GASTRIC CANCER

Relationships between mucinous gastric carcinoma, MUC2 expression and survival

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Abstract

AIM: To investigate the expression of the four secreted gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) in a series of gastric carcinomas, classified according to Laurén's, Mulligan's, WHO and Goseki's classifications, with special attention to all the different components (major and minor) present in tumors and to follow up clinical data.

METHODS: Expression of MUC2, MUC5AC, MUC5B and MUC6 was investigated using immunohistochemistry and in situ hybridization.

RESULTS: Expression of secreted gel-forming mucins in gastric carcinoma was particularly complex, each mucin being not restricted to any histopathological type even considering all components (major and minor) present in a given tumor. There was a worst survival in patients with a higher content of mucus (Goseki II or IV) and high positive MUC2 expression.

CONCLUSION: Complexity of mucin gene expression patterns in gastric cancer may reflect a precise state of differentiation at the cell level not recognized in used morphologic classification systems. High expression of MUC2 was nevertheless associated with mucinous subtype of the WHO classification and with group II of Goseki’s classification identified by the major component of a particular tumor. The quantity and quality of mucus were related to survival.

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Key words: Gastric cancer; Secreted gel-forming mucin; MUC; Immunohistochemistry; in situ hybridization; Survival

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INTRODUCTION

Gastric cancers constitute a highly heterogeneous group of tumors with respect to epidemiology, genetics, histopathology and biological behavior. Numerous histopathological classifications have been proposed to identify the morphological variability in a reproducible manner and to correlate to prognosis. These different classifications include Laurén's, Mulligan's, World Health Organization (WHO), and Goseki's classifications, which all are based at least in part on the quantity of mucus present in an individual tumor[1-3]. The Goseki's classification combines two distinct criteria, e.g. the tubular differentiation and the intra-cellular mucin content[3]. It was shown to be the best classification with prognostic value additional to TNM staging[4]. All histopathological classifications are based on the identification of the major component of the tumor, neglecting the various minor components. However, in pathological practice, assessment of a histopathological type of gastric carcinoma is difficult due to the considerable variation frequently present within an individual tumor.

Epithelial mucins consist of at least thirteen different types that are usually subdivided into secreted gel-forming mucins and membrane-bound mucins[5-7]. The first class includes the four large mucins MUC2, M-
C5AC, MUC5B and MUC6 whose genes are clustered on chromosome 11p15.5 and which are involved in the mucus gel formation. MUC5AC and MUC6 are the major mucin components in normal gastric mucosa. MUC5AC is highly expressed in mucous cells of the superficial and foveolar epithelium, whereas MUC6 is present in mucous neck cells and in mucous glands of cardia and antrum. MUC5AC and MUC6 are also expressed in some gastric carcinomas. Moreover, MUC2 and MUC5B which are absent or barely detectable in normal gastric tissues have been reported in gastric carcinoma. MUC2 expression has been related more specifically to the mucinous carcinomas of WHO classification and to group II of Goski’s classification (high tubular differentiation and high mucin content) whereas MUC5AC has been associated with the group IV of Goski’s classification (poor tubular differentiation and high mucin content). However, coexpression of multiple mucins is frequently observed in gastric carcinomas. Such a complex pattern of mucin expression may reflect the variety frequently observed in gastric carcinomas.

In an effort to bring additional information in understanding the histopathological diversity of gastric carcinomas, we investigated the expression of the secreted gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) in a series of gastric carcinomas with special attention to all the different components (major and minor) present in tumors and to follow up clinical data.

**MATERIALS AND METHODS**

**Tissue samples and histopathological study**

A series of 31 gastric adenocarcinomas was obtained from institutional files. Cases included 7 women and 24 men with a mean age of 65 years-old, from 39 to 89 years. The surgical specimens were quickly immersed in fresh 4 g/L neutral formaldehyde solution (pH 7.4) in phosphate buffer and then were processed for paraffin embedding. Each gastric carcinoma was classified according to each of the following histopathological classifications; i.e. Lauren’s classification, Mulligan’s classification, WHO classification and Goseki’s classification. These classifications are based on the identification and classification of the major component of the tumor on hematoxylin-eosin-saffron (HES) coloration. The Goseki’s classification was realized on an adjacent tumor slide with Periodic Acid Schiff and Alcian Blue combined coloration. The Goseki’s classification combines two distinct criteria, e.g. the tubular differentiation and the intracellular mucin content. Four group are defined: group I, high tubular differentiation and poor mucin content; group II, high tubular differentiation and high mucin content; group III, poor tubular differentiation and poor mucin content; group IV, poor tubular differentiation and high mucin content. The classification proposed by Mulligan recognizes three forms of gastric carcinoma: the intestinal cell type, the mucous cell type and the pylorocarcinoid gland cell type. In this classification the intestinal and mucous cell types are similar to the intestinal and diffuse types defined by Lauren. The pylorocarcinoid gland cell type carcinoma is a distinct group of gastric carcinoma characterized by large cells with clear cytoplasm. The differentiation of each tumor was assessed on the basis of the degree of gland formation as well, moderate, poorly differentiated or undifferentiated. When the tumor presented with a major and one or more minor components, a sample where the different components were present was chosen to further perform the immunohistochemical and *in situ* hybridization studies on serial sections (4-µm thick).

**Immunohistochemistry**

Immunohistochemistry was performed using an automated immunostainer (ES, Ventana medical systems, Strasbourg, France). Primary antibodies were directed against MUC2 (polyclonal, LUM2-3, 1/1000), MUC5AC (monoclonal, 1/2) (Novocastra, New Castle, UK), MUC5B (polyclonal, Lum5B-2, 1/1000), MUC6 (monoclonal, 1/5) (Novocastra). The pretreatment was realized by a steamer for MUC2 (for 1 min 30) or by a microwave for MUC5AC, MUC5B and MUC6 (for 20 min), in citrate buffer (pH 6.0). For polyclonal antibodies, the sections were incubated for 32 min with normal goat serum to block the non-specific antibody-binding sites and endogenous peroxidase activity was suppressed by first incubating the specimen in 30 mL/L hydrogen peroxide. The immunohistochemistry method used a 3-step indirect process based on the biotin-avidin complex. Slides were counterstained with hematoxylin. Negative controls were run by omission of the primary antibody and positive controls with the appropriate tissue (normal colonic mucosa for MUC2, normal gastric mucosa for MUC5AC and MUC6, normal respiratory submucosal glands for MUC5B). The immunohistochemical results were estimated on the whole tumor and on the different components. The immunostaining was scored semiquantitatively as: 0, no immunostained cells; 1, less than 30% of immunostained cells; 2, more than 30% and less than 60% of immunostained cells; 3, strictly more than 60% of immunostained cells.

**In situ hybridization**

*In situ* hybridization was performed as described previously using four 35S-labeled antisense oligonucleotide probes corresponding to each tandem repeat domain of MUC2, MUC5AC, MUC5B, and MUC6. Briefly, tissue sections were deparaffinized, rehydrated, incubated with 2 mg/L proteinase K (Roche Diagnostics, Meylan, France) for 15 min and fixed again in 40 g/L paraformaldehyde in PBS for 15 min. Sections were then immersed in 0.1 mol/L triethanolamine (Sigma, L’Isle d’Abeau Chesnes, France) containing 2.5 mL/L acetic anhydride for 10 min. Sections were prehybridized in 4 × SSPE, 1 × Denhardt’s buffer for 45 min, and hybridized overnight at 42 °C in 20-100 µL of a 4 × SSPE solution containing 500 mL/L formamide, 1 g/L N-lauroylsarcosine, 1.2 mol/L sodium phosphate (pH 7.2), 1 × Denhardt’s buffer, 3 g/L yeast tRNA, 20 mmol/L dithiothreitol, and 125 MBq/L of 35S-labeled oligonucleotide. After post-hybridization washes, slides were dipped in LM-1 emulsion (Amersham, Les Ulis, France), developed 1-3 wk after exposure, and counterstained with methyl green pyronin (Sigma). The following controls were performed: (1) competition studies by treatment of tissue sections with

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Table 1  MUC expression in 31 gastric carcinomas stratified according to Laurén’s classification, Mulligan’s classification and Goseki’s classification n (%)

| Parameter               | n | MUC2 + IHC | MUC2 + ISH | MUC5AC + IHC | MUC5AC + ISH | MUC5B + IHC | MUC5B + ISH | MUC6 + IHC | MUC6 + ISH |
|-------------------------|---|------------|------------|-------------|-------------|------------|-------------|------------|------------|
| Laurén                  |   |            |            |             |             |            |             |            |            |
| Intestinal              | 14| 3 (21)     | 13 (93)    | 8 (57)      | 7 (50)      | 12 (86)    | 5 (36)      | 6 (43)     | 6 (43)     |
| Diffuse                 | 12| 1 (8)      | 6 (50)     | 9 (75)      | 8 (67)      | 9 (75)     | 3 (25)      | 5 (42)     | 3 (25)     |
| Unclassified            | 5 | 3 (60)     | 4 (80)     | 3 (60)      | 1 (20)      | 3 (60)     | 1 (20)      | 1 (20)     | 2 (40)     |
| Mulligan                |   |            |            |             |             |            |             |            |            |
| Intestinal cell         | 18| 5 (28)     | 16 (89)    | 11 (61)     | 8 (44)      | 15 (83)    | 6 (33)      | 7 (39)     | 7 (39)     |
| Mucous cell             | 13| 2 (15)     | 7 (54)     | 9 (69)      | 8 (62)      | 9 (69)     | 3 (23)      | 5 (38)     | 4 (31)     |
| Pylorocardiac           |   |            |            |             |             |            |             |            |            |
| WHO                     |   |            |            |             |             |            |             |            |            |
| Papillary               | 0 | 0 (0)      | 0 (0)      | 0 (0)       | 0 (0)       | 0 (0)      | 0 (0)       | 0 (0)      | 0 (0)      |
| Tubular                 | 14| 3 (21)     | 13 (93)    | 8 (57)      | 7 (50)      | 12 (86)    | 5 (36)      | 6 (43)     | 6 (43)     |
| Mucinous                | 3 | 3 (100)    | 3 (100)    | 2 (67)      | 0 (0)       | 2 (67)     | 0 (0)       | 0 (0)      | 1 (33)     |
| Signet-ring cell        | 12| 1 (8)      | 6 (50)     | 9 (75)      | 8 (67)      | 9 (75)     | 3 (25)      | 5 (42)     | 3 (25)     |
| Undifferentiated        | 2 | 0 (0)      | 1 (50)     | 1 (50)      | 1 (50)      | 1 (50)     | 1 (50)      | 1 (50)     | 1 (50)     |
| Goseki                  |   |            |            |             |             |            |             |            |            |
| Group I                 | 11| 2 (18)     | 10 (91)    | 5 (45)      | 4 (36)      | 9 (82)     | 2 (18)      | 5 (45)     | 4 (56)     |
| Group II                | 5 | 4 (80)     | 5 (100)    | 4 (80)      | 2 (40)      | 4 (80)     | 2 (40)      | 1 (20)     | 2 (40)     |
| Group III               | 9 | 0 (0)      | 4 (44)     | 6 (67)      | 5 (56)      | 5 (56)     | 3 (33)      | 3 (33)     | 4 (44)     |
| Group IV                | 6 | 1 (17)     | 4 (67)     | 5 (83)      | 5 (83)      | 6 (100)    | 2 (33)      | 3 (50)     | 1 (17)     |

1 MUC2 positivity in more than 30% of tumoral cells. IHC: Immunohistochemistry; ISH: In situ hybridization. *P* < 0.05, **P** = 0.01 vs other groups.

RESULTS

Histopathological results

Among the 31 gastric carcinomas, 2 were well differentiated, 11 moderately differentiated, 16 poorly differentiated and 2 undifferentiated. 14 carcinomas were classified as intestinal type in the Laurén’s classification, these tumors being mainly classified as intestinal cell type in the Mulligan’s classification, as tubular in WHO classification and as group I or II in the Goseki’s classification. 12 tumors were classified as diffuse type in the Laurén’s classification, being classified as mucous cell type in the Mulligan’s classification, as signet-ring cell in the World Health Organization (WHO) classification and as group III or IV in the Goseki’s classification. 3 carcinomas, not individualized in the Laurén’s and Mulligan’s classifications, corresponded to the mucinous type of the WHO classification and were classified in group II of Goseki’s classification (Table 1).

Relationship between histopathological classifications and MUC expression

All MUCs were observed in each subtype of gastric carcinomas classified either in Laurén’s classification, Mulligan’s classification, WHO classification or Goseki’s classification (Table 1, Figure 1). MUC2 was the major secreted gel-forming mucin detected in our series of gastric carcinomas. It was detected in 81% of the cases by immunohistochemistry and in 74% of the cases by in situ hybridization. MUC2 immunoreactivity was observed in the cytoplasm, in intracellular mucous vacuoles of tumoral cells, and also in extracellular mucus (Figure 1). Detection of MUC2 was not associated with a particular subtype of gastric carcinoma whatever the classification. However, high MUC2 immunostaining (in more than 30% of the tumoral cells), was significantly associated with the mucinous subtype of WHO classification (P = 0.015) and with the group II of the Goseki’s classification (P = 0.01, Table 1). Although less significant, the absence of in situ hybridization signal for MUC2 was more frequent in the signet-ring subtype of the WHO classification (P = 0.04) and in the diffuse subtype of Laurén’s classification (P = 0.04, Table 1). MUC5AC was detected in 65% of the cases by immunohistochemistry and in 52% of the cases by in situ hybridization. MUC5AC immunoreactivity was localized in the cytoplasm of tumoral cells, without staining of intracellular mucus vacuoles (Figure 1). MUC5AC detection either by immunohistochemistry or in situ hybridization was not associated with a particular subtype of gastric carcinoma classified by Laurén’s, Mulligan’s, WHO or Goseki’s classifications (Table 1). MUC5B was

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detected in 77% of the cases by immunohistochemistry and in 29% of the cases by *in situ* hybridization. MUC5B immunoreactivity was observed in the cytoplasm, in intracellular mucus vacuoles of tumoral cells and in extracellular mucus (Figure 1). MUC5B detection either by immunohistochemistry or *in situ* hybridization was not associated with a particular subtype of gastric carcinoma whatever the classification (Table 1). The discrepancies between the immunohistochemical and *in situ* hybridization results for MUC5B and at a lesser extent for MUC5AC corresponded mostly to cases which presented less than 30% of positive cells by immunohistochemistry and were negative by *in situ* hybridization. MUC6 was detected in 39% of the cases by immunohistochemistry and in 35% of the cases by *in situ* hybridization. The detection was observed in the cytoplasm of the tumoral cells, without staining of intracellular mucus vacuoles (Figure 1). Its expression was not associated with a particular subtype of gastric carcinoma whatever the classification (Table 1). No significant difference was observed for immunohistochemical or *in situ* hybridization detection of MUC2, MUC5AC, MUC5B or MUC6 between well/moderate differentiated carcinomas and poorly/undifferentiated carcinomas. The coexpression of at least two MUCs in the same tumor was frequent either identified by immunohistochemistry (83% of the gastric carcinomas) or *in situ* hybridization (54%). The coexpression of three MUCs in the same tumor was also remarkable either by immunohistochemistry (64%) or *in situ* hybridization (38%). In the series, 22% of the cases presented with the four MUCs detected by immunohistochemistry and 13% by *in situ* hybridization. The coexpression of at least two MUCs in the same tumor was not associated with a particular subtype of the
Goseki's classification.

Relationship between the different components and MUC expression

Among this series of 31 gastric carcinomas, we observed an histopathological heterogeneity in the majority of individual tumors. Indeed, 61% of the series presented with more than one component (one major component and one or more minor components). Given the difficulty to demonstrate a constant correlation between MUC expression and histopathological classifications, we further evaluated the expression of the four secreted gel-forming mucins independently in all the different components, major or minor, classified according to the Goseki's classification, frequently present in a same gastric carcinoma.

When the immunohistochemical labeling was studied separately in each component of each gastric carcinoma classified on the basis of the Goseki's classification, MUC2, MUC5AC, MUC5B, and MUC6 were observed in all the different groups (group I to IV, Table 2). In particular, MUC2 was not restricted to the components classified as group II of Goseki (83% of histopathological components of group II were positive for the immunohistochemical detection of MUC2), but was also detected in components classified as group I (67%), group III (20%) or group IV (69%). Similarly, MUC5AC, MUC5B and MUC6 were detected in components classified as group I, group II, group III or group IV. The comparison between the tumors which presented with a component of Goseki group II (major or minor) and the tumors which presented without this component was not statistically different.

Relationship between TNM and MUC expression

Among this series of 31 gastric carcinomas, 5 were T1, 20 were T2, 4 were T3 and 2 were T4, 2 were Nx, 6 were N0, 15 were N1, 5 were N2 and 3 were N3. The immunohistochemical and in situ hybridization detection of MUC2, MUC5AC, MUC5B or MUC6 was not associated with T1-T2 tumors when compared to T3-T4 tumors, and was not different between N0-N1 tumors in comparison to N2-N3 tumors. Moreover, the detection of two or three MUCs in the same tumor, either by immunohistochemistry or in situ hybridization, was not significantly associated with T1-T2 or T3-T4 tumors, nor with N0-N1 or N2-N3 tumors.

Relationships between TNM, Goseki classification, MUC2 expression and survival

Follow up data showed that there was a significant difference in mean survival time decreasing between the carcinoma patients with T1, patients with T2, patients with T3 and those with T4 tumors (Log rank 9.63, \( P = 0.022 \)). There was also a significant difference in mean survival time decreasing between the carcinoma patients without lymph node metastasis (N0) and those with lymph node metastasis (N+) (Log rank 9.29, \( P = 0.0023 \)). Patients with Goseki II or IV classes had worst survival (mean 24 mo) than those with Goseki I or III classes (mean 51 mo) \( P = 0.0036 \), Figure 2. Patients with positive immunohistochemical MUC2 expression more than 30% had a worst survival (mean 20 mo) than those with positive MUC2 expression less than 30% (mean 39 mo) \( P = 0.0434 \) and those without MUC2 expression 0% (mean 72 mo) \( P = 0.0391 \). Patients with positive immunohistochemical expression of MUC2 less than 30% had worst but not statistically significant, survival than those without MUC2 expression (\( P = 0.0951 \), Figure 3).

DISCUSSION

In the present study, we looked for a correlation between the expression of the secreted gel-forming mucins MUC2, MUC5AC, MUC5B, MUC6 and histopathological classifications (WHO, Laurén, Mulligan and Goseki) in a series of 31 gastric carcinomas with a special attention to all components (major or minor) present in an individual tumor. Expression of MUC2 was frequently observed in all subtypes of gastric carcinoma whatever the classification. However, the high expression of MUC2 was significantly associated with the mucinous subtype of the WHO classification and with the group II of Goseki's classification (high tubular differentiation and high mucin content). A relationship between gastric mucinous carcinomas and group II of their classification has been previously reported by Goseki et al[3]. The WHO and the Goseki's classifications are more precise than the Laurén's and the Mulligan's classifications to classify gastric carcinoma, individualizing the mucinous subtype and group II respectively. Moreover, the Goseki's classification, that combine two distinct criteria, e.g. the tubular differentiation and the intra-cellular mucin content was shown to be the only classification with prognostic value additional to TNM staging[3-4]. The asso-

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**Table 2  MUC expression in each component of gastric carcinoma on the basis of Goseki’s classification n(%)**

| Goseki | n | MUC2 + IHC | MUC5AC + IHC | MUC5B + IHC | MUC6 + IHC | MUC2 + ISH | MUC5AC + ISH | MUC5B + ISH | MUC6 + ISH |
|--------|---|------------|--------------|-------------|-------------|------------|--------------|-------------|------------|
| Group I | 21 | 14 (67)  | 17 (81)      | 12 (57)     | 8 (38)      | 12 (57)   | 7 (33)       | 6 (29)      | 6 (29)     |
| Group II | 12 | 10 (83)   | 10 (83)      | 7 (58)      | 7 (58)      | 8 (67)    | 5 (42)       | 5 (42)      | 7 (58)     |
| Group III | 10 | 2 (20)    | 1 (10)       | 4 (40)      | 3 (30)      | 4 (40)    | 1 (10)       | 1 (10)      | 1 (10)     |
| Group IV | 13 | 9 (69)    | 6 (46)       | 7 (54)      | 5 (38)      | 7 (54)    | 2 (15)       | 4 (31)      | 2 (15)     |

IHC: Immunohistochemistry; ISH: In situ hybridization.
Survival curves for patients divided according to their Goseki classification. Some gastric and colon as well as in intestinal metaplasia of Laurén's or Mulligan's classifications. MUC2 was not significantly associated with the intestinal and unclassified carcinomas of the Laurén's classification. MUC5AC has been also found more frequently expressed in group IV of Goseki's classification, which corresponds in part to the diffuse type of the Laurén’s classification. In our series, the expression of MUC5AC was frequent in the diffuse subtype of the Laurén's classification (75% of the cases in this subtype), in the group IV of Goseki’s classification (83%) and in the signet-ring subtype of the WHO classification (75%) but the comparison with the other subtypes did not reach the significant level. Moreover, the expression of MUC6 in our series was not associated with any histological type of the gastric carcinomas, whatever the classification, in accordance with previous studies. In addition, expression of MUC5B, which before this study had never been extensively studied in gastric carcinoma using a morphological approach, was not found to be associated with particular subtypes of the Laurén’s, Mulligan’s, WHO or Goseki’s classifications.

Given the difficulty to demonstrate a constant correlation between MUC expression and histopathological classifications, we further evaluated the expression of the four secreted gel-forming mucins independently in all the different components, major or minor, frequently present in a same gastric carcinoma. In our series, 39% of the tumors presented one component, whereas 61% presented at least two different components further classified according to the Goseki’s classification. With this approach, none of the gel-forming mucin was restricted to any group of Goseki. In particular, MUC2 was not restricted to the components classified as group II but was also expressed in components classified as group I, III or IV. Furthermore, the comparison between the tumors which presented with a component of Goseki II (major or minor) and the tumors which presented without this component was not statistically different. Thus the association between MUC2 and the group II of Goseki was not reinforced by the study of each component. The best correlation between MUC expression and histopathological classifications was achieved for MUC2 and mucinous carcinomas of WHO classification and group II of Goseki’s classification, these classifications being based on the identification of the major component of the carcinoma.

In this study, we also looked for a correlation between TNM and MUC expression. We did not found any association between expression of MUC (individual MUC or several MUCs) and T or N stage. More particularly, although increased mucin gene heterogeneity has been described in advanced gastric carcinomas, this was not confirmed in our series. A correlation between the
expression of gastric mucins, i.e. MUC5AC and MUC6 and a poorer prognosis has been reported by some authors\cite{27}. Nevertheless, these results were not confirmed by others\cite{17,28}. As expected, we observed a significant difference in mean survival time between the carcinoma patients according to the TNM stage. We observed a shorter mean survival time for patients with gastric carcinoma classified as Goseki II or IV in comparison to patients with Goseki I or III tumors. Interestingly, we also observed a shorter mean survival time for patients with positive MUC2 expression \cite{29} (more than 30% of the cells) in comparison to patients without MUC2 immunohistochemical detection. Thus the quantity and quality of mucus present in gastric carcinoma were related to survival. This association between MUC2 expression and poorer outcome was controversial in the literature\cite{17,20,33}.

In conclusion, expression of secreted gel-forming mucins in gastric carcinoma is particularly complex. Complexity of mucin gene expression patterns in gastric cancer may reflect a precise state of differentiation at the cell level not recognized in the used morphologic classification systems. Nevertheless, this study showed a correlation between a high expression of MUC2 and mucinious subtype of gastric carcinoma that is achieved by considering the major component of the tumor. We also observed a shorter mean survival time in patients with a higher content of mucus (Goseki II or IV) and high positive MUC2 expression.

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