Induction of p53 Up-Regulated Modulator of Apoptosis Messenger RNA by Chemotherapeutic Treatment of Locally Advanced Breast Cancer

Rutger Middelburg,1 Richard R. de Haas,1 Henk Dekker,1 Ron M. Kerkhoven,2 Paula R. Pohlmann,1 Adolfo Fuentes-Alburro,4 Alejandro Mohar,4 Herbert M. Pinedo,1,3 and Jan Lankelma1

1Department of Medical Oncology, VU University Medical Center; 2Central Microarray Facility, The Netherlands Cancer Institute; 3VUmc Cancer Center Amsterdam, Amsterdam, the Netherlands; and 4Instituto Nacional de Cancerosología, Mexico City, Mexico

ABSTRACT

Purpose: In biopsies of patients with locally advanced breast cancer, we investigated the in vivo changes of the gene expression pattern induced by chemotherapy to find genes that are potentially responsible for the efficacy of the drug.

Experimental Design: Early cellular responses to chemotherapy-induced damage, both in vivo and in vitro, were investigated by analyzing chemotherapy-induced changes in gene expression profiles. Core biopsies were taken from nine patients with locally advanced breast cancer, before and at 6 hours after initiation of doxorubicin-based chemotherapy. Both samples were cohybridized on the same microarray containing 18,000 cDNA spots.

Results: The analysis revealed marked differences in gene expression profile between treated and untreated samples. The gene which was most frequently found to be differentially expressed was p53 up-regulated modulator of apoptosis (PUMA). This gene was up-regulated in eight of nine patients with an average factor of 1.80 (range, 1.36-2.73).

In vitro MCF-7 breast cancer cells exposed to clinically achievable doxorubicin concentrations for 6 hours revealed marked induction of PUMA mRNA, as well.

Conclusions: This is the first report describing PUMA mRNA to be up-regulated as a response to chemotherapy in patients. Because PUMA is a known member of the family of BH3-only proapoptotic proteins, this finding suggests PUMA’s potential importance for the response to anticancer drugs.

INTRODUCTION

Locally advanced breast cancer (LABC) consists of a heterogeneous group of breast tumors including stages IIB, IIIA, IIIB, and IIIC, according to the American Joint Committee on Cancer criteria (1). It has been suggested that patients with LABC often have micrometastases at presentation, which seriously reduces the effectiveness of local therapy (2–6). The use of different regimens of systemic chemotherapy, in the treatment of LABC, has therefore been investigated extensively (7–10). Recent studies in this field have investigated the use of four to six cycles of neoadjuvant chemotherapy, consisting of an intermediate-high dose of doxorubicin, cyclophosphamide and granulocyte macrophage colony-stimulating factor (11, 12). This treatment regimen has shown impressive survival rates, especially in those patients treated with six cycles of this chemotherapy.

However, in spite of these advances in the treatment of LABC and the initial high rate of response to this form of chemotherapy, relapses still occur in >25% of the patients (11, 12). These clinical findings clearly indicate that, although the vast majority of tumor cells are killed during the initial treatment, some malignant cells survive and will inevitably lead to a relapse. Understanding the in vivo response of human cancer cells to the exposure to both doxorubicin and cyclophosphamide could not only help us identify those cellular mechanisms responsible for the death of most tumor cells but also those responsible for the survival of a few.

From in vitro studies, it has been known for some time that doxorubicin causes DNA damage by intercalation, metal ion chelation, free radical generation, and topoisomerase II inhibition (13–17). More recently, several molecular pathways have been implied in either the repair of doxorubicin-induced damage or the induction of programmed cell death (apoptosis) in response to this damage (13, 18, 19). Cyclophosphamide is active after activation in liver microsomes to a metabolite with DNA alkylating activity (20). Therefore, it could induce similar, DNA damage–related, cellular responses.

Some reports on microarray studies on long-term responses to doxorubicin treatment of breast cancer have been published (21, 22). However, these studies compared gene expression profiles in samples taken 16 weeks and 21 days, respectively, after initiation of treatment. Because we aimed to identify mechanisms involved in the early response to doxorubicin-induced cellular damage, we needed to analyze gene expression changes that are more rapidly induced. Therefore, we set out to investigate these mechanisms by identifying differences in gene expression profiles between tumor biopsies taken from LABC patients before and at only 6 hours after initiation of doxorubicin-based chemotherapeutic treatment.

MCF-7 breast tumor cells were included as a model to verify that changes in gene expression as found in patients can also occur in vitro, because we would preferentially identify mechanisms...
that can be further investigated in vitro. To our knowledge, no microarray studies on the in vitro effects of cyclophosphamide and its active metabolite have been published, which is probably due to the instability of its active metabolite in culture medium (23). A microarray-based study of doxorubicin induced gene expression changes in MCF-7 breast cancer cells has already been published (24). However, the experimental conditions and microarray platform used in this study did not allow for a direct comparison of these results with the data obtained from our in vivo samples. Gene expression changes in vitro have previously been observed after only a few hours of exposure to a relatively high concentration of doxorubicin (24). However, our studies showed that slow cellular uptake and reduced penetration into certain types of tumor tissue can lead to several hours of delay time before the cellular response to doxorubicin can take effect in a clinical setting (25, 26). Therefore, we obtained our samples at 6 hours after initiation of chemotherapy, to allow for doxorubicin to induce sufficient cellular damage to provoke a detectable response.

MATERIALS AND METHODS

Patients and Treatment. Inclusion criteria for LABC patients whose biopsies were used in this study, were (a) Histologically proven breast cancer, which should be LABC: stages IIB with a primary tumor of >5 cm (T3 tumor), IIIA, or IIB according to the American Joint Committee on Cancer 1997 criteria (27). (b) Adequate hematologic, renal, and hepatic functions ([WBC ≥ 3.0 × 109/L, platelets ≥ 150 × 109/L/I], normal serum bilirubin, normal aspartate amino transferase, alanine amino transferase, and alkaline phosphatase, normal serum creatinine (according to local reference standards). (c) Normal serum bilirubin, normal aspartate amino transferase, and its active metabolite have been published, which is probably due to the instability of its active metabolite in culture medium (23). A microarray-based study of doxorubicin induced gene expression changes in MCF-7 breast cancer cells has already been published (24). However, the experimental conditions and microarray platform used in this study did not allow for a direct comparison of these results with the data obtained from our in vivo samples. Gene expression changes in vitro have previously been observed after only a few hours of exposure to a relatively high concentration of doxorubicin (24). However, our studies showed that slow cellular uptake and reduced penetration into certain types of tumor tissue can lead to several hours of delay time before the cellular response to doxorubicin can take effect in a clinical setting (25, 26). Therefore, we obtained our samples at 6 hours after initiation of chemotherapy, to allow for doxorubicin to induce sufficient cellular damage to provoke a detectable response.

Sample Processing and Cell Culture. The patient biopsies were immediately embedded in compound embedding medium (Tissue-Tek, Sakura, Inc., Torrance, CA), frozen in liquid nitrogen, and stored at −80°C. At least one dozen cryosections (4-5 μm) were cut from each biopsy and were stored at −20°C. One section was stained with H&E and examined by a pathologist to confirm the presence of tumor cells in the biopsy. Biopsies with tumor cells present were selected for further processing (paired biopsies, from nine patients). After removal of the compound embedding medium, samples were homogenized in a microdisembrator (Braun, Melsingen, Germany) and the obtained tissue powder was solubilized in TRIzol LS reagent (Life Technologies, Gaithersburg, MD). Then RNA was isolated according to the manufacturer’s instructions.

For in vitro experiments, MCF-7 cells were grown in Dulbecco’s modified Eagle medium (DMEM, Life Technologies) with 8% heat-inactivated (30 minutes at 56°C) fetal calf serum (FCS) (Life Technologies) under 5% CO2. All experiments were done in triplicate and for each experiment the appropriate volume out of a doxorubicin stock solution was added to the medium to reach final concentrations in the range of 0 to 500 nmol/L. This range corresponds to the blood concentrations found in breast cancer patients after doxorubicin administration (28). After 6 hours medium was removed, cells were washed and solubilized in RNA-Be (Tel-Test Inc., Friendswood, TX). Total RNA was isolated according to the manufacturer’s instructions.

p53 up-regulated modulator of apoptosis (PUMA)–inducible DLD-1 cells (A5-DJYFG) were kindly provided by Dr. B. Vogelstein (John Hopkins University School of Medicine, Baltimore, MD) and were maintained in DMEM with 10% FCS under 5% CO2, supplemented with 0.4 mg/mL G418 (Invitrogen, Leiden, the Netherlands), 0.25 mg/mL hygromycin B (Invitrogen), and 20 ng/mL doxycycline (Invitrogen). The induction of PUMA was started by removal of the doxycyclin.

Gene Expression Profiling with Microarrays. Spotted cDNA microarrays (18K human) were obtained from the Netherlands Cancer Institute central microarray facility. Protocols for sample preparation were also supplied by the NKI-CMF and are shortly described below (also see http://microarrays.nki.nl and ref. 28).

The quality of the total RNA, isolated as described above, was confirmed on an agarose gel. RNA was then amplified using the lin-amp protocol (29). The quality of the obtained amplified-RNA was confirmed on an agarose gel and labeled cDNA was synthesized from this amplified-RNA using Cy5- and Cy3-AP3-DUTP (Amersham, Arlington Heights, IL) and SuperScript II (Invitrogen). The labeled products were then purified (QIAquick PCR Purification Kit, Qiagen, Inc., Chatsworth, CA) and “treated” and “control” samples were cohybridized against the same microarray. Microarrays were scanned on an Agilent DNA Microarray scanner (Agilent Technologies, Palo Alto, CA) and data extraction was done using Imagene 5.1 (BioDiscovery Inc., Los Angeles, CA). Each experiment consisted of two microarrays, to allow for the dyes to be reversed between the “treated” and “control” samples (dye-swap). Weighed data from each dye-swapped microarray were averaged with the original array, as described below, to obtain a better signal to noise ratio and to reduce systemic errors due to cDNA specific dye preferences.
Real-time Quantitative Reverse Transcription-PCR. Total RNA obtained from experiments with MCF-7 cells (extracted as described above) was further purified using the RNeasy Mini kit (Qiagen), according to the manufacturer’s instructions. After digestion of genomic DNA traces (RNase-Free DNase Set, Qiagen) cDNA was synthesized from this RNA (M-MLV RT, Invitrogen). PCR reactions and real-time fluorescence measurements were done with a LightCycler (Roche, Almere, the Netherlands) using the LightCycler FastStart DNAMaster SYBR Green I kit (Roche). The fluorescence was recorded real-time according to the manufacturer’s instructions.

The expression levels of seven well-known housekeeping genes (i.e., GAPDH, ACTB, cyclophilin, 28S rRNA, ARP, U1A, and B2MG) were tested for their stability after doxorubicin treatment. From this test, cyclophilin (peptidylprolyl isomerase A) was determined to be the most reliable housekeeping gene for our experimental conditions. Therefore, PUMA expression levels were determined relative to the levels of cyclophilin. Furthermore, the expression levels of both PUMA and cyclophilin mRNAs were corrected for the efficiencies of the respective PCR reactions. Sequences of the primers (Isogen Bioscience, Maarssen, the Netherlands) are as depicted in Table 1 and annealing temperatures were 65°C for both primer pairs. Specificity of the PCR reactions was verified by melting curve analysis and size separation on an agarose gel.

Statistical Analysis. The obtained microarray data was analyzed using the “The classic R normalization tools” analysis software provided by the NKI-CMF (http://dexter.nki.nl), as previously described by Hughes et al. (28; supplementary material). Normalization was done per subarray with a Lowess factor of 0.5, not including the flagged results. Subsequent analysis of statistical significance of changes was done with f set at 0.135 and L (for combining dye-swap experiments) set at 1.0. The f value was experimentally determined by performing four self-self experiments with pooled amplified-RNA from all patient samples used in this study. For each individual self-self experiment $f = 0.135$ resulted in $<1\%$ false positives at $P < 0.01$.

Immunohistochemical Staining. Cryosections obtained from patient biopsies, or cells grown on microscopy slides, were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes and subsequently in ice-cold acetone for 15 minutes. Endogenous peroxidase activity was blocked by incubation with 3% H$_2$O$_2$ in PBS for 30 minutes. After a 10-minute incubation with 2% normal goat serum (Dako A/S, Copenhagen, Denmark) and 5% bovine serum albumin (Sigma, St. Louis, MO) in PBS, the sections were incubated with 5 µg/mL rabbit anti-PUMA antibody (Abcam Ltd., Cambridgeshire, United Kingdom) in 5% bovine serum albumin in PBS for 2 hours. This antibody was raised against a synthetic peptide (PLPRGHRAPEMEPN) corresponding to amino acids 180 to 193 of human PUMA-α. Incubation with the secondary antibody was done using 5 µg/mL horseradish peroxidase–conjugated goat anti-rabbit antibody (Dako) in PBS with 5% bovine serum albumin for 1 hour. The slides were developed with 0.05% 3,3′-diaminobenzidine (Sigma) and 0.015% H$_2$O$_2$. They were counterstained with Mayer’s hematoxylin (Sigma) and embedded.

Multiplex Ligation-Dependent Amplification. A multiplex ligation-dependent probe amplification procedure has been described recently (30). The method enables relative quantification of up to 40 sequences in a one-tube assay through amplification of sequence specific probe combinations. Recently, method adjustments have been made to allow application of RNA-derived cDNA and the method was tested for the application on low cell number samples (31). In the present study, an apoptosis related probe set including PUMA/BBC3 was used (32) as an extra verification of the results obtained for this gene by using microarrays. Results were expressed as the ratio of the signal of the gene of interest, to the signal of the housekeeping gene GUS-B.

RESULTS

Microarray Analysis of Locally Advanced Breast Cancer Patient Samples. After the analysis of the microarray data, we sorted all data based on the observed number of significant ($P \leq 0.05$) expression changes. One table was constructed to include both the up-regulated genes (more abundantly expressed after initiation of chemotherapy than they were before) and the down-regulated genes (less abundantly expressed after treatment than they were before). All mRNAs that were either changed in less than half of our patients ($\leq 4$ of 9) or were antiregulated (i.e., up-regulated in one patient but down-regulated in another), were subsequently excluded. This selection procedure resulted in a list of candidate genes, which may be involved in the in vivo response to chemotherapy in LABC, as shown in Table 2.

PUMA is the most frequently differentially expressed gene, with a significant up-regulation in eight of nine patients. The second most frequently differentially expressed gene (F-box only protein 32) shows a significant up-regulation in only six of nine patients.

The observed up-regulation of PUMA mRNA in patients was subsequently confirmed, for one of our patients (patient 7, see Table 2), using reverse transcription-multiplex ligation-dependent probe amplification.

Microarray Analysis of MCF-7 Samples. The selection of differentially expressed genes from the data of our experiments with MCF-7 cells was carried out in a similar way as described for the LABC patient data. The major selection criterion was, that only the data from those genes that showed significant ($P \leq 0.05$) up-regulation in three independent experiments were included. Furthermore, because a cutoff point set at $P \leq 0.05$ resulted in 42 significantly down-regulated mRNAs, we only included those genes that were down-regulated with $P \leq 0.01$ in all three experiments. The results of this selection were averaged and sorted for the average, as shown in Table 3. The same cDNA for

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sequences of the primers used for real-time quantitative reverse transcription-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense primer (5'-3')</td>
<td>Antisense primer (5'-3')</td>
</tr>
<tr>
<td><strong>Cyclophilin</strong></td>
<td>tctcttgagcttgctgag</td>
</tr>
<tr>
<td><strong>PUMA</strong></td>
<td>ccaacgctgacaggactgct</td>
</tr>
</tbody>
</table>

**NOTE.** Cyclophilin (peptidylprolyl isomerase A) was determined to be the most reliable housekeeping gene for our experimental conditions, based on a test of the expression levels of seven well-known housekeeping genes.
Table 2 Microarray data from LABC patients at 6 hours after initiation of chemotherapy relative to levels before start of chemotherapy.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Description</th>
<th>No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL688112</td>
<td>PUMA/BBBC3: BCL2 binding component 3</td>
<td>8</td>
<td>2.06*</td>
<td>1.65</td>
<td>1.73*</td>
<td>1.09</td>
<td>1.82*</td>
<td>1.36</td>
<td>1.58</td>
<td>1.75</td>
<td>2.73*</td>
</tr>
<tr>
<td>AA046700</td>
<td>F-box only protein 32</td>
<td>6</td>
<td>1.39</td>
<td>1.34</td>
<td>2.38*</td>
<td>1.69</td>
<td>1.03</td>
<td>1.09</td>
<td>1.48*</td>
<td>1.85*</td>
<td>1.41*</td>
</tr>
<tr>
<td>AA486747</td>
<td>Z39IG: immunoglobulin superfamily protein</td>
<td>6</td>
<td>1.57</td>
<td>1.04</td>
<td>0.79</td>
<td>1.53</td>
<td>1.64</td>
<td>0.85</td>
<td>1.78*</td>
<td>1.26</td>
<td>1.60</td>
</tr>
<tr>
<td>R59136</td>
<td>Z39IG: immunoglobulin superfamily protein</td>
<td>6</td>
<td>2.11*</td>
<td>1.04</td>
<td>0.93</td>
<td>1.80</td>
<td>1.87*</td>
<td>0.85</td>
<td>2.14*</td>
<td>1.29</td>
<td>1.85*</td>
</tr>
<tr>
<td>AI671604</td>
<td>FDXR: ferredoxin reductase</td>
<td>5</td>
<td>1.78</td>
<td>1.27</td>
<td>1.28</td>
<td>1.55</td>
<td>1.88</td>
<td>1.17</td>
<td>1.63</td>
<td>1.96</td>
<td>1.91*</td>
</tr>
<tr>
<td>T50083</td>
<td>CDNA FLJ42250 fis, clone TKIDN2007828</td>
<td>5</td>
<td>1.46*</td>
<td>1.10</td>
<td>1.25</td>
<td>1.27</td>
<td>1.33</td>
<td>1.11</td>
<td>1.15</td>
<td>1.35</td>
<td>1.67*</td>
</tr>
<tr>
<td>AA056013</td>
<td>MAGP2: Microfibril-associated glycoprotein-2</td>
<td>5</td>
<td>1.55</td>
<td>1.29</td>
<td>0.77</td>
<td>0.87</td>
<td>1.70*</td>
<td>1.09</td>
<td>2.09*</td>
<td>1.07</td>
<td>2.19*</td>
</tr>
<tr>
<td>T72089</td>
<td>NMT: nicotinamide N'-methyltransferase</td>
<td>5</td>
<td>1.65</td>
<td>1.02</td>
<td>0.99</td>
<td>0.94</td>
<td>1.80</td>
<td>0.96</td>
<td>2.78*</td>
<td>1.33*</td>
<td>1.23*</td>
</tr>
</tbody>
</table>

**NOTE.** The No. column refers to the number of statistically significant gene expression changes. Numbers refer to patient identification numbers. All data represent expression after / expression before ratios. Genbank accession number of cDNA clone spotted on the microarray.

**Description:** Gene name as registered in the Unigene database (for the Unigene cluster corresponding to the Genbank accession number).

*P ≤ 0.001.
|P ≤ 0.01.
|P ≤ 0.05.

FOS-like antigen 1 has been spotted twice, resulting in two ratios (2.15 and 1.76). Variation of the duplicate spots may be due to differences in the (local) hybridization.

Measured expression levels of PUMA mRNA in MCF-7 cells by real-time quantitative PCR were corrected for the cyclophilin mRNA levels measured in the same sample, and the respective efficiencies of both PCR reactions. Relative expression levels, as determined from these calculations, are depicted in Fig. 1. PUMA mRNA up-regulation, relative to the untreated control, is statistically significant (P ≤ 0.05, n = 3) at all concentrations of doxorubicin at which it was measured. Thereby not only confirming doxorubicin induced PUMA up-regulation at a wide, clinically relevant, concentration range but also confirming our microarray results with an unrelated technique. PUMA mRNA was subsequently measured in two of these three experiments by reverse transcription-multiplex ligation-dependent probe amplification and up-regulation was confirmed.

**DISCUSSION**

We have shown PUMA mRNA to be up-regulated in *vivo* in eight of nine LABC patients treated with an intermediate high dose of doxorubicin and cyclophosphamide. Because we have also shown doxorubicin to cause a dose-dependent up-regulation of PUMA mRNA in MCF-7 breast cancer cells *in vitro*, we propose here that PUMA up-regulation in LABC patients treated with an intermediate high dose of doxorubicin and cyclophosphamide is, at least in part, doxorubicin induced. *PUMA* is the...
only gene identified in this study, which is differentially expressed both after doxorubicin-based chemotherapeutic treatment of breast cancer in vivo and after exposure of MCF-7 cells to doxorubicin in vitro.

PUMA is a member of the BH3-only family of proteins and has recently received much attention as a potential key mediator of cell death induced by a wide range of different apoptotic stimuli (33–36). It is known that PUMA overexpression or PUMA induction by p53 overexpression can induce apoptosis in some cell lines (34, 35). Even a more physiologic role for PUMA in the response to doxorubicin has been suggested in mice and in in vitro models (33, 37) and several reports on in vitro studies showing PUMA mRNA induction by other cellular stressors are also available. These stressors include various other anticancer drugs, such as etoposide, 5-fluorouracil (34, 35), actinomycin D, fludarabine (32), and Velcade (38) and other factors, such as hypoxia and radiation (33, 35).

Among the other genes induced by doxorubicin in MCF-7 cells, we found tumor protein p53 inducible nuclear protein 1 (TP53INP1) and sestrin 2 (SESN2), which are both p53-responsive genes (39, 40). Because p53 stabilization is a well-known response to DNA-damaging agents, we propose here that PUMA induction in MCF-7 cells is also likely to be a p53-mediated process. However, up-regulation of TP53INP1 and SESN2 was not observed in the majority of our patients. This indicates different conditions for regulation of expression of these genes in our patient population, depending on stimulus conditions.

Table 3 Microarray data from MCF-7 cells after a 6-hour exposure to 500 nmol/L doxorubicin (relative to 0 hour)

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Description</th>
<th>Log2Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA459364</td>
<td>TP53INP1: tumor protein p53 inducible nuclear protein 1</td>
<td>2.91</td>
</tr>
<tr>
<td>AA4454079</td>
<td>SESN2: sestrin 2</td>
<td>2.36</td>
</tr>
<tr>
<td>AI688112</td>
<td>PUMA/BBC3: BCL2 binding component 3</td>
<td>2.34</td>
</tr>
<tr>
<td>T89996</td>
<td>FOSL1: Fos-like antigen 1</td>
<td>2.15</td>
</tr>
<tr>
<td>AA1777845</td>
<td>LIMK2: LIM domain kinase 2</td>
<td>1.86</td>
</tr>
<tr>
<td>T89996</td>
<td>FOSL1: Fos-like antigen 1</td>
<td>1.76</td>
</tr>
<tr>
<td>AA679864</td>
<td>DLEC1: deleted in liver cancer 1</td>
<td>1.41</td>
</tr>
<tr>
<td>AA977296</td>
<td>Clone IMAGE: 5314816, mRNA</td>
<td>1.40</td>
</tr>
<tr>
<td>N74524</td>
<td>TUBB5: tubulin, beta, 5</td>
<td>1.39</td>
</tr>
<tr>
<td>AA504246</td>
<td>ATP5H: ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d</td>
<td>1.34</td>
</tr>
<tr>
<td>H13257</td>
<td>FBXO22: F-box only protein 22</td>
<td>1.33</td>
</tr>
<tr>
<td>AA660637</td>
<td>PNRC1: proline-rich nuclear receptor coactivator 1</td>
<td>1.33</td>
</tr>
<tr>
<td>AA670330</td>
<td>C6orf49: chromosome 6 open reading frame 49</td>
<td>1.32</td>
</tr>
</tbody>
</table>

NOTE. Genbank accession number of cDNA clone spotted on the microarray. Description: Gene name as registered in the Unigene database (for the Unigene cluster corresponding to the Genbank accession number).

All data represent average expression after / expression before ratios from three independent experiments, which are significantly different from 1 (P ≤ 0.05).
Fig 2 Immunohistochemical staining of LABC biopsies using a polyclonal antibody, raised against a PUMA-derived peptide and counterstained with hematoxylin. Counterstaining shows cell nuclei of both tumor cells and of stromal cells. Staining of an LABC biopsy taken at 2.5 hours after i.v. injection of doxorubicin (A), and its control without primary antibody (B). Normal breast tissue (C), and its control without primary antibody (D). Breast cancer biopsy of patient 5 (Table 2) before (E), and at 6 hours after (F), start of doxorubicin treatment. DLD-1 cells with PUMA construct, before (G), and after induction of PUMA by withdrawal of doxycyclin. Bar, 200 μm (A–F) and 50 μm (G–H).

and other associated pathways. Besides the p53-independent up-regulation induced by glucocorticoids (41), PUMA could also be up-regulated by p73 (42). The latter could be up-regulated by doxorubicin (43).

Furthermore, immunohistochemical staining suggests PUMA to be expressed exclusively in tumor cells. Therefore, the relative abundances of tumor cells and stromal cells are likely to influence the factor of up-regulation, because this factor is measured in a homogenate of the entire biopsy that also contains the stromal cells, which do not seem to express PUMA. However, the high frequency of up-regulation we found in our patient population clearly indicates that PUMA up-regulation exceeds these variations that occur due to tissue heterogeneity.

Differences in PUMA protein levels, in tumor biopsies from before and 6 hours after chemotherapy, were not detectable. This could be caused by the relatively nonquantitative nature of the immunohistochemical staining and the low intensity, which hampers the detection of relatively small changes. However, a different explanation could also be the absence of such changes, at the early time point at which the biopsies were taken. Although mRNA levels were changed at this time point, changes at the protein level could occur later.

To our knowledge, no data showing PUMA expression to be a clinical response to chemotherapeutic treatment have been published to date. Even the induction of PUMA in a breast cancer cell line has not been previously described, because all previous in vitro studies have used cell lines derived from a different organ. Therefore, we conclude this study to be the first to show PUMA up-regulation as an in vivo process, induced by a chemotherapeutic treatment, and as an in vitro response to DNA damage in MCF-7 breast cancer cells.

Together with literature data showing PUMA mRNA induction to be a potent apoptotic stimulus (33–36), we suggest here that up-regulation of PUMA mRNA could be a key effector mechanism in the induction of tumor cell death by doxorubicin and cyclophosphamide in LABC patients. Furthermore, we propose MCF-7 breast cancer cells to be the model of choice for the further in vitro investigation of the mechanisms involved in this process.

Future studies should aim to further clarify the contribution of PUMA to the in vivo induction of apoptosis, thereby further elucidating the relevance of PUMA up-regulation for the efficacy of chemotherapeutic treatment. Furthermore, extensive in vitro studies into the mechanisms involved in these processes could help identify new molecular targets for therapies aiming to increase chemotherapeutic efficacy.

ACKNOWLEDGMENTS

We thank Dr. Wim de Boer for his skillful statistical advice; Arno Velds, Guus Hart, and Mike Heimeriks for assistance in analysis of microarray data; Dr. Elsken van der Wall for logistic assistance; Drs. Paul van Diest and Wolter Mooi for pathologic examinations; Corine Hess and Fedor Denkers for performing the multiplex analysis; Dr. Victor van Hinsbergh for textual comments; and the colleagues in the laboratory for helpful discussions.

REFERENCES


Induction of p53 Up-Regulated Modulator of Apoptosis Messenger RNA by Chemotherapeutic Treatment of Locally Advanced Breast Cancer


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/11/5/1863

Cited articles
This article cites 38 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/11/5/1863.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/11/5/1863.full.html#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, contact the AACR Publications Department at permissions@aacr.org.