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Genomic and transcriptomic landscape of carcinogenesis in patients with gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS)

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Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) is an autosomal dominant syndrome characterized by polyposis localized in the gastric body and fundus with a strong tendency for adenocarcinoma. The genetic mutations that accumulate during the progression from normal mucosa through polyp to carcinoma in GAPPS remain unclear. We investigated the evolutionary process from normal mucosa to polyp and carcinoma in GAPPS. Through comprehensive mutational and transcriptome analyses, we aimed to provide insights into the biology of this disease. Whole-exome sequencing and RNA sequencing were performed on carcinoma, polyp, and normal mucosa samples from multiple sites from seven patients with GAPPS (n = 54 samples). We comprehensively investigated genomic alterations (including copy number alterations and somatic mutations), clonal architecture, and transcriptome dynamics during carcinogenesis. Genomic evolutionary analysis showed that in GAPPS, somatic mutations of APC occur in carcinoma and polyp while mutations of KRAS additionally occur in carcinoma. We also found the co-occurrence of APC and KRAS mutations in carcinoma recurrently both across cases and within subclones of the same case. The co-occurrence of APC/KRAS mutations may contribute to the carcinogenesis of GAPPS. Our study provides detailed information on the genomic and transcriptomic landscape in GAPPS carcinogenesis, conferring valuable insights into its underlying mechanisms.

genetic | carcinoma

Significance

Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) is an autosomal dominant syndrome characterized by polyposis localized in the gastric body and fundus with a strong tendency for adenocarcinoma. In this study, we demonstrated that somatic mutations of APC occur in carcinoma and polyp, while mutations of KRAS additionally occur in carcinoma. Furthermore, we revealed that APC and KRAS mutations recurrently co-occur in carcinoma both between cases and within subclones of the same case. KRAS mutations could serve as a biomarker for carcinogenesis in GAPPS, potentially contributing to the early diagnosis of carcinoma and determining the appropriate timing for resection.

Gastric cancer (GC) is the third leading cause of cancer-related death and the fifth most common human malignancy worldwide (1). While environmental factors such as *Helicobacter pylori*, tobacco, and a high-salt diet are major contributors to GC development worldwide, up to 5 to 10% of GC cases are estimated to be due to an underlying hereditary susceptibility caused by germline pathogenic variants (2).

Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) is an autosomal dominant syndrome characterized by polyposis localized in the gastric body and fundus with a strong tendency for adenocarcinoma (3). Lifetime risk of GC in GAPPS patients is 13 to 25% (4). First described in 2012, GAPPS is diagnosed via the following clinical and pathological criteria: a) gastric polyps in the body and fundus without colorectal or duodenal polyposis; b) >100 polyps in the proximal stomach in an index case or >30 polyps in a first-degree relative of a patient already diagnosed with GAPPS; c) mostly fundic gland polyps (FGPs), some with dysplasia (or a family member with either dysplastic FGPs or gastric adenocarcinoma); and d) autosomal dominant inheritance. To diagnose GAPPS, other causes of FGPs including proton pump inhibitors (PPIs) and other heritable gastric polyposis syndromes must be ruled out (5). In 2016, Li et al. reported that point mutations in promotor 1B of APC are pathogenic variants of GAPPS (6). Point mutations of promoter 1B of APC including c.-195A>C, c.-125delA, c.-191 T>C, and c.-192A>C are located within a Ying Yang 1 (YY1) binding motif and reduce the expression from promoter 1B by interrupting YY1 binding. A study also reported that a second somatic hit in APC occurred in FGPs in patients with GAPPS, via either loss of the wild-type allele or acquisition of protein-truncating variants (6). This study has enabled the diagnosis of GAPPS through genetic testing. Although awareness of the disease has become more widespread and reports on GAPPS have increased globally (7–9), the genetic mutations that accumulate during the progression from normal mucosa to polyp and eventually to carcinoma in GAPPS remain unclear. In the colon, the concept of the adenoma-carcinoma

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sequence is generally accepted, which involves stepwise progression from normal epithelium to dysplastic epithelium and eventually to carcinoma due to the accumulation of selected genetic mutations, for which preventive polypectomy is common practice (10). In contrast, this concept is not defined in the stomach, and polypectomy is not recommended except for some types of polyps (hyperplastic or adenomas) (11).

In this study, to reveal the evolutionary process from normal mucosa to polyp and carcinoma, we implemented multiple sampling from each of these areas, and conducted whole-exome and RNA sequencing. Through comprehensive mutational and transcriptome analyses, we provided insights into the biology of GAPPS.

Results

Patient Characteristics. The clinical and pathological characteristics of the seven GAPPS patients in three families are summarized in Table 1. The patients' ages ranged from 24 to 57 y; three were men and four were women. All patients were nonsmokers and tested negative for *H. pylori* infection by antibody testing at initial diagnosis and had no history of PPI use. For each family's proband, the diagnosis was reached due to the mother's death from GC, epigastric pain, and endoscopy for a health check-up.

Regarding the timing of surgery: For patients P1 and P2, regular endoscopic surveillance was conducted after the initial diagnosis, and carcinoma was detected by biopsy 1 y later, leading to surgical resection. In patients P2 and P4, carcinoma was detected during the endoscopic examination at the time of diagnosis, and surgery was performed accordingly. For patients P6 and P7, although no carcinoma was detected during the diagnostic endoscopy, prophylactic gastrectomy was performed based on the patients' wishes. In summary, of the seven patients, five were diagnosed with gastric carcinoma. Four (P1 to P4) underwent curative total gastrectomy, and one (P5) was not indicated for resection due to multiple liver metastases and received systemic chemotherapy. The pathological classification of carcinoma in all cases was tubular adenocarcinoma. The pedigree of each family is shown in *SI Appendix*, Fig. S3. Resected specimens and endoscopic images of the cases are shown in *SI Appendix*, Fig. S2.

Pathological Evaluation. For the pathological evaluation of samples collected through macroscopic sampling from carcinoma, polyp, and normal mucosa (body/fundus and antrum) at multiple sites in each case, HE staining was performed on all samples. Histological review was performed by experienced pathologists based on the HE staining (Fig. 1*B*). In carcinoma, the main component was well-differentiated tubular adenocarcinoma. In polyp, the main component was diagnosed as FGP with hyperplasia. Hyperproliferative aberrant pits (HPAP), which is considered a characteristic finding of GAPPS, was also observed among the polyp samples, and one of these samples exhibited low-grade dysplasia (details in *SI Appendix*, Note 1). In addition, the pathological findings of family 2 differed from those of the other families. Carcinoma in family 2 was mainly characterized by dysplasia (earlier stage of cancer). Furthermore, strong lymphocytic infiltration was observed in both the carcinoma tissues and the normal tissues of this family (*SI Appendix*, Fig. S3). To investigate the cause of the progression of normal mucosa to FGPs and carcinoma, we performed WES and RNA-seq on all samples.

Genetic Landscape of GAPPS. To investigate the genomic changes in GAPPS, we analyzed the WES data. First, we confirmed that the germline variant in APC promoter 1B (chr5: 112707527 T>C: c.-191T>C), identified in peripheral blood, was present in all samples. The mean coverage of WES was 175 (144 to 236, *SI Appendix*, Table S1). Following GATK best practices, mutation calling was performed using Mutect2. In total, 2,927 somatic mutations were found across the 54 samples for which WES data were available. The mutation burden (MB) per tissue was in the following order: normal antrum < normal body/fundus < polyp < carcinoma (*SI Appendix*, Fig. S4*A*). In addition, when comparing each family, carcinoma of family 3 exhibited a high MB, which was considered to be attributable to the cancer stage. Meanwhile, it was lower in carcinoma of family 2 than in the other families, with some samples showing levels comparable to those in normal mucosa and polyp (DNA_029, DNA_030) (Fig. 2*A*). To examine mutations in cancer driver genes, we focused on COSMIC 736 cancer driver genes (both Tier 1 and Tier 2 in The Cancer Gene Census, https://cancer.sanger.ac.uk/census) (Fig. 2*A*). The somatic mutation of APC was the most common mutation, which was mutated in 78.6% of carcinomas and 53.8% of polyp samples (Fig. 2*B*). Meanwhile, the somatic mutation of APC was not identified in normal mucosa of the body/fundus and antrum. Following APC, the second most frequently mutated gene was KRAS, which was carcinoma specific. Other than APC and KRAS, no genetic variants were shared between each pathological group except for ZRSR2. In sporadic gastric cancer, TP53 mutations have been reported to have the highest frequency (12–14), but no

Table 1. The clinical and pathological characteristics

Patient	Family	Gender	Age	*H. pylori*	Smoking	Timing of detection	UICC (8th)	Procedure	Timing of gastrectomy	Pathology
P1	1	F	39	Negative	None	Family diagnosis	I	TG	After 1 y follow-up	Tub
P2	1	F	37	Negative	None	Family diagnosis	I	TG	After 1 y follow-up	Tub
P3	2	F	24	Negative	None	Epigastric pain	I	TG	After diagnosis	Tub
P4	2	M	57	Negative	None	Family diagnosis	I	TG	After diagnosis	Tub
P5	3	F	41	Negative	None	Health checkup	IV	CT	None	Tub
P6	3	M	43	Negative	None	Family diagnosis	No malignancy	PTG	After diagnosis	No malignancy
P7	3	M	45	Negative	None	Family diagnosis	No malignancy	PTG	After diagnosis	No malignancy

TG: total gastrectomy; CT: chemotherapy; PTG: prophylactic total gastrectomy; Tub: tubular adenocarcinoma.

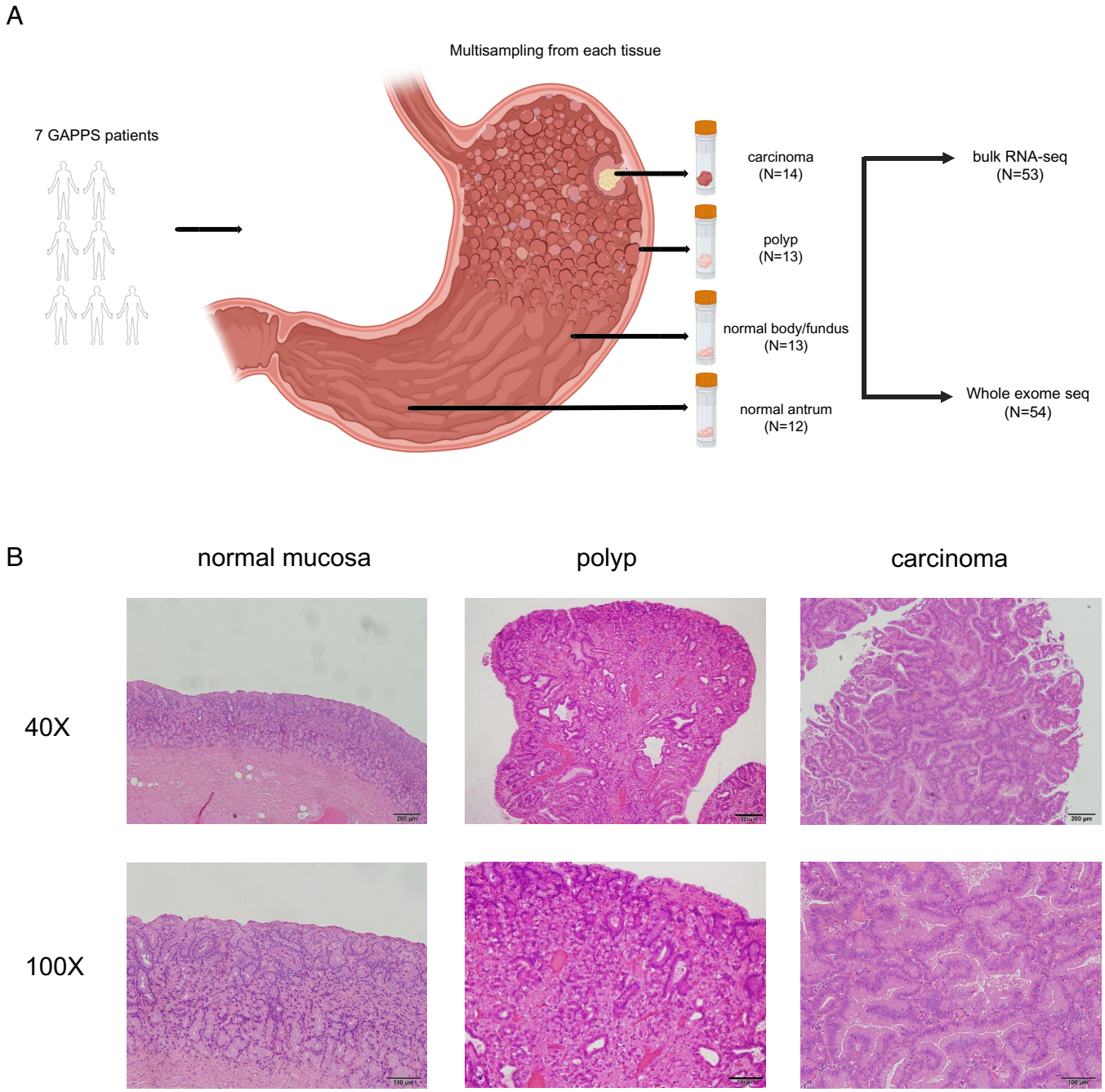


Fig. 1. (*A*) Workflow of this study. Multiple sampling was performed from carcinoma, polyp, normal mucosa of body/fundus, and normal mucosa of the antrum in each case. A total of 54 samples (12 normal mucosas of the antrum, 15 normal mucosas of body/fundus, 13 polyps, and 14 carcinomas) were collected. DNA and RNA were extracted from these samples and submitted for whole exome sequencing and RNA sequencing. For whole exome sequencing, peripheral blood DNA was also submitted as a control. For RNA sequencing, one sample did not pass the quality check. (*B*) Hematoxylin and eosin (H.E.) section of gastric lesions: *Left*: Normal mucosa (body/fundus and antrum), *Middle*: polyp contain fundic gland hyperplasia and dilated foveolar epithelium, *Right*: carcinoma (×40 and ×100 original magnification).

mutation of TP53 was found in the polyp and carcinoma samples of GAPPS. In an advanced case (P5) of carcinoma, mutations in SMAD4, MSH2, PHOX2B, MED12, TGFBR2, and KEAP1 were also observed. The evaluation using PolyPhen-2 (15) to assess how these mutations affect the structure or function of proteins revealed that mutations in MSH2 and PHOX2B were benign, whereas mutations in other genes including SMAD4, TGFBR2, MED12, and KEAP1 were pathogenic (*SI Appendix*, Table S3). Additionally, we investigated whether significantly coaltered genes reported in a recent large-scale study of Japanese gastric cancer (12) were also observed in our GAPPS samples. As a result, we did not observe the co-occurring mutations reported in that study.

Next, we performed copy number analysis from WES using FACETS (16) (*SI Appendix*, Fig. S5). Some recurrent CNAs were observed, but these were most likely to be false-positive results, such as occurring when one attempts to force a call on a copy number change from a sample with no copy number change (details in *SI Appendix*, Note 2). Meanwhile, recurrent CNAs particularly in carcinomas and polyp were not identified. Regarding arm level event or copy-neutral loss of heterozygosity (cnLOH), cnLOH on Chr14 was identified in two carcinoma

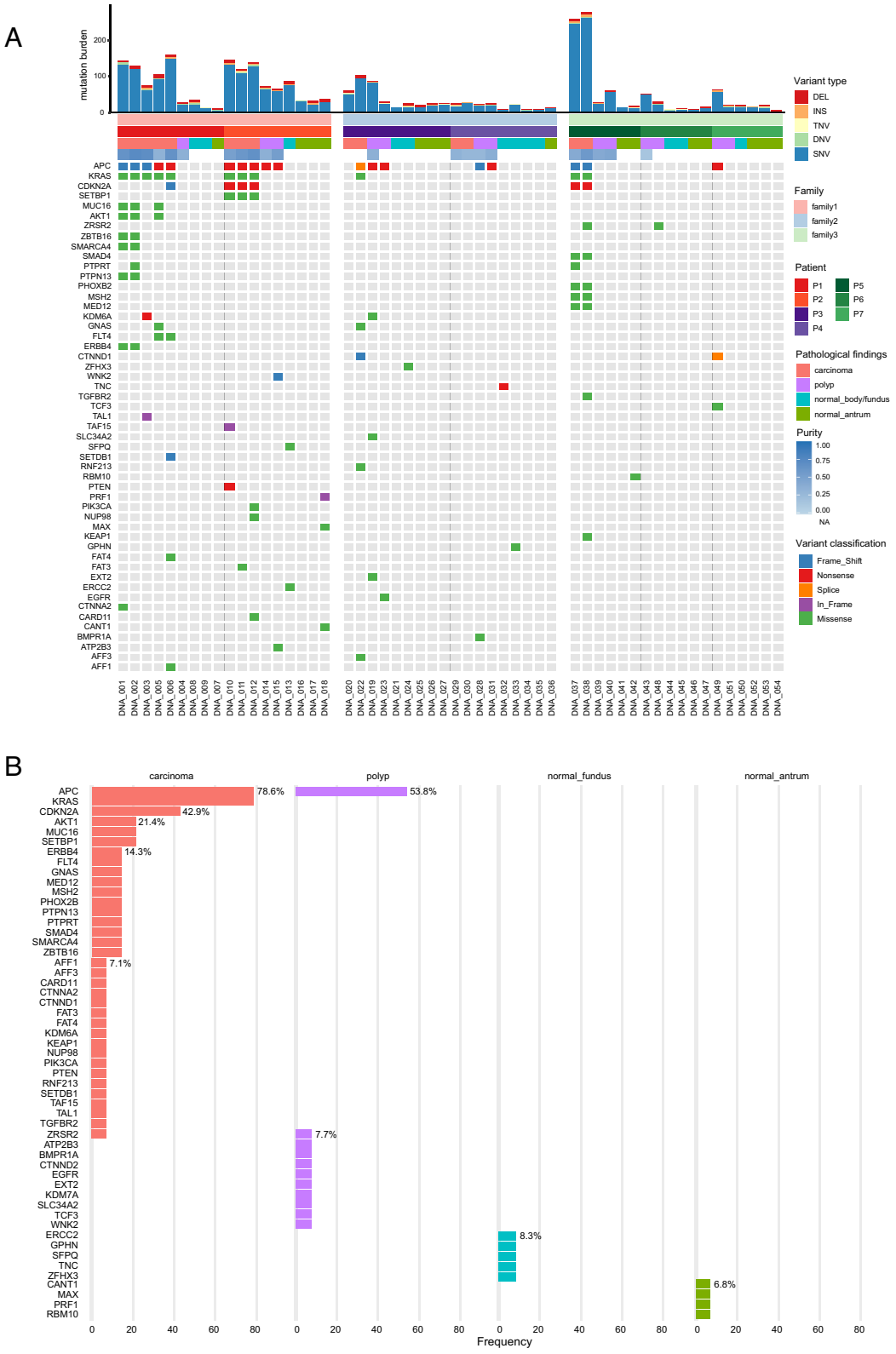


Fig. 2. (*A*) Mutational status of driver genes in the seven patients with GAPPS (including 12 normal mucosas of the antrum, 15 normal mucosas of body/fundus, 13 polyps, and 14 carcinomas). The stacked bar graph in the upper panel represents the MB. The rows in the middle panel, from top to bottom, indicate the family, case, pathological findings, and the purity of the samples as estimated by copy number analysis. The heatmap represents the variant classification of each driver gene with each column corresponding to a sample and each row representing a gene. The color of the squares indicates the mutation classification. (*B*) Mutation frequency of genes in each tissue. Each bar represents the frequency of a specific gene mutation within the respective pathological tissue. Each gene is arranged in order of descending mutation frequency.

samples from P1; however, no tissue-specific events were observed (*SI Appendix*, Table S4). Based on these results, it is suggested that CNAs do not play a role in the carcinogenesis of GAPPS. Purity (tumor content) estimated from CNAs was relatively low in carcinoma of family 2 (Fig. 2*A*). Given the low detection rates of mutations in APC and KRAS, as well as the low MB of these

samples, it was suggested that the purity might be low, leading to insufficient mutation detection.

We used FilterMutectCall to perform screening of mutations called by Mutect2 (17) to analyze only the high-accuracy mutations. However, it is possible that true mutations in samples with low purity were filtered out, so we also checked the unfiltered output of Mutect2 (*SI Appendix*, Fig. S4*B*). Although recurrent mutations were found in some genes (MUC16, MED12, ZFHX3, etc.), this was not a phenomenon specific to samples with low purity, so it is highly likely that this was a false-positive result. However, APC mutations were additionally confirmed only in polyp with low purity, and it was found that APC mutations may occur in 84.6% of polyp. Interestingly, mutations of TP53, which are considered the most common in sporadic gastric cancer, were not detected in mutation without FilterMutectCall. This indicated that compared with sporadic gastric cancer, carcinoma of GAPPS has a distinctive mutational process. We also assessed the MSI status of each tumor sample using the WES data and found that all tumor samples were MSS (*SI Appendix*, Table S5).

APC Was Inactivated by Germline Variant and Somatic Mutation. In previous studies, it was established that a germline APC promoter 1B variant is the causative variant of GAPPS (6). This variant is located in the promoter region and is potentially involved in regulating expression levels. To confirm the reduction of the expression of APC, we compared the expression between the alleles with and without the promoter variant. As we were unable to obtain reads on APC promoter 1B from RNA-seq, we used germline heterozygous variants in APC exons to investigate allele-specific expression bias in each pathological tissue (Fig. 3*A*) (details in *Materials and Methods*). Allelic imbalance, indicating a significant bias toward the expression of one allele, was observed in all tissues, suggesting that the promoter 1B variant causes this decreases in expression of the APC gene. Allelic imbalance was particularly strong in carcinoma and polyp. As reasons for this tendency, the following possibilities were considered (*SI Appendix*, Fig. S6): a) the expression of the allele with the promoter variant is further reduced or b) the expression of the allele without the promoter variant is further increased. To investigate these two possibilities, we examined the relationship between APC expression and the allelic imbalance. Unfortunately, there was no clear difference in expression and the allelic imbalance correlations (*SI Appendix*, Fig. S7), and thus we were unable to verify either of the above possibilities.

Next, we focused on somatic mutation in the APC gene. We examined how somatic mutations affect the function of APC. Fig. 3 *B* and *C* shows the distribution of somatic mutations in APC. All detected mutations were located upstream of codon 1600, especially codons 1450 to 1600, and most of them were nonsense mutations. This is consistent with a study of familial adenomatous polyposis (FAP)-associated FGPs (18, 19), which showed that somatic mutations were most common at codons 1450 to 1556 and that all mutations identified were nonsense mutations. Since the somatic mutation in APC in GAPPS is thought to function in the same way as the somatic mutation in APC in FAP, somatic mutations occurring in this region may function as pathogenic variants for polyp in GAPPS.

Mutations in APC are known to lead to dysfunction in the canonical Wnt cascade, resulting in the stabilization and nuclear translocation of β-catenin in colorectal cancer (20). Therefore, we performed immunohistochemical staining for β-catenin to determine whether this also occurs in GAPPS, but no accumulation of β-catenin in the nucleus was observed (*SI Appendix*, Fig. S8). These results suggest that although the APC mutation is functionally inactivating, it does not appear to contribute to activation of the WNT/β-catenin pathway.

Co-Occurrence of APC and KRAS Mutations. Given that KRAS had the second highest mutation frequency after APC and was mutated specifically in carcinoma, we next focused on this gene. Regardless of the case or family, the AA changes involved one of two well-known hotspot mutations, G12V and G12D, and almost all carcinomas had these KRAS mutations (Fig. 4*A*). Based on the above, we examined the recurrence of APC and KRAS mutation co-occurrence in multiple carcinoma samples. We conducted a phylogenetic analysis on P1, for whom five carcinoma samples were available (*SI Appendix*, Fig. S3). These carcinoma samples were taken macroscopically from different anatomical regions, but it was unclear whether they originated from the same clone or different clones. Therefore, we compared the genetic mutations in each sample. Mutations present in all samples were defined as "shared mutations," those found in some, but not all samples were defined as "branched mutations," and mutations unique to each sample were defined as "private mutations." Based on these mutation patterns, we estimated the clonal origin of each sample. Only one mutation was detected as a shared mutation, but it was also detected in polyp and normal mucosa samples of the same case and was considered to have been mistakenly called as a germline variant (*SI Appendix*, Table S6). Based on these findings, it is suggested that these carcinoma samples are not a single clone, but three subclones, each of which recurrently acquired the co-occurrence of APC and KRAS mutations (Fig. 4*D*). The same analysis was conducted on other cases, but similar results were not obtained because the tumor samples were either a single clone or KRAS mutations were not detected (*SI Appendix*, Fig. S9). In carcinoma samples without KRAS mutation (DNA_020, DNA_029, DNA_030), no APC mutations were found either, and the purity might have been extremely low, suggesting that the co-occurrence of APC and KRAS mutations is almost essential for carcinoma associated with GAPPS.

Comparison of Expression between Tissues and with Sporadic Gastric Cancer. We found that APC mutations play an important role in the change from normal mucosa to polyp and that KRAS mutations do so in the carcinogenesis of polyp. To clarify which pathways and genes are upregulated during the progression from normal mucosa to polyp and carcinoma, we performed pathway analysis using RNA-seq data. First, to investigate the relationship between pathological findings and RNA expressions, we performed principal component analysis (PCA) on all samples (Fig. 5*A*). Similar to the results of the pathological evaluation, PCA also revealed that family 2 is distinct from the other families; while carcinoma and other samples were classified into different clusters in families 1 and 3, all samples from family 2 were classified together in the same cluster. Moreover, when examining each pathological tissue separately, except for the normal antrum, samples from family 2 were classified into a different cluster compared with the samples from the other families. Since pathological evaluations revealed lymphocyte infiltration in family 2 and samples from family 2 were clustered apart from those from the other families in PCA, we estimated the proportions of immune cells in each pathological tissue using the ConsensusTME (15) of CIBERSORT (21) (*SI Appendix*, Fig. S10). Consistent with the pathological findings, high proportions of immune cells, mainly T cells, were found in the carcinoma, polyp, and normal body/fundus of family 2.

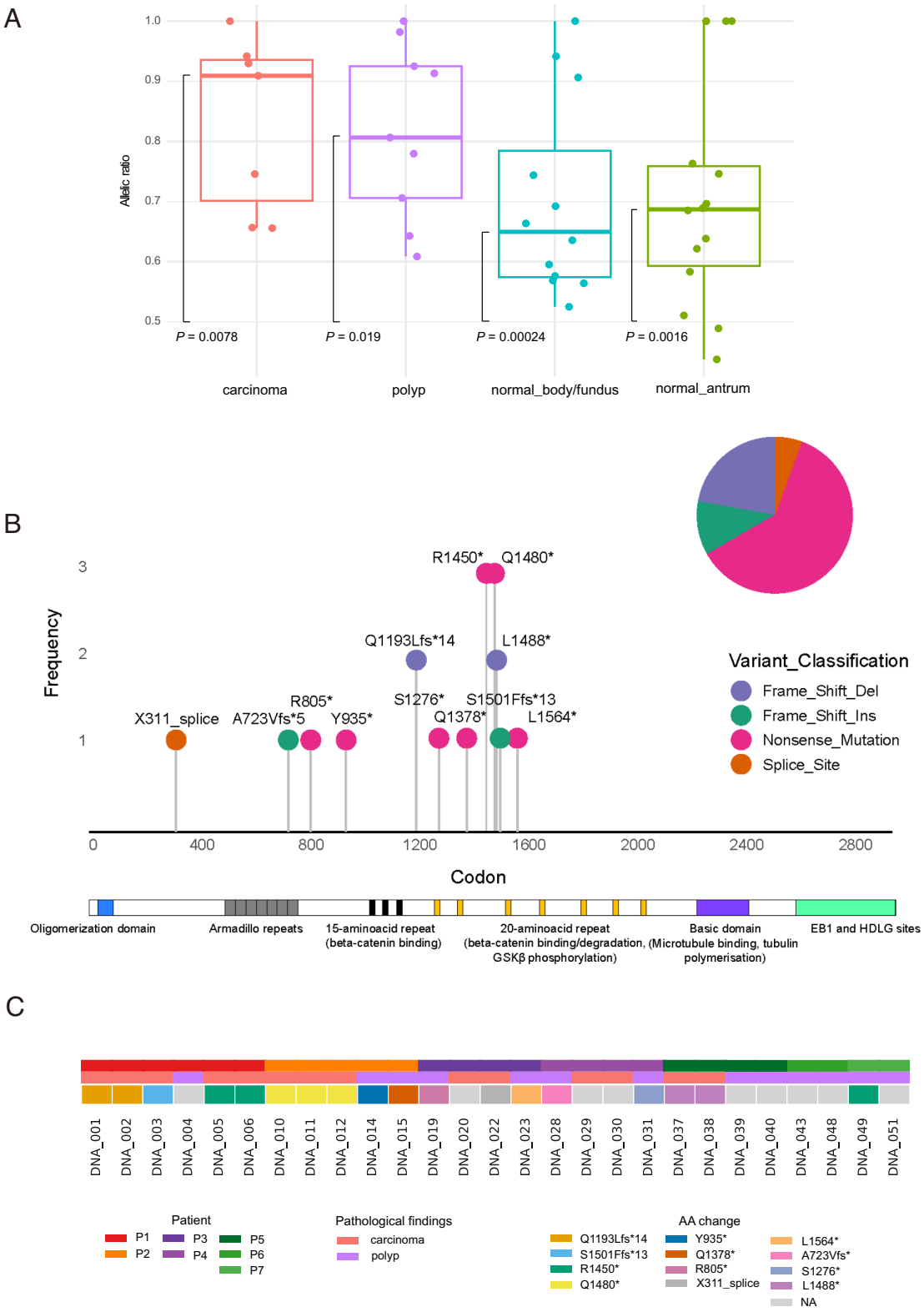
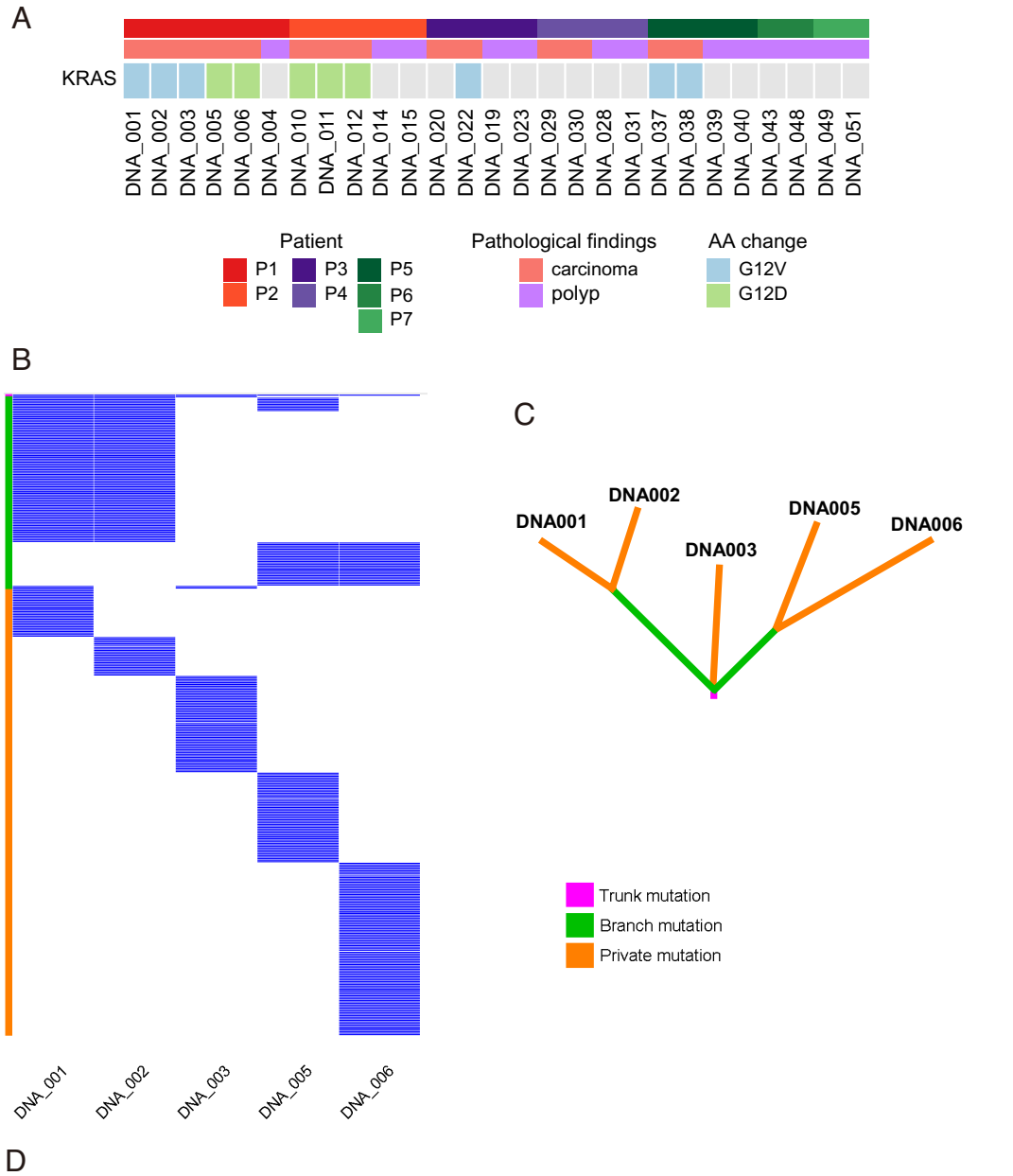


Fig. 3. (*A*) Allelic ratio distribution of each tissue. Each boxplot represents the interquartile range (IQR) with the whiskers extending to 1.5 times the IQR. The central line in each box indicates the median value. Individual data points are shown as dots. (*B*) Distribution of somatic mutations across the coding sequence of APC, with corresponding frequencies and variant classifications. The lollipop plot visualizes the specific codons affected by mutations, with each lollipop representing a mutation at a specific site. The height of the lollipop corresponds to the frequency of the mutation observed in the dataset. The color of each lollipop indicates the type of mutation. The pie chart on the right summarizes the overall distribution of these variant classifications. The gene structure at the bottom highlights functional domains. (*C*) Heatmap illustrating the distribution of APC mutations, highlighting the specific amino acid (AA) changes.

Based on these results, to ensure the purity of the samples, further analyses were performed by extracting samples (framed samples) with matching pathological findings and statistical clustering by K-means (Fig. 5*A*).

Fig. 5*B* shows the pathways that were upregulated in each pathological tissue. Compared with the normal antrum, pathways related to the TCA cycle, metabolism, and mitochondria were enhanced in the normal body/fundus, which is consistent with



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Sample ID	Exome seq	APC					KRAS				
		Position	Ref	Alt	classification	AA Change	Position	Ref	Alt	classification	AA Change
6	DNA001	112839172	AG	-	Frame_Shift_Del	p.Q1193Lfs*14	25245350	C	A	Missense_Mutation	p.G12V
7	DNA002										
11	DNA003	112840091	-	T	Frame_Shift_Ins	p.S1501Ffs*13					
8	DNA005	112839942	C	T	Nonsense_Mutation	p.R1450*			T	Missense_Mutation	p.G12D
9	DNA006										

Fig. 4. (*A*) Heatmap representing the distribution of KRAS mutations, with patient identification, pathological findings, and AA changes. The top row indicates the patient. The middle row indicates the pathological findings associated with each sample. The bottom row indicates the type of KRAS mutation observed in each sample, with specific AA changes represented by different colors. (*B*) Heatmap displaying the presence of mutations across the five carcinoma samples of P001. Each row represents a mutation, and each column corresponds to a sample. The colors indicate the type of mutation: trunk mutations (shared across all samples), branch mutations (shared by a subset of samples), and private mutations (unique to a single sample). (*C*) Phylogenetic tree constructed from the mutation data, showing the evolutionary relationships among the samples. The branches of the tree are colored according to the mutation type. The tree indicates how the different samples have diverged. (*D*) Somatic mutations detected in the APC and KRAS genes across five samples. The table lists each mutation by its genomic position, reference allele (Ref), alternative allele (Alt), mutation classification, and resulting AA change.

the results of previous studies using healthy gastric mucosa (22). This likely reflects that the energy required for acid secretion, which is a primary function of the body/fundus, is produced by the TCA cycle within mitochondria (23). In fact, it is estimated that 30 to 40% of the cytoplasm of parietal cells in the body/fundus is occupied by mitochondria (24). In addition to these pathways, the enrichment of pathways related to the cell cycle including E2F targets and the G2-M checkpoint was observed in polyp. This may reflect the proliferation associated with polyposis. Compared with polyp, in carcinoma, in addition to cancer-related pathways such as epithelial–mesenchymal transition and hemostasis, KRAS_signaling_up was enhanced, consistent with the

Fig. 5. (*A*) PCA plot illustrating the clustering of RNA samples with annotations for pathological findings and family. Each point represents a sample, color-coded according to the pathological findings. The shape of the points indicates the family to which the sample belongs. Black outlines highlight samples where there is concordance between statistical clustering and pathological findings. (*B*) Bar plots illustrating the upregulated pathways in different tissue comparisons (*Top*: normal_body/fundus vs. normal_antrum, *Middle*: polyp vs. normal_body/fundus, *Bottom*: carcinoma vs. polyp), based on pathway enrichment analysis. Each bar represents a significantly enriched pathway, with the x-axis showing the −log10(*P*-value) and the pathways ordered by significance. (*C*) Comparison of hallmark pathway enrichment between TCGA STAD and GAPPS using MSigDB_HALLMARK gene sets. The plot shows normalized enrichment scores (NES) for selected pathways. The size of the dots represents the gene set size, while the color indicates the false discovery rate (FDR) q-value, with red denoting more significant enrichment. (*D*) Enrichment plot for the HALLMARK_KRAS_SIGNALING_UP gene set. The green line shows the enrichment score (ES), with a peak indicating strong enrichment in carcinoma samples. Black vertical lines mark the positions of KRAS pathway genes.

results of the mutational analysis. Gene ontology (GO) analysis was also performed and the results were similar to those of the pathway analysis (*SI Appendix*, Fig. S11).

Next, to clarify the differences between GAPPS and sporadic gastric cancer, we performed gene set enrichment analysis (GSEA) on carcinoma and normal mucosa in GAPPS and sporadic intestinal-type gastric cancer from TCGA-STAD and compared the pathways that are upregulated in each type of cancer. Compared with the levels in their respective normal mucosa, E2F targets, G2-M checkpoint, and mitotic spindle were significantly enhanced in both GAPPS and sporadic gastric cancer. However, KRAS_signaling_up was significantly enhanced only in GAPPS (Fig. 5 *C* and *D*). These results suggest that even in the same intestinal-type gastric carcinoma, the upregulation of KRAS-related pathways is specific to carcinoma of GAPPS.

Discussion

In this study, we revealed the landscape of driver events in the carcinogenesis of GAPPS. We revealed that in GAPPS, somatic mutations of APC occur in both carcinoma and polyp, while mutations in KRAS additionally occur in carcinoma. We also found that the co-occurrence of APC and KRAS mutations recurrently appears in carcinoma, both across different cases and within subclones of the same case.

To date, only one report of mutational analysis involving multiple cases of GAPPS has been published. In that study, WES and whole-genome sequencing failed to identify any novel or rare genetic mutations associated with the carcinogenesis of GAPPS (6). In contrast, the co-occurrence of APC/KRAS mutations was recurrently observed in nearly all carcinoma cases of GAPPS in our study, excluding in low-purity samples. In sporadic gastric cancer, the mutation frequencies of APC and KRAS are 7.7% and 9.4%, respectively, and they rarely co-occur (12). This suggests that the co-occurrence of APC/KRAS mutations contributes to the carcinogenesis of GAPPS and highlights a significant difference in the mutational status between GAPPS and sporadic gastric cancer, despite both being forms of gastric cancer. Regarding KRAS mutations, pathway comparison with TCGA-STAD also showed that KRAS-related pathways were specifically upregulated in GAPPS (Fig. 5 *C* and *D*). Only one report sequenced a carcinoma sample from a single case, which also identified a KRAS mutation (25). In a patient with FAP, KRAS mutation was also detected in both carcinoma and adenoma arising from FGPs (26). These findings support our current findings.

Various reports have highlighted the synergistic effects of the co-occurrence of APC/KRAS mutations in cancer progression. For example, Janssen et al. reported that the increase in Wnt/β-catenin signaling induced by KRAS enhances tumor plasticity and self-renewal capacity, significantly increasing tumor multiplicity and malignant behavior (27). In addition, Phelps et al. reported that the nuclear accumulation of β-catenin and the associated proliferation requires the activity of KRAS (28). However, these reports primarily focus on colorectal cancer, and few studies have explored the synergistic effects of APC/KRAS in gastric cancer. Therefore, further research is needed to investigate the interaction between APC/KRAS mutations in the carcinoma of GAPPS. On the other hand, it remains unclear whether KRAS mutations occur independently of APC mutations or are dependent on them. In our study, most polyps harbored somatic APC mutations, while most carcinomas additionally exhibited KRAS mutations, suggesting that these mutations might occur sequentially. Previous studies, particularly in colorectal cancer, have suggested that APC mutations activate the Wnt pathway, creating an environment that facilitates the acquisition of KRAS mutations and promotes tumor progression (29). However, it is necessary to consider the possibility that each mutation arises randomly, merely appearing sequentially as a result. There is an opinion which insists the stochastic co-occurrence of APC and KRAS mutations during tumor progression (30). In the future, analyses such as lineage tracing will be essential to determine whether KRAS mutations can independently drive tumorigenesis or whether they depend on prior APC mutations.

It is well established that the loss of APC function leads to β-catenin accumulation in the nucleus, contributing to colorectal cancer development (31). However, in the present study, although somatic mutations in APC were observed in polyp/carcinoma, nuclear translocation of β-catenin was not detected. Previous reports confirmed a second hit due to the loss of the wild-type allele or somatic mutations in APC in some FGPs of GAPPS (6). However, reports on the nuclear accumulation of β-catenin are inconsistent, with findings both supporting and refuting such accumulation (25, 32). It is known that APC has multiple β-catenin binding sites including 15- and 20-amino-acid repeats (15R and 20R) (33) and SAMP repeat (34). Additionally, it has been reported that truncated APC resulting from mutation can still regulate the transcriptional activity of β-catenin (35). These findings suggest that the downstream effect of β-catenin may vary depending on the functionality of β-catenin binding sites on truncated APC. On the other hand, in a study investigating the mechanism of FGP formation in FAP patients, although somatic APC mutations were not detected in the FAP patient biobank, elevated Wnt signaling was observed in FGP biopsy samples and organoids derived from the polyps (36). These findings suggest that FGP formation in the gastric fundus may be promoted even in the absence of a so-called "second hit." Furthermore, a recent study identified Wnt-independent growth-promoting genes such as Bclaf3 and Prka, suggesting that FGPs may also proliferate through Wnt-independent pathways (37). Taken together, these observations highlight the complexity of β-catenin regulation in FGP development and suggest that both APC-dependent and Wnt-independent mechanisms may contribute to the pathogenesis of FGPs.

The absence of TP53 mutation, which is the most commonly mutated gene in sporadic gastric cancer, in GAPPS carcinoma can also distinguish between these two types of gastric cancers. However, even among sporadic gastric cancers, there are subtypes such as Gastric Adenocarcinoma of the Fundic Gland Type (GA-FG) that do not exhibit TP53 mutations (38, 39). Thus, the existence of subtypes lacking TP53 mutations, including GAPPS, suggests that TP53 mutation may not be an essential event in gastric carcinogenesis, and highlights the molecular heterogeneity of carcinogenesis pathways in gastric cancer. Additionally, pathogenic mutations in SMAD4 and TGFBR2 were observed in a sample of advanced carcinoma, suggesting the accumulation of mutations that aligns with the concept of adenoma-carcinoma sequence. Jung et al. conducted a mutational analysis with paired sampling of adenomas and carcinomas in sporadic gastric cancer and reported that adenomas and carcinomas evolve in parallel from an early stage of adenoma, rather than through stepwise evolution from adenoma to carcinoma (40). These results suggest that the process of mutation accumulation in GAPPS differs from that in sporadic gastric cancer. Given that this study was conducted with a small number of cases, further investigation with a larger sample size is needed to address this issue.

Previous reports on GAPPS have indicated various heterogeneities among different families as well as within families carrying

the same pathogenic APC variant. In a study involving 24 patients from 8 families, some individuals developed gastric polyposis in their 20s, while others had not developed polyposis even at the age of 65. Additionally, five individuals developed gastric adenocarcinoma, with an age of onset ranging from 29 to 64 y (7). In our study, specific lymphocytic infiltration was observed in the normal mucosa of the body and fundus, polyp, and carcinoma of family 2, which may reflect heterogeneity between families. In addition to sample purity, we investigated potential causes of interfamily heterogeneity by examining clinical backgrounds and MSI status in this study; however, we were unable to identify any clear differences between this family and the others. Further investigations with an increased number of cases are needed.

This study has several limitations. First, this study was conducted at a single institution and involved a relatively limited number of samples. Considering regional and ethnic differences, validation with larger samples from multiple institutions is necessary. Second, the purity of the samples used in this study was relatively low. This study used bulk samples and did not involve sample processing techniques such as laser microdissection to improve purity, to secure enough RNA/DNA for sequencing. Although pathological confirmation was performed before submitting each sequence, the proportions of each tissue component in the samples were not verified. In addition, data on the size of each sample were not available, so we could not discuss the relationship between polyp size and the number of mutations. Analysis using higher-purity samples is necessary to resolve the issues that could not be clarified in this study. Third, we could not determine whether the lymphocyte infiltration, which contributed to the low sample purity, was family specific or caused by other factors. We examined potential causes such as *H. pylori* infection and MSI, but no results were obtained that could explain the differences among families. While tumor heterogeneity could be a contributing factor to the observed interfamily differences, we aim to clarify this through further investigations with an increased number of cases in future studies.

In summary, we presented the comprehensive genomic landscape of GAPPS, investigating the genomic dynamics during carcinogenesis. The results of this study suggest that KRAS mutations may serve as a biomarker for carcinogenesis in GAPPS. In the future, if detection through circulating tumor DNA (ctDNA) becomes feasible, it could lead to earlier cancer detection and enable more appropriate timing for surgical intervention. The findings obtained in this study should deepen our understanding of the pathogenesis of GAPPS.

Materials and Methods

Patients. Seven cases of GAPPS in three families were first diagnosed at Kumamoto University Hospital between October 2015 and November 2020 (Table 1). All of these patients had a point mutation in promotor 1B of APC (NM_001127511: c.-191T>C) confirmed by genetic testing using peripheral blood genomic DNA after genetic counseling. Clinical information and family history were obtained from the medical records. All patients had been followed up periodically. The study was approved by the Institutional Review Board of Kumamoto University Hospital (Genome No. 386). Written informed consent was obtained from all patients.

Sample Collection. In this study, samples were collected from surgically or endoscopically resected tissue specimens. Multiple sampling was performed from carcinoma, polyp, and normal mucosa (body/fundus and antrum) in each patient (Fig. 1*A*). A total of 54 samples were collected: 12 normal sites of the antrum mucosa, 15 normal sites of body/fundus mucosa, 13 polyps, and 14 carcinomas (*SI Appendix*, Table S1). To obtain germline DNA, we collected peripheral blood from all patients before treatment. These samples were briefly stored in liquid nitrogen and then transferred to a freezer set at −80 °C for storage.

Whole-Exome Sequencing (WES) and Somatic Mutation Calling. We performed WES on all samples using DNA from peripheral blood as a control. The DNA from frozen samples was extracted using the QIAamp DNA Mini Kit (Qiagen, Stanford, CA), in accordance with the manufacturer's protocol. Exome regions were captured using SureSelect All Exome V6 (Agilent, Santa Clara, CA), in accordance with the manufacturer's protocol. Then, the resultant products were pair-end sequenced using a NovaSeq 6000 system (Illumina, San Diego, CA), with a read length of 150 bp.

RNA Sequencing (RNA-seq). The RNA from frozen samples was isolated using the RNeasy Mini Kit (Qiagen). Paired-end libraries were synthesized using the NEBNext Ultra II RNA Library Prep Kit (NEB, UK), following the manufacturer's protocol. Then, products were sequenced on the NovaSeq 6000 system (Illumina). Using the software tool RSEM (41), the sequencing reads were aligned to the hg38 reference genome and presented as transcripts per million (TPM).

Hematoxylin–Eosin Staining and Immunohistochemistry. The collected tissues were fixed in neutral buffered formalin and embedded in paraffin. Hematoxylin–eosin staining was performed on 5-μm thick slides according to standard procedures. Immunohistochemical staining was performed using the standard streptavidin–biotin–peroxidase technique and the Dako EnVision FLEX system (Dako, Carpinteria, CA). The antibodies for immunohistochemistry included β-catenin (1:100 dilution, Abcam, USA). Briefly, 5-μm-thick tissue sections cut from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized and rehydrated. After antigen retrieval by autoclaving (121 °C, 15 min in Tris/EDTA buffer pH 9.0), we blocked proteins by incubating sections in peroxidase blocking reagent. Then, we incubated the sections with polymer reagent at room temperature for 20 min. We visualized the staining with 3,3′-diaminobenzidine (DAB) and counterstained the sections.

Somatic Mutation Calling. The single-nucleotide variant (SNV)/indel call analysis pipeline was based on the Genome Analysis Tool Kit (GATK, v4.0; https://software.broadinstitute.org/gatk/) best practices (17). Briefly, sequenced reads were mapped to the human reference genome hg38 using BWA-MEM (42). The mapped reads were sorted and indexed by SAMtools (43), and duplicate reads were marked by Picard MarkDuplicates. Somatic mutations were detected using GATK Mutect2 and FilterMutectCalls (17). The variant call format (VCF) files of somatic mutation were annotated using ANNOVAR (44).

Copy Number Analysis. FACETS (ver 0.6.2), an allele-specific copy number analysis tool, was used to estimate integer copy number calls corrected for tumor purity, ploidy, and clonal heterogeneity (16). Arm- and focal-level copy number alterations (CNAs) with FDR $q < 0.25$ were considered significantly aberrant regions. The presence of CNAs was analyzed not only in carcinoma and FGP but also in normal mucosa (*SI Appendix*, Fig. S1). No CNAs common to normal mucosa were detected (details in *SI Appendix*, Note), and since it is difficult to estimate purity when there is no change in copy number, the purity of normal samples (body/fundus and antrum) was set to NA.

Microsatellite Instability Analysis. Microsatellite instability (MSI) status was evaluated using MSIsensor-pro, a computational tool designed to detect MSI from next-generation sequencing data (45). WES data from paired carcinoma and normal antrum mucosa were used as input. The MSI score was calculated for each carcinoma sample, and samples with a score ≥10 were classified as MSI-high (MSI-H), while those with a score <10 were considered microsatellite stable (MSS), based on previously established criteria (46).

Allelic Imbalance Analysis of APC Gene Expression. To investigate the relationship between APC mutations (germline variant of promoter 1B and somatic mutation) and the expression of each allele, APC allelic imbalance analysis was performed on all RNA samples. From the exome sequence data, it was confirmed that seven cases had the common seven heterozygous SNPs on the APC gene (rs2229992, rs351771, rs41115, rs43437, rs866006, rs459552, and rs465899) (*SI Appendix*, Table S2). There is a very high probability that all seven of these SNP site variants are linked to the APC promoter germline variant c.-191T>C.

By using RNA-seq, we counted the number of reads supported each variant to analyze the allelic expression bias.

Pathway/GO Analysis. Differential expression analysis for RNA was performed using R package edgeR (version 2.10.0) (47). Differentially expressed gene with |log2(FC)| value > 1 and q value < 0.05, considered as significantly modulated, were retained for further analysis. Pathway/GO analysis on the differentially expressed genes was performed using clusterProfiler (version 4.2.0) in R (21).

Data Collection from the Public Database. The RNA expression data and corresponding clinicopathological information of sporadic gastric cancer (TCGA-STAD) were obtained from the UCSC Xena (https://xena.ucsc.edu/). For a more accurate comparison, we extracted the data of intestinal type STAD without *H. pylori* infection (N = 79) and normal mucosa (N = 5) for further analyses.

GSEA. To understand the biological processes involved in different pathological groups, we performed GSEA (https://software.broadinstitute.org/gsea). Hallmarks in GSEA were used to identify predefined gene sets; 5,000 permutations were performed according to the gene set to determine *P*-values. A pathway with a *P*-value < 0.01 and a FDR < 0.25 was considered to be significant, as described in *Results*.

CIBERSORT. CIBERSORT (https://cibersort.stanford.edu/), a deconvolution algorithm based on gene expression, can calculate the composition of immune cells from the gene expression profile of complex tissues (15). We used CIBERSORT software to calculate the infiltration level of 22 immune cells based on the expression data. Subsequently, the ESTIMATE algorithm ("estimate" package in R) was used to calculate the immune score of each patient, and the difference in the immune score between the cluster subgroups was evaluated (21).

Statistics. All statistical analyses were performed in the R statistical environment (version 4.3.1). All statistical tests, unless indicated otherwise, were two-sided tests with default *P* value set to 0.05 as the cutoff to indicate statistical significance.

Data, Materials, and Software Availability. The datasets used in this study have been submitted to the DNA Data Bank of Japan (DDBJ) (https://www.ddbj.nig.ac.jp/index-e.html) under JGAS000843 (48) and will be made publicly available after the publication of the article.

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