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2) Title: Indolent feature of *Helicobacter pylori*-uninfected intramucosal signet ring cell carcinomas with *CDH1* mutations

3) Authors:

Mitsuhiro Nikaido^{1,13}, Nobuyuki Kakiuchi^{1,2,3,13}, Shin'ichi Miyamoto^{1,4}, Tomonori Hirano^{1,2,3}, Yasuhide Takeuchi^{2,5}, Taro Funakoshi⁶, Akira Yokoyama⁶, Tatsuki Ogasawara^{2,3}, Yoshihiro Yamamoto⁶, Atsushi Yamada⁶, Takeshi Setoyama^{1,7}, Takahiro Shimizu¹, Yukari Kato⁸, Suguru Uose⁸, Takaki Sakurai^{5,9}, Sachiko Minamiguchi⁵, Kazutaka Obama¹⁰, Yoshiharu Sakai^{10,11}, Manabu Muto⁶, Tsutomu Chiba^{1,8}, Seishi Ogawa^{2,3,12}, and Hiroshi Seno¹

4) Affiliations:

1. Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan
2. Department of Pathology and Tumor Biology, Kyoto University Graduate School of Medicine, Kyoto, Japan
3. Institute for the Advanced Study of Human Biology (WPI-ASHBi), Kyoto, Japan

4. Department of Gastroenterology, National Hospital Organization Kyoto Medical Center, Kyoto, Japan
5. Department of Diagnostic Pathology, Kyoto University Graduate School of Medicine, Kyoto, Japan
6. Department of Therapeutic Oncology, Kyoto University Graduate School of Medicine, Kyoto, Japan
7. Department of Gastroenterology, Osaka Red Cross Hospital, Osaka, Japan
8. Department of Gastroenterology and Hepatology, Kansai Electric Power Hospital, Osaka, Japan
9. Department of Pathology, Kansai Electric Power Hospital, Osaka, Japan
10. Department of Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan
11. Department of Surgery, Osaka Red Cross Hospital, Osaka, Japan
12. Department of Medicine, Center for Hematology and Regenerative Medicine, Karolinska Institute, Stockholm, Sweden
13. These authors contributed equally: Mitsuhiro Nikaido and Nobuyuki Kakiuchi.

5) Corresponding author information:

Shin'ichi Miyamoto

Department of Gastroenterology, National Hospital Organization Kyoto Medical Center,
1-1 Fukakusa-Mukaihata-Cho, Fushimi, Kyoto, 612-8555, Japan
(Phone) 81-(75)-641-9161, (Fax) 81-(75)-643-4325
Email: shmiyamo@kuhp.kyoto-u.ac.jp

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approval of the submitted versions.

Abstract

Background: In *Helicobacter pylori* (*Hp*)-uninfected individuals, diffuse-type gastric cancer (DGC) was reported as the most common type of cancer. However, the carcinogenic mechanism of *Hp*-uninfected sporadic DGC is largely unknown.

Methods: We performed whole-exome sequencing of *Hp*-uninfected DGCs and *Hp*-uninfected normal gastric mucosa. For advanced DGCs, external datasets were also analyzed.

Results: Eighteen patients (aged 29–78 years) with DGCs and nine normal subjects (28–77 years) were examined. The mutation burden in intramucosal DGCs (10–66 mutations per exome) from individuals aged 29–73 years was not very different from that in the normal gastric glands, which showed a constant mutation accumulation rate (0.33 mutations/exome/year). Unbiased dN/dS analysis showed that *CDH1* somatic mutation was a driver mutation for intramucosal DGC. *CDH1* mutation was more frequent in intramucosal DGCs (67%) than in advanced DGCs (27%). In contrast, *TP53* mutation was more frequent in advanced DGCs (52%) than in intramucosal DGCs (0%). This discrepancy in mutations suggests that *CDH1*-mutated intramucosal DGCs make a relatively small contribution to advanced DGC formation. Among the 16 intramucosal DGCs (median size, 6.5 mm), 15 DGCs were pure signet ring cell carcinoma (SRCC) with reduced E-cadherin expression and a low proliferative

capacity (median Ki-67 index, 2.4%). Five SRCCs reviewed endoscopically over 2–5 years showed no progression.

Conclusions: Impaired E-cadherin function due to *CDH1* mutation was considered as an early carcinogenic event of *Hp*-uninfected intramucosal SRCC. Genetic and clinical analyses suggest that *Hp*-uninfected intramucosal SRCCs may be less likely to develop into advanced DGCs.

Mini abstract: The *CDH1* somatic mutation was considered as an early carcinogenic event in *Hp*-uninfected intramucosal SRCC. *Hp*-uninfected SRCCs may progress slowly and be less likely to develop into advanced DGCs.

Keywords: stomach neoplasm, signet ring cell carcinoma, *Helicobacter pylori*, *CDH1*, whole-exome sequencing

Introduction

Epidemiological studies have reported that *Helicobacter pylori* (*Hp*) infection is the greatest risk factor for gastric cancer [1, 2]. Chronic gastritis caused by *Hp* infection was shown to promote oncogenic mutations [3, 4]. Although most gastric cancers arise in *Hp*-infected stomachs, gastric cancers in *Hp*-uninfected stomachs also occur, with their prevalence reported to account for 0.42–5.4% of all gastric cancers [5]. The prevalence of *Hp* infection is lower in developed countries than in developing countries [6]. In Japan, the prevalence of *Hp* infection and incidence of gastric cancer was previously high. However, in recent decades, its prevalence and mortality rate by gastric cancer have decreased [7-9]. The incidence of *Hp*-uninfected gastric cancers is expected to increase as the *Hp* infection rate declines. However, the molecular basis of gastric cancer in the *Hp*-uninfected stomach is largely unknown. Recent studies showed that somatic mutations accumulate in normal tissues with age [10-14]; however, mutation accumulation in the *Hp*-uninfected normal gastric mucosa has not been reported.

Histologically, gastric cancer is classified into intestinal-type gastric cancer and diffuse-type gastric cancer (DGC) [15]. DGCs include poorly differentiated adenocarcinomas (PDAs) and signet ring cell carcinomas (SRCCs). In *Hp*-uninfected patients, gastric cancers are most often found as intramucosal DGCs, which are

typically 'pure' SRCCs that contain only SRCC cells and no PDA components [5, 16]. *Hp*-uninfected intramucosal DGC is smaller in size than *Hp*-infected intramucosal DGC [16, 17]. Additionally, T1 stage (UICC classification) DGCs were shown to be significantly less invasive in patients without *Hp* infection than in those with *Hp* infection [17].

Hereditary diffuse gastric cancer (HDGC) is mainly caused by a *CDH1* germline mutation [18, 19]. E-cadherin, encoded by *CDH1*, is a cell adhesion molecule that regulates signaling pathways associated with cell proliferation, survival, invasion, and migration. Based on studies of HDGC, DGCs are thought to arise from SRCCs because of reduced cell adhesion associated with the loss of E-cadherin function [19]. Although somatic *CDH1* mutations have been found in 29–56% of sporadic DGCs in previous genetic analyses, the mechanisms of sporadic DGC development remain unclear [20–22]. *RHOA* mutations and *CLDN18-ARHGAP* fusion have also been reported to cause DGC [21–24]. These results were mainly obtained in studies of advanced gastric cancer, whereas few studies have examined intramucosal cancer. Furthermore, previous studies either mainly consisted of *Hp*-infected patients or did not assess the *Hp* infection status. In the present study, we examined the clinicopathological and genetic features of *Hp*-uninfected DGCs. We performed whole-exome sequencing (WES) to investigate the genetic alterations in early and advanced

stage DGCs. These genetic alterations were then compared with those in the *Hp*-uninfected normal gastric mucosa.

Materials and methods

Patient and sample collection

Tumor samples, paired blood samples, and clinical data were collected from patients diagnosed with *Hp*-uninfected DGC and enrolled in the Kyoto University Hospital and Kansai Electric Power Hospital between January 2016 and March 2020. As normal controls, we collected biopsied gastric tissues and peripheral blood from nine subjects without *Hp*-infection or gastric cancer who underwent diagnostic endoscopy for upper gastrointestinal symptoms. All patients and normal subjects provided written informed consent to participate in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of Kyoto University Hospital and institutional board of Kansai Electric Power Hospital.

Certified pathologists (S.M. and T.S.) histologically examined the surgically- or endoscopically-resected or biopsied specimens according to the third English edition of Japanese classification of gastric carcinoma and Lauren's classification [15, 25].

***Hp* infection status evaluation**

Diagnostic criteria for *Hp*-uninfected gastric cancer have not been established. These criteria must be strict to rule out naturally *Hp*-eradicated cases [5]. Patients were defined as *Hp*-uninfected when they met all of the following criteria: (i) no history of *Hp* eradication therapy; (ii) negative results in the *Hp* infection test (serum anti-*Hp* antibody <3 U/mL and/or negative urea breath test result); (iii) absence of *Hp* bacterial body in normal gastric mucosa identified by immunohistochemistry with an anti-*Hp* antibody (Dako, Glostrup, Denmark), Giemsa staining, or hematoxylin and eosin (H&E) staining of formalin-fixed paraffin-embedded (FFPE) sections; and (iv) no endoscopic findings of mucosal atrophy.

Immunohistochemistry

Immunohistochemistry analysis was performed on FFPE specimens using primary antibodies against E-cadherin (clone NCH-38, Dianova, Hamburg, Germany) and Ki-67 (clone MIB-1, Dako). The proportion of tumor cells positive for Ki-67 was measured at the site with the highest number of labeled nuclei. Investigators were blinded to the tumor genotypes.

Tumor-DNA extraction

Ten-micrometer-thick sections were sliced from the FFPE tissue onto membrane

frame slides (Leica, Wetzlar, Germany). Laser capture microdissection of tumor cells was performed using a Leica LMD 7000 instrument. To perform WES, genomic DNA was extracted from the microdissected tumor cells and matched peripheral blood using the GeneRead DNA FFPE Kit and the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), respectively.

Gastric single gland isolation

We performed WES on *Hp*-uninfected normal gastric epithelia for comparison with *Hp*-uninfected DGCs. Normal gastric epithelium is composed of single-layer columnar cells that are compacted into numerous small replication units known as glands. Each gland is replenished by stem cells located at the neck or base of the gland [26]. To evaluate the precise somatic mutation in the *Hp*-uninfected normal gastric epithelia, genomic DNA extracted from fresh single glands was subjected to WES as previously described [11]. Briefly, two biopsies of normal gastric mucosa were obtained from the fundic gland area, at the gastric angle, for each *Hp*-uninfected subject without a history of gastric cancer. A total of 18 samples were obtained from nine subjects. Gastric epithelia were manually dissociated from the lamina propria after treating the gastric mucosa with 20 mM EDTA in PBS at 4°C for 20 min. Subsequently, single glands were picked up under a stereomicroscope. Genomic DNA isolated from each gland was split

into two aliquots, each of which was independently subjected to whole-genome amplification (WGA) using the REPLI-g Single Cell Kit (Qiagen). Both amplified DNA samples were subjected to WES independently. Variants commonly detected in the two split samples were considered as true somatic mutations.

Whole-exome sequencing

WES libraries were prepared using SureSelect Human All Exon V5 (Agilent Technologies, Santa Clara, CA, USA) or xGen Exome Research Panel (Integrated DNA Technologies, Coralville, IA, USA), followed by sequencing of enriched exon fragments using a HiSeq 2500 or NovaSeq 6000 system (Illumina, San Diego, CA, USA) in 100–150-bp paired-end mode as previously described [27]. The target depth was 100×, and the actual depth was 108× on average. Sequencing reads were aligned to the human reference genome (GRCh37), and mutation calling was performed using the Genomon2 pipeline (v.2.6) as previously described [11]. Candidate mutations were adopted using the Empirical Bayesian Mutation Calling (EBCall) algorithm with the following filtering criteria: (i) a sufficient number of reads (total reads ≥8 and variant reads ≥3); (ii) variant allele frequencies (VAFs) ≥0.05 (for FFPE samples), ≥0.25 (for single-gland WGA samples), and <0.02 (for germline control); (iii) a strand ratio not equal to 0 or 1; and (iv) p value by EBCall $\leq 10^{-3.5}$. Putative germline variants were also

excluded by comparing VAFs with matched controls using Fisher's exact test ($p \leq 10^{-1}$). Candidate mutations in tumor samples were validated by PCR-based amplicon deep sequencing [27]. Because of the limited amount of DNA available, validation was performed with three samples, and the true positive rate was 95% (39 of 41).

Driver genes were investigated based on dN/dS, which is the ratio of the number of nonsynonymous substitutions per nonsynonymous site to the number of synonymous substitutions per synonymous site, using dNdScv [28]. We also adopted 69 driver genes previously reported in comprehensive genetic analyses of gastric cancer [21-24] (Table S1). Because mutations in the driver gene had a high prior probability of being true mutations, we included the driver gene mutations found by loosening the filter criteria: (i) VAFs >0.04 ; (ii) p value by EB call $\leq 10^{-3}$; and (iii) a strand ratio = 0–1. Copy number abnormalities were evaluated using WES data with our in-house pipeline 'CNACS' [12].

External dataset

Because we collected only two cases of advanced DGC, somatic mutation data identified by MuTect and clinical information from patients with DGC were downloaded from The Cancer Genome Atlas (TCGA) data portal [23]. Thirty-seven DGC cases from TCGA were reported as *Hp*-negative, and all had T2–T4 tumors (UICC TNM

classification 6th edition). As hypermutated tumors were considered genetically different from our samples, tumors with more than 12 mutations/Mb were excluded from the comparative data [23]. We also used 30 *Hp*-negative advanced DGC data from Hong Kong [22], all of which were DGCs without microsatellite instability. In total, data for 60 *Hp*-uninfected advanced DGCs (two from our cohort, 28 from TCGA, and 30 from Hong Kong dataset) were collected and compared with data for nine intramucosal DGC cases from our cohort. The Hong Kong dataset could not be used for mutation number and mutational signature analyses because of a lack of information about synonymous mutations. Next, 282 single-nucleotide variants (SNVs) from 11 DGC samples, 260 SNVs from 18 normal glands, and 2596 SNVs from 28 DGCs from TCGA were allocated to 65 ‘the Catalogue Of Somatic Mutations In Cancer’ (COSMIC) single base substitution signatures (SBS) using SigProfilerExtractor [29].

Statistical analysis

All tests were two-tailed, and $p < 0.05$ was considered as significant. The linearity of the number of mutations in the normal gastric glands, intramucosal DGCs, and advanced DGCs with age was evaluated based on Pearson’s correlation coefficient in a linear regression model that assumed a zero intercept, because the number of somatic mutations in an exome region at 0 years of age was assumed to be nearly 0,

as previously described [11]. The Mann–Whitney U test was used to compare normal glands, intramucosal DGCs, and advanced DGCs. Driver mutation rates between intramucosal and advanced DGCs were compared by Fisher’s exact test with Benjamini-Hochberg adjustment. $q < 0.05$ was considered to indicate significance. All statistical analyses were performed using R software (version 3.6.3).

Results

Clinical information

The clinical information of 18 patients with *Hp*-uninfected DGCs (median age, 57 years; range 29–78) and nine subjects without *Hp* infection (median age, 44 years; range 28–77) was collected (Table 1). Sixty-seven percent of patients with DGC had a smoking history, and 33% were current smokers (Table 2). Fifty percent of patients with DGC had alcohol consumption habit. Among the 18 patients, 16 were diagnosed with intramucosal DGC (median age, 60 years; range 29–73) and two with advanced DGC. Sixteen intramucosal DGCs were found as flat pale areas upon endoscopy, all of which were resected endoscopically and were found to contain an SRCC component. One DGC localized in the gastric cardia consisted of a mixture of SRCC and PDA components (Fig. 1a and lower panels of Fig. 1b), and the remaining 15 DGCs localized near the gastric angle were ‘pure’ SRCCs scattered around the neck

of the gland (Figs. 1a and upper panels of 1b). These intramucosal ‘pure’ SRCCs had a low proliferation capacity, with a median Ki-67 labeling index of 2.4% (range, 0–15.4%). Five cases with intramucosal ‘pure’ SRCC showed no progression over 2–5 years according to endoscopic image review (Fig. 2).

Genomic analysis of intramucosal DGCs

Tumor cells were dissected by laser microdissection to increase the tumor cell content because the intramucosal DGCs were small (median, 6.5 mm; range, 3–14 mm) and their tumor cells were scattered around the necks of glands. Small tumors with low densities of signet ring cells did not yield adequate amounts of DNA to perform WES. Accordingly, WES was performed for only nine intramucosal DGCs (Table 2). The median amount of extracted DNA was 67 ng (range, 10–309 ng). A total of 239 mutations (median, 20 mutations/exome; range, 10–66) were found (Table S2). The most recurrently mutated gene was *CDH1*; *CDH1* mutations were found in six of nine intramucosal DGCs (67%) and were considered as positively selected by dN/dS analysis ($q < 0.001$). All six cases with *CDH1* mutations had ‘pure’ SRCCs, which were near the gastric angle. For the other genes, dN/dS analysis showed no significant results ($q > 0.05$). However, a search for 69 previously reported driver genes revealed mutations in three genes, *RNF43*, *TGFBR2*, and *FAT4* (Fig. 3a, Tables S1–S3). All

nine cases of *Hp*-uninfected intramucosal DGC had two or fewer driver mutations.

CDH1 mutation is generally known as a loss-of-function mutation [30]. A missense mutation at L581R and loss of heterozygosity were found in Case 2 (Fig. 3a). A missense mutation at L581 was previously reported in a sporadic DGC [20]. Two-hit *CDH1* mutations, including truncating mutations, were observed in Cases 5 and 6 (Fig. 3a). Cases 3 and 5 harbored a mutation at I250 (L249_T253del, I250N, respectively), which was reported as a mutation hotspot in sporadic DGCs and resulted in impaired cellular aggregation *in vitro* [21] (Fig. 3b). Hotspot splice site mutations (c.531+2T>A, c.687+1_687+4del) reported in sporadic DGCs were observed in Cases 4 and 7 [21, 24] (Fig. 3b). These splice site mutations are thought to induce exon truncations in extracellular domain 1 to prevent the homodimerization of E-cadherin according to computer models [21]. Case 13, the only case with PDA and SRCC located at the cardia, did not harbor *CDH1* mutations. Among the three cases without *CDH1* mutations, we detected a frameshift *RNF43* mutation (A146fs) in Case 10, but we detected no driver mutations in Cases 12 and 13. We also searched for germline mutations in *CDH1*, *CTNNA1*, *PALB2*, *BRCA1*, and *RAD51C*, all of which had been reported as causative genes of HDGC using blood DNA [19, 31, 32]. None of the nine subjects possessed pathogenic germline mutations in these genes. These results indicate that somatic *CDH1* mutations can be considered as an early event in

intramucosal SRCC carcinogenesis. E-cadherin expression was immunohistochemically reduced in all eight cases with intramucosal 'pure' SRCCs near the gastric angle but was maintained in DGC without *CDH1* mutations at the gastric cardia (Figs. 1a, b). These data suggest that reduced E-cadherin expression contributes to SRCC development near the gastric angle, whereas the one case of PDA/SRCC at the gastric cardia may have been caused by different mechanisms.

Analysis of advanced DGCs

Genomic DNA extracted from laser capture microdissected SRCC cells in the mucosal part of two advanced DGCs was subjected to WES. For technical reasons, some PDA cells were included. A *TP53* missense mutation at R248W and loss of heterozygosity was found in Case 11 (Fig. 3a, Table S4). Four driver mutations in *CDH1*, *TGFBR2*, *ARID1A*, and *RHOA* were found in Case 14 (Fig. 3a, Tables S3, S4). The *CDH1* mutation at D402V has been reported in sporadic DGCs [20, 33] (Fig. 3b). Neither of the two cases had pathogenic germline mutations in the HDGC-causative genes described above.

To explore the differences in gene alternations between *Hp*-uninfected intramucosal and advanced DGCs, we examined nine intramucosal DGCs and 60 advanced DGCs, including two from our cohort and 58 from external datasets (Table S5). *CDH1*

mutations were more frequent in intramucosal DGCs (6 of 9, 67%) than in advanced DGCs (16 of 60, 27%) ($p = 0.02$, $q = 0.11$, Fig 4a). In contrast, *TP53* mutations were significantly more frequent in advanced DGCs (31 of 60, 52%) than in intramucosal DGCs (0 of 9, 0%) ($p < 0.01$, $q = 0.03$). *CDH1* and *TP53* co-mutated DGC occurred in only 10% (6 of 60) of advanced DGC (Fig. 4b). *CDH1*-mutated/*TP53* wild-type advanced DGC accounted for 17% (10 of 60) and *TP53*-mutated/*CDH1* wild-type advanced DGC accounted for 42% (25 of 60) of total advanced DGCs. The difference of *CDH1* and *TP53* mutation frequencies between intramucosal and advanced DGCs suggests that *Hp*-uninfected advanced DGCs may be more likely develop from precursor lesions other than *CDH1*-mutated intramucosal DGCs.

Comparison of *Hp*-uninfected normal gastric mucosa, intramucosal DGC, and advanced DGC

To investigate the mechanism of *Hp*-uninfected intramucosal DGC development, we compared the mutations and mutational signatures between intramucosal DGCs and normal gastric glands. WES also indicated that mutations accumulated with age at a frequency of 0.33 mutations/exome/year in the normal gastric gland (Fig. 5a, Table S6). The number of mutations in intramucosal DGCs was not significantly higher than that in normal glands when the age of the patient was considered ($p = 0.40$, Fig. 5a).

In contrast, advanced DGCs had a significantly higher number of mutations than intramucosal DGCs and normal glands ($p = 0.001$, $p < 0.001$, respectively, Fig. 5a).

Next, we performed mutational signature analysis, as different mutational processes may contribute to the accumulation of mutations in a cell, with each imprinting a mutational signature on the cell genome. COSMIC SBSs 1, 5, and 40 accounted for the majorities of normal glands and intramucosal DGCs (Fig. 5b). SBS 1 is known as an age-related signature [34]. The underlying mechanism of SBS 1 is likely deamination of 5-methylcytosine at CpG sites, leading to a C>T transition. The relative contributions of SBS 1 did not significantly differ between the normal gland and intramucosal DGC ($p = 0.40$). In addition, the number of mutations allocated to SBS 1 did not differ between the normal gland and intramucosal DGC when considering the age of the patient ($p = 0.92$). SBS 5 is characterized by C>T and T>C transitions [34]. SBS 40 is similar to SBS 5, making it difficult to estimate their separate contributions [13, 29]. Therefore, we counted SBS 5 and SBS 40 together (designated as SBS 5/40). SBS 5/40 also accumulates with age, although the underlying mutational processes are not well understood [34]. The contribution of SBS 5/40 tended to be higher in intramucosal DGCs than in normal glands ($p = 0.07$, Fig. 5c). This trend did not change when the number of mutations allocated to SBS 5/40 was compared while considering the age of the patient ($p = 0.08$, Fig. 5d). This difference in mutational signature spectra

suggests that intramucosal DGC and normal glands undergo different mutational processes despite similar numbers of mutations.

To further investigate the mechanism underlying the progression from intramucosal DGC to advanced DGC, we compared the mutational signatures. The contribution of SBS 5/40 was lower and that of SBS 17b was higher in advanced DGCs than in intramucosal DGCs (Fig. 5b). Although the etiology of SBS 17b is not well understood, its possible link to damage inflicted by reactive oxygen species has been reported [35]. This result suggests that other or additional mutational processes are involved in the development of advanced DGCs compared to intramucosal DGCs.

Discussion

In the present study, we found that 67% (6 of 9) of *Hp*-uninfected intramucosal DGCs harbored *CDH1* somatic mutations, and the six DGCs with *CDH1* somatic mutations were histologically 'pure' SRCCs without PDA components. *Hp*-uninfected intramucosal DGCs harbored similar numbers of mutations as normal gastric glands and few driver mutations other than those in *CDH1*. These results suggest that *CDH1* somatic mutations are driver mutations for the development of *Hp*-uninfected 'pure' SRCCs.

The median Ki-67 index of 'pure' SRCCs was 2.4%, which is similar to that in a

373 previous report, indicating their low proliferative capacity [16]. Interestingly, five cases
374 with ‘pure’ SRCCs whose endoscopic images could be reviewed showed no
375 progression over 2–5 years. These results suggest that *Hp*-uninfected ‘pure’ SRCCs
376 have an indolent feature that makes them less likely to become invasive. Yorita et al.
377 also reported in a retrospective study that *Hp*-uninfected early SRCCs were less likely
378 to be invasive cancers [17]. Furthermore, all 15 ‘pure’ SRCCs, including those that
379 were not genetically analyzed, were located near the gastric angle and characterized
380 by reduced E-cadherin expression. In animal studies, the loss of E-cadherin function
381 due to *Cdh1* deficiency resulted in the development of SRCC-like atypical cells, but it
382 was not sufficient for invasive cancer formation [26, 30]. Factors such as chronic
383 stimulation by *Helicobacter* infection or nitroso compounds and *Trp53* mutations are
384 required for the invasion of SRCC in *Cdh1* knockout mice [26, 30, 36, 37]. The present
385 study showed that 10% of advanced DGCs harbored both *CDH1* and *TP53* mutations
386 in contrast to none of the *CDH1*-mutated intramucosal SRCCs harboring *TP53*
387 mutation. These results suggest that some *CDH1*-mutated intramucosal SRCCs
388 become advanced DGCs with the addition of *TP53* mutations, as shown in animal
389 experiments [26, 36]. This hypothesis is supported by a study showing that p53 is not
390 aberrantly expressed in intramucosal HDGCs, whereas its expression is altered in
391 invasive HDGCs [38]. However, the discrepancy in the *CDH1* and *TP53* mutation

frequencies between intramucosal and advanced DGC suggests that *CDH1*-mutated intramucosal DGCs make a relatively small contribution to advanced DGC formation in the *Hp*-uninfected stomach and that unknown *TP53*-mutated precancerous lesions might exist.

In addition to *Hp* infection, smoking has been reported to increase the risk of DGC compared to in non-smokers [39, 40]. Previous studies reported that the prevalence of smoking was high in patients with *Hp*-uninfected DGCs [41, 42]. However, SBS 4, the signature associated with smoking, was not observed in the present study (Fig. 5b), which is consistent with the findings of a recent genetic study of DGC [43]. Further analysis is required to clarify the carcinogenic effect of smoking on DGC development. Recent studies reported alcohol-related mutagenesis in gastric cancers in patients with alcohol consumption habit and *ALDH2*-defective alleles [12, 43]. However, SBS 16, an alcohol-related mutational signature, was not detected in our cohort (Fig. 5b), possibly because of the small number of mutations detected by WES analysis (Table 2, Fig. 3a).

We confirmed that somatic mutations accumulate with age at a frequency of 0.33 mutation/exome/year in the *Hp*-uninfected normal gastric epithelia. This mutation frequency is lower than that in the normal colon, as previously determined by WES [11]. The predominant mutational signature of normal gastric epithelia without *Hp*

infection was SBS 5/40, followed by SBS 1. This result differs from that observed for the normal colon and small intestine, in which SBS 1 is most predominant [11, 14].

This study had several limitations. First, the cohort size was small. Second, the size of intramucosal DGC lesion in this study was small. The small lesion size may have contributed to the very slow disease progression, such as that observed for SRCC foci found in HDGC [44]. Further studies are needed to evaluate larger-sized early DGCs. Third, even cases without *CDH1* mutation showed reduced E-cadherin expression. We could not determine the mechanism by which E-cadherin expression was attenuated despite the absence of *CDH1* mutations and copy number alternations. Although we performed *CDH1* promoter methylation analysis, we failed to obtain informative results. The methylation rates in intramucosal DGCs were generally low, although they tended to be higher than those in the adjacent normal mucosa (data not shown). Recent genetic analysis of *Hp*-uninfected DGC reported that *CDH1* mutations were found in only one of seven cases, in contrast to the high prevalence of *CDH1* mutations observed in our study [42]. The low amount of input DNA (10 ng in every case) and the long period of paraffin embedding (median 6 years) may have affected the quality of the analysis, resulting in a low frequency of detection of *CDH1* mutations [45].

In conclusion, we demonstrated that *CDH1* somatic mutations are positively

selected as driver mutations in *Hp*-uninfected sporadic intramucosal SRCCs and that *Hp*-uninfected intramucosal SRCCs may progress slowly and be less likely to develop into advanced DGCs.

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Figure Legends

Figure 1 Clinicopathological features of 18 *Hp*-uninfected DGCs

(a) Tumor locations and pathological features. Sixteen cases of intramucosal DGC and two cases of advanced DGC were sampled. The red dots represent intramucosal DGCs. Fifteen of sixteen intramucosal DGCs were concentrated near the gastric angle and were 'pure' SRCCs with reduced E-cadherin immunoreactivity. One cardiac intramucosal DGC consisted of SRCC and PDA components with maintained E-cadherin immunoreactivity. Green ovals represent advanced DGCs. Advanced DGCs consisted of SRCC and PDA components with reduced E-cadherin immunoreactivity.

(b) Representative endoscopic and histological images of intramucosal DGC cases. Upper panels: Images of Case 5. A pale flat lesion was observed at the anterior wall of the gastric antrum, close to the gastric angle, upon endoscopy (yellow arrowhead). H&E staining of the endoscopic submucosal dissection specimen showed that SRCCs were confined to the proliferative zone of the mucosa. Immunohistochemistry for E-cadherin showed weak immunoreactivity in SRCCs. Immunohistochemistry for Ki-67 showed only a few SRCCs positive for nuclear staining.

Lower panels: Images of Case 13. A pale flat lesion was observed at the greater curvature of the gastric cardia upon endoscopy (yellow arrowhead). H&E staining of the endoscopic submucosal dissection specimen showed SRCCs and PDAs in the

mucosa. Immunohistochemistry for E-cadherin showed almost the same immunoreactivity in carcinoma cells as in surrounding epithelial cells. Immunohistochemistry for Ki-67 showed that all carcinoma cells were negative for nuclear staining.

Scale bar indicates 50 μ m.

Figure 2 Changes in endoscopic findings in *Hp*-uninfected intramucosal signet ring cell carcinoma over time

Top panels: Endoscopic images of Case 8. A biopsy from a tiny pale area at the greater curvature of the gastric angle revealed an SRCC in the year 2018. After biopsy, the lesion became indistinct. In 2020, the lesion became visible, and the size of the lesion was almost the same as that observed in 2018.

The second, third, fourth and fifth panels: Endoscopic images of Cases 10, 16, 17, and 18 respectively. Upon retrospective review of the endoscopic images, SRCCs were found that remained unchanged for 5, 3, 2, and 3 years, respectively. Yellow arrowheads show the lesions. The number in the lower right-hand corner represents the year in which the image was taken.

Figure 3 Mutational landscape and *CDH1* mutation sites

(a) Mutational landscape of *Hp*-uninfected nine intramucosal DGCs and two advanced DGCs. Tumor invasion depth, location, histological type, and E-cadherin immunoreactivity are indicated. LOH, loss of heterozygosity.

(b) *CDH1* mutation sites. Alterations in our sequenced tumor samples are plotted above the *CDH1* protein. Somatic mutations in sporadic DGCs that have been repeatedly reported in previous studies are plotted below the *CDH1* protein [Refs. 20, 21, 24, 33].

Sig, signal peptide; Precursor, precursor sequence; EC, extracellular domain; TM, transmembrane domain; Cytoplasmic, cytoplasmic domain; # reported as a germline mutation of HDGC.

Figure 4 Driver gene mutation frequencies in *Hp*-uninfected intramucosal and advanced DGCs

(a) Comparison of mutation frequencies in driver genes between *Hp*-uninfected intramucosal DGCs ($n = 9$) and advanced DGCs ($n = 60$). p and q values were provided for *CDH1* and *TP53* calculated by two-tailed Fisher's exact test with Benjamini-Hochberg adjustment. There were no significant differences in other genes.

(b) Detailed *CDH1/TP53* mutation profiles for *Hp*-uninfected intramucosal and advanced DGCs.

Figure 5 Mutation number and mutational signature analyses of *Hp*-uninfected normal gastric glands, intramucosal DGCs, and advanced DGCs

(a) Correlation between age and number of mutations in *Hp*-uninfected normal glands, intramucosal DGCs and advanced DGCs. The number of mutations in single glands from normal gastric epithelium without *Hp* infection was plotted against patient age ($n = 18$, blue dots). A regression line (blue dotted line) assuming an intercept of zero is shown, with R^2 and coefficient values. Orange and gray dots indicate the number of mutations in intramucosal and advanced DGCs respectively. The two-tailed Mann–Whitney U test for comparison between normal glands and intramucosal DGCs showed no significant difference ($p = 0.40$). In contrast, advanced DGCs had a significantly higher number of mutations than intramucosal DGCs and normal glands ($p = 0.001$, $p < 0.001$, respectively).

(b) Relative contribution of COSMIC SBSs in *Hp*-uninfected normal glands, intramucosal DGCs, and advanced DGCs.

(c) Proportion of mutations allocated to SBS 5/40 in intramucosal DGCs and normal glands. SBS 5/40 in intramucosal DGCs was relatively higher than that in normal glands ($p = 0.07$, two-tailed Mann–Whitney U test).

(d) Correlation between age and number of mutations allocated to SBS 5/40 in normal

662 glands (blue dots) and intramucosal DGCs (orange dots). Blue dotted line shows
663 regression line assuming an intercept of zero for normal glands. Intramucosal DGCs
664 showed a relatively larger number of mutations than normal glands ($p = 0.08$, two-
665 tailed Mann–Whitney U test).

666

Table 1. Patient characteristics

	Intramucosal DGC (n=16)	Advanced DGC (n=2)	Normal gland (n=9)
Sex			
Male	9	1	6
Female	7	1	3
Age, years, median (range)	57 (29-78)	44.5 (37-52)	44 (28-77)
Tumor location			
Cardia	1	0	N/A
Body	3	1	N/A
Antrum	12	1	N/A
Size of tumor, mm, median (range)	6.5 (3-14)	97.5 (50-145)	N/A
Treatment			N/A
Endoscopic resection	16	0	N/A
Gastrectomy	0	1	N/A
Chemotherapy	0	1	N/A
Histology			
Pure SRCC	15	0	N/A
PDA/SRCC	1	2	N/A
Depth of invasion*			
M	16	0	N/A
SE	0	2	N/A
Cases met HDGC testing criteria	6	1	N/A
Cases subjected to WES	9	2	9

DGC, diffuse-type gastric cancer; SRCC, signet ring cell carcinoma; PDA, poorly differentiated carcinoma; HDGC, hereditary diffuse gastric cancer; WES, whole exome sequencing; N/A, not applicable

* According to the third English edition of Japanese classification of gastric carcinoma: M, Tumor confined to the mucosa; SE, Tumor invasion is contiguous to or exposed beyond the serosa

Table 2. Details of DGC patients

Case number	Age	Sex	Family history of GC ^a	HDGC testing criteria	Location	Histology	Size	Depth of invasion ^b	E-cadherin expression	Ki-67 index	WES	Smoking history	Alcohol intake habit ^c	ALDH2 allele deficiency
1	48	M	no		Antrum	SRCC	3	M	reduced	0.0%	N/A	former	light	N/A
2	61	M	no		Antrum	SRCC	13	M	reduced	1.8%	○	current	light	no
3	63	M	yes	○	Antrum	SRCC	14	M	reduced	3.4%	○	current	heavy	hetero
4	56	F	no		Antrum	SRCC	10	M	reduced	0.7%	○	never	never/rare	no
5	73	M	no		Antrum	SRCC	7	M	reduced	0.0%	○	former	moderate	no
6	50	M	no		Antrum	SRCC	12	M	reduced	3.7%	○	former	light	hetero
7	40	M	yes	○	Antrum	SRCC	6	M	reduced	2.9%	○	current	never/rare	homo
8	65	F	no		Antrum	SRCC	4	M	reduced	15.4%	N/A	former	never/rare	N/A
9	55	F	yes	○	Body	SRCC ^d	4	M ^d	N/A	N/A	N/A	never	light	N/A
10	51	M	no		Body	SRCC	5	M	reduced	1.6%	○	current	light	hetero
11	52	F	no		Body	PDA/SRCC	145	SE	reduced	1.0%	○	current	never/rare	no
12	29	F	no	○	Antrum	SRCC	8	M	reduced	0.9%	○	never	never/rare	no
13	61	F	unknown		Caridia	PDA/SRCC	6	M	maintained	0.0%	○	unknown	unknown	no
14	37	M	no	○	Antrum	PDA/SRCC	50	SE	reduced	0.0%	○	never	never/rare	no
15	63	F	no		Body	SRCC	9	M	reduced	9.8%	N/A	never	never/rare	N/A
16	58	M	no		Antrum	SRCC	6	M	reduced	4.4%	N/A	former	never/rare	N/A
17	71	M	yes	○	Antrum	SRCC	6	M	reduced	1.9%	N/A	current	heavy	N/A
18	78	F	yes	○	Antrum	SRCC	6	M	reduced	5.7%	N/A	never	light	N/A

GC, Gastric cancer; HDGC, hereditary diffuse gastric cancer; SRCC, signet ring cell carcinoma; PDA, poorly differentiated carcinoma; N/A, not applicable

^a Family history of GC at any age in first- or second-degree relatives; ^b According to the third English edition of Japanese classification of gastric carcinoma: M, Tumor confined to the mucosa; SE, Tumor invasion is contiguous to or exposed beyond the serosa; ^c Never/rare, <1U ethanol/week; light, 1-8.9 U/week; moderate, 9-18 U/week; heavy, >18 U/week (1 U = 22g ethanol); ^d The biopsy specimen showed SRCC, but the endoscopic resection specimen showed no evidence of tumor, suggesting that the lesion was intramucosal and was completely removed by biopsy.

Figure1

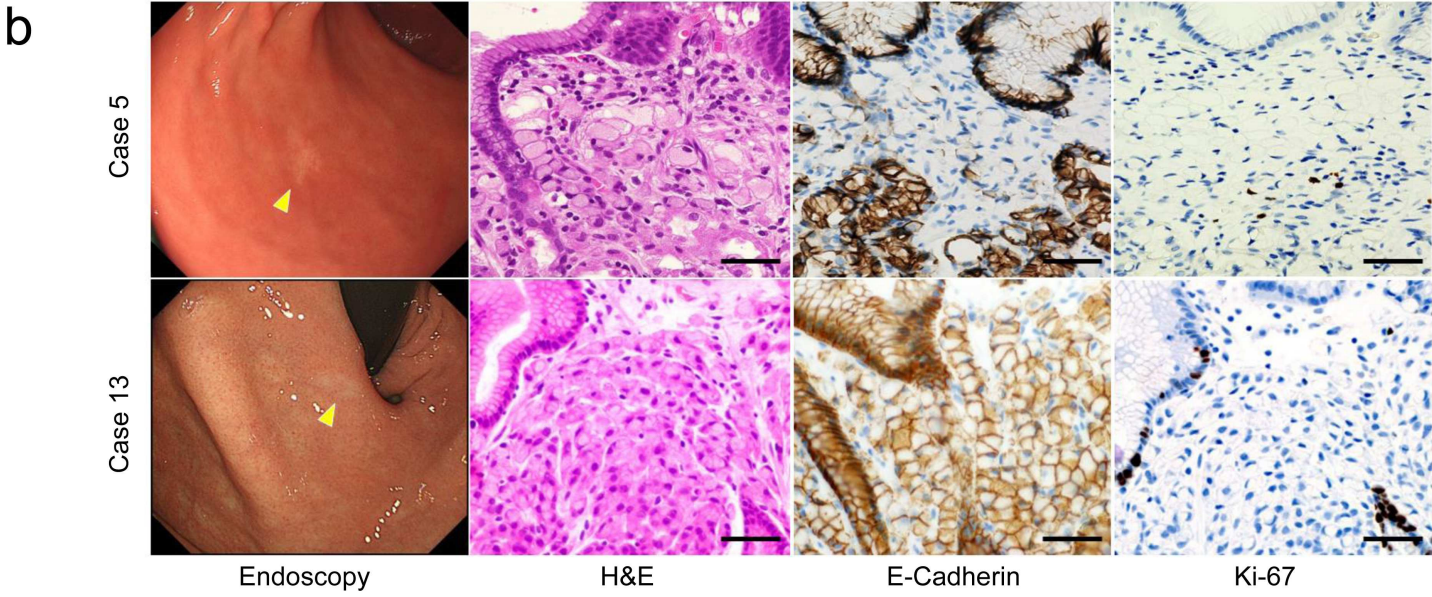
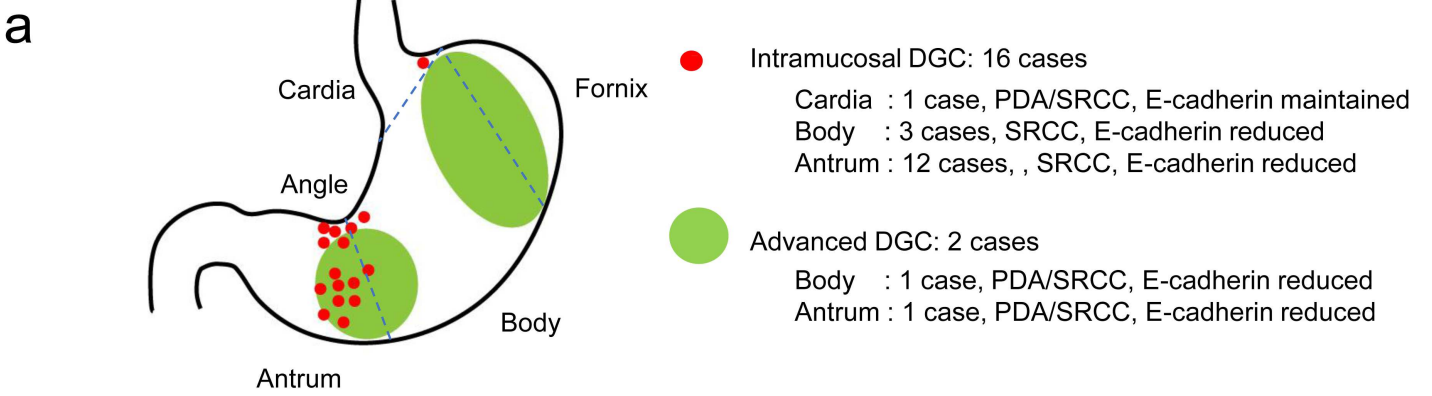


Figure2

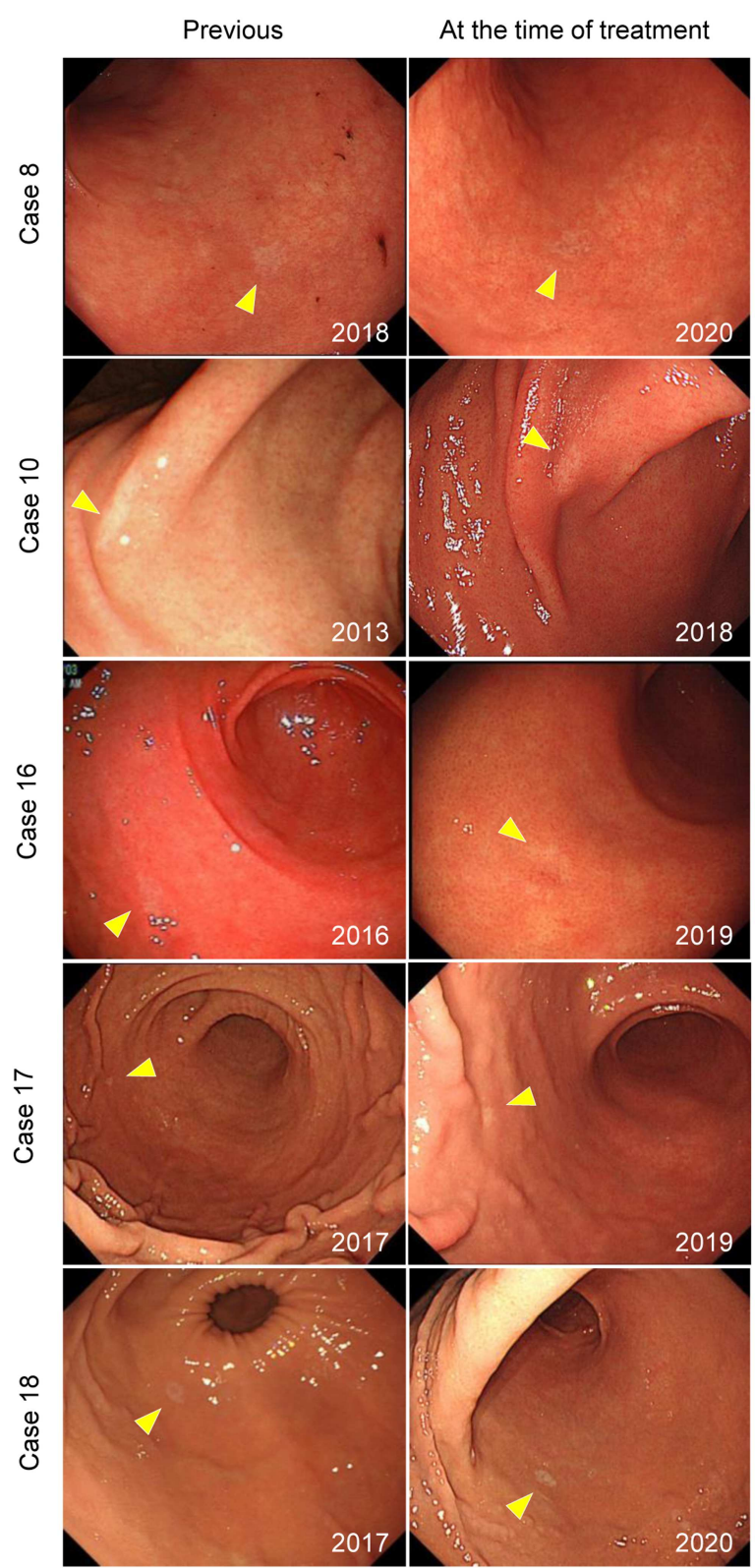
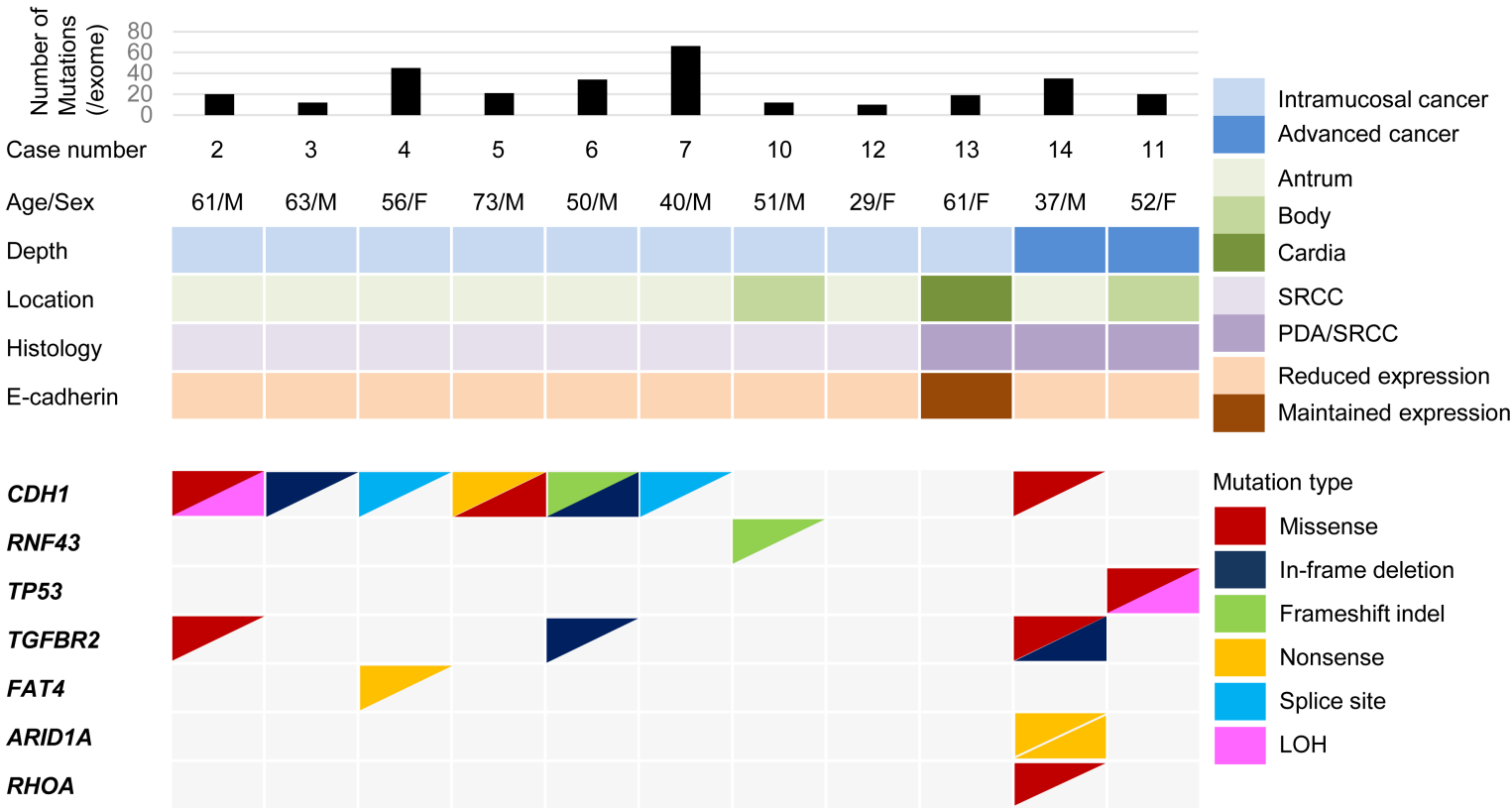


Figure3

a



b

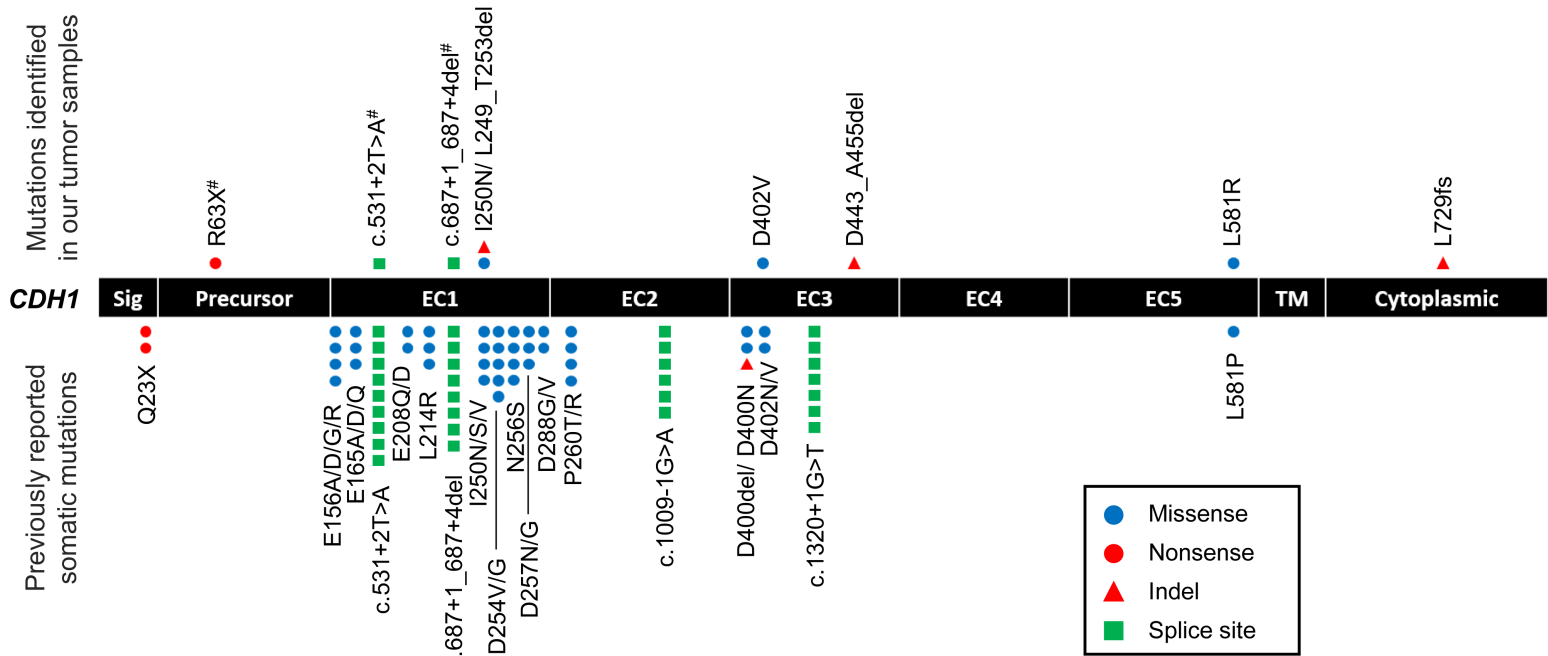
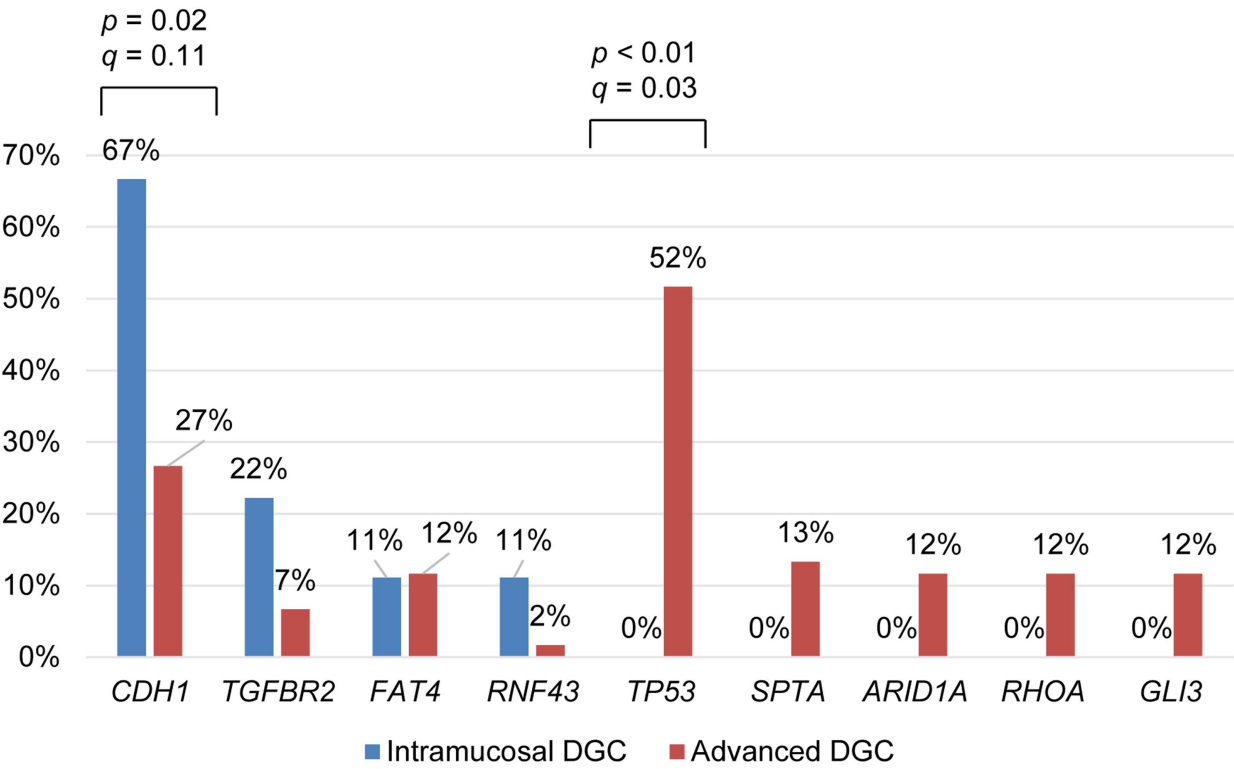


Figure4

a



b

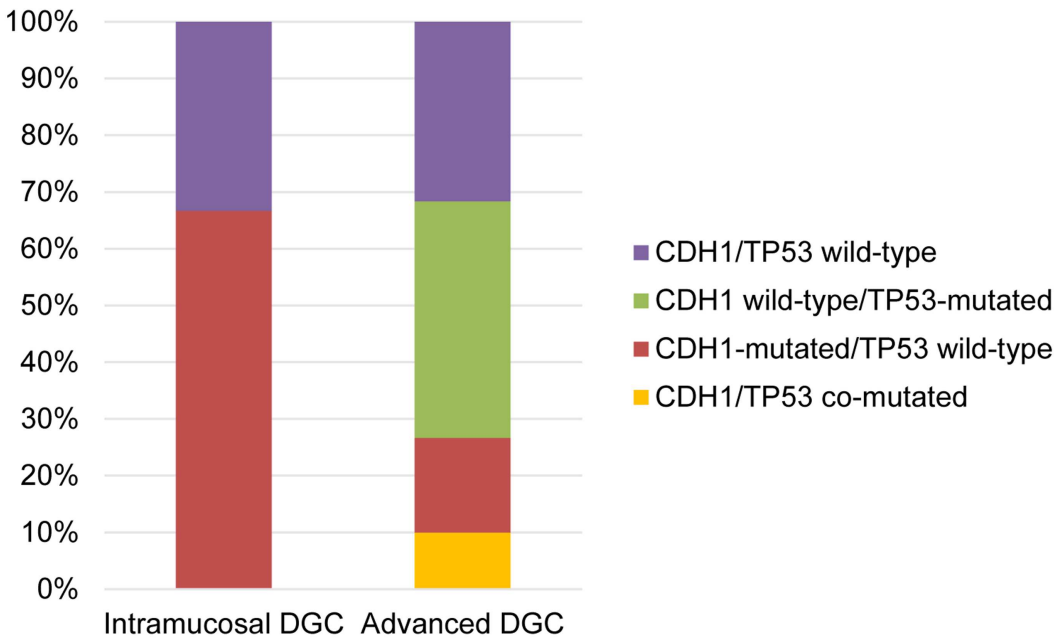


Figure5

