Heterogeneous Protooncogene Amplification Correlates with Tumor Progression and Presence of Metastases in Gastric Cancer Patients

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ABSTRACT

In order to evaluate the relevance of protooncogene alterations in gastric cancer and to specifically relate these alterations to types and stages of the neoplasia, we studied oncogenes of possible interest in gastric tumors with different clinical parameters. Fifty DNAs from primary gastric adenocarcinoma were analyzed, by the Southern blotting technique, for the presence of amplification or rearrangements of seven different protooncogenes: c-myc, c-erbB2, c-Ki-ras, c-Ha-ras, c-N-ras, hst, and c-mos. All the tumors analyzed were histologically classified and staged. Amplification of the following genes was found: c-myc (2 of 50), hst (3 of 50), c-erbB2 (5 of 50), and c-Ki-ras (5 of 50). The simultaneous amplification of hst (3 cases), c-myc (1 of 3), or c-Ki-ras (2 of 3) was observed. Analysis of DNAs from atrophic and metaplastic gastric mucosa (which can be regarded as preneoplastic lesions) of the 10 patients showing gene amplification demonstrated that this was limited to neoplastic cells. Considering protooncogene amplification in general (i.e., involving different genes and occurring to different degrees) and clinical parameters of tumors, we found a statistically significant association between amplification and both tumor progression and presence of metastases. Therefore, at least for the genes analyzed, amplification is a relatively infrequent phenomenon and represents a late event in the temporal development of gastric cancer.

INTRODUCTION

Protooncogenes are cellular genes with a fundamental role in cell growth, development, or differentiation. They can be activated (oncogenes) by a variety of molecular mechanisms resulting in qualitative and/or quantitative changes of their products. These mechanisms include point mutations, rearrangements, and amplification (for a review, see Ref. 1).

Numerous findings suggest that oncogenes are involved in human cancer both in the initial conversion of normal to malignant cells and in the progression of less aggressive cell types to more aggressive ones (1). A specific mutation of the Ki-ras gene has been found in human colon cancer, not only in neoplastic cells but also in premalignant lesions (2). This indicates that the mutation precedes the development of the malignancy. On the other hand, amplification of N-myc gene in neuroblastoma (3) and of erbB2 in breast cancer (4) has been correlated with a more advanced stage of the neoplasia and a poorer prognosis.

Although stomach cancer is a common malignancy in man, little is known about the oncogenes which may be associated with the origin or development of the tumor. Findings on amplification and single base mutations of several oncogenes have already been reported (5–13). However, in most cases the available data derive from sporadic observations, making it difficult to evaluate the relevance of these alterations. More over, no analyses have been made to specifically relate different gene alterations to types and stages of the neoplasia.

We therefore carried out a systematic study on 50 cases of gastric adenocarcinoma characterized by different clinical parameters. DNAs from neoplastic and control tissue samples were hybridized by the Southern blotting technique with probes homologous to seven different protooncogenes: c-myc, c-erbB2, c-Ki-ras, c-Ha-ras, c-N-ras, hst, and c-mos. These genes were chosen since they are possibly involved in stomach cancer, as suggested by previous data, or they are frequently activated in other types of solid tumors.

We found amplification of c-myc, c-erbB2, c-Ki-ras, and hst oncogenes. Our data indicate that amplification is a relatively infrequent phenomenon in gastric cancer, is limited to tumors at advanced stages, and is associated with the presence of metastases.

MATERIALS AND METHODS

Patients. We examined 50 untreated patients affected by gastric adenocarcinoma who were all from the Province of Forlì, a small area of north-central Italy, characterized by an incidence of stomach cancer among the highest in the world (14).

Tissue Samples. Primary tumors were obtained during surgery. Control tissues, both atrophic and metaplastic mucosa, were available in most cases. Tumors were histologically classified and staged according to both the Laurén (15) and the World Health Organization (16) classifications. Tissue fragments were stored in liquid nitrogen until utilized as a source of DNA.

Samples (about 10 ml) of peripheral blood from patients were taken in the presence of an anticoagulant and buffy coat cells were stored at −20°C until utilized for DNA extraction.

DNA Extraction and Southern Blotting. Frozen tissue fragments were converted to fine powder with a Brown Mikro-dismembrator (17). The powder was resuspended in the extraction buffer (18). High molecular weight DNA was isolated by proteinase K digestion and phenol/chloroform extraction (18).

Frozen buffy coat cells were used as a source of DNA, as previously reported (19).

DNA (10 μg) was digested to completion with the EcoRI restriction enzyme, fractionated on 0.8% agarose gel, and transferred to Zetabind filters (CUNO) as described (19). Filters were hybridized, washed, and exposed as reported previously (19). Suggestions from the membrane supplier were followed to remove probes and rehybridize filters.

Probes. The various probes, inserts from recombinant plasmids, were 32P-labeled at high specific activity (>1 × 105 cpm/μg) by nick-translation (20).

The following oncogene-specific DNA fragments were used as probes: 1.4-kilobase Clal-EcoRI fragment corresponding to the III exon of the c-myc gene, from the pMC413RC clone (21); 1-kilobase HindIII v-Ki-ras fragment, from the pHIH3 clone (22); 6.4-kilobase BamHI fragment corresponding to the c-Ha-ras locus from the pT24-C3 clone (23); 1.5-kilobase EcoRI N-ras-specific fragment, from the p52c-v clone (24); 1.1-kilobase EcoRI hst (KS) fragment, from the KS3 clone (25); 2.7-kilobase EcoRI mos-specific insert, from the pHM2A clone (26); 4.6-kilobase HindIII-SalI fragment corresponding to a full-length complementary DNA of the rat neu gene, from the pSV2neuNT clone (27).
RESULTS

We studied a total of 50 tumors from patients with gastric adenocarcinoma. Table 1 shows a clinical classification of the tumors examined.

Genomic DNAs extracted from neoplastic tissue samples were digested with EcoRI and hybridized by the Southern blotting technique to seven different probes, homologous to the following cellular protooncogenes: c-myc, c-erbB2, c-Ki-ras, c-Ha-ras, c-N-ras, hst, and c-mos.

In all cases, probes detected the genomic DNA fragments expected on the basis of restriction maps previously reported (5, 22, 25, 27-30). In 2 cases only was an extra fragment observed in addition to the EcoRI fragment commonly detected by the c-myc probe (5). Its presence in DNA from peripheral blood leukocytes of the same patients indicated that it was due to a genomic alteration rather than to a somatic rearrangement in neoplastic cells. (Molecular characterization of the c-myc variants will be given elsewhere.)

In 13 cases, with probes homologous to c-erbB2, c-myc, c-Ki-ras, and hst genes, we found an increased intensity of hybridization signals (Fig. 1). Rehybridization of all filters with at least three different protooncogene probes allowed us to exclude the possibility that this was due to a difference in the amount of DNA bound to filters (Fig. 2).

An increased hybridization signal could result from a true gene amplification or from the presence in neoplastic cells of extra copies of the chromosome carrying the gene under analysis. We ruled out this last possibility by rehybridizing relevant samples with a second probe lying on the same chromosome. In particular, c-mos was used as a control probe to rehybridize filters with samples showing c-myc amplification since both genes map to chromosome 8. c-Ha-ras was used as a control for hst (both genes lying on chromosome 11), int1 for c-Ki-ras (both on chromosome 12) and c10-HOX 2.1, for c-erbB2 (both on chromosome 17) (see Fig. 2 for an example).

Dilution experiments (Fig. 3) and densitometric scanning allowed us to estimate the degree of gene amplification. This was low (3- to 5-fold) in all samples except two, in which the hybridization signal obtained with the c-erbB2 and c-Ki-ras probes was increased at least 20-fold. The simultaneous amplification of hst (3 cases) and c-myc (1 of 3 cases) or c-Ki-ras (2 of 3 cases) was observed. Amplification was found to occur in tumors of different histological types at progression stages III or IV. Six of 10 DNAs showing amplification were from the 12 patients with metastases. All these observations and a classification of the tumors which were characterized by gene amplification are summarized in Table 2.

Additional analysis of DNA from atrophic and metaplastic mucosa of patients showing gene amplification demonstrated, without exception, that the amplification was only detectable in neoplastic cells (Fig. 3).

DISCUSSION

Using the Southern blotting technique we hybridized 50 DNAs from gastric adenocarcinoma with seven different probes homologous to c-myc, c-erbB2, c-Ki-ras, c-Ha-ras, c-N-ras, hst, and c-mos protooncogenes. The hybridization patterns allow us to exclude the occurrence of gross rearrangements (such as deletion/insertion, translocation) involving the genes analyzed. The two unexpected patterns found with the c-myc probe can be explained by a genomic alteration of the c-myc locus.

Ten of 50 DNAs showed gene amplification involving different protooncogenes, namely: c-myc (4%), c-erbB2 (6%), c-Ki-ras (10%), and hst (6%). Our finding that these genes can be amplified in gastric cancer is in agreement with previous observations (5-7, 31). The amplification frequencies we estimate for c-myc, c-Ki-ras, and hst genes are comparable to those obtained by other authors (31-33) surveying a more limited number of cases. During a recent study of Japanese patients, Yokota et al. (30) observed that the amplification of the c-erbB2 gene is frequent (5 of 13) in tubular adenocarcinoma and absent (0 of 30) in other histological types of stomach cancer. Our studies show an occurrence of only 3 of 41 cases in tubular

Table 1 Classification of tumors

<table>
<thead>
<tr>
<th>Classification</th>
<th>Intestinal</th>
<th>Diffuse</th>
<th>Signet ring</th>
<th>Tubular</th>
<th>Papillary</th>
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<tbody>
<tr>
<td>Lauren's classification (15)</td>
<td>38*</td>
<td>3</td>
<td>2</td>
<td>41</td>
<td>2</td>
</tr>
<tr>
<td>World Health Organization classification (16)</td>
<td>8</td>
<td>22</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimension</td>
<td>T1-T2</td>
<td>T3</td>
<td>T4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphonodal status</td>
<td>N0</td>
<td>N1</td>
<td>N2</td>
<td>N3</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>I-II</td>
<td>III</td>
<td>IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastases</td>
<td>M0</td>
<td>M1</td>
<td>M2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Including 19 poorly differentiated tumors.

Fig. 1. Southern blot analysis of EcoRI-digested DNAs from neoplastic tissues of different patients. The sizes of the fragments hybridizing to the different protooncogene-specific probes are indicated in kilobase on the left side of the autoradiograms. Patterns 4-A, 2-B, 1-C, 2-C, and 2-D are characterized by gene amplification.
ONCOGENES IN GASTRIC CANCER

Table 2

Cases characterized by gene amplification

<table>
<thead>
<tr>
<th>Patient</th>
<th>World Health Organization type</th>
<th>Lauren’s type</th>
<th>Dimension</th>
<th>Lymphonodal status</th>
<th>Stage</th>
<th>Metastases</th>
<th>Amplified oncogene</th>
<th>Degree of amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. C.</td>
<td>M</td>
<td>M</td>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>N&lt;sub&gt;1&lt;/sub&gt;</td>
<td>III</td>
<td>M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>c-myc</td>
<td>3-4</td>
</tr>
<tr>
<td>M. R.</td>
<td>T</td>
<td>I*</td>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>c-myc</td>
<td>3-4</td>
</tr>
<tr>
<td>V. M.</td>
<td>T</td>
<td>I</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N&lt;sub&gt;1&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>c-erb-B2</td>
<td>&gt;20</td>
</tr>
<tr>
<td>R. A.</td>
<td>T</td>
<td>I*</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>c-erb-B2</td>
<td>5</td>
</tr>
<tr>
<td>C. E.</td>
<td>T</td>
<td>I</td>
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<td>N&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>3</td>
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<tr>
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<td>M</td>
<td>I</td>
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<td>N&lt;sub&gt;1&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>c-Ki-ras</td>
<td>2-3</td>
</tr>
<tr>
<td>V. E.</td>
<td>T</td>
<td>D</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>c-Ki-ras</td>
<td>2-3</td>
</tr>
<tr>
<td>L. R.</td>
<td>T</td>
<td>I</td>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>N&lt;sub&gt;0&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>c-Ki-ras</td>
<td>&gt;20</td>
</tr>
<tr>
<td>R. R.</td>
<td>T</td>
<td>I*</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N&lt;sub&gt;1&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>c-Ki-ras</td>
<td>2-3</td>
</tr>
<tr>
<td>N. A.</td>
<td>T</td>
<td>I*</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>c-Ki-ras</td>
<td>2-3</td>
</tr>
<tr>
<td>C. E.</td>
<td>M</td>
<td>I</td>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>N&lt;sub&gt;1&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>hst</td>
<td>5</td>
</tr>
<tr>
<td>L. R.</td>
<td>T</td>
<td>I</td>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>hst</td>
<td>2-3</td>
</tr>
<tr>
<td>B. C.</td>
<td>T</td>
<td>I</td>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>IV</td>
<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>hst</td>
<td>3</td>
</tr>
</tbody>
</table>

* M, mixed or mucinous; T, tubular; S, signet ring; I, intestinal; D, diffuse.
* a,b,c,d Same patients in this table.
* Poorly differentiated.

Fig. 2. Autoradiograms obtained by hybridizing the same filter with hst (A) and c-Ha-ras (B) probes. Lane 5 is characterized by hst gene amplification. This control experiment allows us to exclude both differences in the amount of DNA bound to the filter and the presence in sample 5 of extra copies of chromosome 11, on which hst and c-Ha-ras genes are located.

Fig. 3. c-Ki-ras hybridization patterns of EcoRI-digested DNAs from atrophic (A), metaplastic (M), and neoplastic (N) gastric mucosa of the same patient. The c-Ki-ras gene is only amplified in DNA from cancer tissue (compare lanes 1, 2, and 4, where 10 μg of DNA are loaded). Decreasing amounts of DNA (12.5–1 μg) from cancer tissue are loaded in lanes 3–8 in order to evaluate the c-Ki-ras amplification degree (>20-fold).

Our results also show that gene amplification is limited, in all cases, to transformed gastric mucosa. It is, in fact, completely undetectable in atrophic and metaplastic tissue areas, which can be regarded as preneoplastic lesions. The observed gene amplification should therefore represent a late event in the temporal development of gastric cancer. Obviously, we cannot exclude the presence of a small number of cells, in preneoplastic areas, harboring amplified oncogenes and having some proliferative advantage. These cells would be undetected due to the dilution of their DNA during extraction from the whole tissue sample.

We checked for correlations between protooncogene ampli-
fication and the clinical parameters reported in Table 1 (i.e., tumor type, dimension, stage, lymphonodal status, and metastases). We considered one gene at a time and found no statistically significant correlation, probably because of the low number of cases showing amplification. However, for all genes, amplification was mostly observed in patients with advanced and metastatic tumors. Due to this precise trend, data were pooled to check for correlations between amplification in general (i.e., involving different protooncogenes and occurring to different degrees) and clinical parameters. In this way, although involving oncogenes coding for different functions, amplification was regarded as a general phenomenon deeply affecting cell growth regulation. We found a significant $\chi^2$ value for the association between tumor progression and gene amplification which turned out to be typical of stage IV tumors (9 of 27 versus 1 of 23; $P = 0.025$, 1 d.f.). Moreover, 6 of 10 DNAs showing amplification were from the 12 patients with metastases. The $\chi^2$ for the association between amplification and presence of metastases was highly significant (6 of 12 versus 4 of 38; $P = 0.003$, 1 d.f.).

In conclusion, protooncogene amplification in gastric adenocarcinoma is a relatively infrequent phenomenon and generally occurs at a low degree. Amplification is absent in tissue areas showing preneoplastic characteristics, being limited to advanced or metastatic tumors. If our findings are considered as a whole, amplification seems to represent a late event in the temporal development of gastric cancer and a marker of progression in this type of tumor. We cannot, however, completely exclude the possibility that amplification occurs early in the neoplastic process and results in a more aggressive phenotype. In this case it should represent a negative prognostic marker.

Two groups of patients with advanced tumors showing and not showing amplification are being followed up clinically on a long-term basis. If amplification actually represents a negative prognostic marker, patients with advanced tumors characterized by amplification will have a poorer prognosis. Otherwise, the idea that amplification represents a late event in the development of gastric cancer could be definitively confirmed.

REFERENCES

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