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Relationship of *c-myc* and *erbB* oncogene family gene aberrations and other selected factors to *ex vivo* chemosensitivity of ovarian cancer in the modified ATP-chemosensitivity assay✪★

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A pilot study on relationships of selected molecular factors [*erbB-1*, *erbB-2*, *erbB–3*, and *c-myc* oncogene average gene copy numbers (AGCN); steroid receptors and *pS2* gene expression; tumor cells' DNA values] to the *ex vivo* chemosensitivity of ovarian cancer in a modified adenosine triphosphate cell viability chemosensitivity assay (ATP-CVA), was performed. Despite the relatively small number of patients, numerous correlations among the factors tested were found. Nevertheless, only *c-myc* gene dosage positively affected *ex vivo* chemosensitivity of tumors tested.

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Abbreviations: AGCN, average gene copy number; AUC, area under chemoresponse curve; ATP-CVA, ATP cell viability assay; ddPCR, double differential polymerase chain reaction; ER, estrogen receptor; *HBB,* human β-globin-encoding gene; OC, ovarian cancer; *p*, probability of error that is involved in accepting our observed result as valid; PR, progesterone receptor; RR, *ex vivo* good response rate; *SOD2*, gene encoding human superoxide dismutase; TDC, test drug concentration; TGI, total growth inhibition.

Ovarian cancer is one of the leading causes of death resulting from gynecological malignancies. Patients with ovarian cancer are primarily treated by surgery, which is the first step of therapy during which tumor resection and staging are performed, and then by postoperative chemotherapy, with the exception of patients with early-stage disease. Primary ovarian cancer frequently responds to chemotherapy and remission rates of 60–80% have been observed after platinum-based first-line therapy. Nevertheless, the majority of patients will relapse and die from their disease. The overall 5-year survival is only about 20%. Due to the serious adverse effects of chemotherapy and its costs, it is important to develop efficient methods of prediction of the response to chemotherapy in patients, before introduction of the treatment (Zabel *et al.*, 1992, 1997; Cree & Kurbacher, 1997; Cortazar & Johnson, 1999; Falkiewicz & Vogt, 1999). It is now well known that, in some cases, more effective clinical response to chemotherapy may be achieved both in patients with ovarian (Sevin & Perras, 1997; Zabel *et al.*, 1997) and other cancers (Zabel *et al.*, 1997; Cree & Kurbacher, 1997; Cortazar & Johnson, 1999) by selecting the most active drugs on the basis of *in vitro* chemosensitivity test results for individual patients. However, only one third of the patients entered in prospective trials of *ex vivo* chemosensitivity testing were treated with an *ex vivo* best regimen, as reviewed by Cortazar & Johnson (1999).

The adenosine triphosphate cell viability chemosensitivity assay (ATP-CVA) and its modifications are currently widely used in prediction of chemotherapy response or *in vitro* drug testing in a variety of cancers, due among others, to its high evaluability rate and reliable results irrespective of the presence of non-cancerous cells in specimens evaluated (Falkiewicz & Vogt, 1999). ATP-CVA is able to detect individually, *in vitro*, the majority of patients with an *in vivo* poor or good response to chemotherapy; its sensitivity seems 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).

To the best of our knowledge, ATP-CVA in various modifications was used in a few studies for the *ex vivo* prediction of ovarian cancer patients response to chemotherapy (Gerhardt *et al.*, 1991; Untch *et al.*, 1992; 1994; Sevin *et al.*, 1993; Andreotti *et al.*, 1994; 1995; Kurbacher *et al.*, 1996; 1997; 1998; 1999; Sevin & Perras, 1997), or for chemosensitivity testing in ovarian cancer cell lines, but its results have never previously been correlated to the results obtained for other molecular factors known as having a prognostic or predictive value in ovarian cancer. Nevertheless, the results of ATP-CVA have previously been correleted with *p53* gene expression in breast cancer specimens by Petty *et al.* (1994), and with DNA ploidy and oncogenes' gene dosage abberations and/or overexpression in a primary non-small cell lung cancer (Vogt *et al.*, 1998a) and breast cancer (Falkiewicz *et al.*, 1999; Schlotter *et al.*, 1999). Recently, ATP-CVA alone as a chemotherapy regimen selection, gave very good results in a prospective clinical trial on individualized chemotherapy for 56 recurrent ovarian cancer patients, failing to respond in 1 to 5 prior chemotherapy regimens (Kurbacher *et al.*, 1999): individualized chemotherapy selected by the ATP-CVA produced both an exceptionally high response rate and promising progression-free survival, and median overall survival. In addition, ATP-CVA confirmed resistance in all patients with platinum-refractory disease tested (Kurbacher *et al.*, 1999). Therefore, we performed a preliminary study on the correlation of selected molecular factors (*erbB-1*, *erbB-2*, *erbB–3*, and *c-myc* oncogene average gene copy numbers, AGCN; steroid receptors and *pS2* gene expression; DNA index and proportions of cells in various cell cycle phases) to the *ex vivo* chemosensitivity of ovarian cancer in the modified ATP-CVA.

PATIENTS, MATERIALS AND METHODS

A group of 17 patients with histopathologically documented primary ovarian cancer, diagnosed and treated in the Department of Obstetrics/Gynecology of the St. Elisabeth Hospital, Ibbenbüren, was assessed. The mean age of patients was 67.9 ± 12.7 years (mean ±S.D., range 31–86 years). Relationship between *ex vivo* chemoresponse and molecular factor status was statistically analysed using Statistica for Windows 4.3 software (Statsoft Inc., OK, U.S.A.).

ErbB *family (*erbB-1, erbB-2, erbB-3*) and* c-myc *oncogene average gene copy numbers*. The AGCNs were determined by 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.*, 1998b). Primers for *erbB-1, erbB-2, erbB-3, SOD2, HBB* (Brandt *et al.*, 1995a), and *c-myc* (Beckmann *et al.*, 1999) fragments' amplification were described previously.

Estrogen and progesterone receptor, and* pS2 *gene expression. The expression of the RNA genes was determined by a combined reverse transcription-polymerase chain reaction (RT-PCR). Cancer tissue (about 100 mg) was first homogenized and lysed with the FastPrep™ System, in the presence of a highly denaturing guanidinium isothiocyanate-containing buffer. β-Mercaptoethanol was added to lysis buffer RLT just before use (60 μl + 600 μl RLT buffer). Then, RNA was isolated by the RNeasy Mini Kit (Qiagen, Germany) as described in the manufacturer's handbook for tumor tissues. All steps in the RNeasy protocol are performed at room temperature. In brief, after homogenization and lysis, ethanol (70%, 600 μl) was added to each sample, providing appropriate binding conditions and then the sample was applied to an RNeasy mini column where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA was eluted by 70 μl of RNase-free water, and then centrifuged for 1 min (10000 rev./min). After identification and staining of the isolated RNA, cDNA was prepared by RNA-PCR KIT Version 2.1 by using AMV-reverse transcriptase (TaKaRa, Japan). The successive steps were as follows: step 1: 30°C for 10 min, step 2: 42°C for 30 min, step 3: 99°C for 5 min, and step 4: 4°C for 5 min (Robocycler, Stratagene, Germany). After cDNA production, the detection of estrogen and progesterone receptor gene and *pS2* gene, as well as beta-2 microglobulin reference gene were detected by regular ddPCR, as described above. Proportion of cDNA for genes tested to that of beta-2 microglobulin reference gene was calculated and used for expression analysis. Each PCR run contained as standard control an RNA/DNA from non-cancerous muscle tissue.

DNA values. Tumor tissue was cut up with a scalpel into 1 ml of Partec HR-A solution (Lysing solution, Partec, Germany), then incubated for 10 min on a shaker board at room temperature. 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.* (1994; 1995) was used. Fresh ovarian cancer tissues were treated exactly as described previously (Vogt *et al.*, 1999b). Therapeutic drug preparations from commercial sources were stored and used before expi-

ration dates according to the manufacturer's instructions. Three drug combinations (etoposide alone, 13 cancer cell cultures were tested; paclitaxel/epirubicin, 13 cancer cell cultures were tested; paclitaxel/carboplatin, 15 cancer cell cultures were tested) 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: etoposide 48.0 μg/ml, paclitaxel 13.6 μg/ml, epirubicin 0.5 μg/ml, carboplatin 15.8 μg/ml) (Andreotti *et al.*, 1994; 1995, Kurbacher *et al.*, 1996). Each measurement was performed in triplicate. Percentages of total growth inhibition (TGI) were calculated according to the formula: 1–(MR–MI)/(MO–MI) × 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. Area under response curves (AUC) values for percentage tumor growth inhibition *vs* test drug concentration were calculated using the trapezoidal rule. In addition, the *ex vivo* chemoresponses were categorized as follows: the high chemosensitivity was defined as TGI percentage of more than 70% for 6.25–200% TDC, the intermediate chemosensitivity as TGI percentage of more than 70% for 50–200% TDC and 50–70% for 6.25–25% TDC, the medial chemosensitivity as TGI percentage of more than 70% for 50–200% TDC and <50% for 6.25–25% TDC, the partial chemosensitivity as TGI percentage 50–70% for 6.25–200% TDC, and the chemoresistance as TGI percentage <50% for 6.25–200% TDC. The *ex vivo* good response was defined as high or intermediate chemosensitivity.

RESULTS AND DISCUSSION

To the best of our knowledge, this is the first study on the correlation of the modified ATP-CVA results and solid tumors molecular status, with the focus on ovarian cancer.

No statistically significant correlations between the patients' parameters or tumor staging and chemoresponse to treatment (measured as AUC) were found. A negative correlation between *c-myc* amplification status and patients' age (p = 0.032 in Mann-Whitney U test; *c-myc* AGCN correlation with patients' age: p = 0.055 in Spearman rank order correlation, Spearman R = –0.57) was found. This is a result inverse to those obtained by us in a study of breast cancer (Falkiewicz *et al.*, unpublished) and was not known to us from published data. We have not found any other statistically significant correlation between the patients' parameters, tumor staging and molecular factor status.

The results of the determination of selected molecular factors were presented in Table 1. In agreement with the published cut-off AGCN values for the *erbB* family gene amplifications determined by ddPCR, we have found the amplification of *erbB-1* in no case, the deletion of *erbB-1* in 58.3% of cases (cut-off values of AGCN > 1.6, AGCN < 0.4, respectively (Brandt *et al.*, 1995b)), the amplification of *erbB-2* in 33.3% of cases (similarly to numerous studies reported), and those of *erbB-3* in 9.1% of cases (cut-off values of AGCN > 2.0, and AGCN > 1.75, respectively (Brandt *et al.*, 1995b). The amplification of *c-myc* was found in 16.6 % of cases (cut-off value of AGCN > 2.0) (Berns *et al.*, 1995). Despite the relatively small number of patients, a few correlations among factors tested were found, namely: *erbB-1* AGCN correlates with that of *erbB-3* (p = 0.006), *erbB-3* AGCN correlates with *ER* RNA expressions (p = 0.03), proportion of cells in G_2M phase correlates with *PR* RNA expression (p = 0.03) (Spearman rank order correlation).

In ATP-CVA all 17 tumors tested gave *in vitro* cultures (100%). The results of statistical analysis of correlation between AUCs obtained in ATP-CVA and other factors tested are shown in Table 2. Notable are better correlations obtained between *ex vivo* chemosensitivity and progesterone receptor gene or *pS2*

Table 1. Descriptive statistics of the status molecular factors' in patients with ovarian cancer

Molecular factor	Mean	S.D.	Median	Minimum	Maximum
erbB-1 AGCN	0.43	0.34	0.34	0.00	1.12
erbB-2 AGCN	1.79	0.64	1.72	1.01	2.75
erbB-3 AGCN	1.12	0.30	1.03	0.80	1.83
c-myc AGCN	1.97	1.56	1.46	0.97	6.36
erbB-1 AGCN/*erbB-2* AGCN	0.26	0.31	0.19	0.00	1.10
ER RNA expression	0.68	0.68	0.60	0.00	1.81
PR RNA expression	0.25	0.38	0.00	0.00	1.08
pS2 RNA expression	0.82	1.39	0.00	0.00	4.53
DNA index	1.19	0.33	1.00	0.97	1.80
Proportion of cells in S phase (%)	12.93	8.86	9.88	3.85	32.85
Proportion of cells in G_0/G_1 phase (%)	81.34	9.42	82.52	63.45	93.85
Proportion of cells in G_2M phase (%)	5.73	3.37	4.46	2.10	11.95

For details see Methods

gene expression than between *ex vivo* chemosensitivity and estrogen receptor gene expression, as well as better correlation obtained between *ex vivo* chemosensitivity and *erbB-1* AGCN/*erbB-2* AGCN ratio than between the chemosensitivity and any of the *erbB* genes AGCN alone. Nevertheless, only *c-myc* gene dosage significantly positively affected *ex vivo* chemosensitivity of tumors tested to paclitaxel/epirubicin combination (Spearman R = 0.62). This result is interesting as regards the dual function of the prod-

Table 2. Relationships (*p* values) of AUC for three drug regimens tested to AGCN of *erbB-1*, *erbB-2*, *erbB-3* and *c-myc* oncogenes, estrogen and progesterone receptor gene and *pS2* gene expression, DNA index and proportions of cells in S, G_0/G_1 or G_2M cell cycle phases

Molecular factor	Drug regimen		
	Etoposide	Paclitaxel/epirubicin	Paclitaxel/carboplatin
erbB-1 AGCN	0.57[b]	0.63[b]	0.97[b]
erbB-2 AGCN	0.42[b]	0.94[b]	0.69[b]
erbB-3 AGCN	0.53[b]	0.96[b]	0.88[b]
erbB-1 AGCN/*erbB-2* AGCN	0.43[b]	0.36[b]	0.71[b]
c-myc AGCN	0.73[b]	**0.04**[b]	0.12[b]
erbB-1 AGCN/*erbB-2* AGCN >0.15[a]	0.16[c]	0.20[c]	0.52[c]
ER RNA expression	0.51[b]	0.50[b]	0.95[b]
PR RNA expression	0.06[b]	0.14[b]	0.15[b]
pS2 RNA expression	0.13[b]	0.33[b]	0.49[b]
DNA index	0.31[b]	0.11[b]	0.17[b]
Proportion of cells in S phase	0.78[b]	0.54[b]	0.73[b]
Proportion of cells in G_0/G_1 phase	0.53[b]	0.77[b]	0.87[b]
Proportion of cells in G_2M phase	0.73[b]	0.51[b]	0.20[b]

[a]ratio of a prognostic value according to Brandt *et al.* (1995b); [b]Spearman rank order correlation; [c]Mann-Whitney U test

ucts of the *c-myc* gene which functions both as transcriptional repressor and activator (reviewed by Ryan & Birnie, 1996).

In cultures tested, *ex vivo* chemoresponses categorized as described above (high, intermediate, medial, partial chemosensitivity, and chemoresistance) were not significantly correlated with molecular factors, probably due to the relatively small number of cases studied (not shown). As regards this classification, etoposide produced high, medial, partial response, or chemoresistance in 9.1%, 27.3%, 36.4%, and 27.3% of cases, respectively (RR = 9.1%); combination of paclitaxel with epirubicin produced high, intermediate, medial or partial response, in 7.7%, 7.7%, 76.9%, and 7.7%, respectively (RR = 15.4%); and combination of paclitaxel with carboplatin produced intermediate, medial or partial response, or chemoresistance in 13.3%, 60%, 20%, and 6.6% of cases, respectively (RR = 13.3%). The most effective drug regimen was the combination of paclitaxel with epirubicin, giving the mean AUC of 15790 slightly less effective was a combination of paclitaxel with carboplatin, giving the mean AUC of 14388, the least effective regimen was etoposide alone, giving the mean AUC of 9295. Using ATP-CVA, a great variation in the degree of the efficiency of single drugs and drug combinations in obtaining an *ex vivo* positive response in individual ovarian cancer specimens has been reported (Sevin & Perras, 1997), however, in our study all *ex vivo* chemosensitivity results expressed (as AUCs) strictly correlated with each other (Table 3).

It is currently accepted that disease staging and histological evaluation do not provide sufficient information as regards the response to chemotherapy (Sevin & Perras, 1997). Drug selection is not usually based on individualized *ex vivo* chemosensitivity assays, but on published response rates for clinical trials in which large numbers of patients have been treated with preselected treatment regimens. The best treatment regimen tested has numerous advantages in OC treatment, as paclitaxel and carboplatin have non-overlapping toxicities with a broad range of clinical activity (Schwartz *et al.*, 1998), therefore, the combination may be very useful for the treatment of OC.

Summing up, in the study presented we have obtained results that focused our interest on the role of *c-myc*, *PR* and *pS2* abberrations, and *erbB* oncogene family interrelationships in ovarian cancer chemosensitivity, but no final conclusions may be suggested. The study is rather preliminary and has to be extended before the method presented can become a rational tool in choosing a chemotherapy regimen for OC. The other possibility for obtaining definite results of combined testing may be a combination of other molecular factors with ATP-CVA.

Table 3. Relationships (*p* values) between AUC for three drug regimens tested in patients with ovary cancer

Drug regimen	Etoposide	Paclitaxel/epirubicin	Paclitaxel/carboplatin
Etoposide	–	**0.001**[a]	**0.002**[a]
Paclitaxel/epirubicin	**0.001**[a]	–	**0.002**[a]
Paclitaxel/carboplatin	**0.002**[a]	**0.002**[a]	–

[a]Spearman rank order correlation

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