Circulating Tumour Cells and Survival of Patients with Gastric Cancer

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Abstract. *Background: The prognostic significance of the presence of tumour cells in the blood of gastric cancer patients remains unclear. Their occurrence and its association with the stage of disease and long-term survival was determined. Patients and Methods: Fifty-seven patients with stage I-IV gastric cancer were divided into two groups: these with and these without circulating tumour cells that were identified as cytokeratin positive (CK^+) cells among $CD45^-$ cells (obtained by sorting of $CD45^+$ leukocytes). Results: Tumour cells were detected prior surgery in the peripheral blood of 54.4% patients but no clear association with the stage of disease was observed. After gastrectomy detection rate was 21.1%. There was no significant difference in the 5-year survival of patients, with or without CK^+ in the blood. Conclusion: The presence of circulating tumour cells is of no prognostic value in patients with resectable gastric cancer.*

The surgical removal of primary tumour and regional lymph nodes even in early stages of cancer does not prevent the disease progression (1-6). Over the past decades, both incidence and mortality rates for gastric cancer have decreased worldwide. Nevertheless, gastric cancer is still the second major cause of cancer-related death (2, 4). The major prognostic factors in gastric cancer are: tumour staging according to TNM classification, histological type of tumour according to Lauren, invasion in lymphatic and vascular system and residual tumour presence (3-8). Surgical resection is the cornerstone in the treatment of gastric cancer, but despite the growing number of potentially curative resections, the rate of tumour recurrence, both locoregional and distant, is high (3, 6).

Micrometastases are frequently observed in the lymph nodes and bone marrow even in less advanced stages of different types of cancer (9-17). Spread of tumour cells in gastric cancer occurs *via* both lymphatic and blood circulation, and single cancer cells are detected in lymph nodes and in the blood (9, 14, 16). Moreover, bone marrow, peritoneal cavity and lymph nodes are the reservoirs of these cells, which often show no proliferation (10, 14, 15). The clinical significance and prognostic value of the presence of cancer micrometastases in bone marrow are still under discussion, but in pancreatic, colorectal, breast and gastric cancers it is associated with shortened survival (11, 14, 18). The association between micrometastases in the bone marrow and impaired survival is observed in gastric cancer patients (18); however, paradoxically the presence of tumour cells in the blood just after gastrectomy correlates with a low rate of tumour recurrence (19). Detection of circulating tumour cells in the blood after surgery led to the suggestion that it may facilitate the release of tumour cells into the blood stream, as in gastric and prostate cancer an increased frequency of tumour cells in the blood was observed after surgery (19, 20).

The detection of cancer cells in the blood or bone marrow requires sensitive methods as the frequency of these cells is extremely low. Immunocytochemistry, molecular techniques and immunofluorescence are commonly used for these studies (14-19, 21-26). The detection of disseminated cancer cells is generally based on the expression of tumour-associated markers, *e.g.* in gastric cancer - TAG72, carcino-embryonic antigen (CEA) and mucin (MUC), and the epithelial cell markers: cytokeratins (CK) or epithelial cell adhesion molecule (EpCAM). CK are commonly used for detection of disseminated tumour cells in blood and bone marrow samples (9, 11, 14, 15-19, 23, 24). A combination of

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sorting of CD45$^+$ cells from blood samples, followed by detection of CK$^+$ cells among CD45$^-$ cells or CK18- and CK20-mRNA were used for the detection of cancer cells in patients with gastric cancer prior to surgery (22, 24, 25). Although, both flow cytometry and immunofluorescence used for the detection of cancer cells are sensitive, false-positive or -negative results can still occur. Non-tumour cells expressing CK in the blood and low and/or heterogeneous expression of CK in cancer cells are the most frequent causes of false-positive or -negative results (14).

In this study, circulating tumour cells (CK$^+$) in the blood were detected among sorted CD45$^-$ cells of patients with different clinical stages of gastric cancer before surgery was carried out. Additional peripheral blood samples taken immediately after the surgery and from the vessels draining the tumour site during operation were studied. Analysis of the survival of patients in relation to the presence of disseminated cancer cells was also undertaken.

Patients and Methods

Patients. The analysis included 57 patients who underwent surgery for gastric cancer between 1997 and 1999 at the First Department of Surgery, Jagiellonian University Medical College. Patients who died within 60 days after surgery and patients with secondary malignancies were excluded from the study. TNM/UICC classification criteria according to WHO (1997) were used for tumor staging (7) while histopathological type (intestinal and diffuse) of tumours was classified according to Lauren (8). The intestinal type tumors located in the lower third of the stomach qualified for subtotal gastrectomy, while diffuse type cancers qualified for total gastrectomy. The extent of lymphadenectomy was at the discretion of the surgeon, however the majority of patients underwent D2 lymph node dissection. Provision of postoperative chemotherapy was dependent on the stage of disease. Follow-up clinical examinations were performed every 3-6 months. Analysis of survival was based on mortality data from the Census Registry Office. Since it was not possible to obtain records of cancer - related death for all patients, the data presented is based on overall survival rates. The study was approved by the Ethical Committee of the Jagiellonian University. Written informed consent was obtained from all patients.

Detection of tumour cells. Peripheral blood was collected in EDTA-containing plastic tubes prior to surgery (sample A), immediately after surgery (sample B) and from the vessels draining the tumour site during surgery (sample C).

The pellet of the cells from blood samples were incubated with lysing buffer (Becton-Dickinson, Biosciences, Immunocytometry Systems, San Jose CA, USA) (10 x volume, 10 min, repeated 3-4 times) to remove the erythrocytes. Leukocytes were washed in phosphate buffered saline (PBS) and adjusted to the concentration of 10×10^6 cells/ml after washing with PBS. Cells were stained with anti-CD45 PE (phycoerythrin) conjugated monoclonal antibody (mAb, DAKO, Glostrup, Denmark) and sorted into CD45$^+$ and CD45$^-$ populations using flow cytometry (FACS Vantage SE, BD Biosciences, Immunocytometry Systems, San Jose CA, USA), equipped with TurboSort option and Aerosol Protection System

(Flexoduct International ApS, Greve, Denmark). The ion laser Innova Enterprise II (Coherent, Santa Clara, CA, USA) operating at 488 nm was used as a light source. Sorting was performed using a 70 mm nozzle tip with a drop drive frequency of 65 kHz, 1.5 drop envelopes and "normal-R" sort mode. Sorted CD45$^-$ cells were collected into water-cooled (constant temperature circulator, Neslab Instruments, Portsmouth, NH, USA) polystyrene Falcon 2057 tubes (BD Biosciences, Two Oak Park, Bedford MA, USA) precoated with foetal calf serum to avoid plastic charging and cell attachment to the wall. The CD45$^-$ sorted cells (1×10^6 cells/ml) were used for making the smears (22, 24, 25).

The slides were dried, fixed with an ethanol and acetone mixture (1:1 vol) then stained with A45-B/B3 mAb (Micromet GmbH, Germany), which recognizes common epitopes of CK8, CK18 and CK19, (5 μg/ml) for 30 min, washed and then stained with goat anti-mouse IgG antibody labelled with fluorescein - FITC (working dilution 1:50) for 30 min. After washing with PBS, cover slides were put on and the slides were assayed within 2 days of staining. The CK$^+$ cells were identified by two independent investigators under fluorescence microscopy (Olympus, Japan) and documented with a DP10 camera (Olympus, Japan). The samples were regarded as positive when at least 3 cells CK$^+$ per slide were found. Accordingly, patients were classified into CK$^+$ and CK$^-$ groups.

Statistical analysis. The differences between groups of patients with or without CK$^+$ cells were analysed using Fisher's exact test and the Mann-Whitney *U*-test. Survival data were analysed according to the Kaplan-Meier method and the log-rank test was used for assessment of the differences. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA) software package.

Results

Occurrence of tumour cells in the blood of gastric cancer patients. A total of 57 patients with different clinical stages (I-IV) of gastric cancer were evaluated. The CK$^+$ cells detected in blood were considered as presumptive tumour cells. Tumour cells were detected in 31 patients (54.4%). Table I present the comparision of clinicopathological characteristics of patients with and without circulating tumour cells in the peripheral blood before surgery. Although the percentage of male patients predominated in both groups, the male/female ratio was similar. In both groups, diffuse type cancer according to Lauren's classification slightly predominated over the intestinal type. There were no major differences in the distribution of clinical stages between the groups. In 43 (75.4%) of all cases, lesions infiltrated gastric serosa and/or the surrounding organs (T3-4), and in the remaining 14 patients (24.5%) tumour infiltration was limited to the mucosal, submucosal or muscular layer (T1-2). Lymph node and distant metastases were equally distributed among both groups. Total gastrectomy was performed in 33 patients (57.89%) whereas 23 patients (40.35%), underwent subtotal gastrectomy, with a similar proportion in both groups. Moreover, there were no differences in the percentage of

Table I. *Clinicopathological characteristics of patients with or without circulating tumour cells.*

	CK positive (n=31)	CK negative (n=26)
Age	56.29±12.7	57.70±12.4
Gender		
male	24 (77.42%)	20 (76.92%)
female	7 (22.58%)	6 (23.08%)
Stage		
IA	3 (9.68%)	3 (11.54%)
IB	6 (19.35%)	1 (3.85%)
II	2 (6.45%)	6 (23.08%)
IIIA	3 (9.68%)	2 (7.69%)
IIIB	0 (0.00%)	1 (3.85%)
IV	17 (54.84%)	13 (50.00%)
Depth of infiltration		
T1-2	9 (29.03%)	5 (19.23%)
T3-4	22 (70.97%)	21 (80.77%)
Histolopatological type of the tumour		
intestinal	13 (41.93%)	7 (26.92%)
diffuse	18 (58.06%)	19 (73.08%)
LN status		
N0	12 (38.71%)	9 (34.61%)
N1-3	19 (61.29%)	17 (65.38%)
Distant metastases		
M0	28 (90.32%)	22 (84.61%)
M1	3 (9.68%)	4 (15.38%)
Surgical procedure		
total gastectomy	19 (61.29%)	14 (53.86%)
subtotal gastrectomy	11 (35.48%)	12 (46.15%)
Postoperative chemotherapy	11 (35.48%)	12 (46.15%)

There were no significant differences between CK^+ and CK^- groups for any parameter.

Figure 1. *The occurrence of tumour (CK^+) cells in the peripheral blood of patients with gastric cancer prior to surgery.*

Figure 2. *The frequency of tumour cells detection in the peripheral blood before surgery (sample A), immediately after surgery (sample B) and from tumour-draining blood (sample C).*

patients who underwent neo-adjuvant or postoperative chemotherapy. Therefore these two groups did not show any significant differences in the parameters defined.

There was no clear association of the presence of CK^+ cells with TNM stage, although the highest detection rate was noted in stage I (Figure 1). The analysis of peripheral blood samples prior to surgery (A), immediately after surgery (B) and tumour-draining blood (C) showed a higher frequency of CK^+ cells in sample A: 54.4% as compared to sample B: 21.1% and sample C: 26.8% (Figure 2). An analysis of paired 26 patients in whom all blood samples were obtained and who had tumour cells in the blood prior to surgery (sample A - 100%) was performed (Figure 3, left panel). The data show that immediately after gastrectomy, tumour cells were detected in only 42.3% of patients. Hence, it represents a substantial drop in the blood positivity after surgery. Tumour cells were present in all blood samples at 23.7%, A and B samples at 19.2%, and A and C samples at 26.9% of patients (Figure 3, right panel).
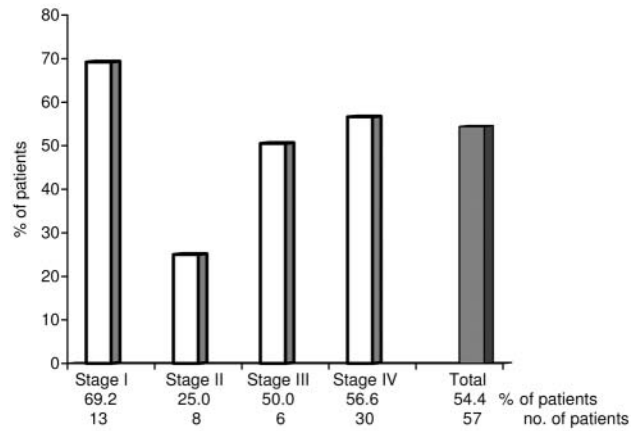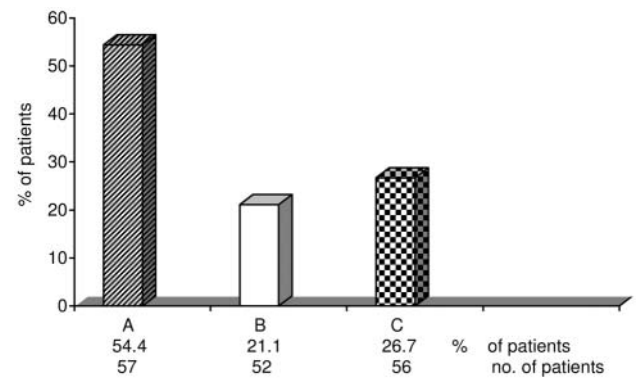
Survival of patients with and without circulating tumour cells. Analysis of overall survival at 5-years showed no differences between patients with or without circulating tumour cells (Figure 4). In patients with stage IV disease, the presence of circulating tumour cells was associated with a shorter survival, but the difference was not significant (Figure 5). Since other prognostic variables among these two groups were comparable (Table I), it seems to suggest that the presence of cancer cells in the blood before surgery is of no independent prognostic value in this limited group of patients with gastric cancer.

Discussion

The presence of tumour cells in the peripheral blood of patients with gastric cancer was assayed before and after surgery, and in the tumour-draining blood. Tumour cells were detected more often in the blood prior to surgery than in the remaining blood samples. Paradoxically, the highest
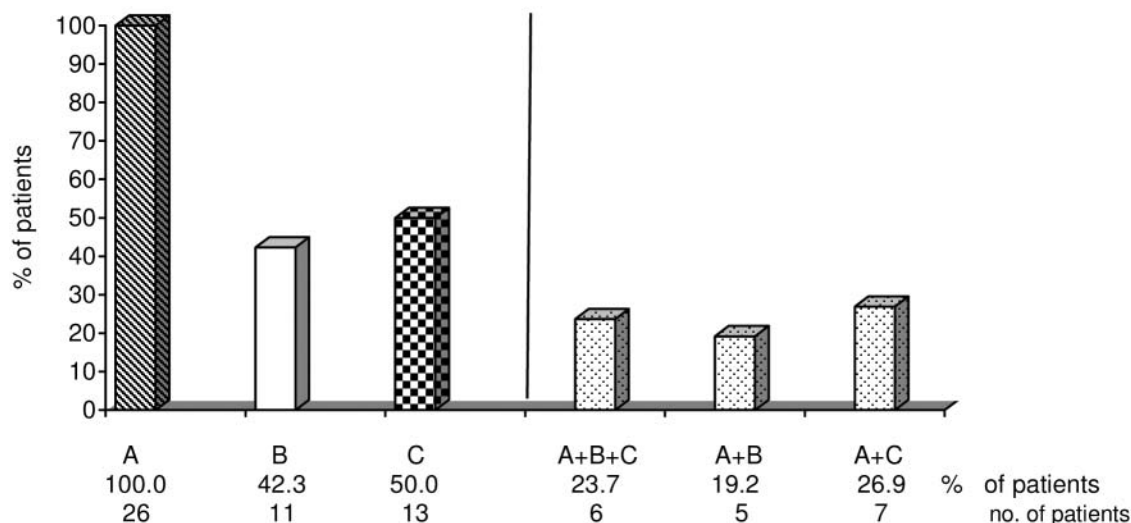
Figure 3. *The subanalysis of the frequency of circulating tumour cells in paired blood samples of 26 patients. Only patients with the presence of CK+ cells in sample A were included.*
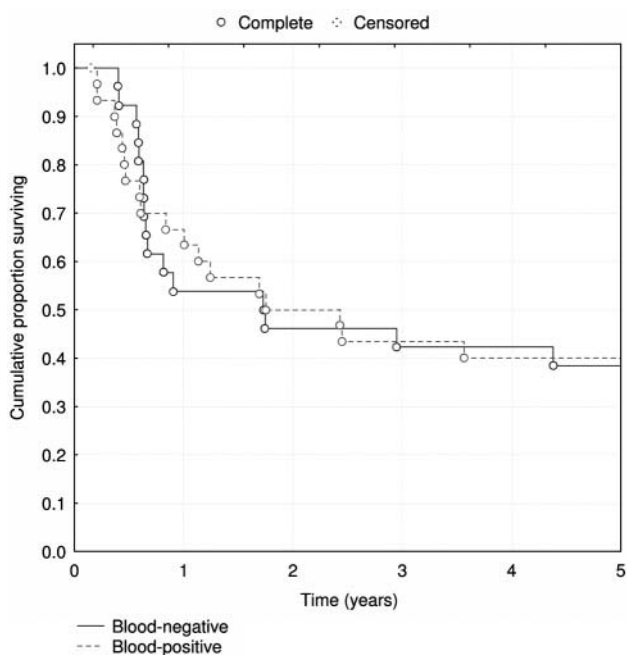

Figure 4. *The overall 5-year survival curve of gastric cancer patients with (n=31) or without (n=26) the presence of tumour cells in the blood.*
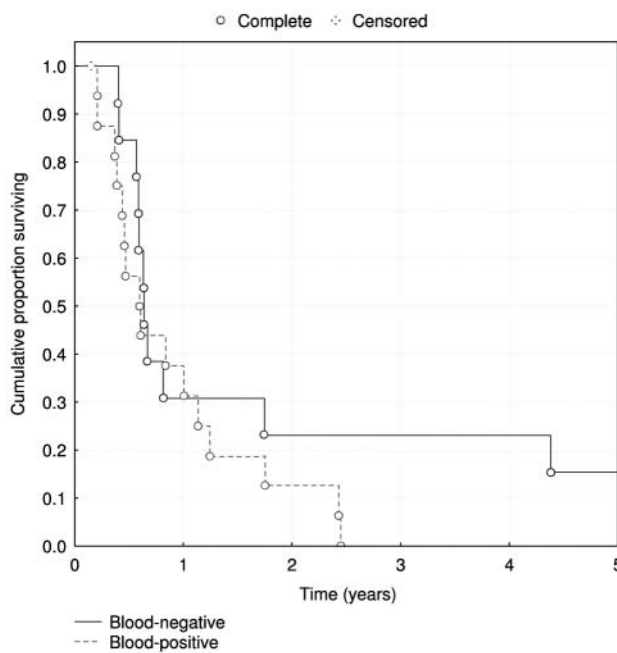
Figure 5. *The overall 5-year survival of stage IV gastric cancer patients with (n=17) or without (n=13) circulating tumour cells.*

frequency of cancer cells in the blood was seen in stage I patients, but overall there was no clear relationship between their occurrence and the stage of disease. The lower frequency of circulating cancer cells in samples taken immediately after surgery suggested that surgery of the primary tumour did not cause the release of cancer cells

into the blood. This is in contrast to other studies demonstrating the presence of CEA-mRNA positive cells in the blood of 46% gastric cancer patients after, but not before, gastrectomy, which disappeared within 2 postoperative days (19). The increase of cancer cells output from the primary tumour during surgery depends on the

performance of surgery and the tumour vascular network which, if at a high density, may facilitate tumour cell migration *via* the blood. Surprisingly, in our studies tumour cells in the tumour draining blood were less frequent than in peripheral blood suggesting that the former may also have arrived *via* the lymphatic circulation. They were detected in 27% of patients which is similar to the 44% detection rate of CEA/CK20-mRNA positive cells in mesenteric blood of patients with colorectal carcinoma (26).

The detection of tumour cells in the blood is difficult and techniques and detection rates vary considerably (13, 14, 17, 19, 21, 23, 26-28). To date only two studies in gastric cancer were reported. One included 30 patients and CK20-mRNA tumour cells in the blood prior to surgery were detected in 17% of patients (27), while in the other, no CEA-mRNA positive cells were found in 59 patients (19). The frequency of gastric cancer cell detection in the present study was much higher, but the sorting of the CD45$^-$ population may have influenced these results as it leads to "concentration" of tumour cells. Furthermore, the use of mAb against several types of cytokeratins may be an important factor in this procedure. However, in patients with colorectal cancer, the use of CK20-mRNA led to a detection rate of 17-48% (27, 28). Although our method is sensitive, allowing the detection of 1 tumour cell per 10^6 - 10^7 leukocytes (22, 25), it should be noted that occasionally the population of sorted CD45$^-$ cells may contain CK$^+$ epithelial cells which leads to false-positive results (14). This is also true for the CK19/CK20-mRNA used for cancer cell detection as it was also found to be present in the blood of 10-20% patients with benign bowel disease (23). However, our unpublished observations indicate that isolated CK$^+$ cells contain MAGE 1 or 2-mRNA. The false-negative results may arise from cancer cell loss during the separation procedures, down-regulation of CK expression by tumour cells, or its heterogeneous expression on tumour cells at different sites in a single patient (14, 15).

The presence of disseminated tumour cells in the blood, bone marrow or peritoneum was found to be an adverse prognostic factor in gastric cancer (6, 10, 12, 14, 18), but the opposite results were also noted (19). In the present study no clear correlation between the presence of CK$^+$ cells in the blood and the 5-year survival of patients was observed. There are only two reports on the prognostic relevance of the presence of circulating cancer cells in patients with gastric cancer. One included 30 patients and showed that the presence of CK20-mRNA was associated with shortened survival (14). The other based on 59 patients, showed no difference in the survival between CEA-mRNA positive and negative patients (19), which is in agreement with our data. These apparent discrepancies are not surprising as tumour cells in the blood are in a transitory stage during their migration to different organs and tissues. In the early stages of disease, the anti-cancer activity of the host may eliminate

cancer cells and they can also undergo spontaneous apoptosis (29). Alternatively, cancer cells expressing some chemokine receptors may migrate to the bone marrow where they can remain for a long time in a dormant state (14, 15, 30). This is associated with the expression of CXCR4 which plays a role in tumour cells homing to bone marrow (31).

Conclusion

The mere presence of circulating cancer cells may not necessarily be associated with the metastatic spread of disease. The present study seems to support this view. We are undertaking a study with a larger group of patients and multiple markers of tumour cells to substantiate these findings.

Acknowledgements

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