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The PAS positive material in gastric cancer cells of signet ring type is not mucin

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ABSTRACT

Purpose: The purpose of this study is to assess the exocrine and neuroendocrine properties of tumour cells in diffuse gastric cancer with signet ring cell differentiation.
Material and methods: Mucin mRNA and protein expressions (MUC1, 2, 3, 4, 5AC, 6 and MUC13) were assessed by immunohistochemistry and in situ hybridization. The neuroendocrine properties were evaluated by protein and mRNA expression of the general neuroendocrine markers chromogranin A and synaptophysin.
Results: No MUC expression was observed in signet ring tumour cells including the amorphous substance in any of the nine cases. All cases showed immunoreactivity to synaptophysin, and seven out of nine cases immunoreactivity to chromogranin A in signet ring and non-signet ring tumour cells. Chromogranin A mRNA expression was observed in tumour cells in all samples with retained mRNA.
Conclusions: The lack of MUC protein and mRNA in signet ring tumour cells suggests the amorphous substance is not mucin. The lack of MUC mRNA expression in non-signet ring tumour cells questions exocrine differentiation in this tumour group. The abundant protein expression of the general neuroendocrine markers CgA and synaptophysin, and mRNA expression in tumour cells strengthens the hypothesis that this tumour group may be of neuroendocrine origin.

Introduction

There are several histological classification systems for gastric adenocarcinoma. The most commonly used classification systems include the WHO and Lauren (1965). Lauren divided gastric adenocarcinoma into intestinal and diffuse type. The diffuse type is characterized by the absence of glandular structures, which are seen in the intestinal group. Epidemiological differences between the diffuse and intestinal types are observed, and the intestinal group is associated with chronic inflammation, often as a consequence of chronic *Helicobacter pylori* infection (Marrelli et al., 2002). Diffuse gastric cancer behaves differently compared to intestinal gastric cancer, and is more common in young females (Borch et al., 2000). The tumour is more aggressive, and preoperative chemotherapy has been shown to be less effective than for the intestinal type tumours (Messager et al., 2011). Distinct genetic differences are also seen between the two groups (Becker et al., 1994; Cervantes et al., 2007; Kuniyasu et al., 1992; Milne et al., 2009; Tahara, 1995; Yokota et al., 1988). Gastric carcinoma has traditionally been viewed as a single but heterogeneous group of tumours, but genomic and molecular analyses both suggest distinct tumour biology for subtypes (Shah et al., 2011).

One such subgroup of the diffuse gastric cancer includes tumours with signet ring cell differentiation. The incidence of signet ring cell carcinoma is increasing, and along with malignant melanoma it has some of the highest rates of increase in incidence among neoplasms (Borch et al., 2000). Signet ring cell morphology is a descriptive term, characterized by an amorphous substance that pushes the nucleus towards one side of the cell (Alos et al., 2005). The amorphous substance is periodic acid Schiff (PAS) positive, and based on the PAS positivity it is thought to be mucin. PAS is however not a specific marker, and it reacts with several substances including carbohydrates, amyloid substance and various glycoproteins (el-Zimaity et al., 1997). Mucins (MUC) are high molecular weight glycoproteins with complex oligosaccharide side chains, and are produced by various secretory epithelial cells. Mucin genes are independently regulated, and

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their expression is organ and cell type specific (Reis et al., 1998). Each mucin gene has a characteristic normal tissue distribution and more than one gene can be expressed in single tissue and at a single cell-level (Reis et al., 1999; Utsunomiya et al., 1998). Several mucin genes have been described, and MUC1–MUC6 are the most extensively investigated MUC proteins in gastrointestinal tumours (Chiaravalli et al., 2009). MUC1, MUC5AC, MUC6 and MUC 13 are expressed in normal gastric epithelium.

Although the amorphous substance in gastric signet ring tumour cells is considered to be of the mucin family, both signet ring and non-signet ring tumour cells have previously shown neuroendocrine differentiation. The general neuroendocrine markers chromogranin A (CgA) and synaptophysin have previously been found in tumour cells in diffuse gastric cancer (Bakkelund et al., 2006; Bartley et al., 2011; Fujiyoshi and Eimoto, 2008; Waldum et al., 1991, 1998). Regenerating islet-derived family member 4 protein (REG-4), which is involved in cell growth and regeneration, is co-localized with CgA in non-neoplastic tissue and expressed in gastric ECL cell carcinoids and in signet ring cell carcinoma (Zhang et al., 2003).

In the present work we wanted to evaluate the amorphous substance in signet ring cells and examine both the exocrine and neuroendocrine characteristics of tumour cells in diffuse gastric carcinoma with signet ring cell differentiation. We thus have examined the most relevant mucins for the gastrointestinal tract (MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC6 and MUC13) by immunohistochemistry (IHC) and also by in situ hybridization (ISH) by the use of a new commercially available in situ hybridization method. We also wanted to evaluate the protein expression of CgA, synaptophysin and mRNA expression of CgA and Reg-4 in tumour cells.

Materials and methods

Consecutive tissue blocks of gastric carcinomas ($n = 9$) were selected from the archive at the Department of Pathology and Medical Genetics St. Olav's University Hospital, Trondheim. These samples had previously been classified as a diffuse gastric cancer according to the Lauren classification. The cases had variable degree of signet ring cells differentiation ranging from 5 to 50%. The study was approved by the Regional Research Ethics Committee.

Immunohistochemistry

Formalin fixed paraffin embedded specimens were cut into 4-micrometer thick sections. The sections were dewaxed and processed for immunohistochemistry (IHC). Heat induced epitope retrieval by microwave cooking was then performed for 20 min in 10 mM Tris–EDTA buffer pH 9 for the following antibodies (MUC1, 2, 3, 4, 5AC, 6 and MUC13, synaptophysin and Ki-67) and citrate buffer pH 6 for the following antibody (CgA). The primary antibodies included rabbit polyclonal anti-synaptophysin (# A0010, Dako, Glostrup, Denmark) 1:400 dilution, monoclonal anti-CgA (#M0869, Dako, Denmark), 1:4000 dilution, monoclonal anti-MUC1 (ab45167, Abcam, Cambridge, UK) 1:200 dilution, monoclonal anti-MUC2 (ab76774, Abcam, UK) 1:500 dilution, monoclonal anti-MUC3 (ab17744, Abcam, UK) pre-diluted, monoclonal anti-MUC4 (ab52263, Abcam, UK) 1:500 dilution, monoclonal anti-MUC5AC (ab80953, Abcam, UK) 1:8000 dilution, monoclonal anti-MUC6 (ab11335, Abcam, UK) 1:8000 dilution, polyclonal anti-MUC13 (ab65109, Abcam, UK) 1:200 dilution, monoclonal Ki-67 (M7249, Dako, Denmark) 1:500 dilution and monoclonal anti-cytokeratin (Clone AE1/AE3, M3515, Dako) 1:50 dilution. All MUC, synaptophysin, Ki-67 and cytokeratin antibodies were applied for 1 h at room temperature (RT), whereas the CgA antibody incubation was performed at 4 °C overnight. Antigen–antibody complexes were visualized by Envision-HRP kit (K5007, Dako, Denmark) followed by DAB + (K5007, Dako, Denmark). For the monoclonal antibodies, mouse IgG1 (X0931 Dako, Denmark) and IgG2a (X0943 Dako, Denmark) were used as negative controls. For the polyclonal antibodies, rabbit serum was utilized.

Periodic acid Schiff (PAS), PAS-Diastase, PAS-MUC and alcian blue staining

PAS staining was performed by the use of Periodic Acid-Schiff kit (395B-1KT, Sigma-Aldrich, St Louis, MO, USA). After deparaffinization and rehydration, the periodic acid solution was applied for 5 min at RT. The Schiff reagent was then applied for 15 min at RT, followed by haematoxylin counter staining. The PAS-Diastase staining was carried out using the same kit, but with addition of alfa-amylase (0.2 g to 40 ml water). The slides where then placed in microwave at 600 W for 25 s and rinsed in water for 5 min. Staining with PAS combined with IHC for each individual MUC antibody was performed as described under immunohistochemistry for each individual MUC-antibody. This was followed by PAS staining utilizing the same PAS kit in order to evaluate the PAS positive amorphous substance. The alcian blue staining (0.1% alcian blue solution pH 2.5) was applied for 30 min at RT and counterstained with nuclear red for 2 min.

In situ hybridization

The method was performed as suggested in the protocol from Advanced Cell Diagnostics (Hayward, California, USA) with modifications. The probes were also delivered from the same company. The accession number of sequences used to generate probes: Chromogranin A: NM_001275.3, NT 313-2008, MUC2: NM_002457.2, NT 14357-15273, MUC4: NM_138297.4, NT 1808-3007, MUC5AC: NM_003403450.1, NT 761-2125, MUC6: NM_005961.2, NT 1245-2375, NM_033049.3, NT 541-1575, Reg-4: NM_032044, NT 287-1330. We were not successful in the assessment of synaptophysin mRNA due to difficulties with the probe. The method was carried out as previously described by us (Sordal et al., 2013). Then main modifications of the protocol are mentioned. Tissue samples were cut in 4-micrometer thick sections. The provided pre-treat 1 solution was applied for 15 min at RT. The tissue sections were boiled at 95 °C for 15 min in the provided pre-treat 2 solution. The pre-treat 3 (protease treatment) solution was then applied at 40 °C. Each individual tumour block was optimized with the protease solution (20–60 min) in order to achieve maximum hybridization signal and to maintain acceptable tissue morphology. The provided probe and probe solution was applied, and slides were covered with agarose gel and placed in a rack at 40 °C for hybridization for 2 h. The amplification steps were done as suggested in the protocol enclosed. Both the 1.0 (4 amplification steps) and 2.0 kits (6 amplification steps) were used. The DAB solution A and B were mixed in equal volume and left on for 10 min at room temperature. A provided positive control probe ubiquitin (UBC), which is a housekeeping gene expressed in most tissues, was utilized. For negative control a sample where the probe was omitted was included to exclude background staining. Also, a sample that had been treated with RNAse for 1 h at 37 °C and probe applied was included as a negative control to ensure that the probe was specifically binding to mRNA. In addition a biotin labelled Poly T probe was included to ensure retained mRNA quality in the tissue samples.

Results

1: HES, PAS, PAS-Diastase, alcian blue, cytokeratin and Ki-67 expression

All cases were assessed with HES (Fig. 1A–B), and all cases had variable degree of PAS positive cells, which were morphologically consistent with signet ring cells (Fig. 1C–D). The same cells were cytokeratin positive (Fig. 1E). These cells were also PAS-Diastase and alcian blue positive. The Ki-67 proliferation index (PI) was assessed, and for five of the cases the PI index was around 35%, and