (nearly 50%); (iii) mostly restricted to the elderly population; (iv) comparatively good prognosis (Inoue et al., 2005). In the present study, we aimed to study the clinicopathological and prognostic significance of MUC2, MUC-4 and MUC-5AC expression in Japanese gastric carcinomas.

**Materials and Methods**

**Patients**

This retrospective study was carried out on curatively-resected gastric cancer specimens collected in Toyama University Hospital from 1993 to 2006. The patients with gastric carcinomas were 132 men and 299 women (38-88 years, mean=66.7 years). Archival materials were obtained from Department of Pathology. In 168 cases, tumor development was accompanied with lymph node metastasis. None of the patients underwent chemotherapy, radiotherapy or adjuvant treatment before surgery. All patients were followed up by consulting their case documents and by telephone.

**Pathology**

All tissues were fixed in 10% neutralized formalin, embedded in paraffin and cut into 4 μm sections stained with hematoxylin and eosin (HE) to confirm the histological diagnosis and microscopic characteristics. The staging for each gastric carcinoma was evaluated according to the Internationale le Contre Cancer (UICC) system indicating the extent of tumor spread (Sobin et al., 2002). Histological architecture was defined in terms of Lauren’s classification (Zheng et al., 2007; 2008). Furthermore, tumor size, depth of invasion, lymphatic and venous invasion, and lymph node metastasis of tumors were determined.

**Tissue microarray(TMA)**

From HE stained sections of the selected tumor cases, representative areas of solid tumor were selected for sampling and two mm diameter tissue cores per donor block were punched out and transferred to a recipient block with a maximum 48 cores using a Tissue Microarrayer (AZUMAYA KIN-1, Japan). Four-jam-thick sections were consecutively cut from the microarrays and transferred to poly-lysine-coated glass slides. HE staining was performed for confirmation of tumor tissue.

**Immunohistochemistry**

Serial sections of TMA were deparaffinized with xylene, rehydrated with alcohol, and subjected to immunohistochemical staining with intermittent microwave radiation as previously described (Kumada et al., 2004). Mouse anti-human MUC-2, MUC-4, and MUC-5AC antibodies (NovoCastra, UK) were used at 1: 100 dilution to detect the respective proteins, with anti-mouse Envision-PO (DAKO, USA) as the secondary antibody. Binding was visualized with 3, 3’-diaminobenzidine and counterstaining with Mayer’s hematoxylin was performed to aid orientation. Omission of the primary antibody was used as a negative control.

Immunoreactivity for MUC-2 and MUC-5AC showed a cytoplasmic pattern, while MUC-4 was localized in the cytoplasm and membrane (Figure 1). One hundred cells were randomly selected and counted from five representative fields of each section blindly by twoindependent observers (Xiao and Zheng). The inconsistent data were confirmed by both persons until final agreements were reached. The expression positivity was graded and counted as follows: 0 =negative; 1 = 1-50%; 2 = 50-74%; 3 ≥75%. The staining intensity score was graded as follows: 1 = weak; 2 = intermediate; and 3 = strong. The scores for MUC-2, MUC-4 or MUC-5AC positivity and staining intensity were multiplied to obtain a final score, which determines their expression as (− = 0; + = 1-2; ++ = 3-4; +++ = 6-9).

**Statistical analysis**

Statistical evaluation was performed using the Spearman correlation test to analyze rank data. Kaplan–Meier survival plots were generated and comparisons between survival curves were made with the log-rank statistic. SPSS 17.0 software was applied to analyze all data and p<0.05 was considered statistically significant.
Results

As indicated in Figure 1, MUC-2 was expressed in goblet cells of intestinal metaplasia. There was strong diffuse expression of MUC-2 in the cytoplasm of gastric carcinoma cells. MUC-2 was positively expressed in 67.4%(62/92) of gastric non-neoplastic mucosa (NNM) and 27.4%(131/431) of gastric cancer, respectively. Statistically, there was MUC-2 overexpression in gastric NNM than carcinoma (p<0.05, Figure 2A). As shown in Table 1, MUC-2 expression was negatively correlated with tumor size, depth of invasion, and TNM staging of gastric cancer (p<0.05). There was higher MUC-2 expression in intestinal- than diffuse-type carcinomas (p<0.05).

As indicated in Figure 1, MUC-4 was positively expressed in goblet cells of intestinal metaplasia, superficial epithelium and gastric carcinoma cells. There was strong diffuse expression of MUC-4 in the cytoplasm and membrane. MUC-4 was positively expressed in 82.3%(76/92) of gastric NNM and 46.1%(152/330) of gastric cancer, respectively (Figure 1B). Statistically, there was MUC-4 overexpression in gastric NNM than carcinoma (p<0.05, Figure 2B). MUC-4 expression was higher in the elder than the younger patients with gastric cancer (p<0.05, Table 2).

As indicated in Figure 1, MUC-5AC was expressed in goblet cells of intestinal metaplasia and gastric carcinoma cells. MUC-5AC was positively expressed in 91.0(81/89) of gastric non-neoplastic mucosa (NNM) and 78.9%(332/418) of gastric cancer, respectively (Figure 1C). Statistically, there was MUC-5AC overexpression in gastric NNM than carcinoma (p<0.05, Table 2). MUC-5AC expression was higher in the elder than the younger patients with gastric cancer (p<0.05, Table 2).

![Image of Figure 1](image1.png)

**Figure 1. The in Situ Expression of MUC-2, MUC-4 and MUC-5AC Protein in Gastric Cancer.** Note: The strong positivity of MUC-2 or MUC-5AC was localized in the cytoplasm, while MUC-4 in the membrane and cytoplasm of gastric intestinal metaplasia of non-neoplastic mucosa (NNM) and cancer.

![Image of Figure 2](image2.png)

**Figure 2. The Ratio Distribution of Different MUC-2, MUC-4 and MUC-5AC Expression Levels in Gastric Cancer.** There was negative(-), weak(+), moderate(++) and strong(+++) expression of MUC-2, MUC-4 and MUC-5AC.

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>MUC-2 expression</th>
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<tbody>
<tr>
<td>n</td>
<td>-</td>
</tr>
<tr>
<td>Age(year)</td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>189</td>
</tr>
<tr>
<td>≥65</td>
<td>242</td>
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<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Male</td>
<td>132</td>
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<tr>
<td>Female</td>
<td>299</td>
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<tr>
<td>Tumor size(cm)</td>
<td></td>
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<tr>
<td>&lt;4</td>
<td>223</td>
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<tr>
<td>≥4</td>
<td>208</td>
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<tr>
<td>Depth of invasion</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>217</td>
</tr>
<tr>
<td>T2-T4</td>
<td>214</td>
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<tr>
<td>Lymphatic invasion</td>
<td></td>
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<tr>
<td>-</td>
<td>271</td>
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<tr>
<td>+</td>
<td>159</td>
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<tr>
<td>Venous invasion</td>
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<tr>
<td>-</td>
<td>370</td>
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<tr>
<td>+</td>
<td>61</td>
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<td>Lymph node metastasis</td>
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<td>-</td>
<td>259</td>
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<td>+</td>
<td>168</td>
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<tr>
<td>0-I</td>
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<td>II-IV</td>
<td>186</td>
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<td>Diffuse-type</td>
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PR, positive rate; T1, carcinoma in situ; T2, lamina propria and submucosa; T3, muscularis propria and subserosa; T4, exposure to serosa; TNM, tumor-node-metastasis.

Table 1. Relationship Between MUC-2 Expression and Clinicopathological Features of Gastric Carcinomas
lymph node metastasis and TNM staging (p<0.05), but positively with MUC-4 and MUC-2 expression (p<0.05).

Follow-up information was available on 431 of the gastric carcinoma patients for periods ranging from 0.2 months to 12.2 years (mean=70.8 months). Figure 3 shows a representative survival curve, stratified according to MUC-2 expression status. Univariate analyses using Kaplan-Meier method indicated no relationship between the three proteins’ expression and cumulative survival rate of patients, even stratified by the depth of invasion (p>0.05).

Discussion

Mucins are high molecular weight O-linked glycoproteins whose primary functions are to hydrate, protect, and lubricate the epithelial luminal surfaces of the ducts within the human body (Zheng et al., 2006). Recent studies have uncovered the unique roles of mucins in the pathogenesis of cancer (Akyürek et al., 2002; Huang et al., 2002; Wang et al., 2003; Wang et al., 2003; Slomiany et al., 2003; Slomiany et al., 2005; Levi et al. 2004; Cozzi et al., 2005; Li et al., 2006; Li et al., 2008; McGuckin et al., 2011). MUC-2 is particularly prominent in the gut where it is secreted from goblet cells in the epithelial lining into the lumen of the large intestine. Here, we found that MUC-2 expression was stronger in the intestinal metaplasia of
gastric NNM than gastric cancer in agreement with the data in ovarian carcinogenesis (Feng et al., 2002), indicating that down-regulated MUC-2 might play an important role in gastric carcinogenesis in agreement with another study (Bu et al., 2010). Additionally, MUC-2 expression was negatively correlated with tumor size, depth of invasion, and TNM staging of gastric cancer, in line with the previous reports (Rakha et al., 2005; İlhan et al., 2010), suggesting that reduced MUC-2 expression might be closely linked to the growth, invasion and progression of gastric cancer. The higher MUC-2 expression in intestinal-than diffuse-type carcinomas gives us a fact that it underlies the molecular mechanisms of the differentiation of both carcinomas, which is remarkably different from the findings of İlhan et al. (İlhan et al., 2010). Mesquita et al found that the promoter Cpg methylation of MUC-2 was closely linked to its down-regulated expression in gastric cancer (Mesquita et al., 2003). A statistically significant association has been identified between rare exonic MUC2-MS6 alleles and the occurrence of gastric cancer, indicating that minisatellite instability might be associated with MUC2 function in cancer cells (Jeong et al., 2007).

MUC-4 is a high-molecular weight membrane glycoprotein and has been reported to play various roles in the progression of cancer, particularly due to its signaling and anti-adhesive properties which contribute to tumor development and metastasis (Carraway et al., 2009). In the present study, it was found that MUC-4 was distributed to the membrane of gastric superficial epithelium and cancer cells. Different from the results of Senapati et al., MUC-4 expression was higher in gastric cancer than its NNM, suggesting that down-regulated MUC-4 might be involved in gastric carcinogenesis (Senapati et al., 2008). Reportedly, epigenetic regulation of the human mucin gene MUC-4 in gastric and pancreatic cancer cell lines involves both DNA methylation and histone modifications mediated by DNA methyltransferases and histone deacetylases (Vincent et al., 2008). Shemirani et al. reported that MUC-4 were expressed 21-fold higher in stage I disease in mucoepidermoid carcinoma, compared to normal tissue by real-time PCR (Shemirani et al., 2011). MUC-4 overexpression is demonstrated in rat cholangiocarcinoma by tissue microarray (Yeh et al., 2009). However, the correlation of MUC-4 expression with aggressive behaviors was not observed in gastric cancer, consistent with other reports (Senapati et al., 2008; Kim et al., 2012), while Tamura et al. found that MUC-4 expression was related to lymphatic invasion and lymph node metastasis of gastric cancer (Tamura et al., 2012). Another group reported found that the increase in the expression and hypomethylation of MUC-4 gene with the progression of pancreatic ductal adenocarcinoma (Zhu et al., 2011).

MUC-5AC is a gel-forming mucin that is secreted from surface mucous cells. Glucocorticoid is required for the expression of Mucin-5AC mRNA and high doses of hydrocortisone suppress its expression (Takami et al., 2012). Mucin-5AC is also expressed in normal endocervical epithelium, small intestine, gastric cells (Lewis type 1) and gastric metaplasia, and it is a one of the major mucus in the ethmoid mucosa (Guillem et al., 2000; Jung et al., 2000). Here, we observed MUC-5AC expression in intestinal metaplasia and cancer cells and found its overexpression in NNM, compared with gastric cancer, suggesting an important role of MUC-5AC downregulation in gastric carcinogenesis. Moreover, a negative link between MUC-5AC expression and aggressiveness of gastric cancer was found, including depth of invasion, venous invasion, lymph node metastasis and TNM staging, which was consistent with the findings about MUC-5AC in pancreatic invasive ductal carcinoma (Jinffeng et al., 2003) and from another group about gastric carcinoma (Wang et al., 2003). Yamazoe et al. (Yamazoe et al., 2010) found that knockdown of MUC-5AC reduced the ability of pancreatic cancer cells to adhesion and invasion, suggesting that MUC-5AC might contribute to the invasive motility of pancreatic cancer cells by enhancing the expression of integrins, MMP-3, VEGF and activating Erk pathway.

In the present study, we found no relationship between MUC-2, 4 or -5AC and the prognosis of the patients with gastric cancer. However, loss of expression of MUC-2 showed significant correlation with poor overall survival in colorectal carcinoma (Kang et al., 2011; Elzagheid et al., 2012). The survival analysis showed that MUC-4 expression was statistically significant risk factors affecting the outcome of the patients with intrahepatic cholangiocarcinoma-mass forming type as an independent risk factor (Shibahara et al., 2004). MUC-4-positive patients had a definite trend towards better survival of laryngeal squamous cancer (Paleri et al., 2004) and gallbladder carcinoma (Lee et al., 2012). In addition, patients with MUC-5AC- positive tumors also had poor clinicopathological parameters and showed shorter survival than those with MUC-5AC-negative gastric cancer (Kocer et al., 2004). MUC-5AC-positive patients showed significant better survival than those MUC5AC- negative patients with pancreatic invasive ductal carcinoma (Jinffeng et al., 2003). Therefore, the prognostic significance of MUC-2,-4, and -5AC in gastric cancer should need further investigation in the future.

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