Clonal analysis of gastric carcinoma and precancerous lesions and its relation to Ki-67 protein expression

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The pathogenesis of intestinal carcinoma is characterized as progressing through multiple steps, which begin with atrophic gastritis followed by intestinal metaplasia, dysplasia and carcinoma. However, the clonal status of gastric precancerous lesions and its association with proliferative kinetics have not been fully understood. In this study, gastric lesions and normal epithelial cells were isolated from formalin-fixed paraffin embedded tissues using a laser capture microdissection (LCM) system, the clonality was analyzed with human androgen receptor gene (HUMARA) polymerase chain reaction (PCR), and the PCR products were examined using Applied Biosystems 3730 DNA Analyzer. The relationship between the clonal status and Ki-67 protein expression was also investigated. Ki-67 was detected by two-step immunohistochemical staining. 5/32 intestinal metaplasia lesions, 10/45 low grade intraepithelial neoplasia, 25/36 high grade intraepithelial neoplasia and 20/20 intestinal gastric carcinoma were of monoclonal origin. Similar to monoclonal inactivation, the expression rate of Ki-67 also increased along the multi-step gastric carcinogenesis. Clonal status was associated with the expression rate of Ki-67 to a certain extent, which may be useful in assessing susceptibility to gastric carcinoma.

Key words: gastric carcinoma; precancerous lesion; clonal analysis; Ki-67

Gastric carcinoma is one of the most common cancers and is a major cause of cancer-related deaths in China. Lauren [1] composed a histological classification of gastric carcinoma that is very useful in evaluating the natural history of gastric carcinoma and is widely applied. According to the classification, lesions are classified into two major types: intestinal and diffuse. Intestinal carcinomas form recognizable glands. In contrast, diffuse carcinomas consist of poorly cohesive cells diffusely infiltrating the gastric wall without gland formation. The pathogenesis of intestinal carcinoma has been characterized as progressing through multiple steps, which begin with atrophic gastritis followed by intestinal metaplasia, dysplasia and carcinoma [2]. However, no preceding stages have been identified in the carcinogenesis of diffuse carcinomas. The early detection and treatment of gastric precancerous lesions are the most important elements in the process of improving gastric carcinoma prevention and cure.

The diagnosis of early gastric carcinoma is a controversial problem. Gastric lesions considered to be high grade dysplasia by Western pathologists are often diagnosed as well-differentiated carcinoma by Japanese pathologists, especially from biopsy specimens [3]. All of the lesions in gastric epithelia including low, moderate and high grade dysplasia and carcinoma in situ are termed intraepithelial neoplasia (dysplasia) [4]. Low grade intraepithelial neoplasia includes low and moderate grade dysplasia, and high grade intraepithelial neoplasia includes high grade dysplasia and carcinoma in situ. One of the problems associated with diagnosing gastric intraepithelial neoplasia is the distinction between regenerative and reactive changes. One essential tenet in defining neoplastic proliferation is that the cells originate from a single cell clone [5]. In contrast, reactive proliferation is polyclonal. Based on analysis of X chromosome inactivation, some cancers as well as many precancerous lesions are monoclonal. Fearon et al [6] reported that all 50 colonic tumors examined, including 20 colonic carcinomas and 30 colonic adenomas, were monoclonal patterns of X chromosome inactivation. Park et al [7] reported that all 25 cases...
of high grade cervical squamous intraepithelial lesions were monoclonal and 54 of 79 low grade squamous intraepithelial lesions were monoclonal. If the cancer arises from multi-step accumulation of genetic changes, there should be a point at which it starts to expand monoclonally. Thus, the clonal status can be used as a biomarker to differentiate reactive proliferation from neoplastic proliferation and provide a more rational basis for diagnosis and treatment. To better understand the pathobiology of gastric carcinoma, the clonal status of gastric precancerous lesions and its association with proliferative kinetics should be investigated.

Clonal status is analyzed using the methylation inactivation pattern of X chromosome on the basis of Lyon hypothesis [8]. One of the two X chromosome alleles of a female cell is inactivated by hypermethylation during the process of embryogenesis, and the methylation is highly conserved within the cell’s somatic heredity. In a monoclonal cell population, all of the cells are derived from a common progenitor and the same alleles are methylated. Therefore, the same alleles are digested by the methylation sensitive restriction enzyme which selectively targets the unmethylated gene region. Afterwards, only the methylated alleles are amplified by PCR utilizing flanking marker gene primers.

Nomura et al [9] have reported that intestinal metaplasia is not a monoclonal lesion. However, the clonal status of intraepithelial neoplasia is unknown. To search for the point at which neoplastic proliferation is substituted for reactive proliferation, we analyzed the clonality of gastric carcinoma and precancerous lesions on the basis of a polymorphism of the X linked human androgen receptor gene (HUMARA).

HUMARA is characterized by a highly polymorphic trinucleotide-repeat (CAG) sequence proximal to the methylation site with a high incidence of heterozygosity, and the methylation inactivation pattern of the X chromosome [10,11,12] The expression of Ki-67 was detected in gastric carcinoma and its precancerous lesions by immunohistochemistry, and the relationship between the clonal status and Ki-67 protein expression was explored to find the biomarker of assessing susceptibility to gastric carcinoma.

Materials and methods

Tissue samples. Formalin-fixed paraffin embedded tissues from 174 cases of gastric endoscopic biopsy and surgically removed specimens, including intestinal gastric carcinoma (N=24), intestinal metaplasia (N=43), low grade intraepithelial neoplasia (N=56), and high grade intraepithelial neoplasia (N=51) in addition to normal gastric mucosa from 27 surgically removed specimens were supplied by the Department of Surgical Pathology of Shanghai Huashan Hospital, Fudan University. The mean age of the patients was 62 years (range 24—83 years), and all of the patients were female. All cases were diagnosed by two experienced pathologists.

Laser capture microdissection and DNA extraction. Five to ten 8 µm thick sections were cut from each block, and sections were mounted on a 1.4 µm membrane with metal frame slides. Paraffin was removed in two steps using xylene and the sections were rehydrated in descending concentrations of alcohol solution sequentially. The samples of intestinal metaplasia were stained with Alcian blue and periodic acid Schiff’s reagent (AB-PAS) to accurately display intestinal metaplasia glands. The acidic mucins (“intestinal” mucins) were stained with Alcian blue and exhibit blue. Neutral mucins exhibited red and were considered PAS positive. The samples of intraepithelial neoplasia and intestinal gastric carcinoma were stained with haematoxylin. The lesions were captured by Lcc1704 Veritas Laser Capture Microdissection and Laser Cutting System (Arcturus Bioscience, U.S.A) accurately with the guidance of haematoxylin and eosin staining section of every sample (Figure 1). The normal gastric mucosa was also captured as a normal control. DNA was extracted with QIAgen DNA Micro Kit (QIAGEN Cat.No.56304).

Enzymatic digestion. Ten microlitres of DNA were digested for 5h at 37°C with 5 units of the methylation sensitive restriction enzyme HpaII (Invitrogen Cat.No. 15209-018) in the presence of 2µl of 10x-REACT 8 enzyme buffer and 13µl DEPC treated water. The negative controls for each test were prepared in identical conditions with the exception of the enzyme. After digestion, the enzyme was inactivated by heating at 95°C for 5 min.

PCR amplification of human androgen receptor gene. The variable trinucleotide repeat sequence ((CAG), repeat n=11-30) of HUMARA exon 1 was amplified by nested PCR. Paired digested and undigested 2µl DNA samples were mixed with 2µl of J0x reaction buffer, 1.6µl of 25mM MgCl2, 2µl of dNTP (2mM each of dATP, dTTP, dCTP, and dGTP), 0.3µl of Taq polymerase(5U/µl, SAGON, China) and 1µl each of outer primers (HUMARA 1A 5'-GCTGTAGAAGGTGCTGTCCCTCAT-3' and HUMARA 2A 5'-GTCCAGACGCTACCAGAAGCTT-3' 10µM each) in a final volume of 20µl. Initial denaturation at 95°C for 5 min and amplification for 25 cycles of 95°C for 45s, 63°C for 50s and extension at 72°C for 60s was followed by terminal extension at 72°C for 10min. Two microliters of the amplification products were used for the second PCR containing 1µl each of inner primers (HUMARA 1B 5'-TCCAGAATCTGTTCTCCAGACGTC-3' and HUMARA 2B 5'-ATGGGCGTTGGGAGACCATCCTC-3'), Reaction system and cycling conditions were identical. HUMARA 1B was labeled at the 5' end with the fluorescent dye FAM.

Genescan analysis of clonality. One microliter of nested PCR products diluted 1:25 with distilled water was mixed with 6.85µl of HiDi formamide (ABI, Cat.No. 4311320) and 0.15µl of ROX500 (internal DNA size markers) (ABI, Cat.No. 401734). PCR products denatured at 95°C and cooled on ice (5 min each) were run in Applied Biosystems 3730 DNA Analyzers (ABI, U.S.A). The GeneMapper Software V4.0 (ABI, U.S.A) analyzed the PCR products by generating peaks; the peak height, peak area, and fragment size were propor-
tional to the concentration of the PCR fragments in the samples given. Heterozygosity at the HUMARA locus was the essential requisite for assessment of clonality. The peak area data were used to calculate a clonality ratio: \( \frac{\text{smaller allele/larger allele}}{\text{undigested}} / \frac{\text{smaller allele/larger allele}}{\text{digested}} \). A clonality ratio less than 0.25 or more than 4, corresponding to an allele reduction of 75 percent or more, was considered as monoclonality.

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed paraffin-embedded sections, using the two-step immunohistochemistry procedure. The primary antibody used in this study was Ki-67 (1:50 diluted, Changdao Biotech, Cat.No. M-0350, China). The supervision anti-mouse detection reagent (HRP) (Changdao Biotech, Cat.No. D-3001, China) was used as the second antibody to avoid problems with endogenous biotin in tissue. The expression rate of Ki-67 was evaluated by calculating the number of Ki-67 expression positive gastric epithelial cells among 1000 randomly counted gastric epithelial cells.

Statistical analysis. The correlation between the clonal status and the progression of the multistep gastric carcinogenesis was analyzed by Fisher’s exact test and the association between clonal status and expression rate of Ki-67 was examined using Spearman rank correlation test. Statistical analyses for correlations between the expression rate of Ki-67 and the progression of the multi-step gastric carcinogenesis were performed using Wilcoxon rank sum test and Kruskal-Wallis H test. Differences were considered statistically significant at \( P<0.05 \) (two-tailed).

Results

Clonality analysis. Informative cases. DNA from 172 of 174 samples was able to be amplified at the HUMARA locus and 34 of these samples were homozygous at this locus. A heterozygosity of 80.23% was identified in our study, which is similar to the result previously described by our group [13]. Nonrandom X chromosome inactivation (also know as X-inactivation Skewing [14–15]) was observed in 5 patients; the
DNA extracted from the nontumor tissue was methylated on the same allele. After digestion with HpaII, one of the alleles almost completely disappeared, indicating a monoclonal pattern of X chromosome inactivation (Figure 2). Therefore, 133 samples which were heterozygous for HUMARA gene and carried the approximately balanced methylation pattern for the alleles in the nontumor tissues could be used in clonality analysis, including intestinal metaplasia (N=32), low grade intraepithelial neoplasia (N=45), high grade intraepithelial neoplasia (N=36) and intestinal gastric carcinoma (N=20). PCR products were analyzed using GeneMapper Software (Applied Biosystems, U.S.A). The internal DNA size standards allowed for precise evaluation of the size of the fragments. We found the size of the alleles ranged from 195 to 250 bp, and the size of the two alleles differed by an integral multiple of 3 bp (representing the CAG unit).

**Evaluation of clonality.** Intestinal gastric carcinoma. Clonality analysis was performed on 20 tumors from the heterozygous patients. All of the samples were found to be demonstrably monoclonal (Table 1, Figure 3A). The presence of stutter peaks was attributed to the slippage of DNA polymerase during the amplification [16, 17], and the dominant peak was chosen to be analyzed.

**Intestinal metaplasia.** Five of 32 informative samples were monoclonal (5/32, 15.63%) (Tab.1). The remaining 27 intestinal metaplasia samples appeared polyclonal; that is, both HUMARA alleles could still be amplified after HpaII digestion (Figure 3B).

**Low grade intraepithelial neoplasia.** Three of 48 heterozygous patients were discarded due to the finding of monoclonality in the nontumor tissues; the skewed X chromosome inactivation pattern mimicked monoclonality and made the clonality results non-informative. Overall, 10 of 45 (22.22%) informative cases displayed a monoclonal pattern of X chromosome inactivation, while 35/45 were polyclonal (Table 1, Figure 3C).

**High grade intraepithelial neoplasia.** Among the 51 samples, 11 were homozygous, 2 samples failed to amplify, and 2 samples displayed the skewed X chromosome inactivation pattern. A majority of the informative samples (25/36, 69.4%) (Table 1) yielded monoclonal patterns of X chromosome methylation (Figure 3D).

The result suggested that monoclonal status increases with the progression of the multi-step gastric carcinogenesis. Significant statistical differences were found among the multiple stages of gastric carcinogenesis (P<0.01), except for the difference between intestinal metaplasia and low grade intraepithelial neoplasia (P=0.47).

**The expression of Ki-67 in gastric carcinoma and its precancerous lesions.** The expression rates of Ki-67 protein in normal epithelia, intestinal metaplasia, low grade intraepithelial neoplasia, and intestinal gastric carcinoma are shown in Table 1. The expression rate of Ki-67 protein was significantly higher in intestinal gastric carcinoma (100.0%) compared to intestinal metaplasia (69.44%) and low grade intraepithelial neoplasia (22.22%).

**Table 1. The incidence rate of monoclonality of gastric carcinoma and its precancerous lesions**

<table>
<thead>
<tr>
<th>Types of lesions</th>
<th>Monoclonal status</th>
<th>Polyclonal status</th>
<th>Total</th>
<th>The incidence rate of monoclonality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal metaplasia</td>
<td>5</td>
<td>27</td>
<td>32</td>
<td>15.63</td>
</tr>
<tr>
<td>Low grade intraepithelial neoplasia</td>
<td>10</td>
<td>35</td>
<td>45</td>
<td>22.22</td>
</tr>
<tr>
<td>High grade intraepithelial neoplasia</td>
<td>25</td>
<td>11</td>
<td>36</td>
<td>69.44  a b</td>
</tr>
<tr>
<td>Intestinal gastric carcinoma</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>100.0  a b c</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>73</td>
<td>133</td>
<td>45.11</td>
</tr>
</tbody>
</table>

a: Compared to the monoclonality incidence rate of intestinal metaplasia P<0.01. b: Compared to the monoclonality incidence rate of low grade intraepithelial neoplasia P<0.01. c: Compared to the monoclonality incidence rate of high grade intraepithelial neoplasia P<0.01.
Figure 3. Representative clonal analysis.

PCR products of increasing molecular mass are shown left to right. Peaks on the right are products of longer repeats of HUMARA, and those on the left are products of shorter repeats. The presence of stutter peaks was attributable to the slippage of DNA polymerase during amplification, and the dominant peak was chosen for analysis.

Morphologically normal gastric epithelia (A-1, B-1, C-1, D-1). Both alleles can be amplified before (A-1, B-1, C-1, D-1) and after (A-2, B-2, C-2, D-2) HpaII digestion, which shows that the HUMARA locus of the 4 cases is heterozygous.

A. Monoclonality of intestinal gastric carcinoma (case 11). Alleles at 208 and 220 bp are detectable before HpaII digestion (A-3), but only the 220bp allele can be amplified after digestion (A-4).

B. Polyclonality of intestinal metaplasia (case 45). Alleles at 222 and 225 bp (B-3) are unchanged (B-4) after HpaII digestion.

C. Monoclonality of low grade intraepithelial neoplasia (case 87). Alleles at 223 and 226 bp (C-3). Following HpaII digestion (C-4), the 226 bp allele cannot be amplified.

D. Monoclonality of high grade intraepithelial neoplasia (case 110). Both alleles (222 and 237 bp) can be amplified before HpaII digestion (D-3), but only the 237 bp allele can be detected after HpaII digestion (D-4).
sia, high grade intraepithelial neoplasia, and gastric carcinoma were 18.0%±4.5%, 25.9%±7.5%, 53.9%±9.3%, 71.5%±7.5% and 78.7%±13.3%, respectively, and the proliferative index was statistically different among the five stages (P<0.05) (Table 2, Figure 4). Similar to the clonal status, the proliferative index increased with the progression of the multistage gastric carcinogenesis. The expression rates of Ki-67 protein in the monoclonal samples of intestinal metaplasia, low grade and high grade intraepithelial

Figure 4. Ki-67 protein expression detection by immunohistochemistry in gastric carcinoma and its precancerous lesions (nuclear staining)
A Normal gastric epithelial ×100.
B Intestinal metaplasia ×100.
C Low grade intraepithelial neoplasia (polyclonal proliferation) ×100.
D Low grade intraepithelial neoplasia (monoclonal proliferation) ×100.
E High grade intraepithelial neoplasia ×100.
F Intestinal gastric carcinoma ×100.
Discussion

Chronic atrophic gastritis, intestinal metaplasia and intraepithelial neoplasia commonly precede intestinal type gastric adenocarcinoma. Endoscopy is widely regarded as one of the most sensitive and specific diagnostic tests for gastric cancer, and is able to detect early lesions. However, sometimes doubts arise as to whether a lesion is a reactive or regenerative proliferation, particularly in small biopsies. This dilemma can be resolved by clonality analysis-neoplastic proliferation is monoclonal, whereas reactive proliferation is polyclonal. The clonality of gastric cancer of the mouse was previously investigated using C3H/HeN↔BALB/c chimeric mice treated with N-methyl-N-nitrosourea. Gastric cancer was found to develop from a signal progenitor cell [18]. Intestinal metaplasia has been extensively studied as a possible premalignant condition in the human stomach [2, 19]. However, intestinal metaplastic glands appear polyclonal [9]. It is therefore important to find the point at which it starts to expand monoclonally along the multi-step gastric carcinogenesis.

To our knowledge, this is the first study investigating the clonality of intestinal type gastric carcinoma and precancerous lesion and its relation to Ki-67 protein expression. The results suggest that monoclonal status increases with the progression of the multi-step gastric carcinogenesis. However, the incidence rate of monoclonality in high grade intraepithelial neoplasia was higher than that in low grade intraepithelial neoplasia and intestinal metaplasia, which indicates that high grade intraepithelial neoplasia is the most critical stage among the multiple steps of progression of gastric carcinogenesis. The results also indicate that the classification of intraepithelial neoplasia—low grade or high grade intraepithelial neoplasia according to WHO classification—is rational. Patients with monoclonal lesions may be susceptible to or already present with invasive cancer, especially in those with lesions of high grade intraepithelial neoplasia. Therefore, the clonality analysis can be used to screen for patients who are predisposed to gastric cancer, so they can be followed up and receive appropriate therapy.

Ki-67, a nuclear antigen, is expressed in all phases of the cell cycle except G0 [20], and therefore can be used as a cell proliferation marker. In our study, we found that the normal gastric epithelial cells showed low proliferative index and intraepithelial neoplasia showed high proliferative index. The expression rate of Ki-67 increased with the progression of the multistage gastric carcinogenesis, and the proliferative index was associated with the clonal status in low grade intraepithelial neoplasia. The proliferation index of the group with monoclonal proliferation was higher than that of the group with polyclonal proliferation in intestinal metaplasia and low grade intraepithelial neoplasia. While the proliferative index of high grade intraepithelial neoplasia was very high, the Ki-67 index of the polyclonal group was higher than that of the monoclonal group. In addition to clonality analysis, the cell proliferative marker Ki-67 was also shown to be useful in the differential diagnosis of reactive proliferation and regeneration proliferation in early stages of precancerous lesions.

Although clonality analysis based on the pattern of X chromosome inactivation is a useful tool, there remain certain limitations. First, it is important to eliminate contamination of nonmalignant cells. To minimize risk of contamination, we used the widely applied Veritas Laser Capture Microdissection and Laser Cutting System [21, 22, 23] to accurately isolate the glands we are interested in. Since the technique is based on the pattern of inactivation of X chromosome, it can only be applied to the analysis of female patients. As gastric cancer is more frequent in males than in females [24], the results must be confirmed by investigation of a large number of cases. Since endoscopy as an effective means of detecting early lesions is regarded as a routine medical examination, there were a large number of endoscopic biopsy samples from Shanghai Huashan Hospital.

Table 2. The expression rate of Ki-67 in gastric carcinoma and its precancerous lesions

<table>
<thead>
<tr>
<th>Types of lesions</th>
<th>cases</th>
<th>The expression rate of Ki-67 (%)</th>
<th>The expression rate of Ki-67 in the monoclonal cases (%)</th>
<th>The expression rate of Ki-67 in the polyclonal cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal gastric epithelia</td>
<td>27</td>
<td>18.0±4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td>32</td>
<td>25.9±7.5 a</td>
<td>28.6±3.3</td>
<td>25.5±8.0</td>
</tr>
<tr>
<td>Low grade intraepithelial neoplasia</td>
<td>45</td>
<td>53.9±9.3 a b</td>
<td>64.8±4.3</td>
<td>50.8±7.8</td>
</tr>
<tr>
<td>High grade intraepithelial neoplasia</td>
<td>36</td>
<td>71.5±7.5 a b c</td>
<td>70.6±8.3</td>
<td>73.7±5.3</td>
</tr>
<tr>
<td>Intestinal gastric carcinoma</td>
<td>20</td>
<td>78.7±13.3 a b c d</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a: Compared to the expression rate of Ki-67 of normal gastric epithelia P<0.01. b: Compared to the expression rate of Ki-67 of intestinal metaplasia P<0.01. c: Compared to the expression rate of Ki-67 of low grade intraepithelial neoplasia P<0.01. d: Compared to the expression rate of Ki-67 of high grade intraepithelial neoplasia P<0.05. e: The expression rate of Ki-67 of the monoclonal cases compared with that of the polyclonal cases in the same lesion type P<0.05.
available for analysis. Another limitation is the significant X-inactivation [14,15], resulting in a normal tissue presenting a monoclonal pattern. Therefore, the normal control in the clonal analysis is indispensable. In order to understand the histogenesis of gastric precancerous and cancerous lesions, the clonality of normal single fundic gland and single pyloric gland was investigated by Nomura et al using HUMARA assay [25]. The study showed that about half of the normal single fundic glands were heterozygic and almost all single pyloric glands were homotypic. Therefore, in each sample, we microdissected the normal gastric mucosa from as many sites as possible, and mixed them together to find the true skewing of X chromosome inactivation.

The PCR products were analyzed by Applied Biosystems 3730 DNA Analyzers, and the peak height, peak area, and product size were given by GeneMapper Software V4.0; the heterozygosity of HUMARA gene and the clonal status of lesions were analyzed from these accordingly. The incidence rate of heterozygosity of HUMARA generated in our study is similar to results previously described by our group [13].

In conclusion, our results indicate that monoclonal status increases with the progression of multi-step gastric carcinogenesis and that clonal status is associated with the proliferative index in the early stages of precancerous lesions.

Reference