Cytokeratin-18 Is a Useful Serum Biomarker for Early Determination of Response of Breast Carcinomas to Chemotherapy

Maria Hägg Olofsson,[1] Takayuki Ueno,[1,2] Yang Pan,[3] Ren Xu,[3] Feng Cai,[3] Heiko van der Kuip,[4] Thomas E. Muerdter,[4] Maike Sonnenberg,[4] Walter E. Aulitzky,[4] Stephan Schwarz,[1] Elina Andersson,[1] Maria C. Shoshan,[1] Aleksandra Mandic Havelka,[1] Masakazu Toi,[2,5] and Stig Linder[1]

Abstract

Purpose: With a widening arsenal of cancer therapies available, it is important to develop therapy-specific predictive markers and methods to rapidly assess treatment efficacy. We here evaluated the use of cytokeratin-18 (CK18) as a serum biomarker for monitoring chemotherapy-induced cell death in breast cancer.

Experimental Design: Different molecular forms of CK18 (caspase cleaved and total) were assessed by specific ELISA assays. Drug-induced release of CK18 was examined from breast carcinoma cells and tissue. CK18 protein composition was examined in serum. CK18 levels were determined in serum from 61 breast cancer patients during docetaxel or cyclophosphamide/epirubicin/5-fluorouracil (CEF) therapy.

Results: Caspase-cleaved CK18 molecules were released from monolayer cultures and tumor organ cultures to the extracellular compartment. CK18 was present in complexes with other cytokeratins in serum. Such CK18 protein complexes are remarkably stable, leading to favorable performance of CK18 biomarker assays for clinical investigations. Docetaxel induced increased levels of caspase-cleaved CK18 in serum from breast cancer patients, indicating apoptosis. CEF therapy led to increases predominantly in uncleaved CK18, indicating induction of necrotic cell death in many tumors. The increase in total CK18 at 24 h of the first treatment cycle correlated to the clinical response to CEF therapy ($P < 0.0001$).

Conclusions: Induction of necrotic cell death may explain the clinical efficacy of anthracycline-based therapy for breast carcinomas with defective apoptosis pathways. We suggest that CK18 biomarkers are useful for early prediction of the response to CEF therapy in breast cancer and may be useful biomarkers for clinical trials.

Chemotherapy induces multiple effects on tumor cells, including apoptosis, necrosis, autophagy, mitotic catastrophe, and senescence (1). The cellular outcome is dependent on several factors, including the type of drug used, the concentration of drug that will reach the tumor cells, and the properties of the tumor and its microenvironment. Apoptosis is a commonly described cellular outcome of treatment with many anticancer drugs (2, 3), and defects in the apoptotic machinery are believed to contribute to therapy resistance (4, 5). However, whether apoptosis is the primary antiproliferative mechanism of anticancer drugs in solid tumors is controversial (6). Other cell death modes than apoptosis are also possible. DNA-damaging agents have been reported to induce a necrotic response, due to hyperactivation of poly(ADP)ribose polymerase and depletion of cytosolic NAD (7). Photodynamic therapy has also been reported to induce necrosis (8). Various agents may also induce improper segregation of chromosomes during mitosis, leading to mitotic catastrophe (1, 9). Mitotic catastrophe is not a form of cell death per se, but rather a trigger for cell death by various mechanisms (9). Different classes of chemotherapeutic agents and ionizing radiation induce long-term growth arrest reminiscent of replicative senescence (10). An understanding of the mechanisms underlying these different outcomes is important to understand the antiproliferative activity of anticancer drugs and for understanding resistance to therapy.

Determining the mode of cell death is not trivial for cultured cells and is very difficult in tumor tissue. We have developed a method based on measurements of different molecular forms of CK18 that can be used to investigate cell death modes of epithelially derived cells *in vitro* and *in vivo* (11). This method is

Table 1. Characteristics of patients treated with neoadjuvant chemotherapy

| Clinical variable	n	Total CK18		Cleaved CK18	
		Median (25th-75th), units/L	P	Median (25th-75th), units/L	P
Tumor size (cm)					
<2	6	338 (276-401)		114 (111-117)	
2-5	22	333 (309-383)	0.0044	110 (110-116)	0.005
>5	15	437 (356-1,115)		140 (115-249)	
Estrogen receptor					
+	27	343 (307-493)		114 (114-134)	
-	13	363 (306-391)	NS	112 (106-131)	NS
Unknown	3	437 (375-457)		118 (102-130)	
Progesterone receptor					
+	28	381 (308-397)		114 (111-122)	
-	12	346 (305-525)	NS	113 (104-138)	NS
Unknown	3	437 (375-457)		118 (102-130)	
Metastasis					
No. nodes					
0	21	322 (263-391)		115 (107-124)	
1-3	14	388 (339-401)	0.0046	113 (109-128)	NS
>4	8	436 (359-635)		118 (90-157)	
Bone metastasis					
+	3	1,871 (759-2641)		276 (138-295)	
-	40	351 (308-410)	0.028	114 (106-127)	NS

Abbreviation: NS, not significant.

based on the measurement of different molecular forms of cytokeratin 18 (CK18) released from dead cells, whereas apoptosis will result in the release of caspase-cleaved CK18 fragments; necrosis will result in release of uncleaved CK18 (11). These forms can be conveniently distinguished by the use of the monoclonal antibody M30, which recognizes a neo-epitope of CK18 generated during apoptosis (12). CK18 is therefore potentially both a quantitative and qualitative biomarker for cell death *in vivo*. Previous investigations have provided evidence that serum CK18 is derived from tumor cells (11, 13) and have been encouraging with regard to the usefulness of serum CK18 as a clinically useful biomarker (13–16). However, a number of issues with regard to the release of CK18 from cells into serum and the clinical utility of CK18 as a response marker remain to be answered. In this study, we examined treatment responses of breast carcinoma to paclitaxel and anthracycline-based therapy *in vitro* and *in vivo*. We provide evidence that increases of serum CK18 during cyclophosphamide/epirubicin/5-fluorouracil (CEF) therapy are associated with clinical responses to CEF therapy. Furthermore, we found that CEF therapy induces a heterogeneous response *in vivo* with regard to cell death mode.

Materials and Methods

Cell culture. MDA-MB-231 breast carcinoma cells were maintained in DMEM supplemented with 10% FCS, L-glutamate, penicillin, and streptomycin at 37°C in 5% CO_2. Tissue culture reagents were obtained from Life Technologies Cell Culture Products. Cells were treated with doxorubicin (Sigma Chemical Company), staurosporine (Sigma), or paclitaxel (Calbiochem) as indicated.

Tissue slice preparation and culture. Primary breast tumors >3 cm were obtained from patients at the Robert Bosch Hospital, Stuttgart, immediately after surgical resection and maintained in organ transportation medium (Euro-Collins) on ice until use. Tissue cores (5 mm in diameter) were prepared using a hand-held coring tool. From the cylinders, tissue slices with a thickness of 200 μm were prepared in cold PBS using a precision cutting tissue slicer (Krumdieck, Alabama Research and Development Corp.). Slices were then individually submerged in supplemented mammary epithelial growth medium as described (17). Incubation was done in 24-well plates at 37°C in a constant atmosphere of 5% CO_2 on a shaking platform. Treatment with drugs started after a recovery period of 24 h and was done for additional 72 h as described (17). In preliminary experiments, done in a panel of breast cancer samples, we found a higher mitochondrial tetramethyl-rhodamine methyl ester accumulation in the tumor cell compartment after a recovery period of 24 h.
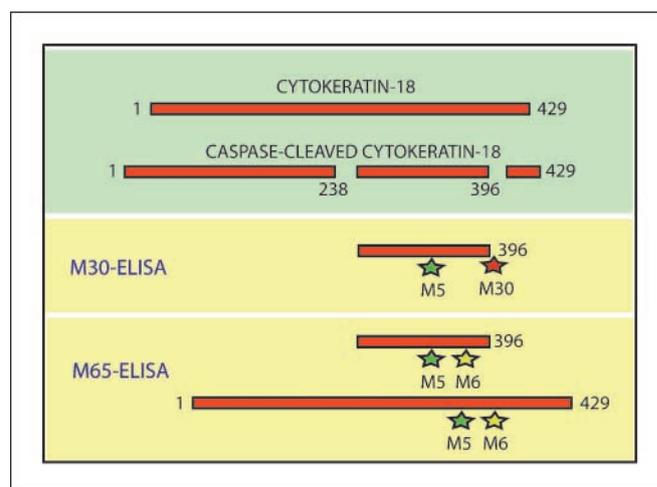
Fig. 1. CK18 is cleaved at Asp[238] and Asp[396] by caspases during apoptosis. The M30-Apoptosense ELISA assay uses antibody M30, which detects a neo-epitope of CK18 formed after caspase cleavage at Asp[396] (12, 17). The M65-ELISA assay will detect all CK18 fragments that contain epitopes in the 300 to 390 amino acid region of the protein (11).

ELISA assays. Caspase-cleaved cytokeratin-18 (CK18-Asp[396]) was determined by the M30-Apoptosense ELISA (ref. 18; PEVIVA AB). The M65-ELISA assay (PEVIVA AB) was used to measure total soluble CK18. ELISA tests for measuring cytokeratin complexes were done by coating different capture antibodies on 96-well Nunc Immuno Module plates overnight at 4°C in PBS at various concentrations. The capture antibodies used for the mixed ELISA assays were purchased from the following commercial sources: monoclonal antibodies for human CK7 (clone 4A39) and CK8 (clone 4A42) from US Biological, and monoclonal antibody for human CK19 (clone 17) from Abcam. The plates were washed thrice with PBST (PBS + 0.1% Tween 20) before incubation with human serum samples for 2 h at room temperature. Horseradish peroxidase–conjugated monoclonal antibody M30 or M5 from the M30 and M65 ELISA kits were used as detection antibody. 3,3′,5,5′-Tetramethyl-benzidine was used as the substrate. The intensity of the signal was determined by measuring the absorbance at 450 nm using the SpectroMax M5 microplate reader (Molecular Device).

Gel filtration. Serum from patients with breast cancer was fractionated on a Superose G200 column in PBS/10% horse serum. Similarly, medium from apoptotic MDA-MB-231 cells was collected and subjected to gel filtration. Fractions were collected and assayed for caspase-cleaved CK18 and CK18 by ELISA.

Patients. Sera were collected from 61 patients with primary breast cancer treated with chemotherapy (preoperative neoadjuvant chemotherapy for 43 patients and postoperative adjuvant chemotherapy for 18 patients) at the Tokyo Metropolitan Komagome Hospital from 1997 to 2003. The characteristics of the patients are shown in Table 1. Tumor sizes were determined by palpation. Patients were treated with CEF (600 mg, 60 mg, and 600 mg/m^2, respectively; average 3.8 cycles). Cyclophosphamide and 5-fluorouracil were given as 45 to 60 min infusions and epirubicin as a bolus injection. The chemotherapy was administered every 3rd week. Sera from cancer patients were collected before each cycle and at various times after each cycle of treatment. Patients with liver and renal dysfunction and other complications were excluded. For all patients, liver, lung, and distant lymph node metastases were diagnosed using computed tomographic scan, and bone metastases were diagnosed using X-ray or bone scintigraphy. For patients with stage II or more, brain metastasis was examined using computed tomographic scan. Of the 43 patients who received neoadjuvant chemotherapy, three patients had bone metastasis and none had other distant metastases. In 18 patients who received adjuvant chemotherapy, major metastatic sites were lung for seven patients, bone for three, liver for three, pleura for one, and lymph node for four. Clinical responses to treatments were evaluated according to the Union Internationale Contre le Cancer criteria. Informed consent was obtained from all patients, and the study was approved by the local institutional review board.

Statistical analysis. Patients' data are presented as median (25th-75th percentile) and graphically displayed by box plots. The Mann-Whitney *U* method was used to test for difference between two groups. The Wilcoxon matched pair signed ranks test was used to examine whether the members of pairs differ in size. The survival analysis was done by the log-rank test and the Cox proportional hazards model. All tests were done using a two-sided α level of 0.05.
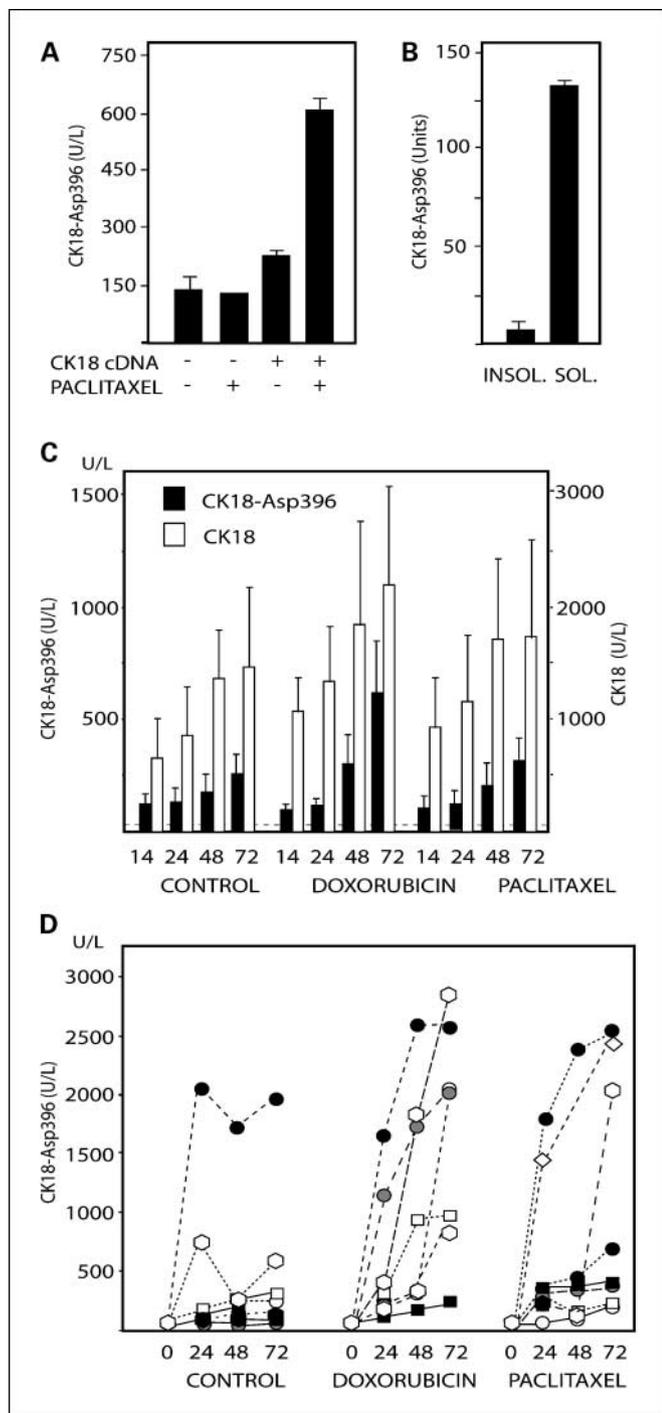
Fig. 2. Release of CK18-Asp[396] from carcinoma cells. *A,* specificity of the M30-ELISA for caspase-cleaved CK18. Mouse embryo fibroblasts were transfected with a cDNA expression plasmid and treated with paclitaxel as indicated. Note that paclitaxel only induces increases in CK18-Asp[396] in transfected cells. *B,* distribution of CK18-Asp[396] epitopes between the insoluble (*INSOL.*) cytoskeleton and the medium after induction of apoptosis. MDA-MB-231 cells were treated with staurosporine (200 nmol/L) for 16 h and the medium was collected. The insoluble fraction was pelleted by centrifugation and washed thrice in PBS/0.5% NP40. The binding of the M30 antibody to the insoluble, nuclear/cytoskeletal fraction was determined by incubation with horseradish peroxidase–conjugated antibody followed by repeated washing and incubation with horseradish peroxidase substrate and expressed in total units (*U*). The total number of units released into the medium was determined by ELISA (U/L corrected with total volume). *C* and *D,* time-dependent increases in release of CK18-Asp[396] from breast tumor organ cultures. Tumor tissue slices were treated with doxorubicin (1 μg/mL) or paclitaxel (6.8 μg/mL) and CK18-Asp[396] was determined in the tissue culture medium. *C,* release of CK18 (*open columns*) and CK18-Asp[396] (*filled columns*) from tumor tissue slices of MDA-MB-231 tumors from severe combined immunodeficient mice (mean values from three slides). Dotted line, baseline activity of tissue culture medium. *D,* release of CK18-Asp[396] from tumor tissue slices from human breast carcinomas. Slices from seven different breast carcinomas were cultured in the presence or absence of drugs (concentrations as in *C*) *in vitro*, and medium was harvested at the indicated times. Points, mean from triplicate determinations. CK18-Asp[396] median levels were 203, 544, and 2,056 units/L in control, paclitaxel-treated, and doxorubicin-treated cultures at 72 h.

Results

Release of caspase-cleaved CK18 molecules from drug-treated cells and tumor tissue. CK18 is a major component of the intermediate filament system of simple epithelial cells. During apoptosis, CK18 is cleaved by caspases at Asp^{238} and at Asp^{396} (refs. 12, 19, 20; Fig. 1). CK18 molecules cleaved by caspases at Asp^{396} ($CK18\text{-}Asp^{396}$) react with the M30 monoclonal antibody and soluble $CK18\text{-}Asp^{396}$ molecules are detected by the M30-Apoptosense ELISA (Fig. 1). Such caspase-cleaved CK18 fragments are convenient biomarkers for apoptosis of epithelially derived cells (11, 18, 21). To formally prove that detection of apoptosis by the M30 ELISA requires CK18 expression, we transfected mouse embryo fibroblasts with a CK18 expression plasmid. As expected, paclitaxel stimulated increases in $CK18\text{-}Asp^{396}$ in transfected, but not in untransfected, mouse embryo fibroblast (Fig. 2A). Induction of activity in the assay was blocked by a caspase inhibitor (data not shown; ref. 11).

CK18 is a constituent of the insoluble cytoskeleton and only a minor fraction is soluble (20). To examine whether most caspase cleavage events will generate soluble CK18 fragments, the fraction of $CK18\text{-}Asp^{396}$ epitopes was determined in the insoluble and soluble fractions after induction of apoptosis in a human breast carcinoma cell line. We found that >90% of the $CK18\text{-}Asp^{396}$ epitopes were present in the soluble fraction (Fig. 2B).

To further study the release of CK18 from tumor cells exposed to cytotoxic agents, we examined organ cultures of MDA-MB-231 breast tumors from severe combined immunodeficient mice. Tumor tissue slices were cultivated in the presence or absence of a taxane (paclitaxel) or an anthracycline (doxorubicin) for 3 days (17). Release of $CK18\text{-}Asp^{396}$ fragments to the culture medium was observed from untreated organ cultures, suggesting spontaneous apoptosis (Fig. 2C). Approximately 2-fold higher levels of $CK18\text{-}Asp^{396}$ were observed in doxorubicin-treated cultures at 72 h, whereas paclitaxel induced weaker increases (Fig. 2C). Similar patterns of release were observed when total CK18 was measured (using the M65-ELISA).

The release of $CK18\text{-}Asp^{396}$ fragments from organ cultures of different clinical cases of breast carcinoma treated with paclitaxel or doxorubicin was examined (Fig. 2D). The median level of $CK18\text{-}Asp^{396}$ was 2,056 units/L after 72 h of doxorubicin treatment, compared with 203 units/L in untreated control ($P < 0.05$, Wilcoxon two-sample test), demonstrating that doxorubicin induced apoptosis in the organ cultures. The median level of $CK18\text{-}Asp^{396}$ in paclitaxel-treated cultures was

Fig. 3. CK18 complexes released from cells into circulation. *A*, Superose G200 gel filtration of tissue culture medium from apoptotic cells or patient serum (*top*) $CK18\text{-}Asp^{396}$ is present in the 10 to 20 kDa and 50 to 100 kDa range in tissue culture medium from apoptotic MDA-MB-231 cells; $CK18\text{-}Asp^{396}$ (*middle*) and CK18 (*bottom*) is present in the 50 to 100 kDa range in serum from a human breast cancer patient. *B* (*top*), plasma levels of the $CK18_{(284-396)}$ fragment after injection into mice. The fragment was synthesized in *Escherichia coli* and injected i.v. into mice. Bottom, plasma was collected after different times and assayed for CK18 (using the M65-ELISA assay): The $CK18_{(284-396)}$ fragment was incubated in mouse plasma at 37°C for the indicated times, and CK18 levels were examined using the M65-ELISA assay. *C*, CK18 is present in complexes with other cytokeratins in serum. Patient serum was analyzed by ELISA using plates coated with increasing amounts (in μg) of CK 8 (*top*), CK7 (*middle*), and CK19 (*bottom*) antibodies. Horseradish peroxidase – conjugated M30 or M5 antibodies were used to show $CK18\text{-}Asp^{396}$ or CK18 in the complexes.
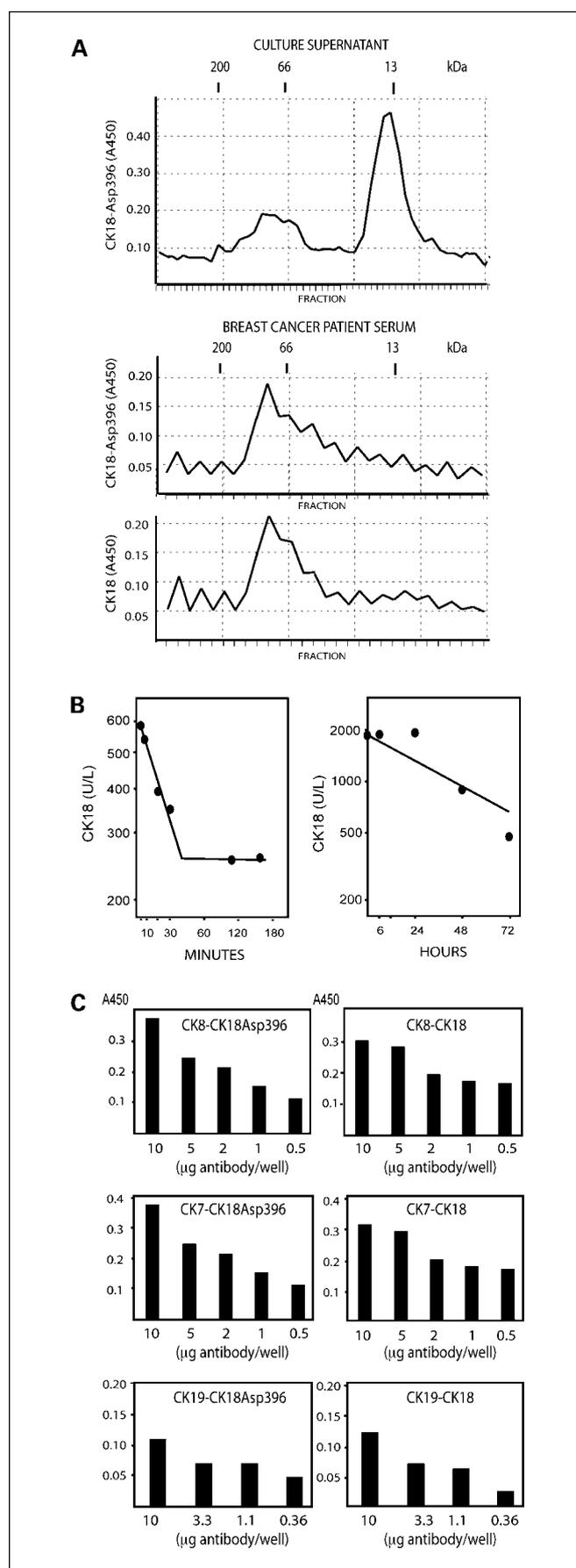
Table 2. Assay precision for the M30 and M65 ELISA

M30 ELISA				M65 ELISA			
Serum sample	Average absorbance	CV%	No. assays	Serum sample	Average absorbance	CV%	No. assays
BRH84384	0.77	17.9	33	BRH84358	0.93	9.16	28
BRH80678	0.57	12.58	28	BRH84384	0.44	11.74	28
BRH84400	0.16	14.03	27	BRH84400	0.21	17.49	23
rCK18 Control	0.68	9.48	36	rCK18 Control	0.48	6.79	26

NOTE: Data were generated over a 8-mo period from two manufacturer's lots using various normal sera as well as the recombinant CK18 standard included in each assay kit.

not significantly different from control cultures at 72 h. Similar patterns of release were observed when total CK18 was measured (not shown).

We considered the possibility that soluble CK18-Asp^{396} fragments may be trapped in tumor tissue. Extensive digestion of tissue organ slices with collagenase did not, however, release significant amounts of activity detected by ELISA (data not shown).

Caspase-cleaved CK18 molecules are present as protein complexes in serum. The molecular composition of soluble proteins containing the M30 epitope was examined. Fractionation of medium from apoptotic cells on Superose G200 revealed one peak in the 10 to 20 kDa region and another in a higher molecular weight region (Fig. 3A, *top*). This pattern was distinct from that observed in sera of cancer patients where the M30 epitope was only found in the 50 to 100 kDa region (Fig. 3A, *middle*). Total CK18 (detected by the M65-ELISA) was found in the same fractions as CK18-Asp^{396} (Fig. 3A, *bottom*).

A 13-kDa CK18 form (CK18 residues 284-396) has been described in culture medium from apoptotic cells (22), consistent with the present findings. Injection of a recombinant 13 kDa fragment i.v. in mice showed a half-life of ~30 min (Fig. 3B, *top*). In contrast, incubation of the 13-kDa fragment in mouse plasma at 37°C *in vitro* showed a half-life of ~48 h (Fig. 3B, *bottom*). CK18 is a 45-kDa protein and the higher molecular weight material in serum were assumed to represent protein complexes. Using the same type of monoclonal CK18 antibodies both on solid phase and for detection in ELISA assays (M5-M5 or M30-M30), signals were detected using serum samples but not using recombinant

CK18 (data not shown), consistent with CK18-CK18 complexes in serum. Signals were also observed in sera using mixed ELISA assays based on antibodies to different cytokeratin types (Fig. 3C). The results show that CK18 (and the CK18-Asp^{396} epitope) can be detected in complexes with CK7, CK8, and CK19 in serum, whereas CK18-CK14 complexes were not detected (not shown). A recombinant CK8 protein was tested in the CK8-CK18 ELISA but did not generate a signal (not shown). These findings suggest that small caspase-cleaved CK18 fragments are rapidly cleared from the circulation and that caspase-cleaved CK18 molecules are present as protein complexes in serum.

Caspase activity has been detected in circulation in patients with malignancies (23). A concern in the analysis of caspase-cleaved fragment in blood as a measure of cellular apoptosis is that cleavage of CK18 might occur in circulation. We addressed this issue using CK18-positive serum samples. Incubation with 1,000 units/mL recombinant caspase-3 for 4 h did not increase the levels of fragments containing the CK18-Asp^{396} epitope (data not shown). Control experiments showed that caspase-3 was active in serum under these conditions (data not shown).

Performance of CK18 assays. The clinical utility of the M30 and M65 ELISAs was investigated and qualified according to available bioassay validation guidelines established by a pharmaceutical industry consortium (24, 25). In brief, ELISA sensitivity, precision, specificity, assay range, reagent stability, sample stability, and variations from multiple blood draws of the same donor were investigated and determined. For example, assay precision profiles for the M30 and M65 ELISA

Table 3. Human serum sample freeze/thaw stability

Freeze thaw cycle	M30 (absorbance/% to control)			M65 (absorbance/% to control)		
	BRH84384	BRH80678	BRH84400	BRH84358	BRH84384	BRH84400
1	0.82/100%	0.58/118%	0.13/87%	0.84/105%	0.38/112%	0.14/108%
2	0.83/101%	0.57/116%	0.13/87%	0.86/108%	0.38/112%	0.14/108%
3	0.81/99%	0.56/114%	0.13/87%	0.87/109%	0.38/112%	0.14/108%
4	0.84/102%	0.59/120%	0.13/87%	0.83/104%	0.36/106%	0.13/100%
5	0.89/109%	0.61/125%	0.13/87%	0.80/100%	0.36/106%	0.13/100%
6	0.80/98%	0.56/114%	0.13/87%	0.79/99%	0.35/103%	0.12/92%
Control	0.82/100%	0.49/100%	0.15/100%	0.80/100%	0.34/100%	0.13/100%

NOTE: Serums samples for this study were generated by thawing at room temperature and refreezing for 24 h at -70°C for each cycle. Controls were the same samples taken directly from -70°C without any freeze-thaws.

assays are shown in Table 2. Both assays were run repeatedly using normal human serum controls of variable basal levels of intact and caspase-cleaved CK18 over a period of 8 months. The assay variability (CV%) ranged from 7% to 18% from two manufacturer's lots. Stability of native caspase-cleaved and intact CK18 proteins were also tested under repeated freeze-thaw conditions. Both forms of CK18 proteins were stable through six freeze-thaw cycles (Table 3). These data show an adequate performance of the assays for clinical studies. Because of the difference between the recombinant CK18 control (amino acids 284-396) and naturally existing serum CK18 analytes discussed above, the quantitation of CK18 should ideally be measured in absorbance instead of the unit based on the standard curve. In the following, CK18 unit is used to describe clinical data as nominal value to be consistent with conventional ELISA assay format so that the ELISA data here are comparable with data from other publications.

Serum CK18 levels during cancer treatment. The levels of total and cleaved CK18 were determined in blood during treatment of 61 patients with breast cancer (patient characteristics, see Table 1). The patients received either anthracycline-based therapy (CEF) or the semisynthetic taxane docetaxel. Blood samples were collected before each cycle of treatment and at 1 and 3 days after treatment. Examples of data from patients are shown in Fig. 4A. The levels of serum CK18-Asp^{396} and total CK18 increased between 24 and 72 h after initiation of treatment with docetaxel (Fig. 4B and C; Table 4). CK18-Asp^{396} levels at 72 h showed a larger spread than the values at 0 and 24 h showing a heterogeneous response between patients. CEF therapy induced more rapid increases in serum CK18-Asp^{396} and total CK18 levels compared with docetaxel. The median levels of total CK18 had increased with 114 units/L (32.7%) at 1 day, whereas CK18-Asp^{396} only increased with 13 units/L (12.9%).

The predominant increases in uncleaved CK18 during CEF therapy indicates a substantial component of caspase-independent cell death in some tumors. As shown in Fig. 4D, a heterogeneous response was observed with regard to the ratio of CK18-Asp^{396} to total CK18 in different patients; some

Fig. 4. Patterns of increase of CK18 during treatment of breast cancer patients. *A,* increases of CK18 (*blue points*; M65-ELISA) and CK18-Asp^{396} (*green points*; M30-ELISA) during CEF therapy of two breast cancer patients. The patients were followed for five or four cycles and showed stable disease (*NC,* no change) or partial response (*PR*). Note that patients received treatment every 3rd week (each cycle was 21 d in total). Samples were run in duplicates. *B,* median (25th-75th percentile) levels of CK18 during CEF or docetaxel therapy. Pretherapy levels and levels at days 1 and 3 are shown. Levels of statistical significance <0.01 (Wilcoxon two-sample test). *C,* median (25th-75th percentile) levels of CK18-Asp^{396} during CEF or docetaxel therapy at days 0, 1, and 3 (the same samples were used as in *B*). Levels of statistical significance <0.01. *D,* increases of levels of CK18 and CK18-Asp^{396} levels in individual patient sera from pretreatment to day 1; for each tumor, CK18 (*blue columns*) and CK18-Asp^{396} values (*red columns*) are shown pairwise. Occasional patients showed decreases in the cytokeratin levels between day 0 and day 1; these decreases are shown as 0.
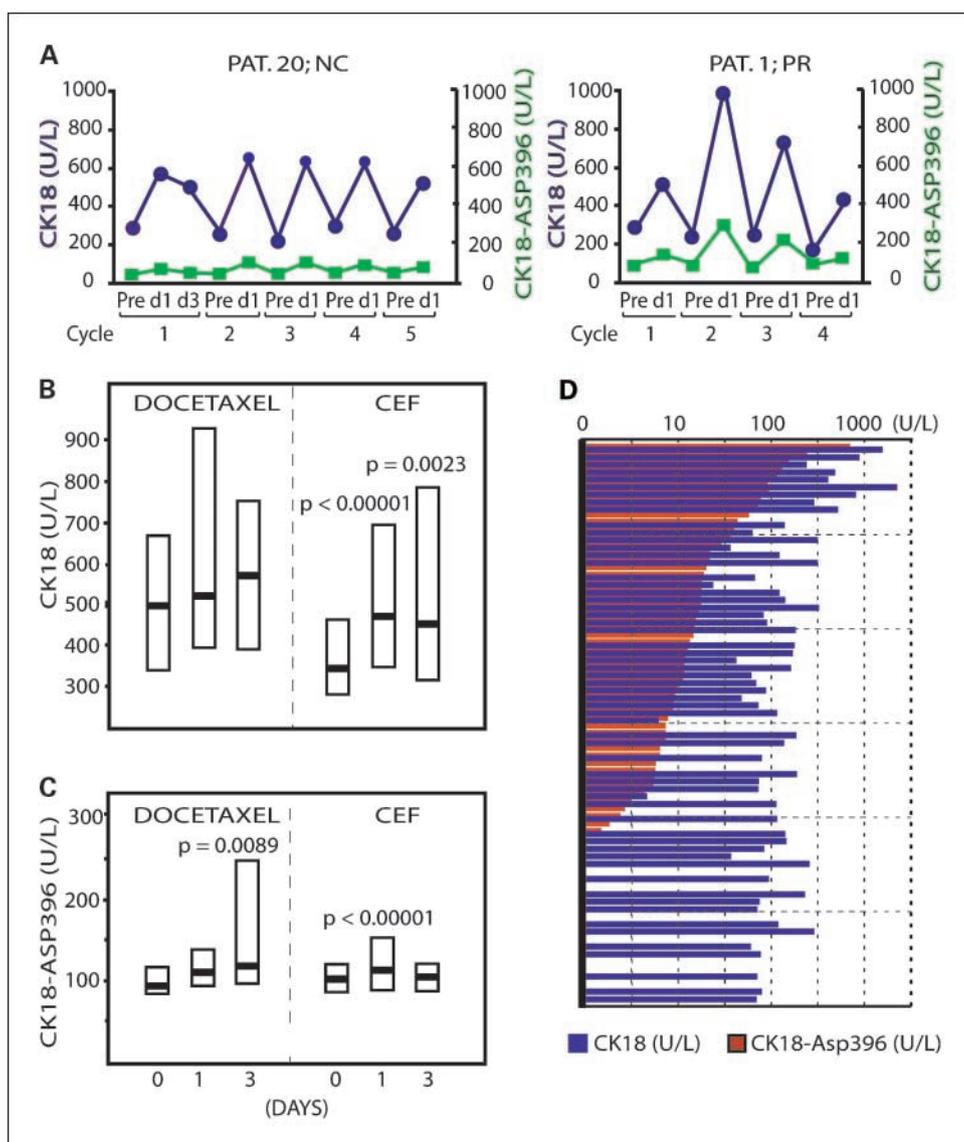
patients showed increases in both markers, whereas others only showed increases in total CK18. This result suggests that CEF therapy induces different death modes in different tumors.

Increases in CK18 are associated with clinical response. We examined the association between clinical response and serum CK18 increases in 43 patients receiving neoadjuvant CEF therapy. Patients normally leave the hospital after drug infusion—leading to difficulties to collect blood samples—but a limited number of paired samples is sufficient to achieve high statistical power using matched-pair statistics. Patients with partial clinical response showed significant increases in total CK18 at day 1 after treatment ($P < 0.0001$, Wilcoxon matched pair test; Fig. 5A). In contrast, nonresponding patients did not show significant changes in total CK18 levels ($P = 0.19$; Fig. 5A). Similar results were obtained using matched pair t test.

Patients who received neoadjuvant therapy were stratified according to the ratios of posttreatment to pretreatment values of CK18 and overall survival plots were constructed (Fig. 5B). A cutoff value of 18% increase in CK18 gave the best prognostic significance for survival ($P = 0.035$ by the log-rank test). The Cox proportional hazards model gave a hazard ratio of 7.28 (95% confidence interval, 0.84-62.9).

Discussion

Previous studies have shown that different anticancer agents induce increases in the levels of caspase-cleaved and total serum CK18 in prostate cancer patients and that serum CK18 is derived from tumor cells (11, 13). These results were promising with regard to the use of serum CK18 as a pharmacodynamic biomarker for tumor cell death. Previous studies have not established whether increases in serum CK18 occurring during treatment are associated with clinical responses because response monitoring is inaccurate in patients with hormone refractory prostate cancer (26). We here studied breast cancer patients receiving neoadjuvant

treatment for local disease and from which accurate clinical data were available. The results show that increases in serum CK18 levels are associated with clinical response to CEF therapy. Interestingly, CK18 increases were not exclusively observed in patients showing clinical response but also in some patients showing stable disease during treatment (Fig. 4A), suggesting that serum CK18 is a sensitive response biomarker. In patients with stable disease, therapy-induced cell death may be balanced by tumor cell regrowth between treatment cycles (27). Both the sensitivity of the assays and the favorable performance characteristics in terms of antigen stability during storage (14) and during freeze-thawing (Table 3) suggest that CK18 biomarkers will be useful for monitoring treatment effects.

Apoptosis has received considerable attention as a major cellular outcome of chemotherapy, including DNA-damaging agents (2). Recent studies have implied that necrosis may also be a possible consequence of treatment (28). Doxorubicin has been shown to induce both apoptosis (29) and necrosis *in vitro* (30, 31). Our studies of tumor organ cultures from seven clinical cases of breast carcinoma showed induction of caspase-cleaved CK18 by doxorubicin in all seven cultures, showing apoptosis. Apoptotic responses were also observed using CEF therapy (cyclophosphamide is converted by the liver into active metabolites; acrolein was used for these studies).[6] In contrast, the *in vivo* CK18 response to CEF therapy was heterogeneous, characterized by increases in caspase-cleaved CK18 in the serum of some, but not all, patients with increases in CK18. This heterogeneous response could be due to defects in apoptosis signaling in some tumors. Furthermore, differences in factors such as tumor hypoxia, nutrition, or variations in the drug concentrations reached in different tumors may also be determinants of cell death mode. It has been reported that DNA-alkylating agents induce a rapid necrotic response due to activation of poly(ADP)ribose polymerase, leading to poly(ADP)ribose polymerase–mediated depletion of β-NAD$^+$ (7). Tumor cells, which are dependent on glycolysis for ATP production, undergo rapid ATP depletion and necrotic death. This response by DNA-damaging agents could be speculated to be more pronounced in hypoxic and poorly nourished tumors. That anthracycline-based therapy may induce a necrotic response is supported by the finding that complete pathologic responses to doxorubicin/docetaxel are associated with the presence of tumor necrosis in tissue sections (32). Induction of a necrotic response could explain the efficiency of chemotherapy in tumors with defective apoptotic pathways (discussed in ref. 7), including efficacy in p53-defective breast cancers (33).

Taxanes induce mitotic catastrophe, characterized by the occurrence of aberrant mitosis followed by cell division. Mitotic catastrophe is not a cell death mode, but will trigger cell death, either by apoptosis or by nonapoptotic mechanisms (1, 34–36). The findings in the present and a previous study (13), demonstrating increases in caspase-cleaved CK18 molecules in serum during docetaxel treatment, shows that this agent induces apoptosis *in vivo* (Table 4). It is likely that the efficiency of microtubule-interacting agents does not rely on the presence of an apoptotic machinery in the target cells;

Table 4. Increased levels of CK18 in patient serum during treatment using different agents

Treatment	Increased levels of CK18 during therapy (%)	
	Caspase cleaved*	Total*
Docetaxel (breast)[†]	19.8 ($P = 0.0089$)	16.5 (NS)
Docetaxel (prostate)[‡]	18.7 ($P < 0.0001$)	21.4 ($P < 0.0002$)
Vinorelbine (prostate)[‡]	7.2 ($P < 0.001$)	6.7 ($P < 0.011$)
Estramustine (prostate)[‡]	-1 (NS)	8.2 ($P < 0.0001$)
CEF (breast)[§]	12.9 ($P < 0.00001$)	32.7 ($P < 0.00001$)

*Increased median levels of CK18-Asp^{396} and total CK18 (measured by the M30-Apoptosense and M65 ELISA assays).
[†] Increase over pretherapy levels at 72 h.
[‡] Increase over pretherapy levels 48 h (prostate data are from ref. 13).
[§] Increase over pretherapy levels at 24 h.
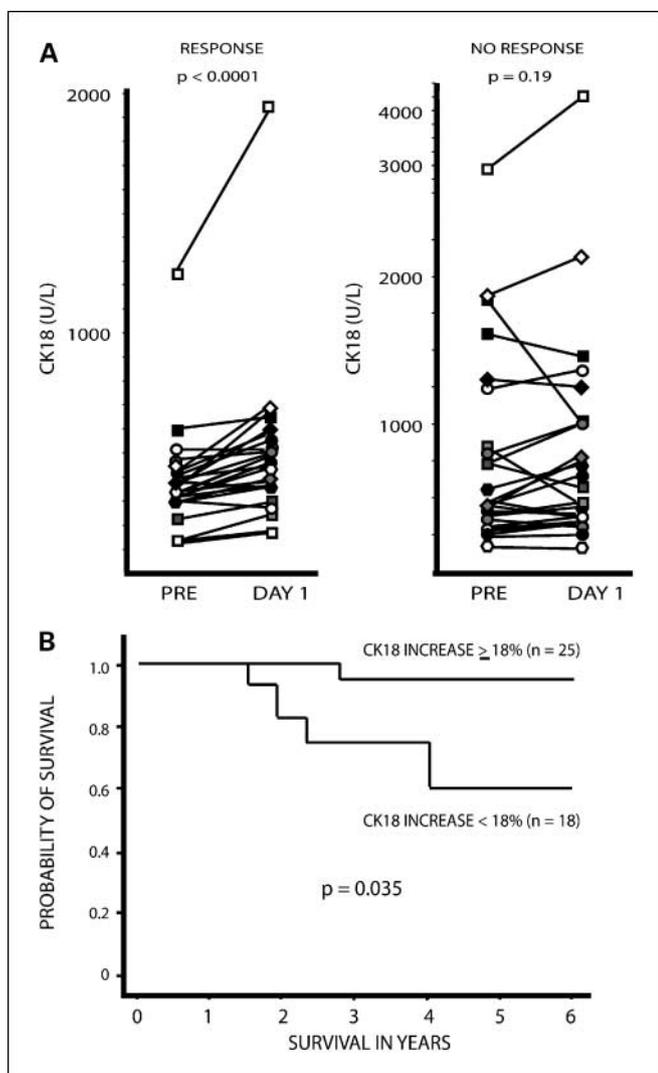
Fig. 5. Association to response. *A,* increases of total CK18 at day 1 of the first CEF treatment cycle are associated with therapy response. Pretherapy and day 1 CK18 levels are shown of patients showing response or no response; *P* values are from Wilcoxon matched pair test. *B,* improved overall survival curves of patients showing >18% increases in CK18 at the first cycle of CEF treatment. Kaplan-Meier plots, *P* value calculated by the log-rank test.

difference in the response to mitotic inhibitors and CEF, whereas mitotic inhibitors induce similar median increases in caspase-cleaved CK18 and total CK18, CEF induces larger increases in total CK18 reflecting a relatively larger proportion of tumors where necrotic cell death occurs.

Our data show that only a minor fraction of caspase-cleaved CK18 will remain in the insoluble, cytoskeletal fraction. Soluble caspase-cleaved CK18 consisted of molecular weight fragments in the 10 to 20 kDa range, which we presume to be monomeric caspase digestion products, and also of higher molecular weight material (50-100 kDa). Only the higher molecular weight material was present in serum from cancer patients, and we suggest that the smaller fragments are being filtered in the kidney glomerulus. The caspase-cleaved CK18 material present in serum reacted with CK7, CK8, or CK19 antibodies, showing that they at least partly are present in complexes. Although the precise nature of these interactions are unknown or if there are any alterations among the cytokeratin complexes under normal or abnormal physiologic conditions, our data showed that the serum CK18 levels from repeated blood draws of healthy donors were fairly stable without any trending (data not shown). The variations from multiple time points in normal donors are consistent with the data reported from cancer patients (15). The presence of caspase-cleaved CK18 in protein complexes is likely to explain the stability of the cleavage products in the circulation and in blood samples, explaining the excellent performance of serum CK18 with regard to stability and yields after repeated freeze-thaw cycles (refs. 14, 15; Table 3). It is likely that many other caspase-cleaved fragments released from apoptotic cells will not show a similar stability. The adequate assay performance adds to the advantages of serum CK18 as a biomarker for rapid monitoring of clinical response to cancer therapy.

We conclude that serum CK18 measurements may be useful for assessing treatment effects. The data suggesting that the initial cell death response determined by CK18 biomarkers is an important determinant of treatment outcome. The method is robust and samples can be frozen and stored before analysis, making the method suitable for multicenter clinical trials of novel anticancer drugs.

if the machinery is not present, cells are likely to die from other mechanisms. It should be noted that we have observed patients where docetaxel induces increases predominantly in total serum CK18 (11). However, there is a quantitative

References

1. Ricci MS, Zong WX. Chemotherapeutic approaches for targeting cell death pathways. Oncologist 2006; 11:342–57.

2. Viktorsson K, Lewensohn R, Zhivotovsky B. Apoptotic pathways and therapy resistance in human malignancies. Adv Cancer Res 2005;94:143–96.

3. Dive C, Evans CA, Whetton AD. Induction of apoptosis—new targets for cancer chemotherapy. Semin Cancer Biol 1992;3:417–27.

4. Eliopoulos AG, Kerr DJ, Herod J, et al. The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. Oncogene 1995;11:1217–28.

5. Reed JC. Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance. Curr Opin Oncol 1995;7:541–6.

6. Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. Nat Rev Cancer 2005;5:231–7.

7. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 2004;18:1272–82.

8. Fabris C, Valduga G, Miotto G, et al. Photosensitization with zinc (II) phthalocyanine as a switch in the

decision between apoptosis and necrosis. Cancer Res 2001;61:7495–500.

9. Weaver BA, Cleveland DW. Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. Cancer Cell 2005;8:7–12.

10. Roninson IB. Tumor senescence as a determinant of drug response *in vivo*. Drug Resist Updat 2002;5:204–8.

11. Kramer G, Erdal H, Mertens HJ, et al. Differentiation between cell death modes using measurements of different soluble forms of extracellular cytokeratin 18. Cancer Res 2004;64:1751–6.

12. Leers MP, Kolgen W, Björklund V, et al. Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. J Pathol 1999;187:567–72.

13. Kramer G, Schwarz S, Hagg M, Havelka AM, Linder S. Docetaxel induces apoptosis in hormone refractory prostate carcinomas during multiple treatment cycles. Br J Cancer 2006;94:1592–8.

14. Cummings J, Ranson M, Lacasse E, et al. Method validation and preliminary qualification of pharmacodynamic biomarkers employed to evaluate the clinical efficacy of an antisense compound (AEG35156) targeted to the X-linked inhibitor of apoptosis protein XIAP. Br J Cancer 2006;95:42–8.

15. Cummings J, Ward TH, LaCasse E, et al. Validation of pharmacodynamic assays to evaluate the clinical efficacy of an antisense compound (AEG 35156) targeted to the X-linked inhibitor of apoptosis protein XIAP. Br J Cancer 2005;92:532–8.

16. Demiray M, Ulukaya EE, Arslan M, et al. Response to neoadjuvant chemotherapy in breast cancer could be predictable by measuring a novel serum apoptosis product, caspase-cleaved cytokeratin 18: a prospective pilot study. Cancer Invest 2006;24: 669–76.

17. van der Kuip H, Murdter TE, Sonnenberg M, et al. Short term culture of breast cancer tissues to study the activity of the anticancer drug Taxol in an intact tumor environment. BMC Cancer 2006;6:86.

18. Hägg M, Biven K, Ueno T, et al. A novel high-throughput assay for screening of pro-apoptotic drugs. Invest New Drugs 2002;20:253–9.

19. Caulin C, Salvesen GS, Oshima RG. Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. J Cell Biol 1997;138:1379–94.

20. Ku NO, Liao J, Omary MB. Apoptosis generates stable fragments of human type I keratins. J Biol Chem 1997;272:33197–203.

21. Linder S, Havelka AM, Ueno T, Shoshan MC. Determining tumor apoptosis and necrosis in patient serum using cytokeratin 18 as a biomarker. Cancer Lett 2004;214:1–9.

22. Rydlander L, Ziegler E, Bergman T, et al. Molecular characterization of a tissue-polypeptide-specific-antigen epitope and its relationship to human cytokeratin 18. Eur J Biochem 1996;241:309–14.

23. Deligezer U, Erten N, Akisik EE, Dalay N. Circulating fragmented nucleosomal DNA and caspase-3 mRNA in patients with lymphoma and myeloma. Exp Mol Pathol 2006;80:72–6.

24. Lee JW, Devanarayan V, Barrett YC, et al. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res 2006;23: 312–28.

25. Lee JW, Weiner RS, Sailstad JM, et al. Method validation and measurement of biomarkers in nonclinical and clinical samples in drug development: a conference report. Pharm Res 2005;22:499–511.

26. Vicini FA, Vargas C, Abner A, Kestin L, Horwitz E, Martinez A. Limitations in the use of serum prostate specific antigen levels to monitor patients after treatment for prostate cancer. J Urol 2005;173:1456–62.

27. Kim JJ, Tannock IF. Repopulation of cancer cells during therapy: an important cause of treatment failure. Nat Rev Cancer 2005;5:516–25.

28. Zong WX, Thompson CB. Necrotic death as a cell fate. Genes Dev 2006;20:1–15.

29. Panaretakis T, Pokrovskaja K, Shoshan MC, Grander D. Activation of Bak, Bax, and BH3-only proteins in the apoptotic response to doxorubicin. J Biol Chem 2002;277:44317–26.

30. Dartsch DC, Schaefer A, Boldt S, Kolch W, Marquardt H. Comparison of anthracycline-induced death of human leukemia cells: programmed cell death versus necrosis. Apoptosis 2002;7:537–48.

31. Mansilla S, Priebe W, Portugal J. Mitotic catastrophe results in cell death by caspase-dependent and caspase-independent mechanisms. Cell Cycle 2006;5: 53–60.

32. Pu RT, Schott AF, Sturtz DE, Griffith KA, Kleer CG. Pathologic features of breast cancer associated with complete response to neoadjuvant chemotherapy: importance of tumor necrosis. Am J Surg Pathol 2005; 29:354–8.

33. Bertheau P, Plassa F, Espie M, et al. Effect of mutated TP53 on response of advanced breast cancers to high-dose chemotherapy. Lancet 2002;360: 852–4.

34. Morse DL, Gray H, Payne CM, Gillies RJ. Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells. Mol Cancer Ther 2005;4: 1495–504.

35. Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L. Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. Cancer Res 1996;56:816–25.

36. Blajeski AL, Kottke TJ, Kaufmann SH. A multistep model for paclitaxel-induced apoptosis in human breast cancer cell lines. Exp Cell Res 2001;270: 277–88.

Clinical Cancer Research

Cytokeratin-18 Is a Useful Serum Biomarker for Early Determination of Response of Breast Carcinomas to Chemotherapy

Maria Hägg Olofsson, Takayuki Ueno, Yang Pan, et al.

Updated version	Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/13/11/3198

Cited articles	This article cites 36 articles, 10 of which you can access for free at: http://clincancerres.aacrjournals.org/content/13/11/3198.full#ref-list-1
Citing articles	This article has been cited by 16 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/13/11/3198.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/13/11/3198. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.