Expression of Activin During and After Chemotherapy in Peripheral Blood of Patients with Primary Breast Cancer

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Abstract. *Background/Aim: Activins are dimeric glycoproteins that play a significant role in reproduction and in endocrine-active tumors. The aim of this study was to evaluate the potential correlation between the concentration of activins (activin A, activin B, and activin AB) in patients receiving adjuvant chemotherapy for breast cancer. Patients and Methods: The serum concentration of activins in 30 patients receiving chemotherapy within the German SUCCESS A study was analyzed using different enzyme-linked immunosorbent assays at three time points: After primary surgery, but before chemotherapy; 4 weeks after the end of chemotherapy; and 2 years after chemotherapy during recurrence-free follow-up. Results: The activin concentration decreased in all patients after chemotherapy. Premenopausal patients had significantly lower concentrations of activin AB during follow-up than postmenopausal women (p=0.037). Thirteen out of 16 premenopausal patients developed chemotherapy-related amenorrhea (CRA) but did not significantly differ in their activin concentrations compared to the other premenopausal women. A positive human epidermal growth factor receptor 2/neu status was associated with a significant reduction of activin AB concentration (p=0.02), and trastuzumab treatment correlated with significantly decreased activin A concentration (p=0.012). Conclusion: Serial measurements of activin A concentration might be used for monitoring trastuzumab treatment. A sudden increase of activin concentration could be an early indicator of disease recurrence.*

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Inhibins and activins are secreted polypeptides of the transforming growth factor-β (TGFβ) superfamily, forming a subfamily of dimeric proteins (1, 2). Inhibins are heterodimers that consist of an α-subunit and one of two possible β-subunits (βA or βB), resulting in the formation of either inhibin A (α-βA) or inhibin B (α-βB), respectively. Activins are either homodimeric, consisting of two identical inhibin β subunits such as activin A (βA-βA) and activin B (βB-βB), or heterodimeric, consisting of two different β subunits such as activin AB (βA-βB) (1, 2). Recently, two additional β-subunits were identified in humans, determined as βC and βE (2), although their precise function in tumorigenesis is not yet clear (3, 4).

Although inhibins and activins have primarily been described in human gonads and identified as modulators of follicle-stimulating hormone production of the pituitary gland (5, 6), they have also been detected in several solid tumor types (5), including endocrine-responsive endometrial (7, 8), ovarian (9-11), and breast (12-15) carcinomas. Their differential expression has suggested their important role in malignant cell transformation (5, 7, 8), as well as possible roles in cancer differentiation, proliferation, and growth (5, 16, 17). Interestingly, TGFβ has been recognized as a tumor suppressor in pre-malignant stages of carcinogenesis, with an additional role as a pro-oncogene in later stages of disease, leading to metastasis (17, 18). Moreover, in experimental settings, inhibition of TGFβ suppressed metastasis to multiple organs (19, 20).

Dimeric activin A is significantly increased in carcinoma tissue and in the serum of patients with breast cancer in comparison to healthy controls (12). After surgical excision of the primary tumor, the serum levels of activin A drop, indicating that the tumor itself is capable of producing activin A (12, 21). Since it can inhibit the proliferation of breast cancer cell lines *in vitro* (22, 23), this glycoprotein is being discussed as an antiproliferative and immuno-

modulatory factor in mammary carcinomas (12). Current knowledge on the potential interactions between activins and growth-promoting oncogenes, how they are expressed in *e.g.* human epidermal growth factor receptor 2/neu (HER2/neu)-positive mammary carcinoma cells, and the influence of specific antibody therapies (*e.g.* trastuzumab) is based on cell-culture experiments (24), while only limited clinical data exist.

We previously demonstrated that chemotherapy significantly reduces inhibin A concentration in serum, possibly reflecting suppression of ovarian function and being a marker for chemotherapy-related amenorrhea (CRA) (25). However, the serological determination regarding activin A, activin B and activin AB is less indicative. Therefore, we performed qualitative and quantitative measurements of these activins in the serum of patients with breast cancer who received adjuvant chemotherapy, analyzing any possible correlation of these glycoproteins with the menopausal status of the patients, CRA, HER2/neu status, and treatment with trastuzumab.

Patients and Methods

Samples. The SUCCESS A study is a multicentric, prospectively randomized German clinical trial in patients with breast cancer comparing the efficacy and compatibility of two different chemotherapy regimens followed by 2- or 5-year bisphosphonate therapy. The aim of the study was the comparison of the recurrence-free survival after randomization of patients who received three cycles of chemotherapy with epirubicin–5-fluorouracil–cyclophosphamide (FEC), followed by three cycles of docetaxel *versus* three cycles of FEC chemotherapy, followed by three cycles of gemcitabine–docetaxel chemotherapy.

Serum samples derived from 30 patients of the SUCCESS A-trial (26) previously used to assess inhibin A concentrations (25) were used in the present study. All study participants had been diagnosed with invasive breast cancer and undergone surgery leading to R0 resection of the primary tumor. According to the study protocol, all of them received adjuvant chemotherapy. For each patient, three serum samples were available at three different time points: before the beginning of chemotherapy, 4 weeks after termination of chemotherapy, and 2 years after chemotherapy. Therefore, a total of 90 serum samples were tested for their activin concentration.

Enzyme-linked immunosorbent sssay (ELISA). For serological measurement of activin concentrations, different 'one-step' sandwich-type ELISAs were used (Active® Free activin A ELISA Kit, REF DSL-10-85100; Active® Free activin B ELISA Kit, REF DSL-10-86100; Active® Free activin AB ELISA Kit, REF DSL-10-86200; Diagnostic Systems Laboratories, Webster TX, USA). This immunoassay used two antibodies which absorb activin at two different sites. The microtitration wells were pre-coated with the primary antibody (100 μl/well). Serum samples tested for activin A or activin AB were diluted 1: 1000 with standard A, while samples tested for activin B, standards, and controls were not diluted. The activin antibody–enzyme conjugate complex contained biotinylated anti-human activin A detection antibody, secondary antibody, and strepatividin labeled with enzyme horseradish peroxidase (HRP). This solution (25 μl for activin A, or 50 μl for activin B and activin AB) was diluted in assay buffer (1: 50) and added to the plates. After incubation at room temperature (2 hours for activin A and activin AB, 3 hours for activin B) and after washing with wash solution, the wells were incubated with the substrate tetramethylbenzidine (TMB, 100 μl/ well) for additional 15 mins. to generate a change of color. An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 620 nm.

The absorbance measured was directly proportional to the concentration of activin present in the samples. A set of activin standards was used to plot a standard curve of absorbance *versus* activin concentration from which the activin concentrations in the serum samples were calculated.

Statistical analysis. For statistical analysis, the statistics programme SPSS 15.0 for Windows, (SPSS Inc, Chicago, IL, USA) was used. The established data of activin concentrations were compared by Friedman test and the Wilcoxon rank-sum test for paired samples. Statistical significance was assumed at values of $p<0.05$.

Table I. *Patient characteristics at primary diagnosis*.

	Number of patients (%)
Age at primary diagnosis[a]	
<52 Years	17 (56.7)
>52 Years	13 (43.3)
Tumor stage (UICC)	
1	11 (36.7)
2a	14 (46.7)
2b	5 (16.7)
Hormone receptor status	
Negative (ER- and PR-negative)	18 (60)
Positive (ER-/PR-positive)	12 (40)
HER2/neu status	
Negative	20 (66.7)
Positive	10 (33.3)
Histological type	
Ductal	24 (80)
Lobular	2 (6.7)
Other	4 (13.3)
Systemic therapy	
FEC-D	18 (60)
FEC-D–G	12 (40)
Endocrine therapy	
Tamoxifen	7 (23.3)
Anastrozol	1 (7)
Goserelin	3 (10)
Pre-menopausal	16 (53.3)
Post-menopausal	14 (46.7)
Chemotherapy-related amenorrhea[b]	13 (81.25)

FEC: 5-Fluorouracil–cyclophosphamide; D: docetaxel, G: gemcitabine. [a]Median age=52 years; [b]chemotherapy-related amenorrhea in 13 out of 16 premenopausal patients. Total rate of amenorrhea after chemotherapy: 27 out of 30 patients (90%).

Table II. *Concentrations of activin A, activin B and activin AB in serum.*

Time	Activin A (ng/ml)	Activin B (ng/ml)	Activin AB (ng/ml)
Prior to chemotherapy	40.19 (±21.28)	30.61 (±44.49)	83.80 (±98.22)
4 Weeks after chemotherapy	42.98 (±23.06)	26.86 (±34.07)	81.15 (±102.77)
2 Years after chemotherapy	37.11 (±20.74)	23.49 (±26.44)	78.95 (±89.21)

Data are presented as mean±standard deviation.

Results

The concentrations of the different activins in the serum of primary breast cancer patients were determined at three different points in time (prior to starting adjuvant chemotherapy, 4 weeks after conclusion of chemotherapy, and 2 years after concluding chemotherapy) and the average values were compared and statistically evaluated with regard to different parameters such as the menopausal status of the patients, CRA, HER2/neu status, and trastuzumab therapy. Additional parameters, such as the age of the patient, histology of the tumor, and hormone receptor status, did not result in any significant differences with regard to the activin concentrations, nor did the comparison of the serum activin concentrations with different endocrine treatments (Table I).

In total, 90 serum samples from 30 female patients with primary breast cancer were analyzed. Fourteen of these patients were postmenopausal when initially diagnosed, while 16 women were premenopausal, of whom 13 developed amenorrhea during the course of the chemotherapy.

The serum activin A concentration at 4 weeks after chemotherapy was similar to that prior to chemotherapy and decreased slightly 2 years after chemotherapy (Table II). The average activin B concentration and, analogously, the activin AB concentration, decreased consistently but not significantly.

The serum concentrations of activin AB were significantly lower in premenopausal patients 2 years after chemotherapy in comparison to the values in postmenopausal patients (p=0.037). There was no significant difference in activin A and activin B (Figure 1).

The serum concentrations of activin AB significantly decreased four weeks after chemotherapy in patients with HER2/neu-positive tumors in comparison to patients with HER2/neu-negative tumors (p=0.02). The concentrations of activin A and activin B were not significantly affected by the HER2/neu status (Figure 2).

Four weeks after chemotherapy, the activin A concentrations in patients who received trastuzumab treatment were significantly higher than in patients without trastuzumab treatment (p=0.012) (Figure 3).

Discussion

Activins were initially isolated from the gonads and identified as hormonal-regulatory factors of the hypothalamic–pituitary–gonadal axis (27). They are expressed by many endocrine and non-endocrine organs, including normal and malignant breast tissue (5, 14, 15, 28, 29). Activins have been associated with important functions in reproduction, cell proliferation, and carcinogenesis in several mammary carcinoma cell lines and mammary carcinoma tissues (5, 21, 23, 30). An *in vitro* analysis of 15 breast cancer cell lines detected activin A expression in only four (22, 31, 32). Interestingly, endocrine-responsive breast cancer MCF7 cells, which have no detectable endogenous activin A, are highly sensitive to the growth-inhibitory effects of activin A (33-35). However, whether this is also true for activin B and activin AB remains to be elucidated.

We previously demonstrated that the inhibin A concentration in serum significantly decreases after chemotherapy (25). However, there are currently no data we are aware of on the chronological progression of activin A, activin B, and activin AB concentrations in serum in patients with primary mammary carcinoma. It is also unknown whether serum activin concentrations are affected by adjuvant chemotherapy, or endocrine, or targeted treatments. We recorded a minimal reduction of these activin concentrations throughout the recurrence-free survival period in patients with primary breast cancer during and 2 years after adjuvant chemotherapy. If this is an effect of chemotherapy-induced direct damage of ovarian cells, paracrine functional effects or even a shift to the productions of other inhibins/activins remains unclear and warrants further research.

Since more and more women are diagnosed with breast cancer before having children, CRA is increasing in significance with regard to the ability to conceive and to the development of postmenopausal morbidities (*e.g.* osteoporosis) (36-39). Several serological parameters have been proposed as prognostic parameters for assessing ovarian function and reserve, including anti-Muellerian hormone and inhibin B (38, 40). The inhibin A concentration in blood serum might also be a possible marker, reflecting suppression

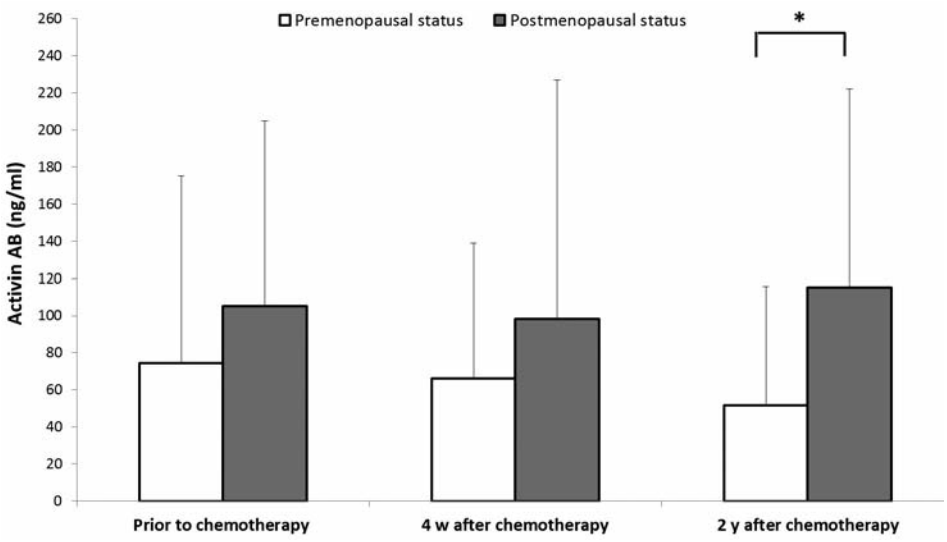

Figure 1. *Comparison of the average values of the activin AB serum concentration with regard to the menopausal status, *p=0.037. Data are presented as mean +/- standard deviation.*

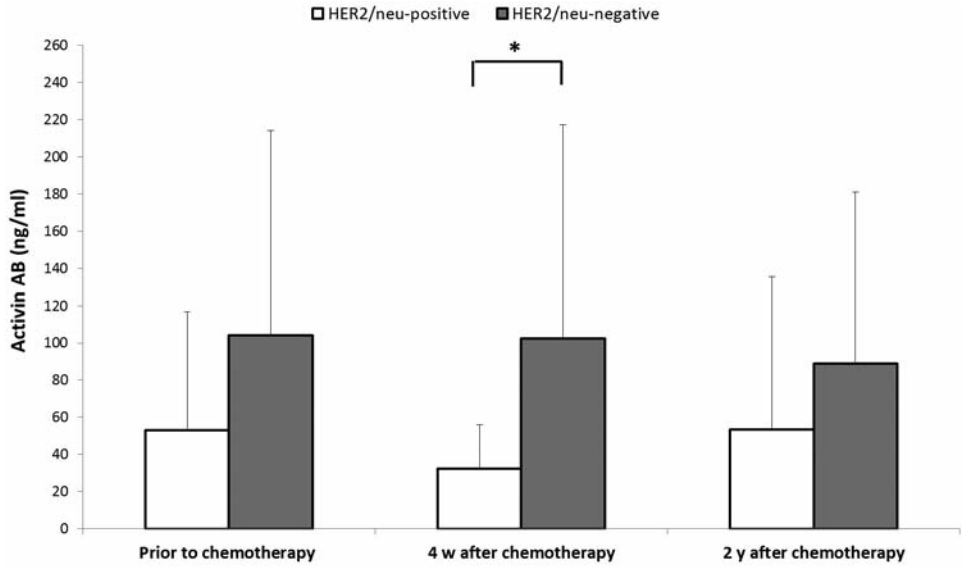

Figure 2. *Comparison of the average values of the activin AB serum concentration with regard to the Her2/neu status, *p=0.02. Data are presented as mean +/- standard deviation.*

of ovarian function (25). The serum inhibin concentration decreases with advancing age and the reduction of ovarian function. Activin concentration, especially of activin A, increases significantly with age but interestingly, more in the last decade of life (41, 42). While the activin concentration in the blood serum does not change significantly immediately after the beginning of menopause, it is significantly increased in cases of hypogonadotropic hypothalamic amenorrhea (43). Whether CRA affects our observed slight reduction of these activin concentrations needs further clarification. Moreover, the comparison of the menopausal status in our analyzed group demonstrates that 2 years after chemotherapy, there was a higher activin AB concentration in postmenopausal than in premenopausal women. The serum concentrations of the different activins decreased overall over time, although without statistical significance. However, serial measurements of the activin concentration in the serum of patients with breast cancer could indicate early onset menopause.

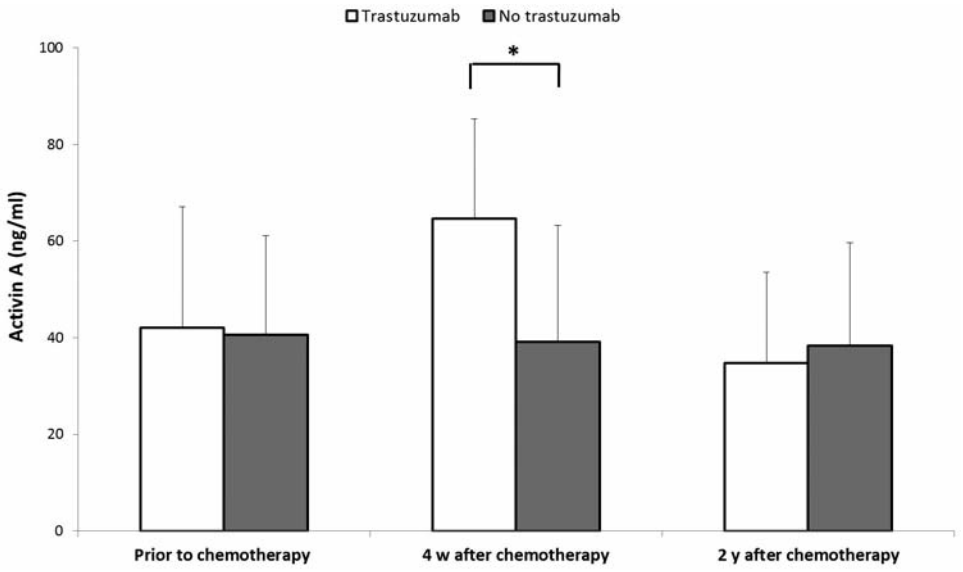

Figure 3. *Comparison of the average values of the serological activin A serum concentration with regard to the antibody targeted treatment with trastuzumab, *p=0.012. Data are presented as mean +/- standard deviation.*

With regard to the HER2/neu status, four weeks after chemotherapy, there was a significantly higher concentration of activin AB in patients with HER2/neu-negative than in women with HER2/neu-positive tumors. A possible explanatory approach for this might be that HER2/neu-positive tumors produce more growth factors and activin production therefore is suppressed, which results in a decrease in its concentration in serum. However, a functional link between HER2/neu and activin AB might be suggested. Interestingly, serum activin A concentration was significantly higher in patients who received trastuzumab treatment in comparison to patients without this targeted treatment. How therapy with trastuzumab might affect the production of activin A has not been fully clarified and requires for additional studies, even if a correlation between the overexpression of the growth factor receptor HER2/neu and the cellular effects of the TGFβ proteins in breast epithelial cells has already been noted (24).

In conclusion, this study demonstrates minimal reduction of the activin concentrations in patients treated for primary mammary carcinoma during and 2 years after adjuvant chemotherapy. Significant results were gathered with respect to menopausal status, with regard to the HER2/neu status, and completion of trastuzumab treatment in cases of HER2/neu-positive tumors. Serial measurements of activin concentrations might, therefore, be used to monitor the efficacy of trastuzumab treatment. However, whether the determination of activin concentration might be helpful in assessing the ovarian reserve or as potential early marker for detecting breast cancer recurrence must be examined in further studies.

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