Genetic analysis of fundic gland-type gastric adenocarcinoma

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Abstract. This study aimed to analyze the molecular characteristics of gastric adenocarcinoma of the fundic-gland type (GAFG) and explore the possible mechanism of tumor development. Samples from 10 Chinese patients with GAFG were collected at the Peking University International Hospital and Liaocheng People's Hospital between January 2015 and March 2022. The nucleic acid sequence of Epstein Barr virus-encoded RNA (EBV-EBER) was detected by *in situ* hybridization. Genetic mutation information for *GNAS*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *TP53*, *APC*, *CTNNB1*, *HER2*, *MLH1*, *MSH2*, *MSH6*, and *PMS2* was obtained by Next-Generation Sequencing, and the relevant literature was reviewed. A total of eight instances of missense mutations were detected, consisting of seven cases with *GNAS* mutations, two cases with *KRAS* mutations, and one case with a *TP53* mutation. Additionally, two patients had simultaneous missense mutations in *GNAS* and *KRAS*. Nonsynonymous mutations in *APC*, *CTNNB1*, *NRAS*, *BRAF*, *PIK3CA*, *HER2*, *MLH1*, *MSH2*, *MSH6*, or *PMS2* were not observed in any cases. In addition, all tumors were EBER-negative. GAFG exhibits diversity at the molecular level, and *GNAS* mutations are more common than *KRAS* mutations, *TP53* mutations, and microsatellite instability. To date, no association between EBV/*HER2* and GAFG has been found.

Introduction

Gastric fundus glands, also known as oxyntic glands, are normally distributed in the fundus and body of the stomach and include the chief, parietal, cervical mucus, endocrine, and stem cells. In 2003 and 2005, Müller-Höcker and Rellecke (1) and Matsukawa *et al* (2) reported on fundic dysplastic polyps of the chief cell-predominant type. Tsukamoto *et al* (3) first described gastric adenocarcinoma with chief cell differentiation in 2007. In 2019, the WHO classification of digestive system tumors (fifth edition) defined intramucosal gastric fundus gland tumors as oxyntic gland adenomas, while those with submucosal infiltration were classified as gastric adenocarcinoma of the fundic gland type (GAFG) (4). GAFG is a rare type of gastric neoplasm with an incidence of <0.1% among the patients undergoing gastroscopy (5), with the majority of case reports originating from Japan. According to previous studies, patients with GAFG are primarily middle-aged or elderly (aged 36-87 years), with a slight male majority (3,6,7). GAFG patients generally have mild symptoms, such as abdominal discomfort and acid reflux, or no symptoms. Additionally, clinical tests have identified only a single case with significant abnormalities, in which slightly increased C-reactive protein and carcinoembryonic antigen levels were observed (1). In brief, no specific clinical manifestations or laboratory test results are known to be associated with the GAFG, to the best of our knowledge.

Endoscopic examinations have revealed that the majority of GAFG cases involve solitary lesions in the upper and middle third of the stomach; multiple lesions were observed in only a small number of individual patients (8). The mean tumor size was ~10 mm, and the maximum reported diameter was 85 mm (9). The lesions may appear as raised, flat, or concave (10-12). Changes in the color of the mucosa, such as from pink to white, yellow, or black, can contribute to an early diagnosis (13). Dilated branching vessels have been observed in approximately half of the reported cases (14). Pathological examination of tumor specimens is necessary for a correct diagnosis. Morphologically, GAFG is divided into three subcategories: Chief cell-predominant (~99% of the reported cases), parietal cell-predominant, and mixed phenotypes (15). Most tumors exhibit mild to moderate dysplasia, even when submucosal infiltration occurs (7). *Helicobacter pylori* infection, intestinal metaplasia, and mucosal atrophy in GAFG are infrequent compared with traditional gastric adenocarcinoma (5,10,16). Immunohistochemistry analyses have revealed the presence of pepsinogen I and mucin-6 (MUC6). Severe cellular dysplasia, lymphovascular invasion, lymph node metastasis, and atypical cellular differentiation may be markers of invasion and have been suggested to indicate poor prognosis (7,9,17-19). High-risk patients can be treated with total or segmental gastrectomy plus lymph node dissection (7,18).

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GAFG has unique pathological features compared to traditional gastric adenocarcinoma; however, few studies have investigated the *GNAS*, *KRAS*, and Wnt signaling pathways at the molecular level in GAFG. Here, molecular analysis of 10 Chinese GAFG specimens was performed and the relevant literature was reviewed to improve our understanding of the molecular characteristics of GAFG. The molecular results were combined with clinicopathological information, first covering EBV infection and *HER2* status, considering that EBV-positive gastric cancer tends to occur in the fundus or body of the stomach (20), and *HER2* has predictive value as it can be used to evaluate the efficacy of trastuzumab and lapatinib in the treatment of *HER2*-positive gastric cancer patients (21). The results of this study have implications for future explorations into the factors underlying tumor occurrence, development, and identification of clinical prognostic biomarkers and potential therapeutic targets. To the best of our knowledge, this is the first study on GAFG in a Chinese cohort. Novel findings surrounding the genetic factors underlying the disease are presented.

Materials and methods

Case selection and clinicopathological characteristics of patients. Tumor samples from 10 Chinese patients with GAFG were collected from Peking University International Hospital and Liaocheng People's Hospital between January 2015 and March 2022. The patients included 4 males and 6 females, aged 46-75 years, with a mean age of 62.5 years. Samples from 9 patients were obtained during complete endoscopic resection, and samples for the remaining one case were obtained by biopsy. The size of the tumors observed during endoscopy ranged from ~0.3-1.2 cm. All postoperative specimens were examined and diagnosed by two senior pathologists. All cases were classified as chief cell-predominant, intruding into the submucosa by 100-300 μm, with mild to moderate dysplasia, with no lymphovascular or perineural involvement. Immunohistochemical investigations found that the tumor cells were diffusely positive for pepsinogen I and MUC6, focally reactive for MUC5ac and H^+/K^+ ATPase, and negative for MUC2 and CD10. Moreover, β-catenin protein expression was observed only in the cell membranes. All samples were identified as negative or partially weakly positive for p53 protein. All tumors were 1-15% diffusely distributed, as measured by the Ki-67 index. After a follow-up period of 16-48 months, no recurrence or metastasis was observed in any of the patients.

Epstein Barr virus-encoded RNA (EBV-EBER) testing. Specimens were fixed with 4% neutral buffered formalin solution at room temperature for 12 h before being embedded in paraffin and cut into 4 μm sections. EBER was detected using an *in situ* hybridization kit (cat. no. ISH-7001, OriGene Technologies, Inc.), and non-keratinizing nasopharyngeal carcinoma was used as a positive control. Finally, the sections were stained with DAB at room temperature for 5 min, counterstained with hematoxylin at room temperature for 5 min, and then observed under a Nikon light microscope (maximum magnification, x400, Nikon Corporation). Positive nuclei were stained brown with DAB and the negative nuclei were stained blue with hematoxylin.

Gene analysis. All tissue samples included in this study were pathologically confirmed to contain at least 20% tumor cells. Tumor tissues embedded in paraffin were cut into wax rolls which were analyzed with Next-Generation Sequencing (NGS, Gene+ Smart Laboratory). This high-throughput DNA panel sequencing technology allowed mutation information for numerous genes to be obtained. Here, *GNAS*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *TP53*, *APC*, *CTNNB1*, *HER2*, *MLH1*, *MSH2*, *MSH6*, and *PMS2*, alongside other genes (see Table SI for a detailed list of all 73 genes) were examined for point mutations, insertions, deletions, fusions, and amplifications; adjacent non-tumor tissues were used as the control.

DNA was extracted from paraffin-embedded tumor tissues using a QIAamp DNA Mini Kit (Qiagen GmbH). DNA was then fragmented into ~300 bp fragments and a library was constructed using the KAPA Library Preparation Kit (Kapa Biosystems, Inc.). The SeqCapEZ Library (Roche Diagnostics) was used to enrich the fragments for the target regions of 73 common genes involved in tumor development. After processing the enriched library using the TruSeq PE Cluster Generation Kit v3 and TruSeq SBS Kit v3 reagent kits (Illumina, Inc.), sequencing was performed using an Illumina HiSeq 3000 sequencing platform (Illumina, Inc.). After removing the terminal connector sequence and filtering out low-quality sequences, the reads were mapped to the human genome. The Genome Analysis Tool Kit (https://www.broadinstitute.org/gatk/; GATK) and MuTect tools were used to detect insertions/deletions and single nucleotide mutations. Contra (22) was used to identify copy number variation detection and BreakDancer (23) was used to detect tumor-related structural variations. The results were manually verified.

Statistical analysis. Statistical analysis was performed using SPSS version 22.0. $P<0.05$ was considered to indicate a statistically significant difference. A student's t-test was used to identify any association between the presence of *GNAS* missense mutations and both the tumor size under endoscopy and the depth of submucosal infiltration.

Results

EBER status. All nuclei stained blue with hematoxylin during EBER testing, indicating all 10 cases were negative for EBER (Fig. 1). The positive control, non-keratinizing nasopharyngeal carcinoma, stained brown due to DAB, as expected (Fig. 2).

NGS analysis. The results of NGS for the detection of mutations are summarized in Table I. A total of seven cases were found to carry *GNAS* missense mutations, and two cases were found to carry *KRAS* missense mutations. Of these cases, two were found to carry both *GNAS* and *KRAS* missense mutations (Figs. 3 and 4). Other instances of missense mutations included two cases carrying an *FGFR* mutation, and one case carrying a *TP53* mutation. Another two cases had no missense mutation. Synonymous mutations were observed in *APC*, *KRAS*, *NRAS*, and *FGFR* (case #3), as well as in *MSH6* and *BRCA1* (case #1). In case #1, *CDK4* amplification with a copy number of 1.4 was detected but not considered relevant. No genetic fusion or frameshift mutations were detected in any of the samples and no mutations

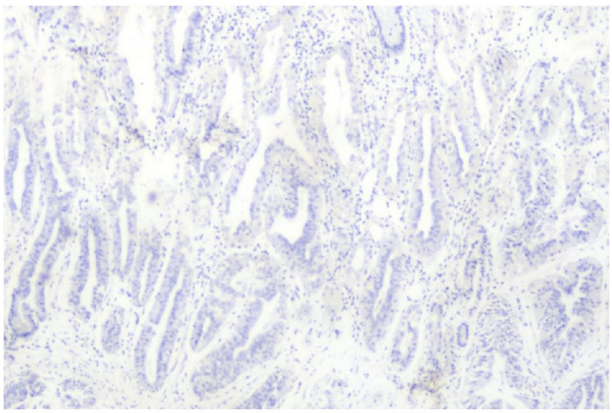

Figure 1. The Epstein Barr virus-encoded RNA status of gastric adenocarcinoma of the fundic-gland type was negative as determined by *in situ* hybridization. The nuclei were stained blue with hematoxylin. Magnification, x40.

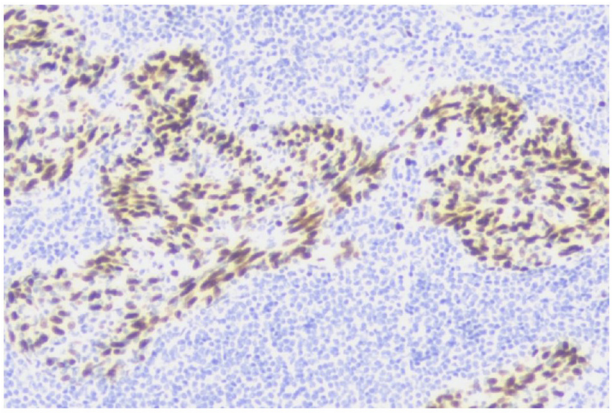

Figure 2. Positive control of Epstein Barr virus-encoded RNA from non-keratinizing nasopharyngeal carcinoma. The nuclei were stained brown with DAB. Magnification, x100.

were detected in *CTNNB1*, *BRAF*, *PIK3CA*, *HER2*, *MLH1*, *MSH2*, and *PMS2*.

Association between GNAS mutations and tumor properties. No significant association was identified between the presence of *GNAS* missense mutations and either the tumor size under endoscopy or the depth of submucosal infiltration (P>0.05).

Discussion

Despite numerous histopathological reports relating to GAFG, to date, few studies have presented data on the molecular features of the disease. More than 300 cases of GAFG have been reported, of which ~one-third have been analyzed by genetic sequencing (7,9,24-30). Considerable research on GAFG has focused on Wnt/β-catenin-related signaling pathways (9,24-27), and mutations in *GNAS* and *KRAS* (7,9,28-30).

β-Catenin is a key protein involved in the Wnt signaling pathway. Typically, cytoplasmic expression of β-catenin is maintained at low levels through degradation. However, when genes related to the Wnt signaling pathway, such as *CTNNB1*, *AXIN*, *APC*, and *PPP2R1A*, are activated by mutations or methylation, β-catenin accumulates leading to nuclear translocation, which in turn activates downstream genes implicated in the occurrence and development of tumors. This pathway has received widespread attention in gastric cancer, and the development of treatments targeting different molecular components in this pathway has been explored (24,31,32). While genetic mutations may not necessarily lead to β-catenin overexpression (25), the mutation rate in GAFG is high and variable. Previous studies have shown that ~85% of GAFG tumors are positive for nuclear β-catenin expression and the mutation rate of Wnt signaling pathway-related genes is ~45% (9,26). The labeling index of nuclear β-catenin immunoexpression, the number of cases in which it is overexpressed, and the mutation rate of related genes are higher in GAFG than in traditional gastric adenocarcinoma (26). However, nuclear β-catenin immunolabeling and related gene mutations were not observed in the present study nor in previously published reports on Chinese patients (33,34). This may be due to the small number of cases, regional factors, or other mechanisms requiring further investigation.

Lee *et al* (25) found that in oxyntic gland adenoma (OGA), the nuclear β-catenin immunolabeling index and the rate of related gene mutations were lower than those in GAFG, with approximate rates of 27% for nuclear expression and 36% for mutations in *APC*, *AXIN*, or *PPP2R1A*. In addition, these measures exhibited no significant correlation with the clinicopathological variables in OGA. However, other studies have noted that nuclear β-catenin staining preferentially appears in deeper sections of tumors (invading surface) (7,9) and that the process of submucosal infiltration may require β-catenin nuclear transition to activate the Wnt signaling pathway (25,26). Due to the lack of overexpression in the samples examined in the current study, similar conclusions could not be drawn.

Murakami *et al* (27) analyzed the methylation of Wnt/β-catenin signal-associated genes (including *sfrp*, *APC*, and *AXIN2*) and found that high methylation levels were more common in GAFG than in OGA, which may be related to the occurrence and progression of GAFG. However, the findings of this study were limited in this regard, as gene methylation testing was not performed.

The missense mutation rates of *GNAS* and *KRAS* from previous sequencing reports on GAFG (7,9,28-30) were analyzed here, resulting in mutation rates of 20.2% (19/94) and 6.2% (5/81), respectively. In particular, all studies reported *GNAS* mutations at base locus 601 or 602 and amino acid locus 201, except for one case that had an additional *GNAS* mutation at base locus 680 and amino acid locus 227 (30). In line with previous results, in the present study, the missense mutation rate of GNAS (70%) was significantly higher than that of KRAS (20%). In traditional gastric adenocarcinoma, *KRAS* mutations are more common and correlate with the clinical stage, differentiation degree, lymph node metastasis, distant metastasis, and depth of invasion, whereas *GNAS* mutations are absent or rare. Mutations of *GNAS* and *KRAS* could present simultaneously in both GAFG and traditional gastric adenocarcinoma (9,35). In the present study, there were two cases of GAFG with missense mutations in both *GNAS* and *KRAS*.

Furthermore, Kushima *et al* (28) and Nomura *et al* (9) evaluated the relationship between the clinicopathological characteristics of GAFG and *GNAS* mutations. They found

Table I. Gene mutations detected in the 10 cases of gastric adenocarcinoma of the fundic-gland type by Next-Generation Sequencing.

Case number	Genes containing missense mutations	Genes containing coding-synonymous mutations	Genes containing gain of function mutations	Genes containing coding segment deletion mutations	Size of the tumor under an endoscope, cm	Depth of submucosal infiltration, μm
1.	*TP53* *FGFR2* *IDH1*	*MSH6* *BRCA1*	*CDK4*	AR	1.2	300
2.	*GNAS* *FGFR1*				0.8	100
3.	*GNAS* *ESR1*	*NRAS* *KRAS* *APC* *CDK6* *PTCH1* *PTEN* *FGFR2* *NF1*			0.6	300
4.					0.4	100
5.					0.4	180
6.	*GNAS*				0.4	280
7.	*GNAS* *MAP2K1*				0.6	220
8.	*GNAS* *FBXW7* *CCND1*				0.3	100
9.	*GNAS* *KRAS*				0.4	300
10.	*GNAS* *KRAS*				0.5	150

that tumors with *GNAS* mutations were more likely to invade the submucosa and were larger than those without mutations, although the differences were not statistically significant. These results suggested that *GNAS* mutations play a role in promoting tumor progression and invasion. Although the missense mutation rate of *GNAS* in the present study was 70% (7/10 cases), there was no significant correlation between *GNAS* mutation status and tumor invasion depth or size. Therefore, the prognostic significance of *GNAS* mutation status in GAFG requires further study and evaluation.

Nomura *et al* (9) reported two cases of *KRAS* mutations in a group of patients with GAFG, where one had the largest tumor size and lymphatic infiltration and the other had the highest submucosal infiltration depth compared to the rest of the cohort. In the present study, the two instances of *KRAS* mutations also occurred in tumors with the highest submucosal infiltration depth (~300 μm). However, OGA occasionally presents with missense mutations in *GNAS* (2/11 cases) (7,30) but never in *KRAS* (0/5 cases) (28,30). These results support the novel idea that the *KRAS* mutations may serve as a more valuable marker of tumor aggressiveness than *GNAS*. Although it is currently impossible to draw conclusions owing to the limited number of samples; this topic warrants further study.

As part of The Cancer Genome Atlas project, researchers have conducted molecular identification in 295 primary gastric cancer samples (20) and classified them into four molecular subtypes: EBV-positive, microsatellite instability, genomically stable, and chromosomal instability. This classification expands our understanding of the pathogenesis of gastric cancer and provides a screening basis for patient stratification, targeted treatment, and clinical trials in patients with gastric cancer. However, the features of each subtype do not accurately reflect the genetic characteristics of GAFG. EBER is the most abundant EBV transcript in long-term latent infections and promotes cell growth, apoptosis inhibition, and immunoregulatory activities through a variety of signal pathways (36). Although GAFG may also develop in the fundus or body of the stomach (5-15), the results from *in situ* hybridization analysis of the 10 samples studied here found all samples to be EBV-EBER negative. In addition, there have been no previous reports of EBV in this

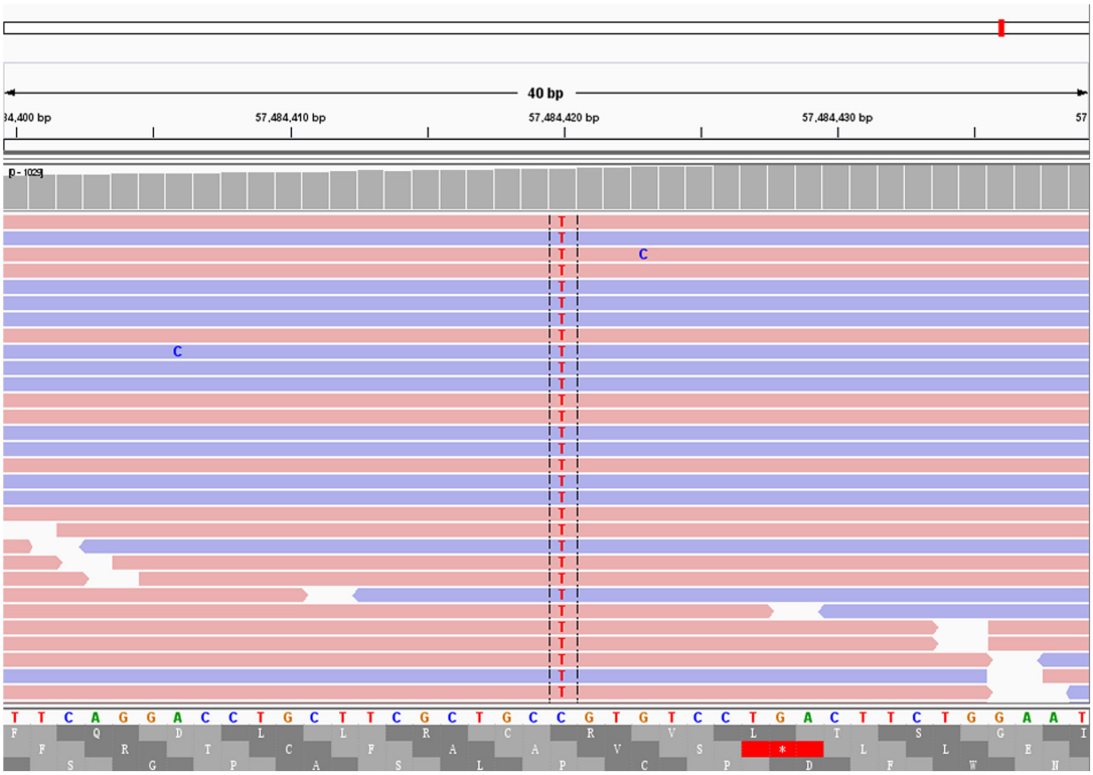

Figure 3. GNAS missense mutation (case #9). The 601st site in the gene sequence was mutated from a cytosine to a thymine.

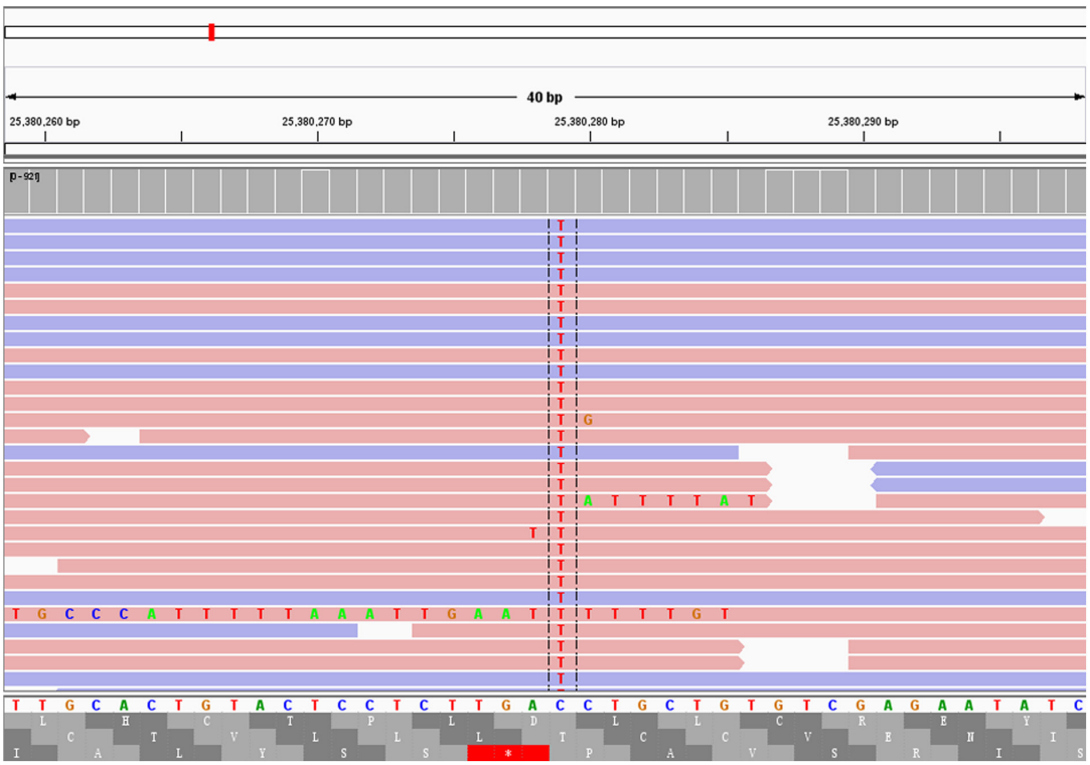

Figure 4. KRAS missense mutation (case #9). The 179th site in the gene sequence was mutated from a guanine to adenine.

type of tumor. Microsatellite instability is more common in the gastric antrum or pylorus, and abnormal DNA repair mechanisms result in a high mutation rate in genes such as *PIK3CA* and *HER2* (37). To date, only one study has identified such mutations in GAFG cases, finding only two cases of *PIK3CA* missense mutations among 34 cases (30). However, these mutations were not accompanied by microsatellite instability as was the case in the gastric antrum and pylorus tumors.

Notably, Yang (38) reported the first case of ulcerative GAFG with microsatellite instability. The lesion invaded the subserosa and exhibited lymph node infiltration and distant metastasis. However, *GNAS* and *CTNNB1* mutations were not detected. The *AXIN2* mutation rate and expression of nuclear β-catenin, as detected through immunohistochemistry, were significantly higher in tumor cells than in normal cells. Moreover, PD-1, PD-L1, and CD8 positivity have been observed in lesions with high microsatellite instability (38). These findings provide crucial information to aid in the discovery of novel targeted therapies. No microsatellite instability was observed in the 10 patients studied in the present report.

A meta-analysis showed that overexpression of *HER2* in patients with gastric cancer was associated with cell proliferation, apoptosis, migration, and a poor prognosis (39). In a retrospective analysis, only three cases of parietal cell-type adenocarcinoma of the fundus were found to be negative for *HER2* by immunohistochemistry or *in situ* hybridization (40,41). In the present study, no *HER2* mutations were detected in the 10 patients with chief cell-predominant GAFG. Whether a negative *HER2* mutation status is associated with early GAFG or a favorable prognosis requires further clarification.

TP53, which encodes p53, plays key roles in cell cycle regulation and apoptosis. Mutations in *TP53* and p53 overexpression are important biomarkers for predicting the prognosis of patients with gastric cancer. However, this has rarely been observed in published GAFG case reports. Of the 10 patients studied here, a missense mutation in *TP53* was found only in one case (case #1). This case showed the deepest infiltration and was negative for p53 protein expression. Compared to traditional gastric adenocarcinoma, the incidence of p53 overexpression in GAFG is extremely low (30). However, the prognostic significance of p53 mutations remain unclear.

In addition, Ke *et al* (42) revealed that the Sonic Hedgehog (Shh) signaling pathway may be independent of the Wnt/β-catenin signaling pathway, which is also involved in the progression and prognosis of GAFG. Ueyama *et al* (30) reported a case of GAFG with a *CDNK2A* missense mutation. The present study showed, for the first time, that *FGFR* and other mutations occur in GAFG. The results of these individual cases highlight the genetic variety of GAFG.

In summary, samples obtained vis endoscopy from 10 Chinese patients with GAFG, a unique pathological tumor type, were retrospectively analyzed. GAFG differs from traditional gastric cancers in that it exhibits diversity at the molecular level, much of which requires further investigation. In the present study, non-synonymous mutations in the Wnt/β-catenin pathway were not detected. The missense mutation rate of *GNAS* was found to be much higher than that of *KRAS*, whereas mutations in *TP53* and microsatellite instability were rare. To date, no study has demonstrated a positive EBV or *HER2* status for GAFG. Although this is the first study of Chinese patients on the molecular factors underlying GAFG, it was limited by the small number of cases. In addition to screening the cases for mutations in a large number of genes, the relationship between the pathogenesis, genetic alterations, and clinicopathological characteristics of GAFG were assessed. Determining the associations between specific genetic alterations and patient prognoses is the aim of future research as no statistically relevant factors were identified in the present study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA005206) and are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human.

Authors' contributions

LL was responsible for the conception and design of the study. XZ, XF, and XZ contributed to the acquisition and interpretation of the data. LL drafted the manuscript. LL and XZ confirm the authenticity of all the raw data. XZ revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by Biomedical Ethics Committee of Peking University International Hospital (approval on. 2020-KY-0011-02); the need for informed consent from patients was waived.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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