DNA Ploidy: A Prognostic Factor of Response to Chemotherapy and Survival in Metastatic Gastric Adenocarcinoma

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Abstract. *Background: Metastatic gastric adenocarcinoma confers a dismal prognosis. Several prognostic factors are needed to distinguish patients that will benefit from chemotherapy. In this setting, the prognostic impact of DNA ploidy is still unclear. Materials and Methods: The records of 61 patients with metastatic gastric adenocarcinoma were retrospectively reviewed. Response to chemotherapy and overall survival (OS) were assessed and correlated to tumour DNA ploidy index, which was calculated by cytometric image analysis. Results: The median value of DNA ploidy index was 2.3. Patients with a low index responded better to chemotherapy than those with a higher index (p<0.01). Nevertheless, when the median value was used as a cut-off, no significant correlation of DNA ploidy index with response to chemotherapy (p=0.41) or OS (p=0.09) was observed. Conclusion: The prognostic role of DNA ploidy in metastatic gastric adenocarcinoma is still debatable. In this study, a low DNA ploidy index was associated with favorable prognosis; however, a suitable cut-off value is not yet available.*

Gastric adenocarcinoma is an aggressive tumour which ranks as the second cause of cancer-specific mortality worldwide, accounting for approximately 8% of new cancer cases (1, 2). Despite advances in the availability of endoscopy and other diagnostic methods, most patients in Europe and the U.S.A. still present with advanced disease at diagnosis. Surgical resection remains the only treatment with curative intent; lamentably, it is relevant to the minority of cases who present with limited-stage disease and are fit for surgery (3).

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In metastatic disease, the therapeutic objectives are to palliate symptoms, increase quality of life and prolong survival. Systemic chemotherapy is the established treatment (4), however, it is of limited value due to the low response rates and severe adverse effects (5, 6). It is therefore important to identify factors that could predict the response to chemotherapy and survival, and furthermore allow for a better stratification of patients in future therapeutic trials.

The prognostic implication of DNA ploidy in human solid tumours was evaluated by Barlogie *et al*. (7) who considered the DNA content as an intrinsic feature of malignant cells and provided data supporting the prognostic value of DNA ploidy. Chromosomal instability, manifested as aneuploidy, is a common cellular aberration in tumours of the gastrointestinal tract. Several studies comment on the adverse effect of aneuploidy on prognosis of patients with colorectal cancer (CRC) (7, 8), however, an expert panel convened by the American Society of Clinical Oncology in 2006 recommended against the routine use of DNA ploidy in early stage CRC (9). A study conducted by Tsavaris *et al*. (10) evaluated the prognostic correlation of DNA ploidy with overall survival (OS) in 226 patients with locally advanced or metastatic pancreatic adenocarcinoma. In multivariate analysis, a low DNA ploidy index, a ratio which expresses the difference in DNA content between the tumour cells and the normal counterparts, had a significant independent favourable association with outcome.

The evidence on the prognostic value of DNA content in gastric cancer remains conflicting, which is thought to be due, at least partly, to objective differences among the various analytical techniques employed. Furthermore, evidence on the role of DNA ploidy as a prognostic factor in patients with metastatic gastric cancer is limited.

In a previous study of our group, a qualitative assessment of DNA ploidy was performed in patients of stage IV noncardia gastric adenocarcinoma (11). A low, intermediate

and high ploidy score defined three prognostic groups. A low DNA ploidy index was correlated to a favourable survival.

In this study, we evaluated the prognostic impact of DNA ploidy index upon response to chemotherapy and OS, and we further investigated whether the DNA ploidy quantification is feasible and provides a cut-off value useful in prognosis.

Materials and Methods

Patients and data sources. Archived data of 61 consecutive patients from a single Oncology Centre were retrospectively reviewed. All patients were diagnosed with metastatic gastric (noncardia) adenocarcinoma and received first-line chemotherapy based on epirubicin, oxaliplatin and capecitabine (EOX) outside of clinical trials.

The patient cohort studied consisted of 55.7% men and 44.3% women. Both the mean and median age were 59 years. Tumour differentiation was grade II in 54.1% of the patients, grade III in 44.3% and grade I in 1.6%. Metastatic disease in the liver was present in 47.5% of the patients, abdominal/peritoneal carcinomatosis in 44.3%, local disease infiltration in 52.5%, lymph node infiltration in 88.5%, lung metastasis in 9.8% and bone metastasis in 5.0% (Table I).

OS was calculated from the time of diagnosis to death due to gastric cancer-related complications. Response to chemotherapy was assessed according to the Revised Response Evaluation Criteria for Solid Tumors (RECIST 2009) (12).

Records with complete data were included in the analysis. The study was approved by the Ethical Committee for Research Projects of Laiko Hospital, Athens, Greece.

DNA image cytometry (DNA ploidy index). For DNA measurements, the Feulgen staining technique was applied which labels DNA as magenta and the intensity of the stain is directly proportional to the amount of DNA present. Formalin-fixed paraffin-embedded tissue sections (6 μm) were de-paraffinized with xylene for 30 min, rehydrated with graded alcohol, and then immersed in 0.1 M hydrochloric acid at 60˚C for 5 min. Slides were then immersed in Schiff reagent for 30 min until the nuclei were stained, and then transferred directly to bisulfate water, followed by rinsing under running tap water. Following dehydration, the samples were treated with xylene, mounted in DPX (a synthetic, non-fluorescent, resinous mounting medium) and dried in the dark. Nuclear morphometry was performed using a Nikon eclipse microscope (Nikon, Tokyo, Japan) connected to a Nikon CCD videocamera and an IBM Pentium 4 PC with appropriate Cell Measurement Software (Image Pro Plus v. 5.1; Media Cybernetics Inc., Silver Springs, MD, USA) as described previously by our group (11).

This analysis configuration permits operator-dependent selection and measurement of DNA content using a magnification of ×200. Areas of the Feulgen-stained sections containing pathological lesions as defined by adjacent haematoxylin and eosin (H&E)-stained slides were selected for DNA content analysis. A total of 200-300 nuclei with clear boundaries appearing to have no loss of membrane integrity were identified for analysis from each tissue sample and overlapping nuclei were excluded. By configuration, the software discharges the majority of overlapping nuclei, internal reference cells are selected and additional non-diagnostic nuclei are discarded by supplementary obligatory visual review. Coefficient of variation of reference cells is automatically limited to 5%. Gray-scale levels in the microscopic image were transformed into digitalized signals and evaluated, with the image analysis system allowing for differentiation between gray level intervals. Cytometrical parameters were calculated automatically by measuring the nuclear integrated optical density (IOD), which represents the cytometrical equivalent of its DNA content (13).

The procedure was performed for all nuclei and the overall mean represented the DNA content, or DNA ploidy index. The mean IOD of control cells (human lymphocytes) served as the diploid standard (2c) and reference for DNA ploidy index calculation for targeted cells. Subsequently, DNA histograms were generated. A tumour was classified as diploid if the DNA ploidy index ranged from 0.9 to 1.1 and the relevant DNA histogram revealed only one peak at 2c, and aneuploid if either of these criteria was not met.

Table I. *Patient and disease features*.

	Number of patients	%
Gender		
Male	34	55.7
Female	27	44.3
Site of metastasis		
Liver	29	47.5
Peritoneum	27	44.3
Lymph node	54	88.5
Lung	6	9.8
Bone	3	5.0
Local failure	32	52.5
Tumour grade		
I	1	1.6
II	33	54.1
III	27	44.3

Statistics. The primary end-points of the study were OS and response to chemotherapy. Descriptive statistics were calculated with the use of mean, median and standard deviation, minimum and maximum values for quantitative measurements and counts/percentages for discrete variables. Chi-square test was used to study bivariate associations between variables, while one-way ANOVA was employed to test differences in distribution between multiple factor levels. The Kaplan–Meier method was used to estimate the effect of the variables on OS. Survival and response rates among categories were compared for statistical differences using log-rank analysis. All analyses were conducted at a 5% significant level using SPSS v16.0 statistical package (IBM Corporation, New York, US).

Results

DNA ploidy index. The mean DNA ploidy index was 2.31 (median 2.31, SD=0.047, range=1.57-3.36). A total of 45.9% of the measurements had a value lower than the median, while 54.1% were equal to or higher than 2.31.

Response rates (RR). We observed 25 out of 61 patients (41%) to have either a complete (CR) or partial (PR) response to chemotherapy, while 17 patients (27.9%) had stable disease

Table II. *One-way analysis of variance of DNA ploidy index values among the groups of response (p-value <0.01).*

	N	Mean	Std. error	Range	95% Confidence interval for the mean
Response	25	2.14804	0.072358	1.566-3.327	1.99870-2.29738
Stability	17	2.32953	0.064836	1.734-2.760	2.19208-2.46697
Progression	19	2.49389	0.089453	1.836-3.356	2.30596-2.68183
Total	61	2.30634	0.047694	1.566-3.356	2.21094-2.40175

(SD) and 19 (31.1%) experienced disease progression (PD). The distribution of the DNA ploidy index among the three groups that were formed according to response to chemotherapy (response, stability, progression) was statistically significant (p-value <0.01) (Table II). Patients that responded to chemotherapy had a lower mean DNA ploidy index than those who did not respond (Figure 1).

Nevertheless, when the median value of 2.31 was used as a cut-off, no statistically significant association of DNA ploidy index with response to therapy was observed (p-value=0.41) (Table III).

Table III. *Groups of response according to DNA ploidy index cut-off, value of <2.3 (p-value=0.41).*

Response type	Ploidy		Total
	<2.3	≥2.3	
Response	56.0%	44.0%	100.0%
Stability	41.2%	58.8%	100.0%
Progression	36.8%	63.2%	100.0%
Total	45.9%	54.1%	100.0%

Overall survival. The mean OS was 49.0 (95% CI=40.7-57.3) months and the median OS was 43.0 (95% CI=26.6-59.4) months (Figure 2). The variance of survival did not achieve statistical significance regarding its association with the DNA ploidy index (p-value=0.09). The mean OS of the patients with a DNA ploidy index <2.3 was 56.9 (95% CI=43.5-70.4) months and the median was 50.0 (95% CI=11.1-88.9) months, while for those with an index ≥2.3, the mean survival was 42.2 (95% CI=32.4-52.1) and the median 32.0 (95% CI=17.9-46.1) months (Figure 3).

Discussion

Following the publication of the Cochrane Review meta-analysis in 2010, first-line chemotherapy in metastatic gastric adenocarcinoma became the cornerstone of treatment, conferring a clear improvement in median survival from 4.3 to 11 months over best supportive care (BSC) (4). Combination chemotherapy based on various synergistic agents represents a therapeutic option that yields increased response rates over BSC; however, whether higher response rates recorded in trials of various chemotherapeutic regimens translate concretely into longer survival and therefore render different trials comparable remains debatable. The REAL study was a landmark trial in metastatic gastric cancer and paved the way for establishing the combination regimen of EOX as the standard-of-care in metastatic gastric cancer. Median survival in patients treated with EOX was longer than treatment with the combination of epirubicin, cisplatin, 5-fluorouracil (ECF) (median 11.2 *vs*. 9.9 months; HR=0.80, 95% CI=0.66-0.97) (14).

Whether the more recently proposed combination of docetaxel, cisplatin, 5-fluorouracil (DCF) is superior to ECF, in terms of OS, remains to be answered. It should be noted that the DCF regimen is highly toxic, causing severe neutropenia (15).

Despite advances in systemic chemotherapy, which include the administration of trastuzumab in patients with HER2-neu-positive cancer (16), the improvement in OS is modest. Despite all the advances in the treatment of metastatic gastric cancer, the life expectancy of these patients is fairly limited and further progress is certainly required. Multi-agent chemotherapy is associated with a high incidence of side-effects and it is important to identify factors that predict survival in order to assist with patient selection and further justify indication and therapeutic value. Moreover, the identification of prognostic factors could lead to a more precise stratification of patients in clinical trials and perhaps indicate hitherto unknown predictive factors.

The prognostic value of DNA content in gastric cancer, as measured by the DNA ploidy index (DNA ploidy), is controversial. Divergent data on DNA analysis are thought to reflect, in part, differences in methodology *i.e*. objective differences in the data obtained by the diverse analytical techniques employed (image cytometry *vs*. flow cytometry). Image cytometry is considered to be superior to flow cytometry, as it allows for direct visualization and selection of tumour cells for inclusion in the DNA measurement (17).

In the late 1980s, Danova *et al*. correlated DNA aneuploidy with poor survival both in early and in advanced gastric cancer

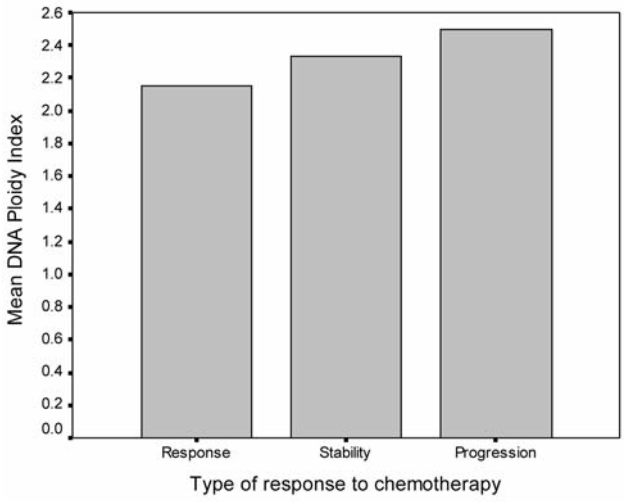

Figure 1. *Response to chemotherapy according to mean DNA ploidy index.*

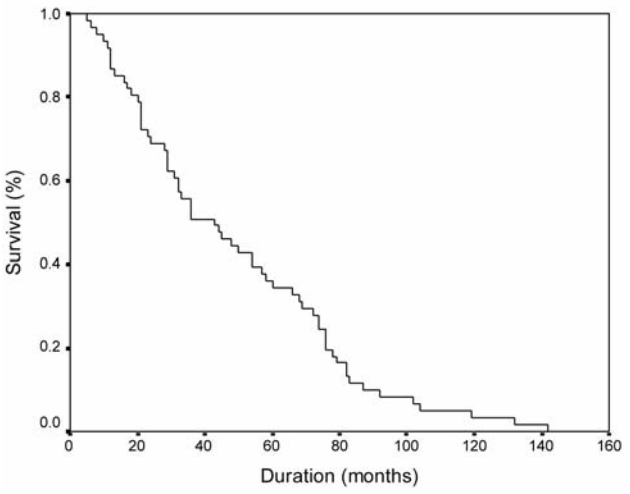

Figure 2. *Overall survival.*

(18). A year later, Baba *et al.* showed that a high DNA ploidy index in patients with advanced gastric cancer was associated with higher incidence of vessel invasion and lymph node metastasis invasion, conferring a poor 5-year survival, especially in elderly patients ($p<0.05$) (19).

Kimura *et al.* analyzed the DNA content of samples from 270 patients with advanced gastric cancer by flow cytometry. A high DNA ploidy index emerged as the third strongest prognostic factor for survival after peritoneal dissemination and presence of liver metastases ($p<0.01$) (20).

The association between tumour histological differentiation and DNA content was noticed by Suh and Min. In their study, patients with poorly-differentiated tumours bore aneuploid DNA, which in turn was associated with poor outcome (21).

Osterheld *et al.* 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 stem lines were found in four cases (26%). Furthermore, analysis of proliferative activity performed on the same samples revealed a higher proliferation rate in DNA aneuploid homogeneous tumours than in aneuploid heterogeneous tumours and heterogeneous tumours that did not overexpress p53 (22). The authors suggested that 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 aneuploid cell clone (22).

In a previous study of our group, 12 potential prognostic variables including tumour DNA ploidy index were evaluated

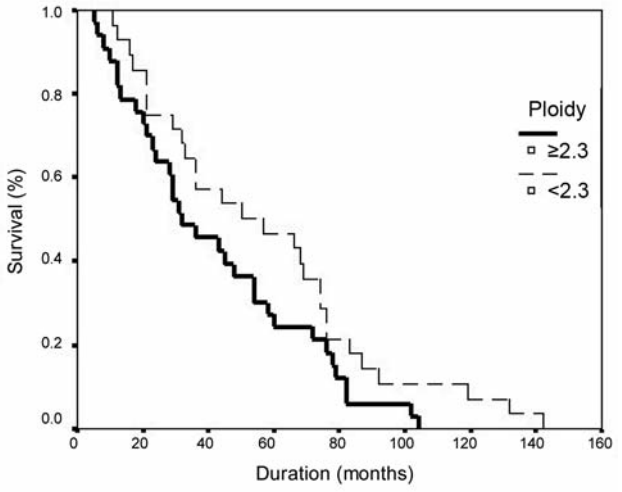

Figure 3. *Overall survival according to DNA ploidy index.*

for their influence on OS by multivariate analysis. DNA ploidy index was assessed by image cytometry and was found to have an independent effect on survival (index 2.2-3.6 *vs.* >3.6, HR=3.059, 95% CI=2.185-4.283, $p<0.001$ and index <2.2 *vs.* >3.6 HR=4.207, 95% CI=2.751-6.433, $p<0.001$) (11).

In the current analysis, patients with a lower median value of DNA ploidy index responded better to standard first-line chemotherapy based on EOX ($p<0.01$). It was also investigated whether a cut-off value of DNA ploidy index would provide a useful measurement in order to distinguish the patients that would respond more or less favorably to

chemotherapy. When the median DNA ploidy index value was used as a cut-off, no statistically significant association of DNA ploidy index emerged with response to chemotherapy (p=0.41). Similarly, defining the median DNA ploidy index value as cut-off, did not achieve significance when correlated to OS (p=0.09).

The limitations of our study would include its retrospective nature and the lack of a unanimously accepted methodology to assess a key indicator, the DNA ploidy index. However, since data from consecutive patients were included, we consider that this inherent risk of bias is limited. The method used to measure the DNA ploidy index is semi-automatic, possibly allowing for more consistent results. This study reports a single-center experience and a widely-accepted and applied first-line treatment (EOX), that in turn could signify a firmer homogeneity of treatment and applicability of the data relevant to patients that undergo this chemotherapy combination. We consider of note that the present study reflects real-life medicine, thus possibly increasing the relevance of the outcomes in current clinical practice. Further studies that ideally could prospectively include more patients would possibly allow for a more definite answer to the prognostic significance of the DNA ploidy index and perhaps also provide a cut-off value. Despite the limitations of this study, we consider that it provides further evidence on the value of the DNA ploidy index, an inadequately studied prognostic factor regarding patients with stage IV gastric cancer in a real-life medical setting.

Conclusion

In the present study, patients with low DNA ploidy index responded better to chemotherapy; however, the median cut-off value of the DNA ploidy index was not found to be suitable for determining response to chemotherapy or overall survival.

Competing Interests

None.

Acknowledgements

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