DNA ploidy in early gastric carcinoma (T1): a flow cytometric study of 100 European cases

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Abstract

DNA ploidy of 100 early gastric carcinomas (T1) was analysed by flow cytometry on archival material from five European centres and was correlated to morphological features and clinical behaviour. Tumours were classified according to the macroscopic appearance, histological type, and growth pattern. Aneuploidy was observed in 39% of tumours. Aneuploidy was more frequent in submucosal than in mucosal tumours (p=0.04), in raised than in flat or ulcerated lesions (p=0.001), and in the intestinal histological than in the diffuse types (p=0.016). The presence of lymph node metastasis in 10 cases had no obvious relation to DNA ploidy. Five related deaths occurred during the follow up (6 months - 16 years) of 84 patients. These results are similar to those reported in a large Japanese series suggesting no major differences between the two populations. Although follow up data were insufficient to relate DNA ploidy to tumour behaviour in this study, the Japanese experience shows that particular attention should be paid to early direction and complete surgical excision of raised intestinal type T1 carcinomas that have a Pen A growth pattern and are aneuploid.

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Numerous studies world wide have indicated that gastric carcinoma confined to the mucosa or submucosa, so called 'early' or T1 gastric cancer, has an excellent prognosis after surgery. Nevertheless, a small proportion of T1 tumours recur, either locally within the gastric remnant or distally as metastases, and studies from Japan have suggested that the morphology and DNA ploidy of the primary neoplasm might be used to predict behaviour. Kodama et al produced a detailed morphological classification of early gastric cancers into superficially spreading ('Super'), penetrating ('Pen'), and small mucosal types. Tumours giving rise to gastric stump recurrence were usually of Super type, while those producing distant metastases were usually of Pen type, and especially of the Pen A subtype that was characterised by raised endoscopic appearance, a well differentiated, intestinal type of histology, and an expansive, penetrating pattern of submucosal infiltration.

Pen A tumours, though forming only 8% of cases in one series, accounted for all early gastric cancer showing venous invasion and 80% of those associated with liver metastases; their 5 year survival rate was 65% compared with 95% for all early gastric cancers. Further studies by the same workers using cell nuclear DNA microspectrophotometry showed that Pen tumours, and again Pen A tumours in particular, were usually of so called 'high ploidy' or aneuploid. Moreover, 17% of small mucosal early gastric cancers showed similar DNA abnormalities and at least two of six aneuploid small mucosal tumours gave rise to distant metastases compared with none of 25 diploid tumours. It was proposed that DNA aneuploidy was an important marker of biological aggressiveness in early gastric cancer. While most T1 tumours were diploid, relatively indolent lesions that were usually cured by surgery unless inadequate excision led to gastric stump recurrence, a minority were aneuploid tumours which were frequently of a differentiated, intestinal pattern that included aggressive, potentially angio-invasive neoplasms with metastatic potential that progressed rapidly from small mucosal tumours to advanced gastric cancer, usually through a Pen A type intermediate.

Other Japanese groups have subsequently confirmed a relationship between tumour DNA content and recurrence, metastatic potential and survival in early gastric cancer using microspectrophotometric analysis of tissue sections but we are aware of only one study in western populations involving 51 Italian cases. It has been suggested that gastric cancer in the west is biologically different from that in Japan. We have therefore carried out a multicentre flow cytometric study of DNA ploidy in 100 European cases of early gastric cancer, relating tumour DNA content to morphological features and clinical behaviour.

Methods

The DNA content of 107 early gastric carcinomas (limited to the mucosa or submucosa) was analysed by flow cytometry using formalin fixed, paraffin embedded material. Seven cases were excluded because of high values of coefficient of variation.

Tissue was obtained from patients submitted to curative gastrectomy for gastric cancer at Hospitalis Civis, Lisbon, Portugal (n=41), University of Wales College of Medicine, Cardiff, Wales (n=27), UMDS Guy's Hospital, London (n=14), Institute of Oncology, Lisbon, Portugal (n=10), and the General Hospital, Birmingham (n=8). Fifty six patients were male and 44 female, with a mean age of 59-3 years (range 27-83 years).

Follow up data greater than 6 months (6-126 months, mean 3-3 years) were available in 84 patients.

Serial sections 3 µm, 40 µm, and 3 µm thick were cut from a representative block of 100 tumours. Histology was assessed on haematoyxlin and eosin stained sections 3 µm
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thick. Carcinomas were classified according to macroscopic appearance (protruded, elevated, flat, depressed, excavated), histological type (papillary, tubular, mucous, signet ring, single cells, neuroendocrine), and according to Kodama’s criteria based on tumour size, growth pattern and depth of invasion: small mucosal M or SM, tumours less than 4 cm confined to the mucosa (M) or invading the submucosa (SM); Super M or SM, superficial spreading tumours larger than 4 cm, confined to the mucosa (M) or invading the submucosa (SM); Penetrating types A and B with expansive (A) or infiltrative (B) invasion of the submucosa, although smaller than 4 cm.

Suspensions of nuclei for DNA flow cytometry were prepared from 40 μm thick sections according to Hedley et al.20 Sections were dehydrated in xylene and rehydrated through serial alcohols to distilled water. The tissue was incubated in 0.5% pepsin solution at pH 1.5 (Sigma Ltd, Poole, Dorset, UK) in a waterbath at 37°C with intermittent vortex mixing for 45 minutes. Digestion was stopped by adding 5 ml of phosphate buffered saline, and the samples were filtered through a 30 μm gauge, centrifuged at 3000 rpm for 10 minutes at room temperature, and most of the supernatant discarded. The pellets were then resuspended using a syringe and needle, and digested with 0.5 ml ribonuclease (Sigma Ltd) in a waterbath at 37°C for 15 minutes. Suspensions were then incubated with 1 ml of 0.1% propidium iodide (Sigma Ltd) solution in phosphate buffered saline for 20 minutes at room temperature before reading.

The DNA content was analysed using an Ortho Cytoflurogaf 50 H (Ortho Diagnostics, Westwood, MA, USA) equipped with a 50 mW argon-ion laser (Lexel, Palo Alto, CA, USA) tuned to 488 nm and measuring forward and orthogonal light scatter and red fluorescence. Between 10 000 and 20 000 nuclei were analysed. A two parameter plot (cytogram) of the peak of the signal from DNA fluorescence versus its integrated area was displayed and a region set to exclude clumped nuclei and debris from further analysis.21 A cytogram of 90° versus forward angle light scatter was then displayed. If two distinct clusters were observed, regions were set on these and separate DNA histograms for each cluster were recorded. If there was only one cluster, one region was set to exclude, as far as possible, degraded nuclei. In 32% of the samples, nuclei from malignant cells could be selected on the basis of light scatter and two DNA histograms could be obtained, one consisting predominantly of normal nuclei, the other predominantly of tumour nuclei. Once the DNA histograms had been recorded, the data were transferred to an IBM PC computer and further analysis was performed using software written by one of the authors (MGO).

The quality of DNA histogram could be quantified by measuring the coefficient of variation (CV) across the G1/G0 peak. The SD was calculated on the computer by setting a region to encompass the G1/G0 peak. The CV is then defined at (100×SD)/peak channel %. The CVs varied from sample to sample, lying for the samples used in the study, between 3-6% and 11% (mean 7-8%). If the CV was greater than 11%, because of excessive degradation of the DNA, the sample was excluded from the analysis. The quality of the material varied depending on the centre from which the blocks were obtained. This observation underlines the importance of careful fixation and handling of the tissues.

The DNA index (DI) for 100 tumours was calculated as the ratio of the DNA content of the tumour cells to the diploid DNA content of the normal cells within the sample. A sample was recorded as aneuploid if the G1/G0 peaks from the tumour cells could be resolved from those of the normal diploid cells (the resolution depended on the CV of the G1/G0 peak).

An estimate of the percentage of cells in the phase of the cell cycle was taken as a measure of proliferative activity. The estimate was made by setting a region on the centre of the DNA histogram to exclude the G1 and G2/M peaks and to include the centre of the S phase clear of the G peaks. The number of nuclei in this region was expressed as a percentage of the nuclei lying within a region set to include all phases of the cell cycle; the S phase was computed by doubling this number. In aneuploid tumours the analysis excluded the diploid G1/G0 peak from any normal nuclei. Any possible contribution from cycling normal cells was ignored. This is unlikely to introduce any major error since normal and abnormal nuclei can be distinguished on the basis of light scatter and we have found that few of the normal cells are in cycle (Ormerod and Imrie, submitted for publication). In the case of diploid tumours, the normal and abnormal nuclei cannot be separated on the DNA histogram and, unless the nuclei could be distinguished on the basis of light scatter, the S phase fraction will have been underestimated because the tumour G1/G0 peak will have been contaminated with nuclei from normal cells. For this measurement to have significance, the sample must have produced a reasonable DNA histogram (CV of the G1/G0 peak 8%). The analysis was not made on histograms whose CVs were larger than this. The S phase fraction was estimated in 68 tumours. It should be noted that, because of the limitations in the method, significance should not be given to a measurement of S phase beyond separating tumours into those with high or low proliferative fractions.

STATISTICAL ANALYSIS

Associations between ploidy and other histological variables were investigated using the chi-squared test. Where frequencies in various cells of a contingency table were small, Fisher’s exact test was used instead.

Results

DNA PLOIDY

Figure 1 shows a typical DNA histogram recorded by flow cytometry; the different phases of the cell cycle are marked on the histogram. There is only one G1 peak resolved and this tumour was scored as diploid (DI=1).

Gut: first published as 10.1136/gut.34.2.230 on 1 February 1993. Downloaded from http://gut.bmj.com/ on September 15, 2023 by guest. Protected by copyright.
Figure 1: DNA histogram from a diploid tumour.

Figure 2 shows an example of an aneuploid tumour (DI=1.6) from which there were two clusters observed in the cytogram of orthogonal versus forward light scatter. By gating on the cytogram of light scatter, the DNA histograms of the two clusters could be recorded separately. The cluster with lower light scatter contained nuclei with a diploid DNA content; that with higher scatter contained predominantly aneuploid nuclei. The latter histogram was used for the calculation of the percentage of cells in S phase.

Sixty one tumours were considered diploid (61%) and 39 aneuploid (39%) (DNA indices 0.9; 1.1–1.8) of which eight showed only a small aneuploid peak.

CORRELATION OF DNA PLOIDY AND CLINICOPATHOLOGICAL PARAMETERS
A significant difference in DNA ploidy was found between tumours confined to the mucosa and those invading the submucosa (28% and 47% aneuploid respectively) (p=0.04).

MACROSCOPIC TYPE
There was also a significant difference between macroscopic types (Table I), the percentage of diploid tumours being higher (64%) in flat, depressed, and excavated types than in the protruded and elevated tumours (39%) (p=0.001).

HISTOLOGICAL TYPE
DNA content also seems to be related to histological types, the signet ring tumours being predominantly diploid (83%) compared with 55% of the tubular types (p=0.016) (Table II).

GROWTH PATTERN
A relationship between DNA ploidy profiles and growth patterns was observed (Table III).

Aneuploidy was found in most of the Pen A tumours (70%) and in 50% of the larger tumours (Super mucosal types) compared with 20% in the small mucosal types (p=0.008).

LYMPH NODE METASTASES AND DIFFERENTIATION
DNA ploidy did not correlate with tumour differentiation or the presence of lymph node metastases that were only found in 10 cases (five diploid and five aneuploid).

S PHASE FRACTION
The S phase fraction estimated in 68 tumours showed a mean value of 8.99% (range 2.0–38.3). No correlation was found between the S phase fraction and any of the other parameters analysed, except DNA ploidy.

Tumours with an S phase fraction <15% were predominantly diploid (47/57) compared with those with S phase >15% (2/11 diploid) (p=0.001). This result was as expected, since in the diploid tumours, if the normal and tumour nuclei could not be separated by light scatter, the DNA histogram would have contained nuclei from normal cells in G1; the S phase would then have been underestimated.

SURVIVAL
Follow up data were not obtained on 12 patients. Eighty eight patients have been followed up and data on survival beyond 6 months was available in 84 (range 6 months – 16 years; mean 3.3 years).

There were five related deaths (interval between diagnosis and death ranging from 1 to 3 years), four postoperative deaths and four deaths from other causes.

Recurrence occurred in three cases (free interval ranging from 6 months to 3 years). Two of the recurrences were in patients with resection margins that had been affected.

Because of the small number of related deaths during this period it is not possible to carry out
TABLE I DNA ploidy related to growth pattern

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Small mucosal M</th>
<th>Small mucosal SM</th>
<th>Super mucosal M</th>
<th>Super mucosal SM</th>
<th>Pen A</th>
<th>Pen B</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>22</td>
<td>9</td>
<td>8</td>
<td>9</td>
<td>3</td>
<td>10</td>
<td>61</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>5</td>
<td>3</td>
<td>8</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>39</td>
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<tr>
<td>Total</td>
<td>27</td>
<td>12</td>
<td>16</td>
<td>18</td>
<td>10</td>
<td>17</td>
<td>100</td>
</tr>
</tbody>
</table>

TABLE II DNA ploidy related to histological tumour type

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Papillary</th>
<th>Tubular*</th>
<th>Mucinous</th>
<th>Signet ring*</th>
<th>Single cells</th>
<th>Endocrine</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>6</td>
<td>30</td>
<td>2</td>
<td>19</td>
<td>3</td>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>4</td>
<td>25</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>39</td>
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<tr>
<td>Total</td>
<td>10</td>
<td>55</td>
<td>6</td>
<td>23</td>
<td>5</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

* p<0.016.

TABLE III DNA ploidy related to macroscopic type of tumour

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Promoted</th>
<th>Everted</th>
<th>Flat</th>
<th>Depressed</th>
<th>Escarinated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>11</td>
<td>3</td>
<td>7</td>
<td>28</td>
<td>12</td>
<td>61</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>15</td>
<td>7</td>
<td>5</td>
<td>8</td>
<td>4</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>10</td>
<td>12</td>
<td>36</td>
<td>16</td>
<td>100</td>
</tr>
</tbody>
</table>

Discussion

Our finding of a 39% frequency of DNA aneuploidy in early gastric cancer, as assessed by flow cytometry studies in archival material, is remarkably similar to the figure of 37% found in the smaller Italian study of Tosi et al. Rather surprisingly, it is also similar to the 45% frequency of DNA aneuploidy that we have found previously by flow cytometry in advanced gastric cancer, a value that is well within the range of 34%-73% described by other European groups. This suggests that major changes in tumour DNA content are unlikely to be of prime importance in the progression from early to advanced gastric cancer. On the other hand, we found DNA aneuploidy to occur significantly more frequently in submucosal tumours compared with intramucosal carcinomas.

Direct comparison of our results with those of most Japanese studies of early gastric cancer is difficult because most have used static rather than flow cytometry – the only large Japanese study to use flow cytometry on archival material was that of Kimura and Yonemura, and although they studied 171 early gastric cancers among a total of 493 gastric tumours, the results for early gastric cancers were not presented separately. Data from studies using different cytometric methods are hardly comparable.

Flow cytometry allows the rapid and automatic analysis of large numbers of cells but it may miss small aneuploid subpopulations that can be picked up by the more sensitive but extremely time-consuming static methods that enable more precise selection of tumour fields for analysis. Indeed we have preliminary data to suggest that static cytometry identifies minority aneuploid populations in advanced gastric cancers more frequently than flow cytometry, although this is not the case for early tumours. This would be in keeping with advanced tumours being larger and more heterogeneous.

Despite the problems in comparing the results of our study with the static cytometric Japanese findings, there are sufficient similarities to suggest that there is no major difference between early gastric cancer in Japanese and European populations. In both groups, abnormal DNA patterns were more frequent in submucosal than mucosal tumours, more frequent in raised than flat or ulcerated tumours, and more frequent in intestinal type (tubular, papillary, and mucinous) tumours than in diffuse type (signet ring cell) tumours. Moreover, the Pen A variety, though uncommon (8% Japanese, 10% European), showed a high frequency of aneuploidy in both populations. While we have insufficient follow up data to comment meaningfully on factors related to prognosis, it is interesting to note that the three cases of local recurrence occurred in aneuploid tumours. We conclude that since morphology and DNA cytometry show no evidence of a fundamental difference between early gastric cancer in Japanese and western populations, the first clinical, pathological, and basic scientific experience of early gastric cancer in Japan is highly relevant to that in Europe. Particular attention should therefore be paid in the west to the early endoscopic or radiological detection and complete surgical extirpation of raised, intestinal type T1 gastric carcinomas that have a Pen A growth pattern and an aneuploid DNA content. These observations need to be tested in prospective studies in well defined cases.

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