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ANATOMICAL PATHOLOGY

Clinicopathological and molecular stability and methylation analyses of gastric papillary adenocarcinoma

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Summary

The molecular alterations and pathological features of gastric papillary adenocarcinoma (GPA) remain unknown. We examined GPA samples and compared their molecular and pathological characteristics with those of gastric tubular adenocarcinoma (GTA). Additionally, we identified pathological and molecular features of GPA that vary with microsatellite stability. In the present study, samples from 63 GPA patients and 47 GTA patients were examined using a combination of polymerase chain reaction (PCR)-microsatellite assays and PCR-pyrosequencing in order to detect microsatellite instability (microsatellite instability, MSI; microsatellite stable, MSS), methylation status (low methylation, intermediate methylation and high methylation level), and chromosomal AI in multiple cancer-related loci. Additionally, the expression levels of TP53 and Her2 were evaluated using immunohistochemistry. GTA and GPA are statistically different in their frequency of pathological features, including mucinous, poorly differentiated and invasive micropapillary components. Clear genetic patterns differentiating GPA and GTA could not be identified with a hierarchical cluster analysis, but microsatellite stability was linked with TP53 and Her2 overexpression. Methylation status in GPA was also associated with the development of high microsatellite instability. However, no pathological differences were associated with microsatellite stability. We suggest that although molecular alterations in a subset of GPAs are closely associated with microsatellite stability, they play a minor role in GPA carcinogenesis.

Key words: Allelic imbalance; gastric cancer; methylation; microsatellite instability; papillary adenocarcinoma.

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INTRODUCTION

Gastric cancer is a major global health threat and the third most common cause of cancer death worldwide.[1] Although in Japan and Korea screening programs using barium photofluorography or endoscopy allow earlier detection, the 5-year overall survival of patients with advanced gastric cancer is still poor.[2] The molecular mechanisms of gastric carcinogenesis are not yet fully understood, but identifying them may help improve therapeutic efficacy and identify strategies for dividing patients into relevant subgroups.[3,4] Recent studies have shown that both genetic and epigenetic alterations are closely associated with gastric carcinogenesis.[3,4] Genetic alterations result in irreversible changes that are responsible for tumour progression,[3–5] and epigenetic alteration through DNA methylation plays an important role in the early phase of carcinogenesis.[3–6] In addition, microsatellite instability (MSI) and microsatellite stability (MSS) phenotypes are mutually exclusive at the genomic level.[4–6]

Gastric cancer occurs in a variety of histological types, each of which shows different features.[7] The treatment of early cancers still depends on the stage of the disease, while advanced and metastatic disease treatment options include Herceptin for HER2 amplified cancers. However, the histological type is also one of the most important factors for determining endoscopic treatment, chemotherapy and patient outcome in gastric cancers.[7–10] The histological type of gastric cancer may have a crucial role in determining treatment strategies.[11–15]

Japanese pathologists preferentially use the Japanese Classification of Gastric Carcinoma for determining the histological diagnosis, but Western pathologists perform histological diagnosis according to the World Health Organization (WHO) classification.[16–18] Although there are fundamental discrepancies between Japanese and WHO histological classifications, common histological types are used in both groups.[17,18] Papillary adenocarcinoma is one of the independent histological types that are commonly accepted by both Japanese and WHO classifications.[7] Previous studies have shown that gastric papillary adenocarcinoma (GPA) is characterised by distinct clinicopathological features such as frequent venous invasion, distant metastasis, and poor prognosis.[19–21] In addition, microsatellite instability (MSI) is commonly found in GPA.[3,4,19] However, the clinicopathological and molecular features of GPA have been not extensively evaluated.

It is widely accepted that there are two distinct types of gastric pathogenesis, the intestinal and diffuse types. The intestinal type includes tubular differentiated adenocarcinoma and papillary adenocarcinoma, and is characterised by gland-forming cells, distant organ metastasis via venous

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invasion, and atrophic gastric mucosa with intestinal metaplasia. The diffuse type is closely associated with non-gland-forming cells, peritoneal dissemination, and non-atrophic gastric mucosa without intestinal metaplasia. These findings indicate that the intestinal type contrasts with the diffuse type in gastric pathogenesis. Therefore, tubular differentiated-type adenocarcinoma was selected as a comparison for identifying genetic alterations of GPA. The purpose of this study is to (1) identify clinicopathological and molecular features of GPA and compare them with gastric tubular adenocarcinoma (GTA), a representative of differentiated-type adenocarcinoma, and (2) examine molecular differences in GPA between MSI-high and MSS phenotypes.

METHODS

Patients

Samples for this study were obtained from 63 patients with gastric papillary adenocarcinomas (GPA) and 47 patients with gastric tubular adenocarcinomas (GTA) diagnosed at the Department of Molecular Diagnostic Pathology, Iwate Medical University, Japan. The clinicopathological features of these patients were obtained from hospital records according to the General Rule for Japanese Research Society for Gastric Cancers.[16] Although tumour stage of the Japanese Research Society for Gastric Cancers was used in the present study, this stage was the same as that of the American Joint Committee on Cancer (AJCC). Our hospital criteria, which are a modified version of the classification system of the Japanese Research Society for Gastric Cancers, were used to make histological diagnoses. The Japanese histological criteria that we used were almost the same as those of WHO.[16] In brief, GPA is characterised by epithelial projections scaffolded around a central fibrovascular core, and GTA consists of tubular structures or cribriform patterns.[7,16] GTA was further subclassified into well-differentiated and moderately-differentiated adenocarcinomas (the former, 24; the latter, 23). We defined GPA as a tumour in which more than 50% of the tumour area contained papillary structures. All the tumours we examined had invaded beyond the muscularis mucosae. Intramucosal tumours were excluded from this study. In the present study, Epstein–Barr virus (EBV) was not examined. However, histological findings that are frequently found in EBV-associated cancer (e.g., medullary carcinoma with lymphoid stroma) were not identified in the present study.

Informed consent was obtained from all patients that we examined, and our study was approved by the ethics committee of Iwate Medical University.

Histological procedures and assessment

The resected specimens were embedded in paraffin and stained with haematoxylin and eosin (H&E) according to routine procedures.[6,17]

We examined specific histological features of each specimen including mitotic figure, poorly differentiated component (PDC), mucinous carcinoma component (MCC), invasive micro-papillary pattern (IMP) and tumour-infiltrated lymphocyte (TIL).[7,17] The presence of a poorly differentiated component and mucinous carcinoma component pattern was recorded if they consisted of $\leq$30% to <50% of the tumour area. However, the presence of IMP was recorded if it made up more than 10% of the tumour area.[6] The definitions of these histological subtypes were described elsewhere.[6,7,17] In this study, several histological components were often seen within the same tumour. In such cases, if the papillary component consisted of more than 50% of the tumour area, papillary carcinoma was diagnosed histologically.

DNA extraction

Microdissection from formalin fixed, paraffin embedded (FFPE) tissue was performed on haematoxylin-stained slides for both tumour and non-neoplastic mucosal tissues. The carcinoma and non-neoplastic mucosal components were microdissected separately. Papillary or tubular components were isolated from the histological section that contained the deepest invasive sites. If tissue other than papillary or tubular components was contained in the histological section, genomic DNA was extracted from only papillary or tubular components that were microdissected as described previously.[22] In brief, microdissected tissue was incubated at 56°C for 12–18 h in 50 μL of buffer containing 0.5% Tween-20 (Boehringer Mannheim, Germany), 20 μg proteinase K (Boehringer Mannheim), 50 mmol/L Trizma base at pH 8.9, and 2 mmol/L EDTA. Proteinase K was inactivated by incubating the samples at 100°C for 10 min.

Immunohistochemical analysis

Sections were cut from the FFPE tissue blocks to a 4 μm thickness for analysis with an extensive panel of immunohistochemical markers, including TP53 (DO7; Dako, Denmark), MUC2 (Ccp58; Novocastra Laboratories, UK), MUC5AC (CLH2; Novocastra Laboratories), MUC6 (CLH5; Novocastra Laboratories), CD10 (56C6; Novocastra Laboratories), and Her2 (polyclonal, Dako). Sections were prepared, dried, deparaffinised and rehydrated before microwave treatment (H2500 Microwave Processor; Bio Rad, USA) in citrate buffer (pH 6.0) for 5 min. An automatic staining machine (Dako Envision+ system) was used for the immunohistochemical procedures. The slides were counterstained in haematoxylin, dehydrated, and mounted.

Analysis of microsatellite instability

PCR-MSI analysis was performed as described previously.[6] Five different loci were assessed for MSI, including all those recommended by the Bethesda panel for colon cancer (BAT25, BAT26, D5S346, D2S123, and D17S250).[23] A tumour was defined as MSI positive when PCR analysis of the tumour sample resulted in an abnormal-sized DNA band compared to the corresponding normal sample at multiple tested markers. MSI positive colorectal carcinomas were used as controls in the study and were divided into two groups, those with high-level instability (i.e., MSI at $\geq$20% of loci) and those with low-level instability (i.e., MSI at <20% of loci) as described previously.[23] Tumours with only one marker displaying an alteration and those previously categorised as MSI low were considered MSI negative (or MSS) in this study.

DNA methylation analysis

The PyroMark Q24 (Qiagen, Germany) system for pyrosequencing was used to assess the DNA methylation status of selected markers. Primer sequences were designed using Qiagen's Pyromark Assay Design 2.0 software.

DNA methylation at the six specific promoters originally described by Yagi and colleagues was quantified.[24,25] Methylation of three markers (*RUNX3*, *MINT31*, and *LOX*) was analysed, and samples with at least two methylated markers were defined as highly methylated epigenotype (HME) tumours. The remaining tumours were also screened for methylation at three other markers (*NEUROG1*, *ELMO1*, and *THBD*) and defined as intermediate methylation epigenotype (IME) tumours if they had at least two methylated markers out of the second set of three markers. Tumours not classified as HME or IME were designated as low methylation epigenotype (LME) tumours.

Analysis of allelic imbalance by polymerase chain reaction

PCR-AI analyses were performed using a thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer, USA) according to previously reported procedures.[26] Allelic imbalances (AI) on chromosomes 1p, 3p, 4p, 5q, 8p, 9p, 13q, 17p, 18p and 22q were examined in paired tumour and normal tissue samples obtained from 63 PGA patients using 22 highly pleomorphic microsatellite markers (D1S228, D1S548, D3S2402, D3S1234, D4S2639, D4S1601, D5S107, D5S346, D5S299, D5S82, D8S201, D8S513, D8S532, D9S171, D9S1118, D13S162, TP53, D18S487, D18S34, D22S274, D22S1140 and D22S1168). Allelic imbalances at these microsatellite markers have been reported frequently in gastric carcinomas.[3,4] Microsatellite sequences were obtained using specific primers reported by the Genome Database (http://gdbwww.gdb.org/gdb/).

The microsatellite weight peaks produced by the normal DNA sample were used to determine whether the cancerous sample was homozygous (one peak) or heterozygous (two peaks). The allelic ratio was calculated as described by Habano *et al.*[27] A tumour was considered to have allelic loss if the allele peak ratio was less than or equal to 0.60, representing an allelic signal reduction of at least 40%.

Hierarchical clustering analysis

A hierarchical clustering analysis was performed to divide all samples into subgroups according to their MSI and AI, with the goal of achieving maximal homogeneity within each group and the highest differences

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