Detection of Disseminated Tumour Cells as a Potential Surrogate-Marker for Monitoring Palliative Chemotherapy in Colorectal Cancer Patients

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In a pilot study the effect of palliative chemotherapy on the detection rates of circulating tumour cells in peripheral venous blood of stage IV colorectal cancer patients was investigated. The results indicate a recruitment of tumour cells during chemotherapy and suggest a poorer survival for tumour cell positive patients. Circulating tumour cells have been shown to be a potential prognostic factor in patients who undergo curative resection for colorectal cancer. The effect of chemotherapy on the detection rates of disseminated tumour cells in blood has not yet been adequately investigated. Objective of this pilot study was to analyze circulating tumour cells in peripheral venous blood of colorectal cancer patients undergoing chemotherapy in order to evaluate its potential value as a surrogate-marker for predicting clinical outcome after chemotherapy.

Our hypothesis was that chemotherapy results in a reduction of the detection rates of circulating tumour cells in colorectal cancer patients. Forty-two Stage IV patients were examined at three different time points before and during palliative chemotherapy for the presence of disseminated tumour cells, using a previously described RT-PCR-assay for cytokeratin 20.

80.1% of the patients showed disseminated tumour cells at least once. Before chemotherapy, patients with multi-organ metastases were positive in 62.5%, patients with locally limited disease in only 14.3%. After the first chemotherapy, the detection rates in the latter group increased to 62.5%, for all patients in the same time from 46.3% to 57.5%. Clinical therapy responders showed an increase in the detection rates from 28.5% before to 71.4% after chemotherapy. In contrast, chemotherapy had no effect on tumour cell detection rates of patients with progressive disease (57% before vs. 60% after therapy). Patients with detected circulating tumour cells showed a shorter overall survival than patients without circulating tumour cells (83 vs. 53 weeks). Clinical therapy responders on average lived only 3 weeks longer than non-responders.

In contrast to the original hypothesis, our data suggest a recruiting of circulating tumour cells during chemotherapy in advanced colorectal cancer. Further investigations are needed to clarify the potential role of circulating tumour cells for monitoring chemotherapy in these patients.

Key Words: Disseminated tumour cells, Colorectal cancer, CK-20-RT-PCR, Chemotherapy, Prognosis

Since Ashworth first described circulating tumour cells in 1869 (1), detection of disseminated tumour cells has recently come again into scientific focus as more sensitive and specific detection methods have become available (2). Clinical studies investigating isolated tumour cells have shown a prognostic significance for different gastrointestinal cancers including colorectal cancer (3,4,5). The occurrence of isolated tumour cells is now more adequately defined as minimal residual disease (MRD) (6,7,8,9). The detection of disseminated tumour cells could be the basis for molecular staging of gastrointestinal cancers and may contribute to a more precise prediction of the individual risk of a specific patient. This again may result in optimisation of oncologic treatment.

Until now the effects of cytostatic agents on disseminated tumour cells in the blood of patients with colorectal cancer have not been adequately investigated. Circulating tumour cells, however, may potentially be appropriate markers for monitoring the effects of
chemotherapy and may even help to predict drug sensitivity in this context.

The majority of currently performed tests for predicting sensitivity to cytostatic agents make use of cell cultures. But, although some authors found a correlation of gene expression (10,11) and enzyme levels (12-17) to cytostatic efficiency, most clinical studies failed to show any prognostic significance for these assays (13).

Determination of free serum DNA (18) and established tumour markers (19) partially allow monitoring of chemotherapy, however, they cannot adequately predict the patient's response to chemotherapy.

Recently, a Histoculture Drug Response Assay with very promising results was presented. Biopsies of gastrointestinal tumours in advanced stages were cultured and incubated with 5-fluorouracil and cis-diaminodichloroplatinum. In vitro inhibition significantly correlated to the clinical response to chemotherapy (20), however, this test has not gained acceptance in the routine management of patients undergoing chemotherapy for colorectal cancer.

Aim of our investigations was to evaluate whether tumour cell detection in venous blood can contribute to the monitoring of the effects of chemotherapy in colorectal cancer patients.

Patients and Methods

Informed consent was obtained from 42 colorectal cancer patients (all UICC stage IV) treated with palliative chemotherapy at the oncological outpatient's unit of the Gastroenterological Department of the University of Heidelberg. The study protocol was approved by the Ethics Committee.

Staging was performed before the beginning of chemotherapy and again after approximately three months to determine the response to therapy. Staging was carried out routinely by clinical examination, abdominal ultrasound, chest X-ray and serum tumour markers and, if necessary, also by computed tomography of the region of interest. Response to therapy was defined according to Miller et al. (21).

Treatment Dosage and Schedules. Patients received chemotherapy as follows: for Fu/Fo (according to the Mayo-Protocol), calcium-folinate 50mg and 5-fluorouracil 450mg/m² were administered on day 1 to 5 for a 4-week-cycle (n=12 patients). For ARD (Ardalan high-dose-fluoruracil protocol) calcium-folinate 500mg/m² over 2 hours and 5-fluorouracil 2600mg/m² over 24 hours were given weekly for 6 weeks in cycles of 50 days (n=13). In combination with Irinotecan 80 mg/m² (CPT/ARD), fluorouracil dosage was reduced to 2000 mg/m² (n=8). For FOLFOX Oxaliplatin 85mg/m² in 500 ml 5% glucose over 2 hrs. on day 1, calciumfolinate 500mg/m² over 2 hrs. on day 1 and 2 and 5-fluorouracil 3000mg/m² over 48 hrs. were repeated every two weeks (n=7).

One patient was treated weekly with Irinotecan 100 mg/m² over 30-45 minutes as mono therapy.

One patient received chronomodulated (LEVI protocol) Oxaliplatin 100 mg/m², calcium-folinate 1200 mg/m² and 5-fluorouracil 3000mg/m² over 96 hours.

Blood Samples. Three 10-ml peripheral venous blood samples were taken in sodium-EDTA tubes from each patient. The first before infusion of the medication, a second the morning after, and a third approximately one week later. All samples were diluted with 10ml of phosphate-buffered saline. After density centrifugation through Ficoll-Paque (Amersham Pharmacia Biotech AB, Upsala, Sweden) (30 minutes, 400g), mononuclear cells were harvested from the interphase and washed twice in PBS. The cell pellet was then shock frozen in liquid nitrogen and stored at -80°C.

RNA extraction and RT-PCR. RNA from the cell pellets was extracted using a commercially available RNA extraction kit (Life Technologies Gibco BRL, Eggenstein, Germany) in accordance with the recommendations of the manufacturer as previously described (22) and kept in RNAse-free water. CK 20-RT-PCR was performed according to our previously described protocol (22). PCR-products were analyzed by electrophoresis on 2% agarose gels, made visible by UV-light and documented photographically. RNA quality and performance of reverse transcription of all analyzed samples was confirmed by RT-PCR amplification of glyceraldehydes phosphate dehydrogenase (GAPDH) transcripts.

Results

From 119 blood samples three had to be excluded due to poor RNA quality since they showed negative signals in amplification of the housekeeping gene GAPDH. Of the 116 remaining samples 59 were positive in the RT-PCR assay (50.9%).

80.9% of the patients (34/ 42) showed an amplification of CK 20 mRNA in at least one out of the three sampling times; 30.3% of patients were positive only once, 33% twice and 15% at all times.
**Table I** - Detection rate of circulating tumour cells in relation to the extent of metastases before and after beginning of chemotherapy

<table>
<thead>
<tr>
<th></th>
<th>solitary-organ metastases</th>
<th>multi-organ metastases</th>
<th>locally metastasized</th>
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<tbody>
<tr>
<td>before therapy</td>
<td>9/16 56.3%</td>
<td>10/16 62.5%</td>
<td>1/7 14.3%</td>
</tr>
<tr>
<td>after first medication</td>
<td>8/15 53.3%</td>
<td>11/15 73.9%</td>
<td>5/8 62.5%</td>
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**Table II** - Detection rates of circulating tumour cells for all patients in correlation to the sampling times

<table>
<thead>
<tr>
<th>detection rate of circulating tumour cells</th>
<th>percentage</th>
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<tbody>
<tr>
<td>3 times cumulative before therapy</td>
<td>34/42 80.1%</td>
</tr>
<tr>
<td>1 day after starting therapy</td>
<td>19/41 46.3%</td>
</tr>
<tr>
<td>third sample</td>
<td>23/40 57.5%</td>
</tr>
<tr>
<td></td>
<td>17/33 51.5%</td>
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The detection rate of 62.5% (10/16) in the group with diffuse metastases (more than one distant organ affected) was only marginally higher than in the group with solitary organ metastases (only liver or lung) (9/16= 56.3%), but clearly higher than in the group with only locally metastasised tumours (peritoneal carcinomatosis, in some cases combined with unresectable local disease but no metastases in distant organs) (1/7=14.3%) (Fishers exact test p= 0.07) (Table I).

From day 1 to day 2 we noticed an increase of the tumour cell detection rate from 46.3% to 57.5% (Table II).

The analysis of the sub-group of patients with only peritoneal metastases revealed an increase in the detection rate from 14.3% on day 1 to 62.5% on day 2. However, this difference failed to reach statistical significance probably due to the low number of patients in this group (n=7) (Table III).

The percentage of positive patients rose from 28.5% to 71.4% in the minor response group within the first two days. None of the originally positive patients later turned negative.

Concerning the survival-data, one patient was lost to follow up. The median follow-up of the remaining 41 patients was nearly two years until all patients had died. The survival-times of the patients ranged from 8 to 109 weeks, the average was 58.8 weeks. Tumour cell negative patients lived longer than positive patients. Median survival-time was 83 vs. 53 weeks (Logrank test p= 0.34) (Fig.1).

The Responder group had a survival benefit of only 3 weeks compared to the Nonresponders (58.6 weeks vs. 55.1).

**Discussion**

The results of this pilot-study contradict our primary hypothesis. Apparently, chemotherapy does not decrease circulating tumour cells in the peripheral blood of metastasized colorectal cancer patients. On the contrary, we could find a trend towards an increased recruitment of disseminated tumour cells after chemotherapy. In patients with metastases in one or several distant organs, we found circulating tumour cells with a comparable frequency before and after chemotherapy. Among patients without distant organ

**Table III** - Detection rates of circulating tumour cells in relation to the patients’ response to chemotherapy

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<thead>
<tr>
<th></th>
<th>minor response</th>
<th>no change</th>
<th>progress</th>
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</thead>
<tbody>
<tr>
<td>cumulative 3 samples</td>
<td>5/7 71.4%</td>
<td>5/7 71.4%</td>
<td>19/22 86.4%</td>
</tr>
<tr>
<td>before therapy</td>
<td>2/7 28.5%</td>
<td>4/7 57.1%</td>
<td>12/21 57.1%</td>
</tr>
<tr>
<td>day after first medication</td>
<td>5/7 71.4%</td>
<td>4/7 57.1%</td>
<td>12/20 60.0%</td>
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metastases, but also in a palliative situation (peritoneal carcinomatosis), circulating tumour cells before cytostatic therapy were rare (14.3%). After chemotherapy, however, the tumour cell detection rate increased considerably and was then comparable to the detection rate in the metastasised group (62.5%). A possible explanation why we only found a noteworthy increase in the tumour cell detection rates in this latter group is that the initial tumour load in the blood compartment was low in this group. This contributed to the recruitment of tumour cells becoming apparent. Other studies have shown that the detection rate of disseminated tumour cells is especially high in patients with distant metastases (23). These metastases are thought to be caused by hematogenic tumour cell dissemination and therefore the high initial detection rate in the group with distant metastases seems well explicable. However, as the initial detection rate was so high a recruitment of tumour cells during chemotherapy would have been difficult to detect in this setting. Our findings correspond to results from others showing a significant spread of tumour cells in patients with advanced malignancies undergoing chemotherapy with whole body hyperthermia (24).

Our results suggest a possible recruitment of isolated tumour cells during chemotherapy. The cytostatic damage of solid tumour masses through chemotherapy may result in the mobilisation of single cells or clusters which then leave their formation and consecutively reach the blood circulation. This concept of tumour cell mobilisation is substantiated by the results of others who were able to directly visualise this effect of
chemotherapy by immunohistochemistry (25). Our findings that more aggressive chemotherapy in general seemed to lead to a stronger recruiting of tumour cells than milder regimes, (data not shown) further support this concept.

In our study, mobilisation of tumour cells was most evident in those patients who showed a minor response. In the group of patients whose tumour growth did not cease despite chemotherapy, the changes in tumour cell detection were only marginal. It would have been interesting to see what effect chemotherapy would have had on tumour cell detection in cases of remission, but unfortunately none of the patients in our group had a remission.

There is little data on the effect of chemotherapy on disseminated tumour cells in colorectal cancer and due to differing detection methods the data are difficult to compare. The potential value of detection of isolated tumour cells is documented by a single case report in the literature. A chemotherapeutically treated patient with colorectal cancer was regularly tested for circulating tumour cells by a CEA-RT-PCR-assay. The positive assay result ultimately disappeared after the patient went into remission (26). These findings are supported by our own data on tumour cell detection rates of patients with rectal cancer having undergone neoadjuvant chemoradiation for locally advanced rectal cancer (T4) or for very low rectal tumours intended for sphincter preserving surgery. Compared to patients not having undergone neoadjuvant therapy, stage-corrected results showed a decrease of tumour cell detection in the neoadjuvant group. Logistic regression analysis confirmed tumour cell detection in the bone marrow of the neoadjuvant group to be a significant prognostic factor for survival (27). This seems somewhat in contradiction with the results of the presented pilot study, however, may well be explained on the basis of different sampling times. The samples in the neoadjuvant study were obtained intraoperatively, 4 to 6 weeks after completion of the neoadjuvant therapy. We hypothesise that the recruitment is an early event during chemotherapy, later the tumour cell detection rates decrease, especially in those patients responding to treatment.

There is somewhat more data on breast cancer and other malignancies. Sabbatini et al. (28) reported on the detection of circulating tumour cells in breast cancer patients using Maspin as a marker. After chemotherapy, elevated detection rates were observed (not significant) resulting in the authors concluding that tumour cells were mobilised.

Patients with different types of cancer, who underwent a conditioning chemotherapy before the harvesting of stem cells for autografting after high dose chemotherapy, were examined immunocytochemically by Brugger et al. (25). They found two peaks in tumour cell mobilisation: an early one which was thought to be induced by an immediate response to cytostatic damage; and a second one, together with the stem cell peak, only in patients also suffering from an affection of the bone marrow as a consequence of the malignant disease.

Wong et al. (29) used semi-quantitative RT-PCR for alpha fetoprotein and albumin to look for disseminated cells of hepatocellular carcinomas. They were able to show that surgical and radiological therapy lead to an increase in tumour cell detection rate as was shown for resection of liver metastases (23).

For tumour cell detection with enhanced specificity Smith et al. (30) made use of CK 19 and the Abelson Onkogene and additionally compared it to conventional immunocytochemistry. The decrease of tumour cell detection correlated with the response to chemotherapy in breast cancer patients. Thus, this method of tumour cell detection may be helpful in monitoring the course of therapy but the above cited study also showed that this marker was not suited to predict clinical outcome in this setting.

In our study, detection of circulating tumour cells was the only factor showing a statistic trend in the logistic regression analysis in regard to the patients' survival: positive patients on average died four months earlier than negative patients. However, the wide range of survival times in both groups probably prevented those results from showing any statistical significance. As causes for death are often not only related to tumour progress (31) but also to other multiple factors including general comorbidities (32) or adverse effects of chemotherapy (33, 34), this wide range is hardly surprising. Moreover, the prognosis also depends on further treatment and its duration, which was not investigated in this pilot study (35). Nevertheless, no other factor including objective response to therapy and outcome after the first three months, was able to predict survival. Especially, the survival difference between responders and non-responders amounted to only three weeks.

The third sampling time did not contribute to clarifying the life cycle of circulating tumour cells under chemotherapy and could have been omitted. In the long run, we would expect disseminated tumour cells to disappear in cases of remission but not in cases of persisting solid masses as was the case in all our patients without exception.
We cannot prove that the trends shown in our data are not merely due to sampling errors. However, we have in the meantime investigated over 30 patients with colorectal cancer on four different points in time to further investigate the so-called sampling error and we have not seen any differences in the detection rates in correlation to the time of sampling (data not shown). Therefore, we assume that we are indeed seeing real effects of chemotherapy on tumour cell detection when the detection rates vary so much. Nevertheless, tumour cell release into the blood stream is probably not a continuous process, therefore, further investigations with larger numbers of patients and more frequent sampling times are necessary to exclude sampling errors (36).

Based on our preliminary findings, there is a clear need to further investigate tumour cell dissemination in patients with an assumed lower level of circulating tumour cells undergoing chemotherapy, e.g., patients undergoing neoadjuvant chemotherapy and patients with advanced disease without affection of distant organs. This would probably greatly increase the chances to demonstrate significant tumour cell recruitment during chemotherapy.

Conclusions

Chemotherapy does not result in a decrease of disseminated tumour cells in patients with stage IV colorectal cancer, but on the contrary may result in a recruitment of disseminated tumour cells. The employment of semi-quantitative modern detection techniques such as Real-Time-PCR (37,38) may be of great future value in this setting as it may allow more precise quantitative evaluation of the effects of therapy.

References


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