IN VITRO CHEMO-SENSITIVITY ASSAY GUIDED CHEMOTHERAPY IS ASSOCIATED WITH PROLONGED OVERALL SURVIVAL IN CANCER PATIENTS

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The aim of the study. The overall survival (OS) of patients suffering from various tumour entities was correlated with the results of in vitro-chemosensitivity assay (CSA) of the in vivo applied drugs. Material and methods. Tumour specimen (n=611) were dissected in 514 patients and incubated for primary tumour cell culture. The histocytological regression assay was performed 5 days after adding chemotherapeutic substances to the cell cultures. n=329 patients undergoing chemotherapy were included in the in vitro/in vivo associations. OS was assessed and in vitro response groups compared using survival analysis. Furthermore Cox-regression analysis was performed on OS including CSA, age, TNM classification and treatment course.

Results. The growth rate of the primary was 73-96% depending on tumour entity. The in-vitro response rate varied with histology and drugs (e.g. 8-18% for methotrexate and 33-83% for epirubicine). OS was significantly prolonged for patients treated with in vitro effective drugs compared to empiric therapy (log-rank-test, p=0.0435). Cox-regression revealed that application of in vitro effective drugs, residual tumour and postoperative radiotherapy determined the death risk independently.

Conclusions. When patients were treated with drugs effective in our CSA, OS was significantly prolonged compared to empiric therapy. CSA guided chemotherapy should be compared to empiric treatment by a prospective randomized trial.

Key words: chemo-sensitivity in vitro, survival prediction, chemotherapy, drug sensitivity, cancer

The “valley of death” has nowadays become the synonyme of failure to translate the growing knowledge about cancer biology into approved new therapeutics (1). This accounts for more than 90% of new molecular entities (NME). One of the major reasons is that approval is costly and demands an epic amount of time, especially during the Phase III prospective controlled (randomized) trial (PCT) (2). PCTs are considered the gold standard for comparing two treatments, because observed and unobserved covariates can be balanced between the treatment groups. However, ethical and methodological concerns on this dogma have already been raised in the 1980ies (3). In fact, many thousands of patients suffering from solid tumours have been recruited for chemotherapy trials and many of them died on side effects or chemoresistance with only modest if at all prolongation of the median overall survival (OS) or progression free survival (PFS) (4, 5). However, in practice Benson and Hartz have shown, that observational studies do not overestimate treatment effects compared to PCTs in a meta-analysis of various reports (6). This was confirmed by other investigators (7). Observational studies may be conducted with lower costs and in a more natural patient’s environment than is possible in PCTs (8). Complex and successful...
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Critical strategies have been developed in the recent past to reduce bias in observational studies (9). Moreover, data assessment, interdisciplinary and international communication has become easy with new information technologies and hence the design of reliable observational or non-randomized multi-center studies is no longer dependent on cost and standardization issues.

Individualization of therapy has always been in the focus of tremendous research efforts considering the large patient- and tumor-side variations. Genetic mapping and other molecular-pathologic investigations are now performed routinely for many entities, and targeted drugs have been developed (10). However the idea of personalized oncology was already set into reality as early as in the 1970ies using in vitro chemosensitivity assays (CSA). The aim of CSA is to predict in vivo response and resistance to chemotherapeutics in analogy to antibiograms. Since the first reports of oncobiograms (11) and the human tumour clonogenic assay (12) appeared, different methods were established to determine in vitro chemosensitivity. Some of these methods are, for instance, taking measurements of radiolabeled DNA-precursors, the fluorescent cytoprint assay (FCA), the differential staining cytotoxicity assay and the ATP-cell viability assay (ATP-CVA) (13), which has shown a predictive accuracy for in vivo sensitivity of 30-86% and for in vivo resistance of 92% in an analysis of 258 in vitro/in vivo associations for various tumour entities (14). Similar results were reported by other authors (15-18).

Up to date two reviews have summarized the results of (randomized or non-randomized) prospective trials investigating CSA-guided chemotherapy (19, 20). It became obvious that in vitro and in vivo effects are strongly associated. However, despite promising results regarding OS compared to empiric therapy, exploding costs due to “high-volume” quantitative assays, differing response variables (response rates, PFS or OS) and ethical concerns when CSA-guided chemotherapy does not correspond to empiric treatment prevented further distribution of this method. Moreover, primary culture is sometimes not successful due to infection, non-growth or fibroblast overgrowth.

CSA have been performed at the Surgical Department of the Leipzig University Hospital, Germany, from the early seventies until 2000 (11). Nevertheless, patients were always treated according to current guidelines. A large observational dataset was collected including in vitro/in vivo correspondance with long-term follow-up despite the fact that not only the health care system but also the political and administrative systems changed radically during this time. Moreover, new, more expensive, quantitative and complex CSA techniques were developed by other groups. In addition, the statistical instruments that allow to reduce the bias influencing in vitro/in vivo correlation to such a level that reliable conclusion may be drawn occurred only in the recent past.

Considering the urgent need for chemoresistance prediction in cancer patients, our aim was to assess whether patients treated with in vitro effective substances using oncobiograms survived longer than patients treated with in vitro resistant or non-tested (empiric) substances. Our analysis was based on the Leipzig observational study because it is the largest long-term in vitro/in vivo dataset available up to now.

MATERIAL AND METHODS

Tumor specimens and in vitro assays

Asservation of tumour cells was performed 611 times in 514 patients from June 1995 until April 2002. The clinical observation continued until August 2002 (0-80 months, median 13 months, standard dev. 17.8 months). We chose the cytohistological regression assay established by Schönfelder (1). Tumour tissue was dissected and incubated for 24 hours in culture flasks with RPMI 1640 medium (10% fetal calf serum, gentamycin 40 mg/l) at 37°C, 95% oxygen, 5% CO2. The medium was changed after adherence of tumour cells and the cells were incubated further for 5 days in microtiterplates (MTP). Then the chemotherapeutics were added, using the calculated maximal plasma concentrations (5-fluorouracile (5-FU), 20 µg/ml, methotrexate (MTX), 12 µg/ml, cyclophosphamide (CPA), 30 µg/ml, epirubicine (EPI), 2.4 µg/ml, vinblastine (VBL), 0.2 µg/ml, doxorubicine (DOX), 0.9 µg/ml, taxole (TXL), 2.4 µg/ml or vincristine, 0.03 µg/ml). The MTPs were further incubated for 72 hours. After
staining by Giemsa the histocytological evaluation was performed and the assays were compared to the untreated control (fig. 1a), while using the following criteria to classify the in vitro response:

1. Resistant (R): No changes in histological architecture and maximally light changes in the cell membrane (fig. 1b).
2. Weak sensitivity (W): minimal changes of the cell membrane, of the cytoplasm, of the nucleo-plasmatic ratio (NPR) and of the histological architecture (fig. 1c).
3. Moderate sensitivity (M): strong changes of the cell membrane, of the cytoplasm and of the NPR, the histological architecture is strongly changed but identifiable (fig. 1d).
4. Strong sensitivity (S): strong changes of cell membrane, cytoplasm and NPR, the histological architecture is destroyed (fig. 1e).

Assessment of clinical data

To evaluate the clinical course, the initial TNM classification as well as surgical procedures, chemotherapy, radiation, staging procedures and PFS and OS were assessed. Postoperative (adjuvant or palliative) chemotherapy was performed in 183 patients.

The standard chemotherapies for the most tumour entities consisted of drug combinations. This makes it difficult to relate in vitro sensitivity to in vivo effects, because one never knows exactly at which extent the drugs contributed to success or failure. Therefore we divided patients into two groups – the first containing those who received one or more in vitro effective drugs (corresponding to “Strong sensitivity”, see Subsection 2.1.) during the postoperative follow-up. The second group underwent empiric (in vitro tested but not ef-

![Fig. 1a. In vitro chemosensitivity assay example: control](image1)

![Fig. 1b. In vitro chemosensitivity example: resistance](image2)

![Fig. 1c. In vitro chemosensitivity example: weak sensitivity](image3)

![Fig. 1d. In vitro chemosensitivity example: moderate sensitivity](image4)

![Fig. 1e. In vitro chemosensitivity example: strong sensitivity](image5)
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Statistics

To compare in vitro chemosensitivity between subgroups Fisher’s exact test was applied. Survival analysis was conducted using Kaplan and Meier statistics. Postoperative overall survival (OS) was defined as the time from surgery until death (failure) or last follow-up (censored observation). Progression free survival (PFS) was calculated as the time from surgery until progress or tumour related death (failure) or until death due to other reasons or last follow-up (censored observation). Survival curves were compared by applying the exact log rank test.

A Cox-regression analysis was performed including the above mentioned factors in order to assess those factors which contributed most and independently to death hazard. Thereby a stepwise backward algorithm was used for model reduction. Furthermore the OS was compared by the log-rank-test and depending on the grouping by in vitro/in vivo correlation into “sensitive” (patients were treated with in vitro effective drugs) and all other patients, whereby the model was stratified by histologic diagnosis. The R software with the “survival” package was used for statistical calculations (21, 22).

A matched-pairs-analysis balancing the independently contributing risk factors between the groups was furthermore performed in order to reduce confounding, and survival was calculated again with matched pairs. The “MatchIt” package was used (23).

Ethics

Since this analysis was retrospective and purely observational, no ethical committee had to be involved. Patients gave their consent allowing us to archive the clinical data, which were saved and treated according to the guidelines set down by the state of Saxony, Germany. These data are exclusively used for the follow-up and treatment following the respective international guidelines and for the statistical analysis of this investigation.

RESULTS

In vitro tests

We evaluated the descriptive statistics of the CSA in order to allow comparison with other investigations. Growth rates and in vitro response rates grouped by tumour entities and drugs are listed in tab. 1. The growth rate ranged from 73% in rectum carcinoma cells up to 96% for CUPS (overall 77%). Reasons of non-growth were infection (20%), primary non-growth (prolonged transfer from theatre to the lab in single cases or entirely necrotic material) or apoptosis under in vitro conditions.

Table 1. Growth rates and in vitro sensitivity of tumour specimens grouped by histology and drug. All data except n (number of assays) are shown in %

<table>
<thead>
<tr>
<th>Tumor entity</th>
<th>n</th>
<th>Growth rate</th>
<th>In vitro response to chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5-FU</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>63</td>
<td>76</td>
<td>44</td>
</tr>
<tr>
<td>CUPS</td>
<td>25</td>
<td>96</td>
<td>36</td>
</tr>
<tr>
<td>Soft tissue sarcoma</td>
<td>76</td>
<td>84</td>
<td>33</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>39</td>
<td>82</td>
<td>42</td>
</tr>
<tr>
<td>Mamma carcinoma</td>
<td>146</td>
<td>77</td>
<td>48</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>76</td>
<td>74</td>
<td>57</td>
</tr>
<tr>
<td>Pancreatic carcinoma</td>
<td>14</td>
<td>86</td>
<td>45</td>
</tr>
<tr>
<td>Rectum carcinoma</td>
<td>26</td>
<td>73</td>
<td>50</td>
</tr>
</tbody>
</table>

conditions (60%), lacking evidence of viable malignant cells in 2%, overgrowth by monocytes or fibroblasts in 4%. Other reasons were responsible in 14% of cases. There was no remarkable influence of histological type, tissue type (primary tumour, recidive tumour, lymph node, metastasis, effusion), localisation, pre-operative chemotherapy or radiation on growth rate (multiple and simple logistic regression). The in vitro response rates varied depending on tumour entity and drug (e.g. 8-18% for MTX and 33-83% for EPI).

Patient data descriptive statistics

The patients were treated with the following chemotherapeutic regimens (with adjuvant or palliative intention). Colon cancer: 24 patients were treated with chemotherapy post-operatively: 5-fluorouracil (5-FU)/Leucovorin or Ardalan 14x, doxorubicin (DOX)/cisplatin/cyclophosphamid 1x, DOX 1x, epirubicin (EPI) 2x, oxaliplatin 1x, oxaliplatin/5-FU 1x, vinblastin (VBL) 4x). CUPS: 2 patients received chemotherapy (unknown regimen). Soft tissue sarcoma: The protocols used were 5-FU/Leucovorine or Ardalan 2x, CEVAIA 1x, CYVADIC 5x, DOX 1x, HD-CWS 96 1x, ifosfamid (IFO)/DOX 4x, VBL 3x, vincristin (VCR) 1x, Cytoablation protocol 1x. Gastric cancer: Postoperative chemotherapy consisted of 5-FU/Leucovorin 1x, ELF-protocol 7x and VBL monotherapy 3x. Breast cancer: 44 patients received postoperative chemotherapy: with Ardalan-regimen 1x, CMF 18x, Docetaxel 2x, DOX 1x, EC protocol 11x, IFO/DOX 1x, navelbine 4x, VBL 1x, Bendamustin 1x, 5-FU/Leucovorin 2x, Taxol 1x, Vinblastin 1x. Malignant melanoma: 7 patients were treated postoperatively with: BHD protocol 1x, BOLD protocol 1x, dacarbazine 1x, regional hyperthermal perfusion 1x, vinblastin 1x, vincristine 1x, vindesine 1x. Rectum carcinoma: 8 patient underwent chemotherapy after being operated: 2 with Ardalan protocol, 3 with camptothecin, 5-FU/Leucovorin, 5-FU alone and de Gramont protocol were used once. 2 patients suffering from Pancreatic cancer were treated with chemotherapy (1x ELF protocol, 1x 5-FU/leucovorin).

Survival data and response rate, as assessed by 1st staging, for patients undergoing palliative chemotherapy are listed in tab. 2. The survival rates differed among tumour entities. The response rates on chemotherapy varied depending on histology (from 9% for colon cancer up to 83% for breast cancer).

In vitro /in vivo associations and overall survival

Of the initially 614 CSAs, n=504 patients remained after elimination of multiple CSA in the same patient. Furthermore we excluded all patients suffering from entities with n<10. Breast cancer (n=146), colorectal cancer (n=89), soft tissue sarcoma (n=79), malignant melanoma (n=76), gastric cancer (n=39), cancer of unkown primary site (CUPS, n=25), thyroid cancer (n= 15), pancreatic cancer (n=14), renal cancer (n=12) patients were included into further analysis. The in vitro/in vivo correlation, as described above revealed the following groups: “sensitive” (according to “strong sensitivity” of the CSA, n=54), “moderately sensitive” (n=10), “weakly sensitive”

Table 2. Clinical data of patients undergoing palliative chemotherapy

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Gender: male/female (n)</th>
<th>Age median (years), (range)</th>
<th>OS after 1 year</th>
<th>PFS after 1 year</th>
<th>Response rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon Ca</td>
<td>8/8</td>
<td>63 (37-77)</td>
<td>16</td>
<td>93</td>
<td>81-100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16</td>
<td>93</td>
<td>81-100</td>
</tr>
<tr>
<td>STS</td>
<td>8/10</td>
<td>59,5 (39-99)</td>
<td>18</td>
<td>82</td>
<td>63-100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18</td>
<td>82</td>
<td>63-100</td>
</tr>
<tr>
<td>Gastric Ca</td>
<td>5/6</td>
<td>63 (43-74)</td>
<td>11</td>
<td>67</td>
<td>36-97</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td>67</td>
<td>36-97</td>
</tr>
<tr>
<td>Malignant</td>
<td>0/20</td>
<td>62,5 (33-74)</td>
<td>20</td>
<td>100</td>
<td>nieustalony / undefined</td>
</tr>
<tr>
<td>Melanoma</td>
<td></td>
<td></td>
<td>20</td>
<td>100</td>
<td>nieustalony / undefined</td>
</tr>
</tbody>
</table>

| Abbreviations: Ca – cancer, STS – soft tissue sarcoma, CI – 95% confidence interval, Stag. – Staging. The survival is shown as percentage of (overall or progression free) survival after 1 year. The CI of survival could not be calculated for breast cancer. The response rate is calculated as percentage of cases with complete or partial remission during 1st performed radiological staging |
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(n=0), “resistant” (n=29). The remaining patients who did or did not receive postoperative chemotherapy were classified as “empiric”.

A Cox-regression analysis was performed including: histology, age, TNM classification (pT pN cM G R), postoperative chemotherapy, postoperative radiation (yes or no respectively), in vitro/in vivo classification into “sensitive” group (see above). n=197 patients with 55 events remained after casewise MV deletion. The full model was stepwise backward reduced using the “step”-function of the “survival”-package of the freely available R software.

Table 3 shows the significantly contributing risk factors of the the Cox regression analysis. Treatment with in vitro effective drugs (“sensitive” group) is the only factor that decreases the death hazard significantly and independently in this analysis.

Therefore we further compared the OS in the correlation groups corresponding to the CSA response. The Kaplan-Meyer-curves are shown in fig. 2. The best OS is achieved by the “sensitive”-group. When comparing the OS of the “sensitive” group with all other groups stratifying the patients by histology the logrank test revealed a tendentially prolonged OS of the “sensitive” group (logrank test, p=0.080, see fig. 2). Nevertheless, age (55 years in the “sensitive” group vs. 60 years in the remaining patients, t-test, p=0.012), postoperative chemotherapy (since the “sensitive” groups is per se associated with chemotherapy) and postoperative radiation (p<0.001) differed between the groups. In order to further reduce confounding, we performed a matched-pairs-analysis with balancing of these variables. After matching n=39 patients were classified into the “sensitive” group and n=39 to the remaining groups. Age, histology, pT, pN, CM, postoperative radiation did not significantly differ between the groups, and all patients were treated with postoperative chemotherapy. Again a survival analysis of the matched groups was performed (fig. 3). The OS of the “sensitive” group was significantly prolonged compared to the remaining groups (logrank test, p=0.0063). It is obvious that, the lower the in vitro effect of the drug, the shorter the OS.

We can summarize that the use of in vitro effective drugs during postoperative chemotherapy was associated with prolonged survival independent from histology and other factors.

Table 3. Results of the Cox-regression analysis. Abbreviations: postop CT – postoperative chemotherapy. Treatment with an in vitro effective drug is the only factor decreasing the death hazard. The likelihood ratio test (p=0.00056), the Wald test (p=0.00059) and the logrank test (p<0.0001) achieved significance

<table>
<thead>
<tr>
<th>Variable</th>
<th>exp (coef)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.036</td>
<td>0.012</td>
</tr>
<tr>
<td>pNX</td>
<td>3.271</td>
<td>0.0078</td>
</tr>
<tr>
<td>G2</td>
<td>2.357</td>
<td>0.0367</td>
</tr>
<tr>
<td>G3</td>
<td>4.103</td>
<td>0.0046</td>
</tr>
<tr>
<td>G4</td>
<td>7.901</td>
<td>0.0059</td>
</tr>
<tr>
<td>Postop CT</td>
<td>2.108</td>
<td>0.0802</td>
</tr>
<tr>
<td>“Sensitive”</td>
<td>0.260</td>
<td>0.0053</td>
</tr>
</tbody>
</table>
DISCUSSION

The aim of the present investigation was to determine whether OS can be prolonged in cancer patients undergoing chemotherapy with in vitro effective drug based on the CSA of primary tumour cell cultures. The growth rates (73-96%) were equal to or higher than what has been determined in other assays (i.e. 57-82% in (14), 87% for ovarian cancer in (15), 40-70% in (13), 66-90% in (3). They differed markedly depending on histological diagnosis. Two- or threefold splitting of the primary cell culture was necessary. The results of the assay were available after 10-14 days. The in vitro concentrations of the drugs were equalized to the calculated maximal plasma concentration during chemotherapy (11). The in vitro sensitivity differed substantially depending on histology and the drug administered (see tab. 1). A comparison to other assays could be made based on ranking of tumor entities by in vitro sensitivity. The investigations of Bertelsen et al. (14) showed a sensitivity ranking of soft tissue sarcoma (STS) > ovarian cancer > colon cancer > breast cancer > melanoma. Andreotti et al. (15) reported similar results. Nevertheless, it is not possible to draw any concrete conclusions based merely on mean in vitro sensitivities with regard to the expected in vivo effects of chemotherapeutic substances. This may be explained by molecular heterogeneity of tumours of the same histological origin (24).

Campling et al. (25) reported on a significant decrease of chemosensitivity in patients after chemotherapy compared to untreated patients. Effects posed by preoperative chemotherapy on in vitro resistance could not be shown in our investigation. Comparing growth rate, reliability and practicability, we can state that the semiquantitative oncobiograms are much simpler and less expensive to perform than any quantitative method. However, the assay depends on subjective evaluation of the in vitro response by the investigator. As can be seen in fig. 1, this might be learned with a short learning curve.

Only about 30% of all patients could be included in the in vitro/in vivo associations according to the above mentioned criteria. A similar percentage was reported by Bertelsen et al. (14). Cox-regression, survival analysis and matched-pairs-analysis were performed in order to reduce any bias. These investigations revealed strong evidence that treatment with in vitro effective may prolong survival in cancer patients suffering from different malignant diseases, despite the fact that the study design comprised a plenty of limitations: The retrospective nature and radical administrative changes of the health care and general political system made it difficult to assess follow-up data at a unified qualitative standard in the 1990ies. Patients underwent surgery with primary cell culture preferentially when the disease was already progressed, therefore the cohort may not have been representative for tumour patients in general. Most patient received multimodal and multidrug therapy, therefore only in vitro sensitivity could be correlated to the in vivo behaviour. Depending on the histologic diagnosis, the CSA may not have tested all substances which were applied afterwards according to treatment guidelines. However, the matched-pairs-analysis revealed that when the most important covariats were balanced between the in vitro/in vivo correlation group, the OS was unequivocally prolonged in the “sensitive” group.

When comparing the evidence base for CSA guided therapy with that of empiric therapy in the current literature (see Introduction section), it seems that the empiric selection of chemotherapeutic multidrug regimens by PCTs is not fully justified. Oncobiograms provide a simple, inexpensive and reliable oppor-
tunity to test the drug sensitivity in an individual before the chemotherapy starts.

Future investigations may determine on how much profit a representative tumour patient cohort may gain from CSA guided treatment, and in this regard, CSAs may have their place as an adjuvant investigation for second- or third-line chemotherapies. The optimal study design would be therefore a multicenter prospective observational study with modern chemotherapeutic regimens, followed, if appropriate, by a therapeutic trial.

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