c-myc Oncogene gene dosage, serum CEA and CA-15.3 antigen levels, and cellular DNA values in relation to *ex vivo* chemosensitivity of primary human breast cancer[✲][★]

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A pilot study on relationships of selected molecular factors (*c-myc* oncogene average gene copy numbers (AGCN); serum CEA and CA 15.3 antigen levels; tumor cells' DNA values), to the *ex vivo* chemosensitivity of primary female human breast cancer in a modified adenosine triphosphate cell viability chemosensitivity assay (ATP-CVA),

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was performed. Four drug combinations were tested. A group of 75 cases of female primary breast cancer was assessed. Numerous correlations were found among molecular factors tested but none, with the exception of tumor grading, of these reflected *ex vivo* chemosensitivity of tumors tested. The results suggest that the parameters tested may not be important factors related to adjuvant chemoresponsiveness of primary human breast cancer to tested drug combinations.

Breast cancer is still a leading cause of death among women concerning the neoplastic disease around the world. Patients with operable breast cancer are primarily treated by surgery, and then by postoperative hormonal therapy, radiotherapy and/or chemotherapy, with the exception of patients with early-stage disease. The necessity of choosing a good adjuvant treatment for breast cancer patients, e.g. an effective chemotherapy regimen, is one of the key problems in the therapy. One of the ways of selecting the proper treatment is the analysis of clinicopathological data, as well as the status of some molecular factors known as having a predictive value in breast cancer, e.g. status of *erbB-2* oncogene and/or its product (recently discussed by Clark, 1998). The other factors studied in the case of breast cancer (without established predictive value, however), are other oncogenes [e.g. *myc* family (Ryan & Birnie, 1996)] and serum markers, e.g. CEA and CA-15.3 antigens which, used in combination, correlate to breast cancer dissemination and may be useful markers of the effectiveness of therapy used, as well as markers of disease relapses (Molina & Gion, 1998; Robertson *et al.*, 1999).

It is currently accepted that cancer patients' clinical and histopathological evaluation does not provide sufficient information as regards the response to chemotherapy. Drug selection for individual patients is usually based on reported response rates for clinical trials (so-called empiric treatment), but not on *ex vivo* individualized chemosensitivity assays. The response rates are usually better after treatment with the *ex vivo* selected chemotherapy regimens than with empiric ones, however, only one third of patients entered in prospective trials of *ex vivo* chemosensitivity test-

ing were treated with an *ex vivo* best regimen, as reviewed by Cortazar & Johnson (1999).

In, at least, some cancer patients, more effective clinical response to chemotherapy may be achieved by selecting the most active drugs on the basis of *ex vivo* chemosensitivity test results for individual patients (Zabel *et al.*, 1992; 1997; Cree & Kurbacher, 1997; Cortazar & Johnson, 1999; Kurbacher *et al.*, 1999). One of the *ex vivo* assays widely used in prediction of chemotherapy response, or *in vitro* drug testing in a variety of cancers, is the adenosine triphosphate cell viability chemosensitivity assay (ATP-CVA) and its modifications (Cree & Kurbacher, 1997; Falkiewicz & Vogt, 1999). It has a high evaluability rate and its results are independent of presence of non-cancerous cells in specimens evaluated (Cree & Kurbacher, 1997; Falkiewicz & Vogt, 1999). ATP-CVA is individually able to detect the majority of patients with *in vivo* poor or good response to chemotherapy; its sensitivity appears to be more than 85% and its specificity not less than 80%, with the true positive predictive value of more than 92% and true negative predictive value of more than 70% (Cree & Kurbacher, 1997; De Vita, 1997; Falkiewicz & Vogt, 1999).

As we hypothesize, even better results may be obtained when the *ex vivo* chemosensitivity assay will be combined with assessment of molecular factors with established or probable predictive value in individual patients with cancer. Therefore, we performed a preliminary study on the correlation of selected molecular factors [*c-myc* oncogene average gene copy numbers (AGCN); serum CEA and CA-15.3 antigen levels; tumor cells' DNA values] with the *ex vivo* chemosensitivity of ovarian cancer in the modified ATP-CVA.

PATIENTS, MATERIALS AND METHODS

A group of 75 female patients with primary, histologically confirmed, invasive breast cancer, diagnosed and treated in the Department of Obstetrics/Gynecology of the St. Elisabeth Hospital, Ibbenbüren, was assessed. The mean age of patients was 57.2 ± 11.4 years (mean ± S.D., range 34–81 years). The tumors assessed were classified according to the TNM classification of breast cancer. Tumor staging was: pT1: 47%, pT2: 39%, pT3: 5%, pT4: 9%. Tumor grading was as follows: G1: 4%, G2: 40%, G3 or G4: 56%. Nodal status classification was: pN0: 65%, pN1: 32%, pN2: 1%, unknown nodal status: 1%. One patient had detectable metastasis (pM0: 99%, pM1: 1%); 20% of patients were premenopausal, 7% peripausal, 73% postmenopausal.

Statistical analyses were performed using Statistica for Windows 4.3 software (Statsoft Inc., OK, USA).

c-myc *Oncogene average gene copy numbers (AGCN).* The AGCNs were determined using double differential polymerase chain reaction (ddPCR). The general ddPCR reaction conditions, reproducibility and clinical usefulness have been described elsewhere (Brandt *et al.*, 1994; 1995a; 1995b), and we used the ddPCR and electrophoresis conditions exactly as previously described (Vogt *et al.*, 1998a). Primers for *SOD2* and *HBB* (Brandt *et al.*, 1995a), and for *c-myc* (Beckmann *et al.*, 1999) fragments amplification have been described previously.

Serum carcinoembryonic antigen (CEA) and carbohydrate antigen 15.3 (CA-15.3). The protein concentrations in serum were determined using commercially available CEA and CA-15.3 enzyme-linked immunosorbent assays (Abbott), in accordance with the manufacturer's instructions.

DNA values. Tumor tissue was cut up with a scalpel to 1 ml of Partec HR-A solution (Lysing solution, Partec, Germany), then in-cubated for 10 min on a shaker board at room temperature. The cell suspension obtained was filtered through a 50 μm nylon mesh, 3 ml of Partec HR-B solution (Partec, Germany) was added, and the solution was incubated for 10 min at room temperature (DAPI-Staining). Analysis was performed directly with the Partec PAS Flow-Cytometry Analyzer using the HBO-Lamp excitation and Modfit or DPAC-Software for Cell Cycle Analysis and Ploidy Determination. The diploid region was defined as a DNA index from 0.95 to 1.05 and the tetraploid region as being from 1.9 to 2.1.

Adenosine triphosphate cell viability chemosensitivity assay (ATP-CVA). For ATP-CVA, a slightly modified method of Andreotti *et al.* was used (1994; 1995). Fresh breast cancer tissue specimens were treated as described by Vogt *et al.* (1999a). Therapeutic drug preparations from commercial sources were stored and used before expiration dates according to the manufacturer's instructions. Four drug combinations were tested: 1 — cyclophosphamide/metotrexate/5-fluorouracil (CMF), 62 cancer cell cultures were tested; 2 — 5-fluorouracil/epirubicin/cyclophosphamide (FEC), 65 cancer cell cultures were tested; 3 — paclitaxel/epirubicin (PE), 67 cancer cell cultures were tested; 4 — paclitaxel/mitoxantrone (PMi), 47 cancer cell cultures were tested. Cyclophosphamide was used in all cases as its active metabolite, 4-hydroxycyclophosphamide. The drugs were used in six dilutions, corresponding to 200%, 100%, 50%, 25%, 12.5% and 6.25% of each drug standard test drug concentration (TDC). 100% TDC values used were as follows: 4-hydroxy-cyclophosphamide 3.0 μg/ml, metotrexate 2.8 μg/ml, 5-fluorouracil 22.5 μg/ml, epirubicin 0.5 μg/ml, paclitaxel 13.6 μg/ml, mitoxantrone 0.65 μg/ml) (Andreotti *et al.*, 1994; 1995; Kurbacher *et al.*, 1996a). Each measurement was performed in triplicate. Percentages of total growth inhibition (TGI) were calculated according to the formula: $1 - (MR - MI)/(MO - MI) \times 100 = \%TGI$; where:

MR = mean counts for replicate test drug cultures; MI = mean counts for maximum inhibition control cultures; MO = mean counts for no inhibition control cultures. Areas under response curves (AUC) values for percentage tumor growth inhibition *vs* test drug concentration were calculated using the trapezoidal rule.

RESULTS AND DISCUSSION

The descriptive results of the determination of selected molecular factors in the patients studied are presented in Table 1. Some interesting statistically significant relationships

2.0) (Berns *et al.*, 1995). A correlation between *c-myc* amplification status and patients' age was found ($p = 0.047$ in Mann-Whitney U test; *c-myc* AGCN correlation with patients' age: $p = 0.035$ in Spearman rank order correlation, Spearman R = 0.24). This result is the opposite of that obtained by us in a study of ovarian cancer (Vogt *et al.*, accompanying paper) and was not known to us from published data. We also found a negative correlation between *c-myc* amplification status and fraction of cells in G_0G_1 phase ($p = 0.044$ in Mann-Whitney U test; *c-myc* AGCN correlation with the G_0G_1 cell fraction: $p = 0.014$ in Spearman rank order correlation, Spearman R = -0.30) and a positive correlation with the proportion of

Table 1. Descriptive statistics of the molecular factors status in the patients studied

Factor	Mean	S.D.	Median	Minimum	Maximum
c-myc AGCN	2.24	2.15	1.42	0.00	15.32
CEA	2.57	6.277	0.95	0.40	34.00
CA-15.3	23.47	25.69	16.70	6.20	141.90
DNA index	1.48	0.45	1.52	1.00	3.12
Proportion of cells in S phase (%)	10.77	8.63	8.30	1.00	37.68
Proportion of cells in G_0/G_1 phase (%)	80.57	9.40	81.24	54.40	96.24
Proportion of cells in G_2M phase (%)	9.51	5.62	8.16	0.30	27.62

For details see Methods

among the patients' and tumor parameters were found and summarized in Table 2.

The *c-myc* gene amplification determined by ddPCR was found in 24 cases (32.0%) (according to the published cut-off value of AGCN >

cells in S phase ($p = 0.004$ in Mann-Whitney U test; *c-myc* AGCN correlation with the S phase cell fraction: $p = 0.012$ in Spearman rank order correlation, Spearman R = 0.29), but not with DNA index ($p = 0.96$ in Mann-Whitney U

Table 2. Some significant relationships (*p* values) between the patients' parameters, parameters of tumors, and status of molecular factors

Relationships analysed	*p* value
Tumor grading & patients' age	0.039[a]
Tumor grading & CEA level	0.046[b]
Tumor grading & DNA index	0.015[a]
Tumor grading & proportion of cells in G_0G_1 phase	0.043[a]
Tumor grading & proportion of cells in S phase	0.017[a]

[a]Kruskal-Wallis ANOVA test; [b]median test

test; *c-myc* AGCN correlation with DNA index: $p = 0.82$ in Spearman rank order correlation). What one may suspect is that the fraction of cells in G_0G_1 phase significantly negatively correlates with their fraction in S phase or in G_2M phase, as well as with DNA index; DNA index correlates positively with the fraction of cells in S phase.

In ATP-CVA, all 75 tumors tested gave the *in vitro* cultures (100%). Chemoresponse *ex vivo* of the tumors assessed was not affected by the molecular parameters tested. However, tumor

relation between areas under chemoresponse curves (AUC) obtained in ATP-CVA and other factors tested are shown in Table 3.

The most effective drug regimen in the study was the PMi combination, giving the mean AUC of 17091, slightly less effective was the PE combination, giving the mean AUC of 16332, a worse regimen was the FEC combination, giving the mean AUC of 14528. The least effective regimen was the CMF combination, giving the mean AUC of 10735. In the study, all the *ex vivo* chemosensitivity results

Table 3. Relationships (*p* values) of AUC for four drug regimens tested to *c-myc* oncogene average gene copy numbers (AGCN), serum CEA and CA-15.3 antigen levels, and tumor cells' DNA values

Factor analysed	Drug regimen			
	CMF	FEC	PE	PMi
c-myc AGCN	0.86[b]	0.92[b]	0.44[b]	0.50[b]
c-myc amplification[a]	0.37[c]	0.55[c]	0.42[c]	0.91[c]
CEA	0.32[b]	0.78[b]	0.96[b]	0.70[b]
CA-15.3	0.43[b]	0.85[b]	0.51[b]	0.86[b]
DNA index	0.81[b]	0.31[b]	0.25[b]	0.71[b]
Proportion of cells in S phase	0.29[b]	0.80[b]	0.19[b]	0.95[b]
Proportion of cells in G_0/G_1 phase	0.50[b]	0.77[b]	0.26[b]	0.56[b]
Proportion of cells in G_2M phase	0.63[b]	0.49[b]	0.34[b]	0.57[b]
Tumor grading	0.77[d]	**0.03**[d]	**0.01**[d]	**0.21**[d]
pT classification	0.40[d]	0.98[d]	0.78[d]	0.75[d]
Patients' menopausal status	0.27[d]	0.31[d]	0.74[d]	0.89[d]

[a]AGCN > 2 according to Berns *et al.* (1995); [b]Spearman rank order correlation; [c]Mann-Whitney U test; [d]Kruskal-Wallis ANOVA test

grading is the single factor found to significantly influence the *ex vivo* chemosensitivity of cancerous cells, but not in every drug regimen. The results of statistical analysis of correlate strictly with each other (Table 4), despite the reported great variability in the effectivity of single drugs and drug combinations in breast cancer

Table 4. Relationships (*p* values) between AUC and four drug regimens tested

Drug regimen	CMF	FEC	PE	PMi
CMF	–	**0.00001**[a]	**0.001**[a]	**0.001**[a]
FEC	**0.00001**[a]	–	**<0.000000**[a]	**0.000001**[a]
PE	**0.001**[a]	**<0.000000**[a]	–	**<0.000000**[a]
PMi	**0.001**[a]	**0.000001**[a]	**<0.000000**[a]	–

[a]Spearman rank order correlation

specimens tested *ex vivo* (Kurbacher *et al.*, 1996b).

Various modifications of ATP-CVA have been applied in some studies for the *ex vivo* prediction of breast cancer patients' response to chemotherapy (Hunter *et al.*, 1993; Petty *et al.*, 1994; Köchli *et al.*, 1993; 1995; Cree *et al.*, 1996; Kurbacher *et al.*, 1996a; 1996b; 1999), or for chemosensitivity testing in breast cancer cell lines (several examples). We recently tested the combinations of ATP-CVA with cellular DNA parameters, oncogenes' gene dosage abberations and/or overexpression in a primary non-small cell lung cancer (Vogt *et al.*, 1998b) and ovarian cancer (Vogt *et al.*, 1999b). To the best of our knowledge, prior to our recent studies (Falkiewicz *et al.*, 1999; Schlotter *et al.*, 1999), ATP-CVA results in breast cancer have only once been correlated with molecular factors known as having a prognostic or predictive value in breast cancer, namely with *p53* gene product expression, in a study which demonstrated a correlation between p53 protein expression and chemosensitivity of human breast cancer specimens (Petty *et al.*, 1994).

In conclusion, the lack of correlation of *c-myc* AGCN, *c-myc* amplification status, serum CEA and CA-15.3 antigen levels, or proportions of cells in various cell cycle phases with chemosensitivity to all tested drug combinations, found in the present study, may suggest that the parameters tested may not be important factors related to adjuvant chemoresponsiveness of primary human breast cancer to tested drug combinations. In order to obtain useful and valuable results of combined testing we recently analysed the combination of other molecular factors (namely *erbB* oncogene family gene dosages and *erbB-2* expression) with ATP-CVA in the breast cancer (Schlotter *et al.*, 1999). The results of the study are promising and suggest that *erbB-2* expression (but not *erbB* family gene aberrations), may be effectively combined in *ex vivo* chemoresponsiveness prediction of primary human breast cancer, but this preliminary suggestion awaits further clinical confirmation.

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