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X染色体不活化を用いた胃正常腺管,腸上皮化生腺管のクローナリティの解析

野村幸正

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INTRODUCTION

Metaplasia is defined as the conversion of one differentiated cell type to another, and occurs in post-natal life.[1,2] Intestinal metaplasia in the stomach is one of the commonest metaplasia in human.[2] The intestinal metaplastic gland is reported to initially arise from the proliferating zone at the neck of a normal gastric glandular tubule.[3] Once cells of intestinal type arise, they replace normal glandular cell types throughout the gland.[3] Macroscopically, intestinal metaplastic lesions most commonly arise at the antrum, which is normally covered by pyloric glands, and the intermediate zone, which is normally covered by a mixture of fundic and pyloric glands and as aging progresses the lesion expands.[3,4]

Intestinal types gastric cancer has been reported to be surrounded by an intestinal metaplasia, which may be due to one of three reasons.[5,6] (1) The intestinal metaplasia is a direct precancerous lesion; (2) the intestinal metaplasia causes an appropriate milieu for carcinogenesis, perhaps by raising the pH of gastric juice, thus improving the growth conditions for some bacteria which produce mutagens; or (3) the intestinal metaplasia is just a paraneoplastic lesion resulting from the same mutagenic stimuli which gave rise to the cancer.[5]

Intestinal metaplasia can be classifed histologically into two types, the complete type (Type I) and the incomplete type (Type IIa and IIb).[7-12] The complete-type is associated with the intestinal marker enzymes sucrose α-D-glucohydrolase, α,α-trehalase, aminopeptidase, and alkaline phosphatase.[13] Tissue of this type contains goblet cells and Paneth's cells but not sulphomucin.[13] The incomplete type is associated with sucrose α-D-glucohydrolase, aminopeptidase, goblet cells, and sulphomucin but not with α,α-trehalase, alkaline phosphatase, or Paneth's cells.[13] The type IIb intestinal metaplasia, which is one incomplete type and secretes sulphomucins, is especially known to accompany the intestinal type of gastric cancer.[7-15]

A "neoplasm" can be defined as a clone of cells distinguished from other tissues by autonomous growth and somatic mutaions.[14] Some cancers have been reported to be monoclonal, based on analysis of X-chromosome inactivation and this is compatible with somatic mutation theories of carcinogenesis.[14-17] Some lesions which are called precancerous have also been reported to be monoclonal.[18,19] Fearon et al. (20) have reported that all colonic cancers and colonic adenomas are monoclonal using X-chromosome methylation. However, Novelli et al. (21) have reported that the earliest adenomas in familial adenomatous polyposis coli are polyclonal. If the cancer arises from multiple step accumulation of genetic changes, there should be a point at which it starts expanding monoclonally.[14] If the intestinal metaplasia is a direct precancerous lesion, it is possible that it has already expanded monoclonally.

To know the monoclonal expansion of some lesions, it is important to know the clonality of the normal tissue originally existed there. In some tissues, there are reported to be monoclonal patches.[21,22] When there is a monoclonal lesion, it cannot be taken to be monoclonally expanding lesion, unless it is larger than the original monoclonal patches. Because there is a possibility that the lesion occurs by a phenotypical change of a stem cell of the patch. The existence of monoclonal patches which is composed of several glands have been reported in both gastric and colonic mucosa using chimeric mice.

Each gland in the gastrointestinal tract is known to have self-regeneration systems. In the normal gastric mucosa, a glandular tubule, which is composed of a gastric pit in the upper part and a gastric gland in the lower constitute an inseparable structural unit.[23-25] In this report, we'd like to use the word "gastric gland" for containing both gastric pit and true gland. The intestinal metaplastic glands have been reported to arise from gradual changes of the phenotypes of the cells constituting the gastric gland.[3] So we analyzed the clonality of normal gastric glands as well as intestinal metaplastic glands.

In recent years, clonal analysis has become possible utilizing X-chromosome methylation.[27] Some specific CpG sites of one of the two X-chromosome alleles of a

female cell are reported to be methylated in the early stages of development and methylation is fixed within the cell's somatic heredity.[28, 29] In a monoclonal cell population that has proliferated from one cell, all the methylated X-alleles are of the same origin, paternal or maternal.[27-29] If the two X-chromosomes are distinguishable, a cell population can be said to be polyclonal or possibly monoclonal.[27] We have used the term "homotypic" for a cell population which is methylated on the same allele, and "heterotypic" for a mixed cell population which is methylated on different alleles in this report, because some cell populations, which have arisen from plural cells that are methylated on the same allele by chance, are homotypic although they are polyclonal.

We analyzed the clonality of fundic gastric glands, intestinal metaplastic glands, and intestinal metaplastic glands based on a polymorphism of the X-linked phosphoglycerate kinase gene (*PGK*) or human androgen receptor gene (*HUMARA*) and X-chromosome methylation.[30-33] And to confirm the clonality of normal gastric glands, we analysed the clonality of gastric mucosa of transgenic mice which carried X-linked *lacZ* gene.[34, 35] The single intestinal metaplastic glands were analyzed separately based on histologic types, whether they were complete or incomplete type, and the locations, antrum or the intermediate zone in the stomach to determine whether a certain area of intestinal metaplasia is a clonal expansion of a metaplastic gland.

MATERIALS AND METHODS

Clonal analysis of normal gastric gland

Specimens

Thirty gastrectomy specimens were obtained from 30 female patients with gastric carcinoma who had been operated upon at the Nationl Cancer Center Hospital East from May 1995 to January 1996. All specimens were examined by the Tes-Tape method to exclude intestinal metaplasia (36). Normal fundic and pyloric mucosa were punched out by a 6 mm-diameter dispopunch (Maruho Co.,Osaka, Japan) from regions as distant as possible from the cancer. One-half of each punched out tissue

sample was studied histologically, and the other half was used for the isolation of glands.

Gland isolation

Single gastric tubules were obtained by the modified method of Cheng (37). Briefly, the tissue sample was incubated for 30 minutes at 37°C in 5 ml of Hanks' balanced salt solution containing 30 mM EDTA, and shuffled mechanically. The isolated glands were picked up by 27G needles under a stereomicroscope.

DNA extraction

Genomic DNA was extracted from isolated single glands with DNA extractor WB kit (WAKO Purechemicals, Osaka, Japan). Finally, DNA extracted from single gland was suspended in 20 µl of 1mM Tris·HCl buffer (pH 7.4) containing 0.1 mM EDTA. The amount of DNA extracted from a single gland was estimated at about 4- 8 ng.

Pre-digestion with HpaII and (or) RsaI

For each DNA sample, 2 enzymatic digestions were performed before PCR amplification. Three µl (0.6-3.0 ng) of DNA was mixed with 1 µl of enzyme solution containing 1.25U of *Rsa*I (with or without 1U of *Hpa*II) and digested for 12 hr at 37°C. The amount of DNA extracted from a single gland was too small to amplify without digestion. However *Rsa*I, which digests many sites of genomic DNA without cutting the amplification loci, enabled the amplification of the small amount of DNA.

PCR amplification of PGK gene

Amplification of a portion of the *PGK* gene, including exon 1, was performed using nested primers 1A, 2B and 2A, 2B, essentially as described by Gilliland et al. (31)with a modification of the primer 2B instead of 1B in the 1st PCR. A schematic representation of the *PGK* gene is shown in Fig. 1. This locus has two *Hpa*II sites which are completely methylated on the inactivated alleles and not methylated on the active alleles, and polymorphic *Bst* XI site which enables to distinguish paternal and maternal alleles. About 30% females are reported to be heterozygous about this *Bst* XI

site. The first PCR reaction was performed in a volume of 20 μl containing 4μl of DNA solution (0.6-3.0 ng DNA) digested by restriction enzyme; 4 pmol of primers 1A and 2B,respectively; 4 nM of dNTP (Takara Shuzo Kyoto, Japan), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 0.5U Taq polymerase (Takara Shuzo, Kyoto, Japan). Samples were amplified using a Perkin-Elmer/Takara thermal cycler for 32 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, with an initial denaturation step at 95°C for 2.5 min, and a final extension step at 72°C for 7 min. One-five hundredth volume of this PCR mixture was added into the 2nd PCR reaction mixture. The 2nd PCR reaction was performed in a volume of 40 μl containing the 1st PCR product; 8 pmol of internal primers 2A and 2B,respectively; and 8nM of dNTP, 1.5 mM $MgCl_2$, 50 mMKCl, 10 mM Tris-HCl (pH 8.3), and 1U Taq polymerase, respectively, under the same conditions as the 1st PCR for 24 cycles. One-tenth of the 2nd PCR products were digested with 2U of *Bst*XI (Toyobo, Tokyo, Japan) at 50°C for 2-3 hr. *Bst*XI-digested products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. Tubes of external control containing 0.6 ng and 3.0 ng of DNA obtained from gastric cancer cell line MKN45 were used in all series of this method, to confirm that only the methylated alleles were amplified in the PCR.

PCR amplification of HUMARA gene

A schematic representation of the *HUMARA* gene is shown in Fig. 2.[32] It has polymorphic CAG repeat, which is repeated between 16 to 29 times in Asian people.[38] More than 90% of Asian females are heterozygous for this repeat. There are two methylation senseitive *Hpa*II sites near this repeat, and these sites are reported to be completely methylated on the inactivated X-allele and completely unmethylated on the active allele.[32,33] When we perform PCR for the locus containing both these *Hpa*II sites and the CAG repeat after digestion with *Hpa*II, we can only amplify the methylated alleles. If the cell population is monoclonal, a single PCR product is obtained, because all the methylated alleles have the same number of CAG repeats. If it

is polyclonal, two kinds of product originating from both the maternal and paternal alleles are obtained. Clonal analysis was performed essentially based on the method reported previously.[32,33] Amplification of a portion of the *HUMARA* gene in exon 1 was performed using primers AR1 and AR2, essentially as described by Mutter et al.(33). AR 1 was labelled at the 5'end with Cy5. Tubes of external control containing 0.6 ng and 3.0 ng of mixed DNA obtained from the blood of a healthy woman and a healthy man were used in all series of this method, to confirm that only the methylated alleles were amplified. DNA samples were diluted to between 0.6 ng and 3.0 ng per tube. The PCR products were analyzed using an automated sequencer (ALFred, Pharmacia) and quantified using the Fragment Manager software package (Pharmacia).

Clonal analysis of gastric mucosa of mice

X-chromosome-inactivation mosaics

Mouse line H253 carries a transgenic X-chromosome-linked marker: 14 tandem copies of a 8.9-kb fragment containing the promoter of the mouse housekeeping gene 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, linked to the *E. coli lacZ* gene.[34,35] The HMG-CoA-lacZ fusion gene structure is discribed in Fig. 3. HMG-CoA reductase is involved in cholesterol synthesis, cholesterol homeostasis, and cell proliferation. Thus this enzyme promotor was used to produce ubiquitous β-gal expression in cells of H253 transgenic mice. Since the transgenic marker is linked to the X-chromosome, mosaics can be produced. This is accomplished by mating male H253 mice to wild-type females. This results in female progeny that are hemizygous for the *lacZ* marker. Because the transgene is present on only one of the two X-chromosomes, X-chromosome inactivation results in the random inactivation of the marker in about half the cells of the embryo. After the X-chromosome inactivation, hemizygous females consist of two cell populations: one that express β-gal and one that does not. These two cell populations can then be used in the clonal analysis. Three hemizygous female H253 mice of 6 weeks old were used.

Fixation and histology

Stomachs were removed and postfixed after cardiac perfusion with 4% (wt/vol) paraformaldehyde/ 0.2% glutaraldehyde in fixing buffer (0.1M phosphate buffer, pH7.4/2 mM $MgCl_2$ / 5 mM EGTA). The fixed stomachs were washed in washing buffer (fixing buffer containing 0.01% sodium deoxycholate and 0.02% Nonidet P-40) and incubated overnight at 37°C in a 5-bromo-4-chrolo-3-indolyl β-D-galactopyranoside (X-Gal; Sigma) solution [0.1% X-Gal / 2 mM $MgCl_2$ / 5 mM EGTA / 0.01% sodium deoxycholate / 0.02% Nonidet P-40 / 5 mM $K_3Fe(CN)_6$ / 5 mM $K_4Fe(CN)_6$ in 0.1M phosphate buffer (pH 7.4)]. After rinsing with buffer, the tissues were embedded in wax and sectioned into 5μm parallel to surfice of epithelium.

Clonal analysis of single intestinal metaplastic gland

Specimens

Three gastrectomy specimens were obtained from three female patients with gastric cancer who had been operated on at the National Cancer Center Hospital, East, Japan, in April 1996. All three cases (C1, 2, 3) were heterozygous for the *HUMARA* gene.

The specimens were examined by the Tes-Tape method with trehalose and sucrose; lesions of intestinal metaplasia were detected with sucrose and separated into complete type and incomplete type with trehalose.[36] The intestinal metaplastic mucosa was punched out by a 6mm-diameter dispopunch (Maruho Co., Osaka, Japan) from the large intestinal metaplastic lesion in the antrum or the intermediate zone. The punching-out was performed as distant as possible from the cancer. One-half of each punched out tissue sample was examined histologically and the other half was used for gland isolation.

Gland isolation

The tissue sample was incubated for 30 minutes at 37°C in 5 ml of Hanks' balanced salt solution containing 30 mM EDTA, and the isolated glands were picked up by needles under stereomicroscopic observation.[37] All the glands were embedded frozen into OCT compound Tissue-Tek (Miles Inc., Elkhart, USA) and each isolated gland was sectioned longitudinally into 5 μm slices until the goblet cells could be seen. The

slices were stained with hematoxylin and eosin, and we confirmed that the glands were intestinal metaplastic by microscopic verification of the presence of goblet cells. The remaining glands in the OCT compound were thawed and picked up under a stereomicroscope.

Clonal analysis

All the intestinal metaplastic glands were analysed with the *HUMARA* gene.[32,33] The procedure from the pre-digestion with the restriction enzymes to the electrophoresis is the same described for the single gastric normal glands. All the glands were analyzed more than twice and the results were all accorded.

RESULTS

Clonal analysis of normal gland

Representative isolated glands from normal fundic and pyloric mucosa are shown in Fig.4. Among the single fundic glands, 67% of the glands had branches, and 33% were straight. Among the single pyloric glands, 54.5% of the glands had branches, and 45.5% were straight. The length of both the single fundic glands and the single pyloric glands was about 500μm.

Among 30 patients, 9 patients were heterozygous for the *PGK* polymorphism. Normal fundic glands were obtained from 6 of these patients, but the other 3 had intestinal metaplasia all over the specimens. From the 6 specimens, 80 glands were obtained. Representative results of the clonal analysis with the *PGK* gene are shown in Fig. 5. When DNA was not digested with *Hpa*II, two peaks were observed in heterozygous patients. When the samples were heterotypic (mixed cells of different allelic methylation), two peaks were retained even after *Hpa*II digestion. When the samples were homotypic (cell populations of the same allelic methylation), one of the peaks decreased significantly in the *Hpa*II-digested sample. From conditional studies, more than 20% contributions of the minor type of DNA can be detected as heterotypic in this way of analysis (the data not shown). Forty-three (54%) glands were made up

of the cell populations of the same allelic methylation (homotypic) and 37 (46%) glands were made up of the mixed cells of different allelic methylation (heterotypic).

It is very important to ascertain whether the same high frequency of heterotypia mentioned above can also be observed using another X-linked locus. To this end, we employed *HUMARA* locus.[32,33] Representative results of the clonal analyses with *HUMARA* gene are shown in Fig.6. Specimens from 6 patients were analyzed, and 5 patients were found to be hemyzygous at *HUMARA* locus. Of the total of 61 glands isolated from the specimens of the 5 patients, 37 (61%) were homotypic, and 24 (39 %), heterotypic.

Thirty-one fundic glands from 3 patients were examined for both loci. Among them, 14 glands were shown to be homotypic, and 17 glands were heterotypic for both loci. All the outcomes were accorded for the *PGK* locus and the *HUMARA* locus. Furthermore, the homotypic glands showed linkage of *PGK* and *HUMARA* in the same allele. For example, glands of one patient showing a shorter band in *PGK* showed a longer one in *HUMARA*, while those showing a longer band in *PGK* showed a shorter one in *HUMARA*. It was indicated that the variant type of *PGK* gene which showed the shorter band and the longer repeat of *HUMARA* gene were on the same allele, in this patient, while the wild type of *PGK* gene and the shorter repeat of *HUMARA* were on the other allele. Glands No.1-6 in Fig.5 and No.1-6 in Fig.6 are the same glands.

Only 1 specimen yielded information on normal pyrolic glands by *PGK*. Of the 7 glands taken from this specimen, 6 (86%) were homotypic and 1 (14%) was heterotypic. Of the 74 pyloric glands obtained from the 4 specimens which yielded information on the *HUMARA* gene, 71 (96%) were homotypic and 3 (4%) were heterotypic. The examples are shown in Fig. 7. Six pyloric glands were examined for both loci. All the outcomes of pyloric glands were also accorded by the *PGK* locus and the *HUMARA* locus.

The results of the clonal analysis of single gastric normal glands are summarized in Table 1. There were no relations between the branching of the glands and the clonal type.

To confirm the existence of the heterotypic glands, we analysed the sectioned view of the stomach of hemizygous H253 mouse. The representative results are shown in Fig. 8. There were heterotypic glands in both fundic and pyloric mucosa. The rate of heterotypic glands was 11% in fundic mucosa and 6% in pyloric mucosa.

Clonal analysis of intestinal metaplastic gland

Thirteen punched out lesions were obtained from three specimens. All the lesions, which were analyzed histologically, contained intestinal metaplasia that were typed by the Tes-Tape method.[13 36] A representative isolated single intestinal metaplastic gland and the sectioned view are shown in Fig. 9. Almost all the isolated intestinal metaplastic glands were simple straight glands, while about half of the isolated fundic and pyloric glands had branching. Between 1 to 10 glands which had goblet cells were obtained from each punched out lesion (Table 2). There was no contamination with interstitial tissues in all the sectioned views of the isolated glands (Fig. 9). Paneth cells could not be detected in the sectioned views of isolated glands.

Clonality of intestinal metaplastic glands were analyzed with *HUMARA* locus. The representative results are shown in Fig. 10. All 86 DNA samples were analyzed twice, and all the outcomes were in accord. The results of the clonal analysis of the single intestinal metaplastic glands are summarized in Table 2. From case 1 (C1), only incomplete type intestinal metaplastic lesions in the antrum were obtained. Four types of lesions, the complete-type in the antrum, the complete type in the intermediate zone, the incomplete type in the antrum, and the incomplete type in the intermediate zone, were obtained from cases 2 and 3. Two lesions only contained either type of homotypic glands; the incomplete type C2-3 (Case2-lesion3) in the antrum and the incomplete type C2-6 in the intermediate zone. Only one or two glands were obtained

from these lesions. Most of the punched-out lesions (11 out of 13) contained heterotypic or both types of homotypic glands.

The total number of the clonal types are summarized again in Table 3. In the complete type in the antrum, 17 glands were homotypic and 5 glands were heterotypic. In the complete type in the intermediate zone, 10 were homotypic and 11 were heterotypic. In the incomplete type in the antrum, 6 were homotypic and 18 were heterotypic. In the incomplete type in the intermediate zone, 12 were homotypic and 7 were heterotypic. Of a total of 86 glands, 45 (52%) were homotypic, and 41 (48%) were heterotypic. Although there were some deviations, there was no correlation between the clonal type and the histologic type or the location of the lesion. One gland out of 8 from one punched out lesion of complete type in the intermediate zone (C2-2) showed a loss of heterozygosity. This gland showed one peak in the ALFred pattern even before digestion with *Hpa*II, and was excluded from the results of the clonal analysis.

We obtained three intestinal metaplastic glands showing replication errors (RER) from the punched-out lesion C1-2, which was an incomplete type intestinal metaplastic lesion in the antrum (Fig. 11). When PCR was done without prior *Hpa*II digestion, all the glands of this case (C1) showed two peaks as seen for C1-1-1 (Case1-Lesion1-Gland1) in Fig. 11. However, three glands (C1-2-1, 2, 3) from one punched out lesion showed one additional peak of a longer repeat, indicating a replication error of the *HUMARA* gene. Although all three of these glands showed the same type of RER, clonal analysis of them indicated that each of the three had a different methylation pattern after digestion with *Hpa*II (Fig. 11). The examination of these three glands was performed in triplicate with the same results. These three glands were also excluded from the clonal analysis. They had no histological differences from the other intestinal metaplastic glands.

Discussion

In clonal analysis it is important to rule out contamination by interstitial tissues.[20] In this analysis, we used the gland isolation technique reported by Cheng et al. (37). The isolated glands were confirmed to contain no interstitial tissues by being sliced and stained with HE. Even though contamination could not be completely ruled out, they were found to have a contamination rate of less than 20% of all cells. We used this technique on colonic crypts, and they were all homotypic as Ponder et al. (21) and Fearon et al. (20) have reported. Some clonal analyses have used microdissection in cases where there was no alternative approach possible.[39] The gland isolation technique that we employed is both useful and reliable because DNA can be obtained from unfixed cells, but one disadvantage of this technique is that it is impossible to reconstruct the positional relationships of neighboring glands.

About half of the single fundic glands were homotypic in *PGK* or *HUMARA* locus, while the other half were heterotypic. About 4% of the single pyloric glands were homotypic, too. The arising way of glands may be different between stomach and colon, because colonic crypts have been reported to be all monoclonal. The existence of heterotypic glands in stomach contradicts the results of clonal analysis using chimeric mice.[22] We confirmed the existence of heterotypic glands in H253 mice, ruling out the difference among the species. One of possible reasons for this discrepancy may be that in chimeras, strain differences prevent the successful cooperation of progenitor cells in forming glands.

At least 50% of the intestinal metaplasia in the present study was polyclonal in origin, even when they were separated into the smallest lesion, a gland. This findings was quite unexpected and is probably concordant with the recent suggestion by Bjerknes et al. (40) that some crypt in dysplastic adenoma of familial polyposis patients are polyclonal. In addition, most of the small punch out lesions of intestinal metaplasia were a mixture of glands originating from different cells. Therefore the present data indicate that intestinal metaplasia is a polyclonal lesion. Slack et al.(1 2) has proposed that one tissue can be replaced by another, either by a switch of developmental commitment, or by colonisation with cells of different origin, and that

only the former is a true metaplasia. The present data indicate that the intestinal metaplasia is a true metaplasia, because each intestinal gland is known to be monoclonal with respect to X-chromosome methylation and the intestinal metaplasia could not be explained by the expansion of intestinal mucosa into the stomach from the duodenum.[21] The intestinal metaplasia is thought to arise by a switch of developmental commitment, but the switching has to occur in plural neighboring stem cells to become polyclonal.

Most of the punched-out lesions (11 out of 13) contained glands originating from different cells. The remaining 2 lesions gave only 1 or 2 glands. From these findings, the intestinal metaplasia may not expand monoclonally larger than 6 mm in diameter. In this respect, the intestinal metaplasia is not a direct precancerous lesion expanding monoclonally, even when the size of the patch is compared with that of normal mucosa. Ochiai et al.(41) has found some clonal expansion of intestinal metaplasia with a p53 mutation, indicating monoclonality. Therefore, some fraction of intestinal metaplasia could be a monoclonal expansion, but our present data indicate that intestinal metaplasia in general is not monoclonal. However, in intestinal metaplasia cells may be in a metastable phase and are highly succeptible to subsequent monoclonal multi-step carcinogenesis.[14]

The intestinal metaplasia type IIb, which have sulphomucin, is reported to have a strong correlation with intestinal type gastric cancer.[7-12] In this report we used a gland isolation technique that uses EDTA treatment, the tissue had to be kept unfixed.[37] Therefore, the α,α-trehalase activity was adopted to classify the intestinal metaplasia and the type IIb could not be separated from type IIa, because the mucin staining technique requires fixation of the tissue. Although we could not differentiate intestinal metaplasia IIa and IIb, there was no punched-out lesion contained more than 3 glands of the same one cell origin, excluding the possibility of monoclonal expansion of intestinal metaplasia.

The intestinal metaplastic gland is reported to arise by gradual change of a normal gland.[3] In the present investigation, however, about half of the intestinal

metaplastic glands were heterotypic even when they were in the antrum surrounded by pyloric glands which were homotypic in 96%. We could explain the existence of the heterotypic and homotypic intestinal metaplastic glands among the fundic glands, which are heterotypic in 40%, by the gradual change of normal glands. But a simple gradual change could not explain the difference of the heterotypic glandal rates between pyloric glands and intestinal metaplastic glands in antrum, as X-chromosome inactivation is reported not to change even in stem cells.[28,29]

We could postulate three explanations for the differnce of heterotypic glandal rates between pyloric glands and intestinal metaplastic glands in antrum. One possible explanation is that originally heterotypic glands are favorable to be changed into intestinal metaplastic glands. Schmidt et al.(42) reported that the intestinal crypts in chimeric mice are of polyclonal origin and the purification of crypt cells occurs within two weeks after birth. From this observation, the heterotypic glands may be infantile and may have an ability to change the phenotypes.

Another possible explanation is that intestinal metaplasia might be formed through a process which incorporates a second stem cell, either from outside the normal gland or from outside of the cell regeneration system of a normal gland, namely a pluripotential dormant cell. In this mechanism, it is possible that the preexisting normal homotypic pyloric gland is gradually replaced by intestinal type cells. We know that a considerable portion of intestinal metaplastic glands show both gastric and intestinal phenotypes.[3] Therefore, our present observation is consistent with previous observations. In addition, we know that even a normal intestinal crypt is formed as a polyclonal cell population in prenatal gut.[42] It is possible that the generation of intestinal metaplasia imitates ontogeny.[2] The purification mechanisms, which Schmidt et al. (42) have said, might explain the existence of both homotypic and heterotypic intestinal metaplastic glands. The intestinal metaplastic glands may arise polyclonally and may be purified into monoclonal populations. But as we took all the punched out lesions from large, possibly fused, intestinal metaplastic areas in this experiment, we could not confirm this purification process with time.

There is the third possible explanation. If we postulate that X-chromosome inactivation is not determined in the stem cell of an intestinal metaplastic gland, a heterotypic gland might potentially arise from a single stem cell. The methylation of autosomal genes is known to be disturbed in the carcinogenesis process.[43] There might be a mechanism of methylation instability in the process of intestinal metaplasia. Our observations of the three neighboring glands showing RER (Fig. 11) support this idea. Because RER is a rare event, it is unlikely to occur independently in three different neighboring cells. Thus, we assumed that the three glands had originated from the same stem cell. However, we observed different methylation patterns among the glands.

We do not presently have any solid data to determine which of above two theories is more likely. Somatic mutations, such as RER in an intestinal metaplastic gland, might be a key to answering this question. Instability of X-chromosome methylation, or possibly inactivation, may represent a new field of research on the differences in cancer susceptibility between men and women.

Conclusion

1. It is less probable that intestinal metaplasia expands monoclonally to a large region. All intestinal metaplasia are not precancerous lesion from the standpoint of the clonality.

2. At least half of the intestinal metaplastic glands are polyclonal even in the antrum where original normal glands are almost monoclonal. Change of the gland from normal to intestinal metaplastic cannnot be explained by a simple transformation of the gland.

3. There may be a X-chromosome inactivation instability in the process of generating the intestinal metaplasia.

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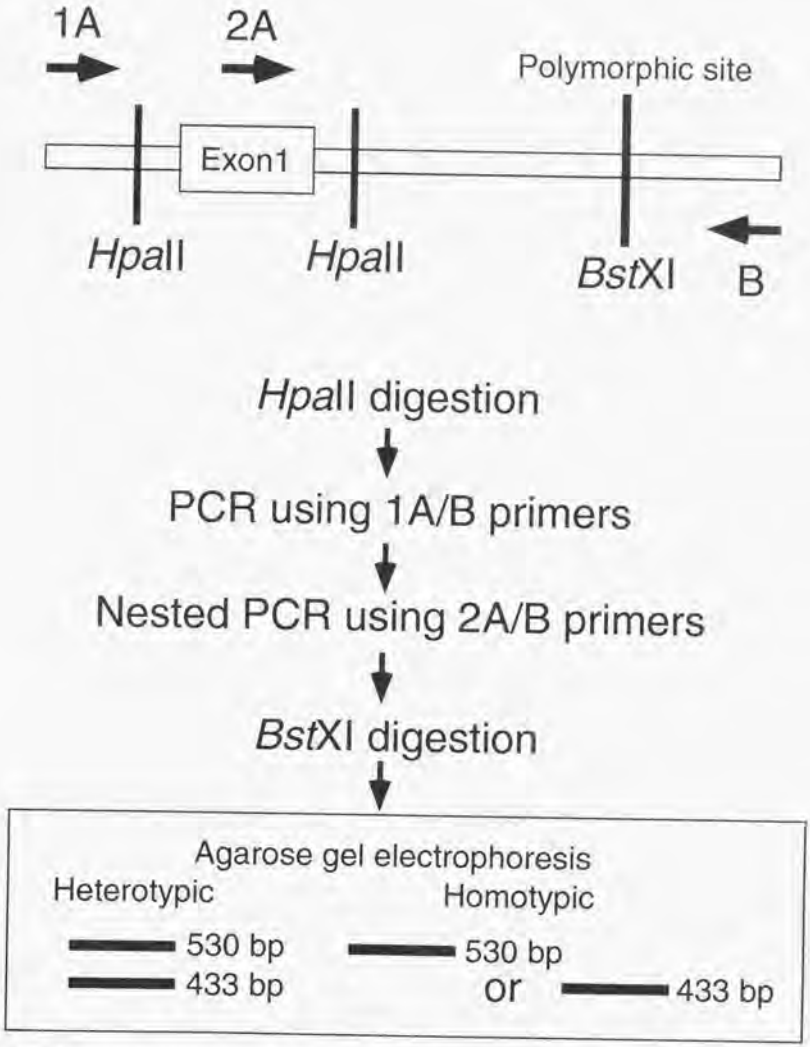


Fig. 1 Schematic representation of PGK gene; there are polymorphic *Bst* XI site and two *Hpa*II sites which are methylation sensitive. The *Hpa*II sites are completely methylated on inactive X-alleles and unmethylated on active X-alleles. DNA was digested with *Hpa*II, and PCR was performed for the locus containing the two *Hpa*II sites and the polymorphic *Bst*XI site. Then, PCR products were digested with *Bst*XI and were electrophresed. Heterotypic samples showed two bands and homotypic samples showed one band.

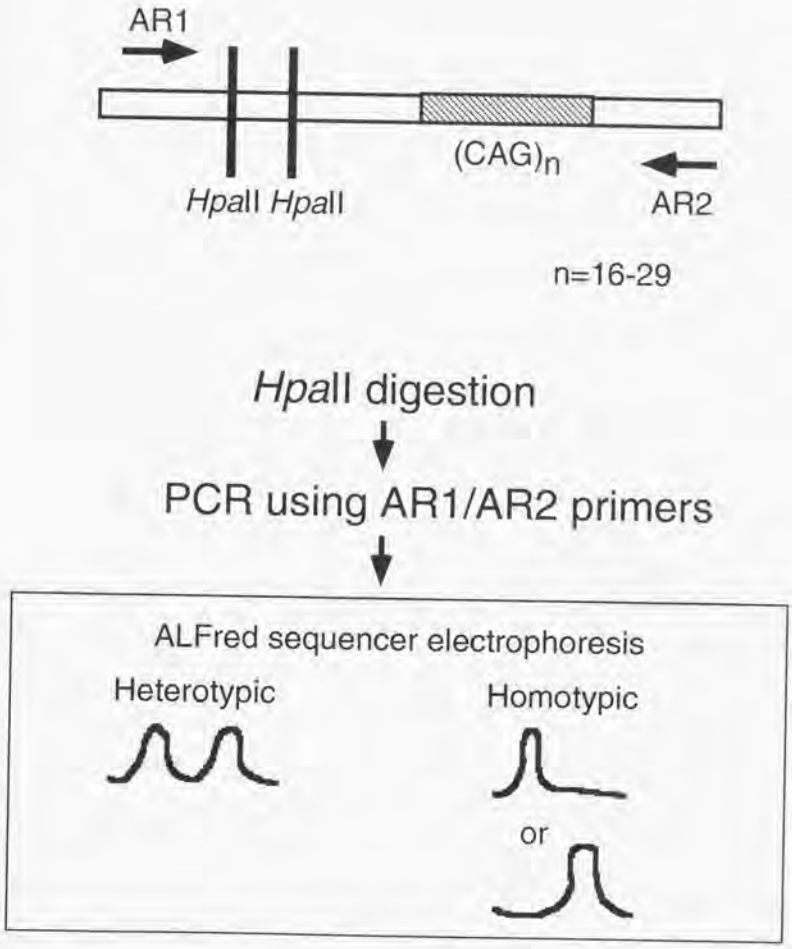


Fig. 2 Schematic representation of *HUMARA* gene; There are polymorphic CAG repeat and two *Hpa*II sites which are methylation sensitive. These sites are completely methylated on inactive X-alleles and unmethylated on active X-alleles. Repetition number of the CAG is from 16 to 29 in Asian people. DNA was digested with HpaII, and PCR was performed for the locus containing both the two *Hpa*II sites and the repeat. PCR products were electrophoresed. Heterotypic samples showed two peaks and homotypic samples showed one peak.

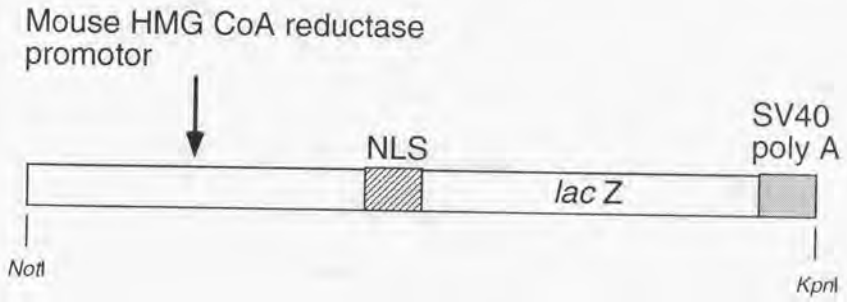


Fig. 3 *HMG CoA-lacZ* fusion gene structure. The 8.9 kb *Not*I-*Kpn*I linear fragment excised from the vector consists of 5.6 kb of the mouse 3-hydroxy-3-methylglutarylcoenzyme A reductase gene sequence and 3.3 kb of *lac*Z gene. A nuclear location signal (NLS) coding for amino acid residues 127-147 of SV40 large T antigen is fused to the 5'-terminal of the *lac*Z gene to provide a β-galactosidase hybrid protein with nuclear localization.
X-linked *lac*Z transgenic mouse carries 14 tandem copies only on the X-chromosome.
NLS: nuclear location signal

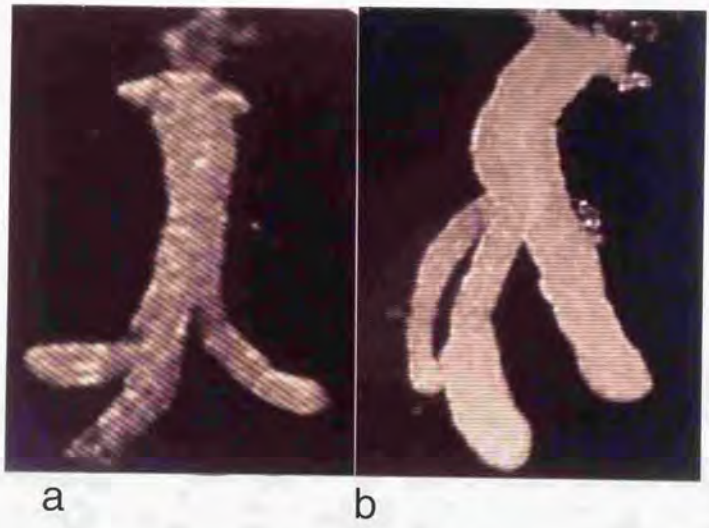


Fig. 4. Stereoscope feature of isolated single glands. a, Fundic gland (*70). b, Pyloric gland (*70). Sixty-seven % of the fundic glands had branches, and 33% were straight. Among the single pyloric glands, 54.5% of the glands had branches, and 45.5% were straight. The length of both the single fundic glands and the single pyloric glands was about 500mm.

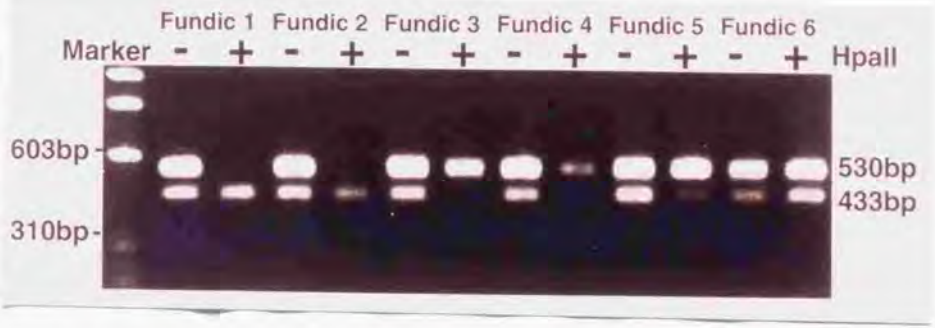


Fig. 5. Clonal analyses of single fundic glands by *PGK*. Glands No.1- 4 were shown to be homotypic and glands No. 5 and 6, heterotypic. One of two bands in the lane of *Hpa*II digested No. 5 was weak, but it was stronger than control sample, which was mixed DNA of a female and a male.

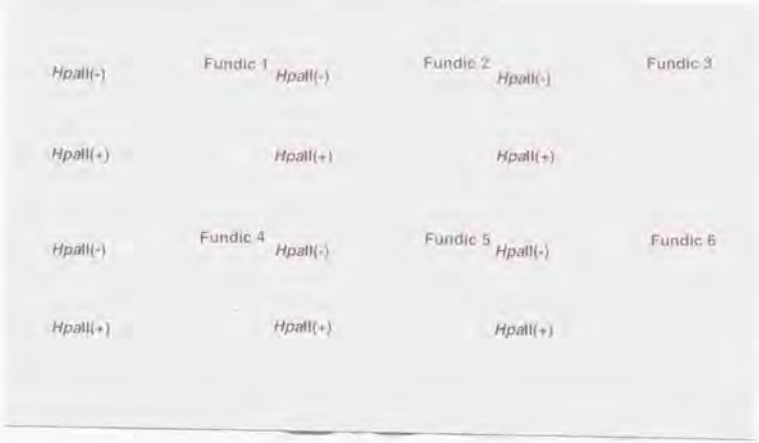


Fig.6. Clonal analyses of single fundic glands by *HUMARA*. All the samples were analyzed in pairs of those not digested with*Hpa*II and digested with *Hpa*II. In ALFred pattern, the PCR product of heavier molecular weight is on the right, and the lighter is on the left. The right peaks are the products of longer repeats of *HUMARA*, and the left are of shorter repeats. Fundic 1-6 in this figure and Fundic 1-6 in Fig.5 represent the same glands, respectively. Glands No.1- 4 were shown to be homotypic and glands No.5 and 6, heterotypic. *Hpa*II(-), not digested with *Hpa*II; *Hpa*II(+), digested with *Hpa*II.

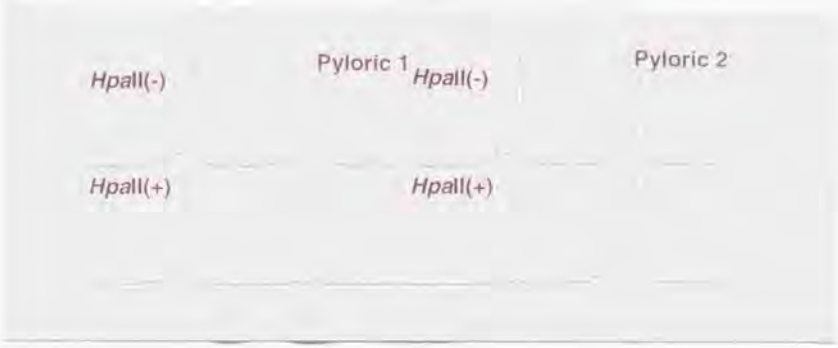


Fig.7. Clonal analyses of single pyloric glands by *HUMARA*. All the samples were analyzed in pairs of those not digested with *Hpa*II and digested with *Hpa*II. In ALFred pattern, the PCR product of heavier molecular weight is on the right, and the lighter is on the left. The right peaks are the products of longer repeats of *HUMARA*, and the left are of shorter repeats. Pyloric 1 shows homotypic gland which is methylated on the allele of shorter repeat, and pyloric 2 shows homotypic gland methylated on the allele of longer repeat. About 96% of the pyloric glands were homotypic. *Hpa*II(-), not digested with *Hpa*II; *Hpa*II(+), digested with *Hpa*II.

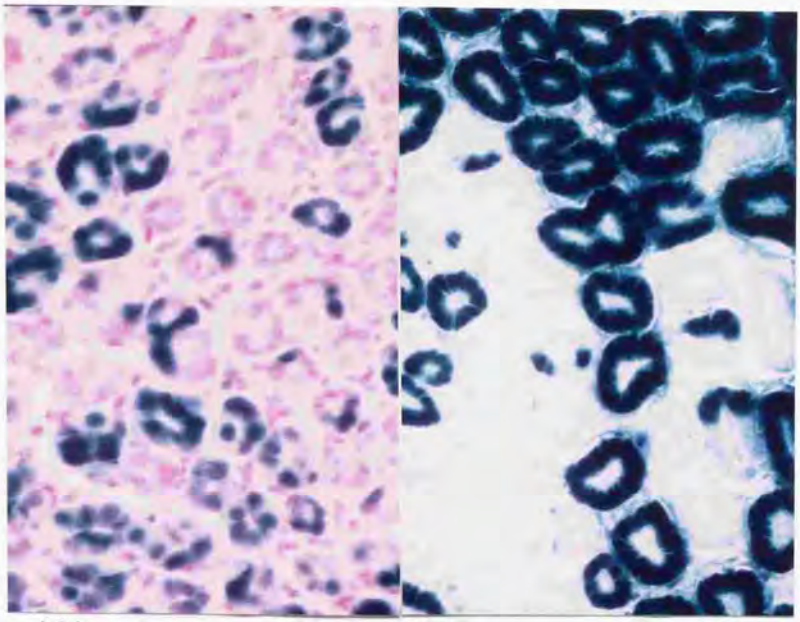


Fig. 8. Cross-sections through glands in stomach of 6 weeks old female heterozygous H253 mice. The H253 mouse has 14 tandem repeats of the construct of Fig. 3 on the X-chromosome. Cells which kept the transgenic allele active are blue and which inactivated that allele are not blue. (A) Fundic glands. (7μm, 60*) Counter stained with nuclear fast red. (B) Pyloric glands. (10μm, 40*) There were heterotypic glands in both glandal fields.

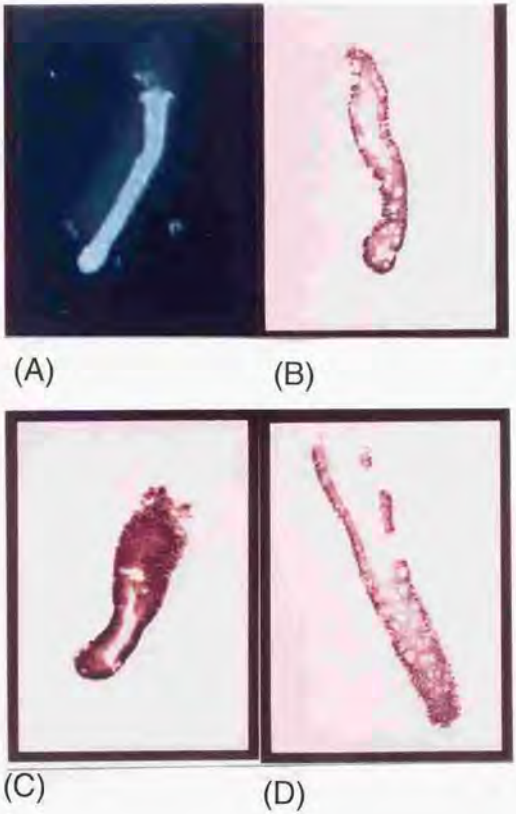


Fig. 9. Single intestinal metaplastic gland;
(A) A feature under a stereomicroscope; magnification *70. Almost all the glands had no branches. (B)-(D) Sectioned and hematoxyline and eosin (HE.) stained features of the gland under a microscope, magnification *100. These three glands showed heterotypic, though there were no interstitial tissues.

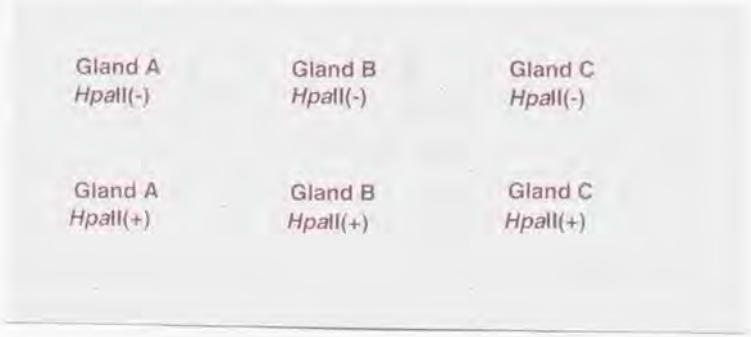


Fig. 10. Representative clonal analysis;
All the samples were analyzed in pairs of those not digested with *Hpa*II and digested with *Hpa*II. In ALFred pattern, the PCR product of heavier molecular weight is on the right, and the lighter is on the left. The right peaks are the products of longer repeats of *HUMARA*, and the left are of shorter repeats. Gland A, heterotypic. The results of digested with *Hpa*II are the same with no digestion with *Hpa*II. Gland B, homotypic of longer allelic methylation. Gland C, homotypic of shorter allelic methylation. *Hpa*II(-), not digested with *Hpa*II; *Hpa*II(+), digested with *Hpa*II.

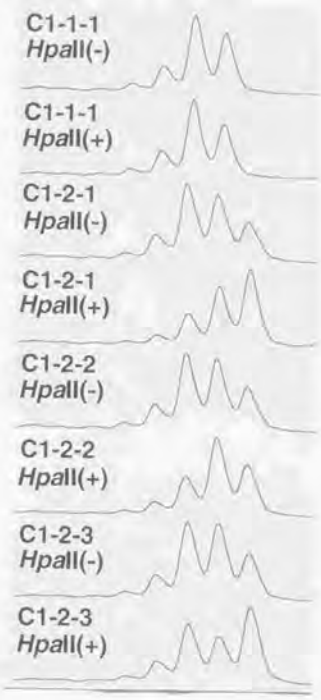


Fig. 11. Results of the three glands which showed replication errors (RER); Gland No. C1-1-1 is a control gland from the same case, which is heterotypic and does not show RER. The results of the three glands (C1-2-1, C1-2-2, C1-2-3) have one more small peak on the right when they are not digested with *Hpa*II. The results all differ when they are digested with *Hpa*II.
No. C1-2-3, gland No.3 of lesion No. 2 of case No. 1.

Table I *Gland type analyzed by PGK and HUMARA*

	PGK		*HUMARA*	
	Homotypic	**Heterotypic**	**Homotypic**	**Heterotypic**
Fundic gland	**43 (54%)**	**37 (46%)**	**37 (61%)**	**24 (39%)**
Pyloric gland	**6 (86%)**	**1 (14%)**	**71 (96%)**	**3 (4%)**

Table 1 Fifty-four percent of fundic glands were homotypic and 46% were heterotypic analyzed with the *PGK*. Sixty-one percent of fundic glands were homotypic and 39% were heterotypic analyzed with the *HUMARA*. In pyloric glands 86% were homotypic and 14% were heterotypic analyzed with the *PGK*, and 96% were homotypic and 4% were heterotypic analyzed with the *HUMARA*.

TABLE II *Details of clonality of intestinal metaplastic glands obtained from each small punch out*

	Punched out No.	*Homotypic (longer peak)*	*Homotypic (shorter peak)*	*Heterotypic*	*Total*
Complete type Antrum	C2-1	6	1	2	9
	C3-1	2	2	0	4
	C3-2	0	6	3	9
Complete type Intermediate zone	C2-2	1	0	6	7
	C3-3	5	3	0	8
	C3-4	0	1	5	6
Incomplete type Antrum	C1-1	0	2	5	7
	C2-3	0	1	0	1
	C2-4	0	0	6	6
	C3-5	1	2	7	10
Incomplete type Intermediate zone	C2-5	2	1	5	8
	C2-6	0	2	0	2
	C3-6	0	7	2	9

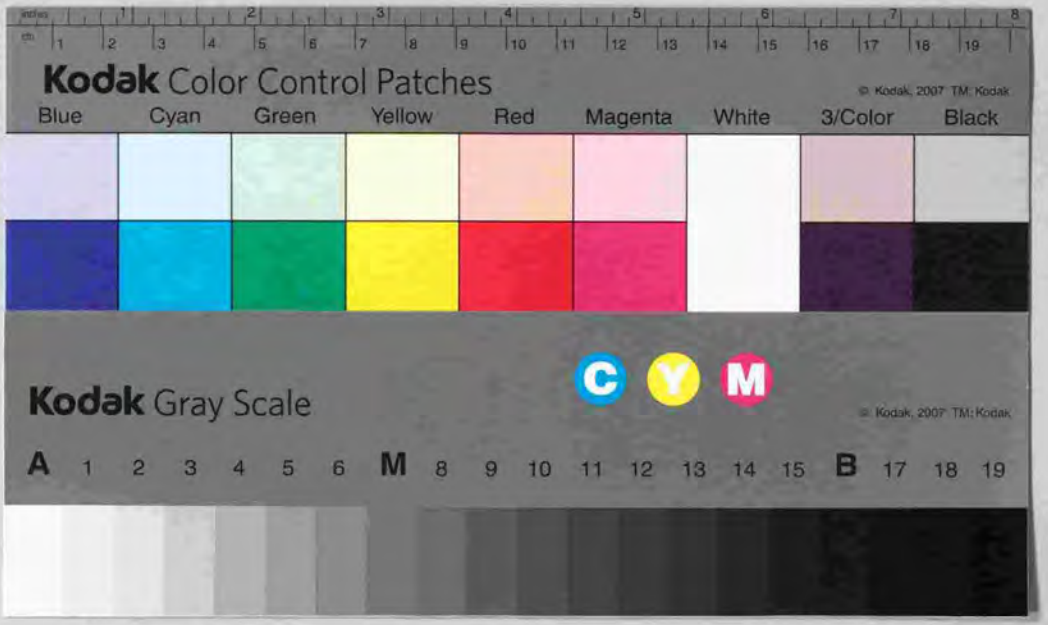
C2-1=case number 2-punched out number 1

Table 2 Most of the small punched out (6mm in diameter) did not composed of glands originated from a single cell, irrespective of histologic types and locations. The punched outs numbered C2-3 and C2-6 contained glands of the same homotypes only. But only one and two glands were obtained from these punched outs. respectively.

Table III *Summary of clonal analysis of single intestinal metaplastic gland*

	Complete type		*Incomplete type*		
	Antrum	*Interm.*	*Antrum*	*Interm.*	*total*
Homotypic	17	10	6	12	45 (52%)
Heterotypic	5	11	18	7	41 (48%)

Table 3 In the complete type in the antrum, 17 glands were homotypic and 5 glands were heterotypic. In the complete type in the intermediate zone, 10 were homotypic and 11 were heterotypic. In the incomplete type in the antrum, 6 were homotypic and 18 were heterotypic. In the incomplete type in the intermediate zone, 12 were homotypic and 7 were heterotypic.
Of a total of 86 glands, 45 (52%) were homotypic, and 41 (48%) were heterotypic. Although there were some deviations, there was no correlation between the clonal type and the histologic type or the location of the lesion.


Kodak Color Control Patches
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Red
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White
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