Overexpression of p73 as a Tissue Marker for High-Risk Gastritis

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Abstract

Purpose: Histologic assessment of high-risk gastritis for the development of gastric cancer is not well defined. The identification of tissue markers together with the integration of histologic features will be required for this assessment.

Experimental Design: Matched tumor/nontumor adjacent mucosa (NTAM) of 91 early gastric cancer and 148 chronic gastritis cases were evaluated for histologic characteristics (atrophy, intestinal metaplasia, chronic inflammation, polymorphonuclear infiltration, and Helicobacter pylori) by the Sydney System. Atrophy risk assessment was also evaluated by the Operative Link on Gastritis Assessment (OLGA) staging system. Eight tissue markers (BRCA1, HSP90, STAT1, FHIT, EGFR, p73, p53, p16INK4a) and EBV were also evaluated by tissue microarray/immunohistochemistry/in situ hybridization platform. Data were analyzed by contingency tables (2 × 2) using Fisher’s exact two-tailed test (P < 0.001) and integrated by Significance Analysis of Microarrays (SAM) and clustering analysis.

Results: Histologically, NTAM have severe intestinal metaplasia/chronic inflammation and severe atrophy assessed by Sydney and OLGA staging systems. H. pylori infection was similar in both groups, and EBV was found only in 5.5% of the tumor samples. Overexpression of p73 was higher in NTAM (50.5%) than in chronic gastritis (10.8%; P < 0.0001). Integration of histologic features and tissue markers showed that overexpression of p73, severe atrophy, and OLGA stage 4 were the most relevant features in NTAM. Clustering analysis correctly assigned NTAM and control cases (P < 0.0001).

Conclusions: Overexpression of p73 should be considered for the assessment of high-risk gastritis. SAM allows the integration of histology and tissue markers for this assessment. Clin Cancer Res; 16(12); 3253–9. ©2010 AACR.

Gastric cancer is the second leading cause of cancer-related deaths and the fourth most common malignancy worldwide (1). The incidence of gastric cancer shows considerable geographic variation. The East Asia, Eastern Europe, and Latin American regions still remain highly prevalent areas, whereas mortality from gastric cancer is declining globally (2). In spite of convincing evidence that gastric cancer is causally linked to Helicobacter pylori and consensus conference recommended population-based screening and treatment in highly prevalent areas (3), screening tests for the early detection of gastric cancer are needed. We recently found that DNA methylation of Reprimo was present in 90% (pair tumor and plasma samples) of gastric cancer cases but only in 10% of plasma from healthy donors (4). These findings support the idea that DNA methylation of Reprimo could be a potential noninvasive marker for the early detection of gastric cancer. However, early detection of this malignancy also requires histologic assessment of premalignant conditions. In spite of that the histologic features of noninvasive neoplasia (formerly dysplasia) are well defined (5), whereas criteria for risk assessments of premalignant conditions such as chronic gastritis are not (6, 7). In this scenario tissue markers, such as overexpressed proteins, have recently been explored as candidates for risk assessment (8). However, integration with the histologic features of premalignant conditions will be required to assess the potential clinical impact of these tissue markers. In this study, we have identified the overexpression of p73, intestinal metaplasia, and severe atrophy assessed by the
Sydney and Operative Link on Gastritis Assessment (OLGA) staging systems as being associated with high-risk chronic gastritis.

Materials and Methods

Clinical samples
Between 1984 and 2005, 91 surgically resected early gastric cancer cases from the Department of Pathology–Pontificia Universidad Católica de Chile (PUC) and the Instituto Chileno Japones de Enfermedades Digestivas–Hospital Clínico San Borja Arriaran (ICHJED-HCSBA), Santiago, Chile, and from Hospital Max Peralta-Cartago, Costa Rica were collected. All cases were surgically resected by total gastrectomy and were classified as early gastric cancer in accordance with the depth of invasion as proposed by the Japanese Research Society for Gastric Cancer (9). A total of 148 cases with history of symptomatic chronic gastritis were used as controls. These control cases were proposed by Rugge et al. (12–14). The evaluation was made by double-blind independent pathologists who were unaware of clinical data and histologic diagnoses.

Histologic variables
Paraffin blocks were cut and stained with H&E and Giemsa stains for histologic evaluation and H. pylori detection. Early gastric cancer cases were classified by histologic type (intestinal or diffuse) according to Lauren classification (11) and location. Chronic inflammation, polymorphonuclear activity, atrophic gastritis, intestinal metaplasia, and the presence of H. pylori were evaluated in nontumor adjacent mucosa (NTAM) from early gastric cancer and chronic gastritis controls according to the Updated Sydney System (10). For atrophy risk assessment, we also applied the new histological staging system, OLGA, proposed by Rugge et al. (12–14). The evaluation was made by two double-blinded independent pathologists who were unaware of clinical data and histologic diagnoses.

Tissue microarray construction
Tissue microarrays (TMA) were done by using a Manual Tissue Array II instrument (Beecher Instruments) as previously described (15). In brief, paraffin blocks from 91 paired tumor/NTAM and from 148 endoscopic gastric biopsies were selected, cut, and stained with H&E for the best histologic area. After whole-section glass slide evaluation, tissue area was selected for placement into the TMA by a circling on the glass slide and was identified in the corresponding paraffin block. A 1-mm stylet in the inner diameter was used to take two cylindrical core biopsies from each tissue block (donor block), with subsequent arraying into a new recipient paraffin block. In this way, all paired tumor and NTAM from early gastric cancer and endoscopic gastric biopsies from control cases were held in seven recipient blocks. An adequate case was defined as a tumor occupying ≥10% of the core area. Each case was in duplicate to avoid the loss of tissue during the cutting of the block. Slides (4 μm) were cut from each tissue array block, deparaffinized, and dehydrated for H&E and immunohistochemical staining.

Immunohistochemistry and in situ hybridization
Eight biomarkers were selected based on genes whose alterations in gastric carcinoma play a significant role in the promotion of chronic gastritis (STAT1; ref. 16), gastric epithelial cell response to H. pylori infection (p73; ref. 17), prognosis (FHIT, p16INK4a; refs. 18–21), early onset (BRCA1; ref. 15), and emerging molecular target for therapy (HSP90, EGFR; refs. 22, 23). In addition, the tumor suppressor gene p53 was also included because it is the most common genetic abnormality in gastric cancer (24). Immunohistochemistry (IHC) was done on 4-μm-thick section TMA blocks as previously described (15). Antibodies used in this study were STAT-1 (clone Rbx, Abcam), p73 protein α (clone 24, Novocastra), FHIT (clone SMP472, LabVision), p16 (clone 6H12, Novocastra), BRCA1 (clone SG-11, Zymed), HSP90 (clone JPZB4, Vision Biosystems/Novocastra), EGFR (clone 31G7, Zymed), and p53 (clone BP53-12-1, Biogenex). The results of the immunostaining were considered to be positive if ≥10% of the cells were stained in the nucleus (BRCA1 and

Translational Relevance
Recently, we found that DNA methylation of Reprimo could be a potential noninvasive marker for the early detection of gastric cancer (Clin Cancer Res. 2008;14:6264-9). However, early detection also requires risk assessment of premalignant conditions at tissue level. Histologic criteria for risk assessment are not well defined. Tissue markers, such as overexpressed proteins, have been recently explored as candidates for high-risk gastritis. Here, we have identified that among eight tissue markers (BRCA1, HSP90, STAT1, FHIT, EGFR, p73, p53, and p16INK4a), p73 was overexpressed in tumor and nontumor adjacent mucosa at a similar level in early gastric cancer cases in comparison with chronic gastritis cases (P < 0.0001). Integration of histologic features and tissue markers showed that overexpression of p73 and Operative Link on Gastritis Assessment (OLGA) stage 4 were the most relevant features in nontumor adjacent mucosa. Our data suggest that overexpression of p73 might have a role in the assessment of high-risk gastritis for gastric cancer development.

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H. pylori (rabbit polyclonal; clone CH-20 429, Novocastra) was also done using a similar protocol as described with a slight modification in the antigen retrieval method (Pepsin, 10 minutes at 37°C). In situ hybridization analysis for EBV was done using fluorescein-labeled peptide nucleic acid probe specific for EBER-1 and the DAKO hybridization kit according to the manufacturer’s instructions. Evaluation was made by

**Table 1.** Histologic characteristic of NTAM from early gastric cancer cases and chronic gastritis controls

<table>
<thead>
<tr>
<th>Histologic characteristics</th>
<th>NTAM (n = 91)</th>
<th>Controls (n = 148)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Chronic inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>9 (9.9)</td>
<td>61 (41.2)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Mild</td>
<td>27 (28.7)</td>
<td>66 (44.6)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>25 (27.5)</td>
<td>16 (10.8)</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>30 (33)</td>
<td>5 (3.4)</td>
<td></td>
</tr>
<tr>
<td>Polymorphonuclear activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>47 (51.6)</td>
<td>81 (54.7)</td>
<td>0.06</td>
</tr>
<tr>
<td>Mild</td>
<td>31 (34.1)</td>
<td>54 (36.5)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>9 (9.9)</td>
<td>8 (5.4)</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>4 (4.4)</td>
<td>3 (2)</td>
<td></td>
</tr>
<tr>
<td>Atrophy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>32 (35.2)</td>
<td>125 (84.5)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mild</td>
<td>17 (18.7)</td>
<td>18 (12.2)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>16 (17.6)</td>
<td>3 (2)</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>26 (28.6)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>20 (22)</td>
<td>142 (95.9)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mild</td>
<td>26 (28.6)</td>
<td>3 (2)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>20 (22)</td>
<td>3 (2)</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>25 (27.5)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>H. pylori</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>51 (56)</td>
<td>100 (67.5)</td>
<td>0.049</td>
</tr>
<tr>
<td>Negative</td>
<td>40 (44)</td>
<td>43 (29)</td>
<td></td>
</tr>
</tbody>
</table>

*Fisher’s exact test, two-sided P values for P.

**Table 2.** Tissue marker expression in tumor and NTAM from early gastric cancer cases and superficial gastritis mucosa from control cases

<table>
<thead>
<tr>
<th>Marker</th>
<th>Tumor (n = 91)</th>
<th>NTAM (n = 91)</th>
<th>Controls (n = 148)</th>
<th>P*</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Positive (%)</td>
<td>Positive (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCA-1</td>
<td>61 (67)</td>
<td>60 (66)</td>
<td>73 (49.3)</td>
<td>0.123</td>
<td>0.004</td>
</tr>
<tr>
<td>HSP-90</td>
<td>88 (97)</td>
<td>90 (99)</td>
<td>141 (95.3)</td>
<td>0.249</td>
<td>0.103</td>
</tr>
<tr>
<td>STAT-1</td>
<td>87 (95.6)</td>
<td>88 (97)</td>
<td>135 (91.2)</td>
<td>0.278</td>
<td>0.057</td>
</tr>
<tr>
<td>FHIT</td>
<td>83 (91)</td>
<td>82 (90)</td>
<td>123 (83.1)</td>
<td>0.194</td>
<td>0.05</td>
</tr>
<tr>
<td>EGFR</td>
<td>26 (28.5)</td>
<td>20 (21)</td>
<td>11 (7.4)</td>
<td>0.08</td>
<td>0.002</td>
</tr>
<tr>
<td>p73</td>
<td>38 (41.7)</td>
<td>46 (50.5)</td>
<td>16 (10.8)</td>
<td>0.058</td>
<td>0.000001‡</td>
</tr>
<tr>
<td>p16</td>
<td>10 (11)</td>
<td>3 (3.3)</td>
<td>0 (0)</td>
<td>0.031</td>
<td>0.054</td>
</tr>
<tr>
<td>p53</td>
<td>20 (22)</td>
<td>2 (2.2)</td>
<td>0 (0)</td>
<td>0.000021‡</td>
<td>0.1439</td>
</tr>
</tbody>
</table>

*Tumor versus NTAM.
†NTAM versus control.
‡Fisher’s exact test, two-sided P values for P.
two double-blinded independent observers who were unaware of clinical data and histologic diagnoses.

Statistical analysis and integration approaches
Histologic and tissue marker data were evaluated by contingency (2 × 2) table using Fisher’s exact test for categorical variables to determine differences between tumor and NTAM from early gastric cancer cases and control cases. All of the values were two-tailed, and statistical significance was defined as $P < 0.001$. Integration of histologic and tissue markers was done by Serial Analysis for Microarray (SAM), a multiple testing approach method that has been extensively applied in genomic research (25). Although SAM was originally designed for continuous variables, here we adapted it to dichotomous variables (0 or 1), so we were able to combine histologic and tissue marker variables. Data found by SAM were confirmed by logistic regression, a multivariate method for analysis of binominal-distributed variables (26). Finally, we carried out an unsupervised hierarchical clustering analysis (HCA) to explore the reliability of self-classification of early gastric cancer/NTAM and control cases, using the previous integrated histologic and tissue marker analysis. HCA is another genomic method previously used in TMA/IHC studies including gastric cancer (27–29). Both SAM and clustering analysis were done by using MultiExperiment Viewer 4.4 software (http://www.tm4.org/mev/; refs. 30–32).

Results
Histologic characteristics of NTAM from early gastric cancer and chronic gastritis control cases
According to Lauren classification, 72 early gastric cancers were intestinal type and 19 were diffused type. Table 1 details the histologic characteristics of NTAM and controls cases. NTAM has moderate to severe atrophy, intestinal metaplasia, and chronic inflammation in comparison with controls. These differences were highly

![Fig. 1.](image-url)

A, representative examples of positive and negative immunostaining for eight tissue markers and EBER-1 expression by in situ hybridization ($×$100) in tumor and NTAM from early gastric cancer cases and chronic gastritis controls. B, high magnification ($×$400) of (a) BRCA1- and (b) p73-positive controls, (c) p73-positive NTAM, (d) FHT-negative control, (e) FHT-negative NTAM, (f) p16-positive NTAM, (g) p16-positive tumor, and (h) positive EBV staining. No cases of chronic gastritis cases were positive for p16, p53, and EBER by in situ hybridization.
significant ($P < 0.001$). Polymorphonuclear activity was predominantly negative or mild in both groups (85.7% versus 91.2%, respectively). Twenty (58.8%) of 34 NTAM but only 1 (1.3%) of 75 chronic gastritis cases were OLGA stages 3 to 4 ($P < 0.001$). No differences were found according to Lauren histologic classification and/or tumor location. The prevalence of *H. pylori* infection was similar in both groups.

**Tissue marker expression in tumor and NTAM from early gastric cancer and chronic gastritis control cases**

Seven array blocks containing 660 cores and representing 91 early gastric cancers (91 paired tumors and NTAMs) and 148 chronic gastritis control cases were built and tested by IHC for eight tissue markers. A total of 646 (98%) cores were correctly evaluated by H&E examination. Missing cores were usually caused by exhaustion of tissue material. Table 2 shows a comparison of tissue marker expression among tumor and NTAM from early gastric cancer cases and chronic gastritis control cases. Figure 1 shows representative examples of positive and negative immunostainings of tumor/NTAM and control cases. In early gastric cancers a similar tissue marker profile was found between tumor and NTAM with the exception of p53, which was significantly overexpressed in tumors ($P < 0.001$). The comparison of tissue marker profile between NTAM and chronic gastritis control samples shows significant differences in p73 protein expression (50.5% versus 10.8%). These differences were highly significant ($P < 0.0001$). Comparisons of tissue marker profiles were not associated with location or tumor histology (data not shown). Sensitivity and specificity for association between p73 protein expression and early gastric cancer were 74.2% and 74.6%, respectively. EBV detection by EBER1 expression was found only in tumor tissue from 5 (5.5%) of early gastric cancer cases.

**Integration of histologic variables and tissue markers**

To integrate histologic variables and tissue markers we applied SAM, a popular method for expression profile analysis that can be adapted to other types of experimental data such as survival time or tumor stage (25). As seen in Fig. 2, SAM shows, in a graphical representation, that the chronic gastritis control group is characterized only by histologic features such as the lack of intestinal metaplasia, atrophy, and chronic inflammation. However, NTAM is characterized by overexpression of p73 and OLGA stages 3 and 4 intermingled with severe atrophy, intestinal metaplasia, and chronic inflammation according to the Sydney System. These data were confirmed by logistic regression analysis (data not shown).

**Self-classification of cases by clustering analysis**

Next, to explore the possibility that this integrated approach was also able to self-classify NTAM and chronic gastritis, we carried out an unsupervised clustering analysis. This widely used method in cDNA microarray analysis has also been used in TMA/IHC in several tumors including gastric cancer (27, 28). Thus, as shown in Fig. 3, this
Discussion

In this study we have found differences in histologic features such as atrophy, intestinal metaplasia, and chronic inflammation between NTAM and chronic gastritis by the Sydney System. The new OLGA staging system for atrophy risk assessment successfully assigned stages 3 and 4 to NTAM cases.

Although differences in expression of p73 have been described between tumor and chronic gastritis (33, 34), the most important and novel finding in this study is that the expression of p73 in NTAM is significantly higher than that in chronic gastritis. Because atrophy is considered a preneoplastic field for gastric carcinoma development, overexpression of p73 could be useful for risk assessment in cases of severe atrophy as shown by the OLGA staging system. Besides gastric carcinoma, overexpression of p73 has been described in other neoplasms such as prostate, lung, bladder, and renal carcinoma. In these malignancies, however, it has to be shown at tissue level, as we have shown here, that such overexpression occurs in a preneoplastic lesion at the same frequency as that in tumors. Therefore, p73 overexpression might emerge as a tissue marker not only for gastric carcinoma but also for other malignancies. We have also found differences in accumulation of p53 in tumor versus NTAM as has been described previously (35, 36). This finding supports the concept that inactivation of p53 occurs at early stages of gastric carcinoma.

Having found that p73 is a potential marker for risk assessment of chronic gastritis and that the new OLGA staging system successfully assigned stages 3 and 4 to NTAM cases, we attempted to integrate histologic features and tissue markers by using a systems pathology approach (37–41). To this end we applied SAM, a multiple testing approach that has been used mostly in genomic research (37). Therefore, SAM shows the admixture of the classical Sydney System and the new OLGA staging system for atrophy risk assessment. Furthermore, this novel approach confirms that overexpression of p73 might become the most significant feature that identifies high-risk chronic gastritis in cases of severe atrophy. These data can be obtained by logistic regression, a multivariate method of analysis of binomial-distributed variables (26). One of the main advantages of SAM, however, is that it gives a sequence of all tested variables and graphical representation showing the strength of variables from each group. Similarly, unsupervised HCA was also able to self-classify NTAM and control cases. In summary, our findings have shown that integration of histologic and tissue marker variables is possible. The overexpression of p73, being present mostly in NTAM from early gastric cancer, but rarely in chronic gastritis controls, suggests a potential role of p73 as a tissue marker of high-risk gastritis for development of gastric cancer. Finally, the goal of systems pathology is to present the data in an integrated way, which might be useful in switching from an interpretive and subjective morphologically oriented approach to a more objective, evidence-based tissue marker approach (39–41).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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