DNA-ploidy in advanced gastric carcinoma is less heterogeneous than in early gastric cancer

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Abstract. This analysis of DNA-ploidy heterogeneity in advanced gastric carcinomas is consistent with the hypothesis of the emergence of a single aneuploid cell clone as a crucial mechanism in the progression from early gastric carcinoma to advanced gastric cancer.

The prognostic value of DNA-ploidy in gastric cancers has been a matter of controversy. Tumour DNA-ploidy heterogeneity, the presence within the same tumour of multiple stemlines differing in DNA content, has been described in various tumours including gastric cancers. The occurrence of such heterogeneity has been accepted as an explanation for the divergent DNA-ploidy results in this type of tumours.

A previous study of early gastric cancers suggested that in pure diploid superficial carcinomas, genetic instability might lead to a cell clone which has undergone a ploidy shift and is more aggressive. If so, this would initially result in DNA-ploidy heterogeneity. Proliferative dominance of the aneuploid clone could eventually evolve to a homogeneous aneuploid tumour.

In order to test this hypothesis, we studied DNA-aneuploidy and DNA-ploidy heterogeneity in advanced gastric carcinomas. We performed DNA cytophotometry on multiple samples collected from 16 advanced gastric carcinomas and found 15 DNA-aneuploid tumours (94%) and one diploid tumour. Multiple DNA-stemlines were found in 4 cases (26%). Analysis of proliferative activity performed on the same samples revealed higher proliferation rate in DNA-ploidy homogeneous tumours than in aneuploid heterogeneous tumours. Heterogeneous tumours did not overexpress p53. These results confirm that DNA-aneuploidy is frequent in advanced gastric cancer and demonstrate that a majority of these aneuploid tumours are not DNA-ploidy heterogeneous. Furthermore, the higher proliferative activity in homogeneous-aneuploid carcinomas and their more frequent overexpression of p53 support the hypothesis that in gastric cancer tumour progression implies the development of a dominant and more aggressive (higher proliferative activity, p53 overexpression) aneuploid cell clone.

Keywords: Gastric adenocarcinoma, heterogeneity, DNA ploidy, prognostic factors

1. Introduction

Several studies have reported a correlation between DNA-ploidy and prognosis in gastric carcinoma [1,11–13,26,29,34]. Korenaga et al. suggested that DNA-ploidy is a major determinant of survival. Others have indicated that there is no correlation between prognosis and DNA-ploidy [5]. These contrasting results could be explained by intratumoral heterogeneity in DNA-ploidy. The presence of intratumoral heterogeneity in DNA-ploidy is generally accepted, and has been described in various tumours [2,3,6,7,10,14,17–24,30,31,33–35]. Several studies have now been performed on this subject, comparing the DNA content of tumour cell populations in multiple samples of the tumours. DNA-heterogeneity has been estimated to run between 20 to 60% in gastric cancers and 7 to 30% in colorectal carcinomas [7,10,20,30] and to reach 54% in oesophageal carcinomas [35]. Some authors have indicated that DNA content heterogeneity is better correlated to tumour progression in gastric carcinomas than presence of aneuploidy [7,28].
In a previous study, we determined that ploidy heterogeneity is present in early gastric carcinoma (EGC) and this heterogeneity occurs more frequently in tumours invading the submucosa. This led us to hypothesise that genome instability plays an important role in gastric carcinoma progression [20]. On the basis of these results we considered the possibility that in DNA-diploid early gastric carcinomas, instability of the genome leads to the emergence of aneuploid cell clones, reflected in DNA-ploidy heterogeneity. Overgrowth of a dominant clone during neoplastic progression eventually could lead to DNA-homogeneous aneuploidy.

In order to confirm this hypothesis, a series of advanced gastric carcinomas was analysed for DNA-ploidy. DNA-ploidy heterogeneity was assessed through the analysis of multiple tumour samples. We also studied whether a correlation exists between ploidy and proliferative activity or p53 overexpression.

2. Material and methods

2.1. Material

Sixteen cases of advanced gastric carcinoma (gastric carcinoma with invasion of at least the muscularis propria) diagnosed in gastrectomy specimens obtained between 1993 and 1999 were used for this study. The nine more recent cases underwent histopathological mapping in detail (cartography), which allowed detailed histological analysis of the tumours and measurement of the size (Fig. 1), following the protocol of Mori [15,16]. The other seven cases were routinely sampled. All available gross and histological material was reviewed. For the nine cases with cartography, one of every two blocks was sampled for cytophotometric DNA-ploidy analysis. For cases without systematic cartographic tissue sampling, the number of blocks for the analysis was calculated as a function of the maximal area of the tumour (one block per 1.5 cm²) depending on the number of available blocks. In addition, one block of normal gastric mucosa was sampled for each case. When a case presented lymph node metastases, metastatic tumour tissue was also sampled. Hematoxylin and eosin stained slides were screened to identify the different layers of the gastric wall. For selective evaluation of DNA-ploidy, the intramucosal, submucosal components, the muscularis propria and subserosa when available, were separately sampled on the corresponding paraffin block by means of a dissecting microscope to account for the depth of infiltration. Overall 176 paraffin blocks were used resulting microdissection in 425 tumour cell samples.

Fig. 1. AGC in the resected specimen prepared for cartography. The lesion is evident in the resected specimen. The tumour containing the surrounding wall was cut into multiple slices parallel to the lesser curvature at interval of 4 to 5 mm.

2.2. DNA-ploidy analysis

All 425 tissue samples were processed following the technique of Hedley, which provided a cytospin of each sample [9]. For Feulgen staining, the cytospins were first hydrolysed in 5 N HCl at 25°C for 45 minutes. The hydrolysis was stopped by rinsing in distilled water. The specimens were then stained with Schiff reagent (Schiff reagent for microscopy, No 1.0933, Merck) for 60 minutes. Sulfite rinse was used. After dehydration in a graded series of alcohols and xylene the cytospins were coverslipped using Eukitt mounting medium. DNA analysis was performed on Feulgen-stained cytospins using an image analysis system (SAMBA, Alcatel TITN Answear, Grenoble, France). A minimum of 200 nuclei was measured per cytospin (300 nuclei for most cases). Only well preserved nuclei were selected by the operator using the gallery program of the SAMBA system. As controls we used 30 to 50 human lymphocytes from a separate slide containing thymus specimen (external reference). At least 6 tissue polymorphonuclear leukocytes were used as internal reference. The coefficient of variation of the DNA index thus measured was below 5%. Histograms exhibiting a G0/1 main peak at 2C were considered to be diploid. DNA index (the mean nuclear DNA content of the G0/1 compartment of a cell population divided by the DNA content of the G0/1 compartment of a similarly
processed group of known diploid cells) for diploidy ranged between 0.85 and 1.15. Considering that in normal tissues and most low-grade or slowly proliferating neoplasms, approximately 85% of the cell population constitute the G0/1 peak and 15% of the cells are in the S-phase and G2/M phases, we only considered as aneuploid the samples in which more than 15% of the cells had a DNA-index in excess of 1.15 [4,20,25]. A sample was considered tetraploid when more than 15% of the cells had a DNA index between 1.70 and 2.30. Similarly, sample was classified as triploid when more than 15% of the cells had a DNA index between 1.3 and 1.70. All other samples displaying more than 15% of cells with a DNA-index over 1.15 including single cells with DNA content above the highest possible G2M-region but not corresponding to a defined peak were considered as a cell population with an abnormal content of DNA arbitrary designated hyperploid (Fig. 2). For statistical purposes the tumours were classified into two groups, diploid and non-diploid (aneuploid). A tumour was considered diploid when all the samples were diploid. A tumour was considered aneuploid when at least one sample was aneuploid (heterogeneous) or all samples were aneuploid (homogeneous). This resulted in different patterns: diploid, aneuploid heterogeneous (Fig. 3) and aneuploid homogeneous (Fig. 4). We assigned a main DNA-ploidy pattern for each case according to the predominant DNA-ploidy pattern.

2.3. Immunohistochemistry

Immunohistochemical analysis was performed on parallel tissue sections, collected from all the blocks before microdissection, using the Mib-1 antibody (1 : 50), for Ki-67 and the D0-7 antibody (1 : 400) for p53. After deparaffinization and rehydration, the 4 μm sections were treated with 0.5% H2O2 in methanol for 10 min to inhibit endogenous peroxidase. Depending on the antibody, antigen retrieval was performed and the sections were preincubated for 15 min in phosphate buffer saline (PBS) containing 5% albumin from bovine serum (BSA) and then incubated with the relevant antibody at room temperature for 60 min, washed with PBS and subsequently incubated with the second biotinylated antibody at RT for 30 min. Antibody binding was revealed using the streptavidin–biotin-complex-peroxidase (Dako) solution, washed in running water, counterstained with hemalun and mounted. The negative control consisted of omission of the primary antibody.

Proliferative activity was established through the Ki-67 (Mib1) labeling index and computer analysis (Immunostiqic system, Zeiss). Multiple microscopic fields (200×) of each tumour were analysed. The account from the field with the highest proliferative activity was taken for statistical analysis.

p53 staining results were read semiquantitatively by two independent observers. Samples were classified into two categories according to a distribution proposed by Symmans [32]. The first group comprised the cases without or with rare p53 positive cells (negative), and those with a low proportion of positively stained nuclei (less than 50% positive cells); the second group was formed by cases displaying a high proportion of positively stained nuclei (more than 50% of positive cells).

2.4. Statistical analysis

Possible correlations between DNA-ploidy, intratumoral DNA-ploidy heterogeneity and histological type of tumour, depth of invasion, TNM stage, proliferative activity and p53 overexpression were analysed by means of the Fischer’s exact test. A p value of less than 0.05 was considered statistically significant.

3. Results

We studied 16 patients (9 women and 7 men) with an average age at gastrectomy of 65.5 years. The histological pattern for each tumour was determined according to the WHO classification. The depth of infiltration was classified according to the TNM system (Classification of malignant tumours, fifth edition, 1997). The cases are summarized in Table 1.

14 tumours out of 16 were poorly differentiated (grade 3), 1 was moderately differentiated (grade 2) and 1 well differentiated (grade 1). Six were of the intestinal type and 9 of the diffuse type. One tumour showed a mixed type. Only two tumours were free of lymph node metastases; most displayed one or more metastatic lymph nodes (2N0; 6N1, 5N2, 3N3). Seven carcinomas were limited to the muscularis propia (7T2); seven invaded the serosa (7T3) and two extended to the adjacent structures (2T4). The surface area of the tumours varied between 3 × 2.5 and 15 × 17 cm.
Fig. 2. Histograms illustrating intratumoral DNA-ploidy different results: * a diploid case, ** an aneuploid hyperdiploid case, *** an aneuploid triploid case and **** an aneuploid tetraploid case.

Fifteen tumours were aneuploid (94%), only one was diploid. Of the aneuploid tumours 7 were hyperdiploid, 6 triploid and 2 tetraploid. Four tumours were heterogeneous; all were diffuse carcinomas. The lymph node metastases found in three heterogeneous aneuploid cases were diploid in 12 lymph nodes and aneuploid in 2 lymph nodes. Lymph node metastases from all other (homogeneously aneuploid) cases were aneuploid.

Regarding p53 immunohistochemistry, 7 tumours showed more than 50% positive cells and 9 cases presented less than 50% of positive cells. The 4 ploidy heterogeneous cases showed a low proportion of p53 positive nuclei. Of the 11 ploidy homogeneous cases, 7 cases showed a high proportion of p53 positive cells; this difference was statistically significant \( (p = 0.05, \text{ Table 2}) \).

Proliferative activity according to the Ki-67 labeling index was lower than 50% in the diploid tumour and in 3 of the 4 ploidy heterogeneous tumours. Of the ploidy homogeneous tumours only one had a labeling index lower than 50%; this difference was statistically significant \( (p = 0.03; \text{ Table 3}) \).
4. Discussion

The malignant potential of many different tumours has been correlated with DNA ploidy: aneuploid tumours tend to be more aggressive than diploid tumours [3,7,10,14,17–24,27,28,30,31,33,35]. Several authors have reported that DNA-ploidy status has prognostic value in gastric cancer [1,11–13,26,29]. Other studies have shown no correlation between clinicopathological findings or therapy response and DNA-ploidy status [5]. One mechanism responsible for these inconsistencies could be a sampling error due to the presence of ploidy status heterogeneity between different subpopulations of cells in one tumour. In fact, heterogeneous DNA-ploidy, the existence of multiple stemlines differing in DNA content, has been found in 20–60% of gastric cancers [7,10,20,30] and furthermore...
Fujimaki et al. (1992) reported that DNA-index-ploidy (multiple clones differing in DNA index in one tumour) heterogeneity was higher than that of DNA-ploidy heterogeneity (multiple clones, either aneuploid or diploid) [7]. In an effort to resolve the controversy we determined DNA-ploidy status and also whether or not heterogeneity existed, by measuring DNA content in multiple samples from a series of resected gastric cancer specimens.

In a previous study [20], we could establish that DNA-aneuploid early gastric carcinomas are frequently heterogeneous for DNA-ploidy and that such heterogeneity occurs more frequently in tumours invading the submucosa (13% in intramucosal tumours and 44%...
Table 1
Pathological data and DNA ploidy in advanced gastric cancers

| Case | Age/Sex | Type       | Grade* | TNM     | Surface (cm²) | Ploidy** | Heterogeneity | Ploidy of N*** | Mib1/Ki67**** | p53***** |
|------|---------|------------|--------|---------|---------------|----------|---------------|---------------|----------------|----------|
| 1    | 82/M    | diffuse    | 3      | T2N1Mx  | 40            | D        | no            | NS            | 42%            | L        |
| 2    | 69/F    | diffuse    | 3      | T3N3Mx  | 77            | A, H     | yes           | 9D and 2H     | 41%            | L        |
| 3    | 41/F    | intestinal | 3      | T3N0Mx  | 22.5          | A, T     | no            | –             | 64%            | H        |
| 4    | 70/M    | diffuse    | 3      | T3N1Mx  | 48            | AT       | no            | NA            | 72%            | L        |
| 5    | 83/F    | intestinal | 3      | T2N1Mx  | 24.75         | A, H     | no            | 1H            | 56%            | H        |
| 6    | 59/M    | mixte      | 3      | T3N2Mx  | 35.75         | A, T     | no            | 1T            | 75%            | L        |
| 7    | 57/F    | diffuse    | 3      | T4N2Mx  | 81            | A, T     | no            | not valuable  | 32%            | H        |
| 8    | 62/M    | intestinal | 2      | T2N0Mx  | 22.5          | A, TT    | no            | NS            | 78%            | H        |
| 9    | 89/F    | intestinal | 3      | T2N2Mx  | 81            | A, H     | no            | 1T            | 84%            | L        |
| 10   | 55/M    | diffuse    | 3      | T3N2Mx  | 255           | A, H     | yes           | 2D            | 45%            | L        |
| 11   | 68/M    | intestinal | 1      | T2N1Mx  | 7.5           | A, T     | no            | 1H            | 93%            | H        |
| 12   | 60/M    | diffuse    | 3      | T3N3Mx  | 177           | A, H     | no            | 1H and 1T     | 81%            | L        |
| 13   | 53/F    | diffuse    | 3      | T4N1Mx  | 29.25         | A, H     | yes           | NA            | 90%            | L        |
| 14   | 75/F    | intestinal | 3      | T3N2Mx  | 27.5          | A, TT    | no            | NA            | 90%            | H        |
| 15   | 55/F    | diffuse    | 3      | T3N2Mx  | 36            | A, H     | yes           | 1D            | 33%            | L        |
| 16   | 59/F    | diffuse    | 3      | T2N1Mx  | 14            | A, T     | no            | 1T            | 83%            | H        |

*Grade: 1: well differentiated; 2: moderately differentiated; 3: poorly differentiated.
**Ploidy: D: diploid; A: aneuploid; H: hyperdiploid; T: triploid; TT: tetraploid.
***Ploidy of N (lymph node metastases): NA: not available (usually insufficient material); D: diploid; H: hyperdiploid; T: triploid; TT: tetraploid.
****Mib1/Ki67: per case field with % of maximal proliferative activity.
*****p53: High (H): more than 50% positive cells; Low (L): less than 50% positive cells.

Table 2
Ploidy pattern and p53 expression

|                  | Low expression p53 | High expression p53 | Total of cases |
|------------------|--------------------|---------------------|----------------|
| Heterogeneous    | 4                  | 0                   | 4              |
| Homogeneous      | 4                  | 7                   | 11             |
| Total of cases   | 8                  | 7                   | 15             |

p = 0.05.

Table 3
Ploidy pattern and proliferative activity

|                  | Ki67* > 50% | Ki67* < 50% | Total of cases |
|------------------|-------------|-------------|----------------|
| Heterogeneous    | 1           | 3           | 4              |
| Homogeneous      | 10          | 1           | 11             |
| Total of cases   | 11          | 4           | 15             |

p = 0.03.

*Labeling index.

In our 16 cases of advanced gastric carcinoma only one case was diploid (6%). DNA-ploidy heterogeneity was found in 4 aneuploid cases (26%). This heterogeneity for DNA-ploidy was not significantly correlated with grade (p = 0.5), or depth of invasion (p = 0.09), or histological type (p = 0.09) as previously described [8].

We earlier reported that early gastric carcinomas are more frequently diploid (53%) [20]. This finding is consistent with our hypothesis of stepwise ploidy progression: from diploid in most early gastric cancer to aneuploid but heterogeneous in infiltrating early gastric cancer to aneuploid but homogeneous in advanced gastric cancer.

The analysis of lymph node metastases also supports this hypothesis: metastases were aneuploid and homogeneous. DNA measurements in lymph node metastases revealed a heterogeneous ploidy pattern only in primary tumours heterogeneous for DNA-ploidy.

To explore more in detail the relationship between the pattern of intratumoral heterogeneity for DNA-
ploidy and genome instability, we have correlated different DNA-ploidy patterns with the Ki-67 labeling index and the degree of p53 immunoreactivity. Our results show that homogeneous-aneuploid cancers have a higher proliferative index than heterogeneous-aneuploid cancers. These findings support the hypothesis that a single more aggressive neoplastic cell clone is responsible for progression of the tumour. This is also supported by the observation that p53 expression was higher in homogeneous-aneuploid tumours.

In conclusion, this study: (1) confirms that most advanced gastric cancers are aneuploid; (2) demonstrates that these tumours are rarely heterogeneous for DNA-ploidy.

These findings are consistent with the hypothesis of the emergence of a single aneuploid cell clone as a crucial mechanism in the progression from early to advanced gastric cancer. More frequent p53 overexpression and higher proliferative activity is in agreement with the notion that this dominant clone is more aggressive.

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