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14. ABSTRACT
The objectives of our research during that last funding year (2005-2006) are: 1) identification of new tumor markers for early stage breast cancer (ductal carcinoma in situ, DCIS); 2) optimization of methods and molecular beacon design for the detection of the levels of tumor marker gene expression, and 3) development of multiplex quantum dot (QD) technology for detection of the level of protein expression in single cell level. The ultimate goal is to develop a multiplex fluorescence cellular imaging approach for simultaneous examination of the levels of gene and protein expression in a single cell level. From our study, we found that in addition to Her-2/neu, survivin and cyclin D1, three more tumor markers are highly expressed in human DCIS tissues, including HIF-1alpha, epidermal growth factor receptor, and urokinase plasminogen activator receptor (uPAR). We have developed antibodies or targeting peptide-conjugated QDs to detect the expression of those tumor markers in human breast cancer cells. At present, we are in the process of starting the feasibility phase of the clinical trail to determine whether we are able to detect breast cancer cells in fine needle biopsy and ductal lavage samples from breast cancer patients.

15. SUBJECT TERMS
Breast tumor markers, Her-2/neu, Cyclin D, survivin, EGFR, uPAR, molecular beacons, quantum dots, molecular imaging of cancer cells, Early detection of breast cancer, identification of ductal carcinoma in situ, ductal lavage, Fine needle aspiration, women at high risk of breast cancer

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Introduction:

This ongoing research project aims to develop novel fluorescence cellular imaging approach for early detection of breast cancer. We are using fluorescence imaging probes to detect the levels of expression of several biomarker genes for breast cancer cells to determine if identification of the gene expressing cells in a single cell level in fine needle aspiration or ductal lavage samples obtained from breast cancer patients or high risk women could increase the specificity and sensitivity of the detection. Although we have developed methods to detect cells expressing three tumor marker genes including Her-2/neu, cyclin D1 and survivin, we have been continuing to identify new biomarkers for the early stage breast cancers (DCIS). To increase the sensitivity and specificity, we have also developed quantum dots (QDs)-labeled fluorescence probes to detect the expression levels of those proteins in the cells. At present, we are in the process of conducting the feasibility phase of the clinical trial to determine the specificity and sensitivity of our detection method in clinical samples obtained from breast cancer patients.

Body of the progress report

1. Identification of new biomarkers for ductal carcinoma *in situ*.

It is well known that human tumors containing heterogeneous cell population expressing various biomarkers. To increase the sensitivity and specificity of early detection of breast cancer cells, we have examined 16 human DCIS tissues and identified several new biomarkers for breast cancer cells at DCIS stage. First, we found that the level of hypoxia-inducible factor 1 alpha (HIF-1α) is [illegible] undetectable in normal breast ductal epithelial cells. Interestingly, we found that this upregulation is not necessarily correlated with tumor hypoxia since many of HIF-1α positive cells were detected in DCIS lesions containing a single cell layer of ductal epithelial cells (Figure 1). We also found high levels of HIF-1α in solid type DCIS lesions where the tumor hypoxia is present.

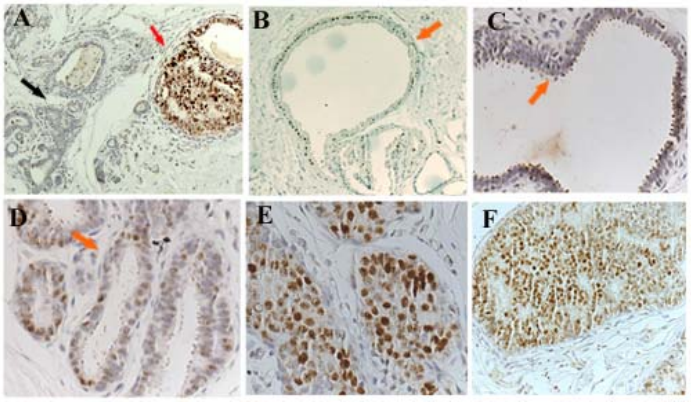

Fig 1. Examination of HIF-1α in a human DCIS tissue by immunohistochemical staining.

We further found that upregulation of HIF-1α in DCIS tissue occurs before survivin expression, suggesting the importance of the HIF-1α in the progression of breast cancer (Figure 2). Importantly, we found that there is a nice correlation between the levels of HIF-1α and survivin expression in DCIS tissues (Figure 2). Recently, we have identified a novel pathway showing that activation of EGFR signal pathway increases the level of HIF-1α in normoxic tumor cells and HIF-1α then up-regulates survivin gene expression (Peng X et al, JBC, 281(8), 25903-25914, 2006). To determine if the survivin/HIF-1α pathway also plays an important role in the progression of DCIS of breast, we examined the levels of expression of those biomarker genes in a series of cell lines that represent normal, premalignant, DCIS and invasive stages of breast cancer development. As shown in Figure 3, we found that Her-2/neu, EGFR, HIF-1α and survivin were highly expressed in human DCIS cell line. Additionally, results from immunofluorescence staining also showed that urokinase plasminogen activator receptor (uPAR) is also expressed in DCIS tissues (Figure 4). Therefore, in addition to three tumor markers that were originally proposed for this study, we will add EGFR, HIF-1α and uPAR as biomarkers for cancer cell detection. We will use QD-labeled with antibodies to EGFR, Her-2/neu, cyclin D1, survivin and HIF-1α to detect cells highly expressing those

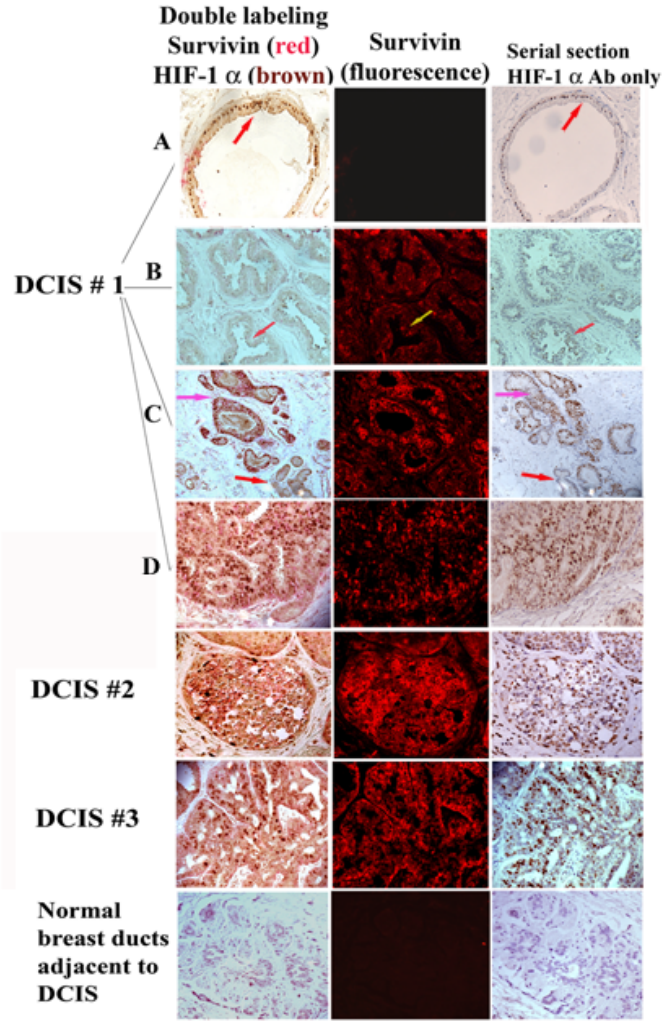

Fig 2. Co-localization of survivin and HIF-1α expression in DCIS tissues.
HIF-1α-Ab positive tumors were labeled with an HRP-rabbit anti-mouse secondary antibody followed by DAB substrate (brown). Survivin-Ab positive cells were detected using an alkaline phosphatase-labeled donkey anti-goat antibody followed by alkaline phosphatase substrate kit 1, which gave rise to red staining under light microscope and red fluorescence observed with fluorescence microscope.

proteins. We have developed an ATF-QD which contains the amino-terminal fragment of uPA, a receptor binding domain for uPAR, to detect cells expressing uPAR.

2. Update on clinical trial: feasibility phase

As the development of new methods and biomarkers in our research laboratory, we feel that it is necessary to incorporate those new strategies into our clinical trial. We have revised the clinical trial protocol and submitted to the Human Investigation Committee of the DOD for approval. The procedure for human trial will be the same. The only modification is that after obtaining the cell samples, the slides will be labeled with molecular beacons and QD-labeled antibodies. We will select quantum dots and fluorescence dyes that emit at different wavelengths, which allow us to determine the levels of survivin, HIF-1α, EGFR, Her-2/Neu and cyclin D1 mRNAs and proteins in a single cell (Figure 5).

Please find the detailed description of changes in Appendix (revised human protocol and consent form).

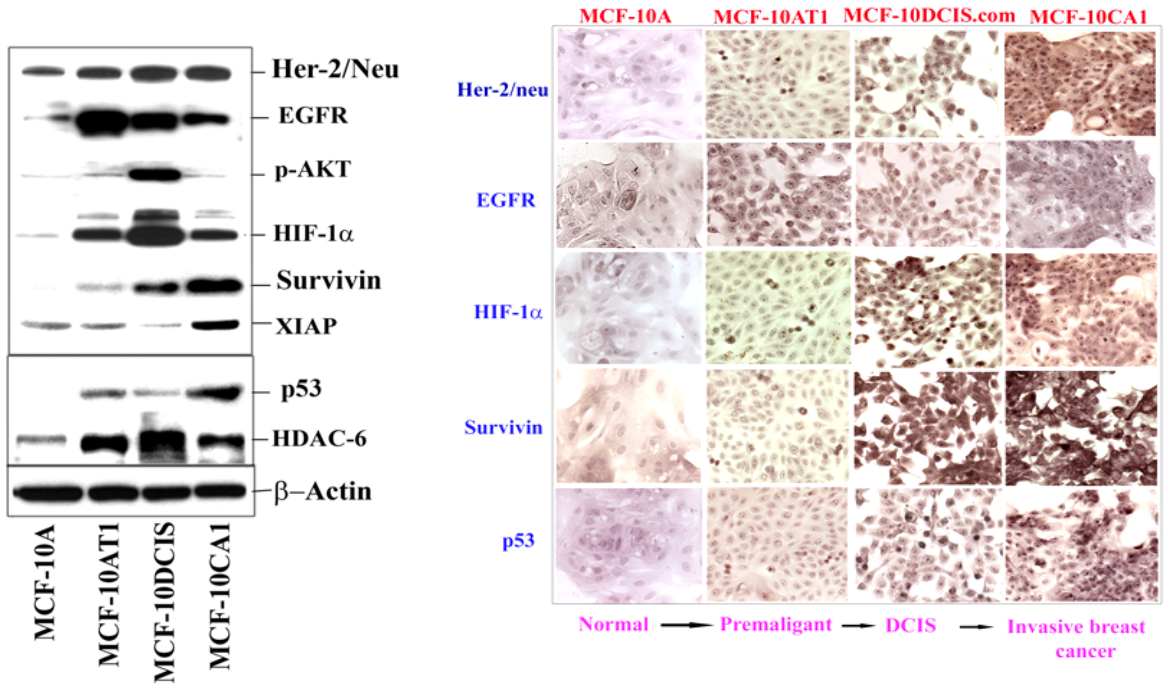

Fig 3. Detection of cellular factors regulating HIF-1α/ survivin pathway in the MCF-10 cell lines.
Right panel: Western blot analysis of the levels of Her-2/neu, EGFR, p-AKT, HIF-1α, survivin, XIAP, mutant p53 and HDAC6 proteins in MCF-10 series of cell lines cultured under normoxic condition. **Left panel**: Immunohistochemical staining of above markers in the MCF-10 serial cell lines. DAB substrate was used and positive cells were stained as brown. Hematoxylin was used as counterstaining (Blue).

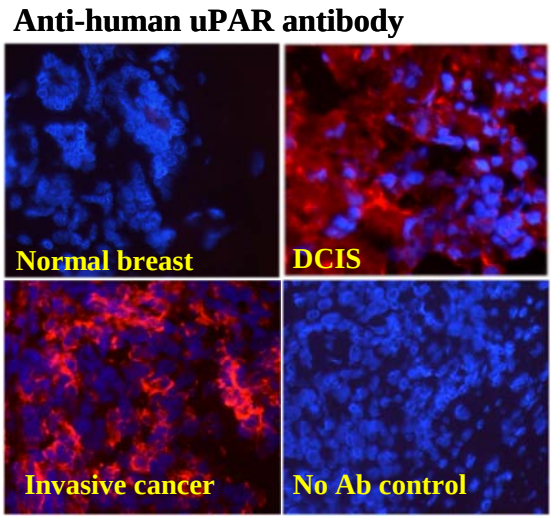

Fig. 4. Detection of uPAR expression in DCIS and invasive breast cancer tissues

Frozen tissue sections of breast DCIS and invasive cancer were labeled with an anti-uPAR antibody. Red: uPAR positive cells, Blue: Hoechst 33342 nuclear staining.

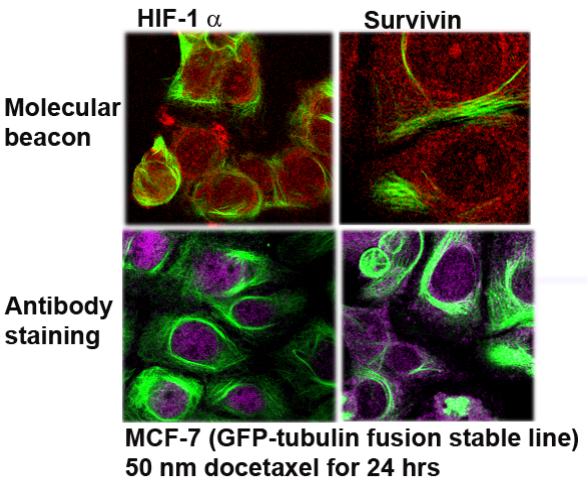

Fig. 5. Simultaneous detection of survivin and HIF-1α mRNA and protein levels using survivin and HIF-1α MBs and antibodies to survivin and HIF-1α.
MB: Red
Antibody: Purple
Green: GFP

Key Research Accomplishments:

Publications:

With the funding support from DOD, we have successfully established methodologies and protocols for detection of breast cancer by fluorescence imaging of tumor marker genes. We have published a research article in Cancer Research (Peng XH et al. Real-Time Detection of Gene Expression in Cancer Cells using Molecular Beacon Imaging: New Strategies for Cancer Research. Cancer Research, 65 (5), 1909-1917, 2005). We have also identified a novel signal transduction pathway in breast cancer cells, which provides new biomarkers for detection of breast cancer cells in DCIS stage (Peng XH et al. Cross-Talk Between Epidermal Growth Factor Receptor and HIF-1 Signal Pathways Increases Resistance to Apoptosis by Upregulating Survivin Gene Expression, Journal of Biological chemistry, 280(36), 25903-25914).

Patent applications:

We have also filed a patent application on the use of this novel technology for cancer cell detection (U.S. patent (# 60/63266) entitled "Methods and applications of molecular beacon image for cancer cell detection"). Additionally, the Idea Award from DOD supported us, in part, to develop tumor targeted, multifunctional nanoparticles for detection and treatment of breast cancer. Based on our study, we have filed two patent applications: 1) U.S. patent (60/676,812) entitled "Targeted Multifunctional Nanoparticles for Cancer Imaging and Treatment; 2) U. S. provisional patent, (# 60/797,613) entitled "Nanostructures, methods of synthesizing Thereof, and methods of use thereof".

Presentations in national and international conferences:

We were invited to present our work in the Fifth Annual Meeting of the Society for Molecular Imaging and the Second Annual Meeting of American Academy of Nanomedicine. We will also give an invited talk in 2006 International Conference on Bio and Pharmaceutical Science and Technology.

Reportable Outcomes

1. **Research progress:**
    - **Identification of new biomarkers for DCIS**

- **Development of multiplex fluorescence QD approach for detection of the protein levels of biomarkers**

2. Publications:

Peng XH, Cao ZH, Xia JT , Carlson W. G, Lewis MM, Wood, WC and Yang L. Real Time Detection of Gene Expression in Cancer Cells using Molecular Beacon Imaging: New Strategies for Cancer Research. Cancer Research, 65 (5), 1909-1917, 2005.

Peng X, Karna P, Cao Z, Jiang B, Zhou M, and Yang L. Cross-talk between epidermal growth factor receptor and HIF-1 signal pathways increases resistance to apoptosis by upregulating survivin gene expression. Journal of Biological Chemistry, (36):25903-14, 2006

3. Patent applications:

U.S. patent, 60/439,771, full patent application filed in January, 2004,
International patent filed in January, 2005.
Methods of detecting gene expression in normal and cancerous cells.

U.S. patent, full patent application (# 10/542,117) filed on July 15, 2005.
Methods and applications of molecular beacon image for cancer cell detection.

Above two patents have been licensed by Alvitae Pharmaceuticals, San Francisco, CA.

U.S. patent, full patent application (60/676,812), file on May 2, 2006
Targeted Multifunctional Nanoparticles for Cancer Imaging and Treatment.

U. S. Provisional patent, (# 60/797,613), filed on May 4, 2006
Nanostructures, methods of synthesizing Thereof, and methods of use thereof

4. Newly awarded research funding:

Emory-GA Tech Nanotechnology Center for Personalized and Predictive Oncology
NIH NCI Center of Cancer Nanotechnology Excellence (CCNE) 1 U54 CA119338-01
Funding for total of 6 projects and 5 cores:
10/2005- 9/2010
(CCNE PIs: Drs. Shuming Nie and Jonathan Simons)

Project 1. Quantum Dots and Targeted Nanoparticles Probes for Tumor Imaging
(Co-PI, 20%), (CO-PIs: Dr. S Nie, **L Yang**, H. Mao)

Project 5. Nanotherapeutics. Multifunctional Nanoparticles for Drug Delivery and Targeting

Golfers Against Cancer Foundation (Winship Cancer Institute Seed grant)

(PI: L Yang)
Development of Targeted Superparamagnetic Iron Oxide (IO) Nanoparticles for *in vivo* Imaging and Therapy of Pancreatic Cancer. 12/2005-12/2006.

Conclusions

In summary, we have development novel multiplex fluorescence cellular imaging approach for detection of early stage breast cancer cells. We are in the process of enrolling the patients for human trial.

Appendix

Research Article: Cancer Research, 2005

JBC 2006

Revised Human protocol

Revised consent form

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 36, pp. 25903–25914, September 8, 2006

Cross-talk between Epidermal Growth Factor Receptor and Hypoxia-inducible Factor-1α Signal Pathways Increases Resistance to Apoptosis by Up-regulating Survivin Gene Expression*

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Although increasing evidence supports a link between epidermal growth factor receptor (EGFR) signaling and resistance to apoptosis, the mechanism by which the EGFR signaling pathway inhibits apoptosis is not well understood. In this study, we found that epidermal growth factor (EGF) stimulation increased the level of expression of the inhibitor of apoptosis protein survivin in breast cancer cells but not in normal mammary epithelial cells. We further demonstrated that activation of survivin gene expression is mediated by oxygen-independent hypoxia-inducible factor (HIF)-1α up-regulation in EGF-treated cancer cells. EGFR signaling activated the phosphoinositide 3-kinase/AKT pathway, subsequently increasing the level of HIF-1α under normoxic conditions. HIF-1α then activated survivin gene transcription through direct binding to the survivin promoter. Furthermore, we found that overexpression of HIF-1α small interfering RNA blocks EGF-induced survivin gene up-regulation and increases apoptosis induced by the chemotherapy drug docetaxel. However, transfection of a plasmid expressing HIF-1α gene activates survivin gene expression and reduces the apoptotic response. Our results demonstrate a novel pathway for EGFR signaling-mediated apoptosis resistance in human cancer cells. Although the role of HIF-1α in regulating cell survival under hypoxic conditions has been studied extensively, our results show that normoxic breast cancer cells utilize cross-talk between EGFR signals and HIF-1α to up-regulate the anti-apoptotic survivin gene, providing a strong rationale for the targeting of HIF-1α as a therapeutic approach for both hypoxic and normoxic tumor cells. Understanding key molecular events in EGFR signaling-induced apoptosis resistance should provide new information for the development of novel therapeutic agents targeting EGFR, HIF-1α, and/or survivin.

The EGFR[2] signaling pathway plays a key role in the regulation of cell proliferation, survival, and differentiation (1, 2). It has been shown that the level of EGFR is up-regulated in many human tumor tissues. Activation of EGFR signaling has been associated with highly aggressive cancer types and poor responses to therapeutic agents (3–7). Prior preclinical and clinical studies have shown that blocking the EGFR signaling via monoclonal antibodies or inhibition of the EGFR tyrosine kinase with small molecules reduces the growth of breast cancers and sensitizes responses to chemotherapy (8–10).

Recently, we and others have shown that activation of the EGFR signaling pathway leads to the up-regulation of survivin, a member of the inhibitor of apoptosis (IAP) protein family (11–14). Survivin is broadly expressed in fetal tissues but is undetectable in the most normal adult tissues (15). However, a high level of survivin is found in most common tumor types, including over 70% of human breast cancer tissues at all stages of cancer development (16–18). It has been shown that in breast cancer cells, levels of survivin expression correlate with susceptibility to apoptosis (17). At present, the mechanism by which this up-regulation of survivin occurs in tumor cells having activated EGFR signaling is not fully understood. Recent studies have suggested, however, that activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway by EGFR signaling causes up-regulation of survivin expression (12, 13). It is still unknown how PI3K/AKT signaling leads to survivin gene transcription.

Several studies have shown that under normoxic conditions, activation of EGFR signaling also increases the level of hypoxia-inducible factor 1α (HIF-1α) through the PI3K/AKT pathway (19–21). HIF-1α, a member of the basic helix-loop-helix-PAS protein family (22), normally becomes highly up-regulated under hypoxic conditions, mostly as a result of inhibition of protein degradation. HIF-1α can then activate transcription of many genes that are critical for continued cellular function

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[2] The abbreviations used are: EGFR, epidermal growth factor receptor; IAP, inhibitor of apoptosis; EGF, epidermal growth factor; HIF, hypoxia-inducible factor; Doc, docetaxel; HRE, hypoxia-responsive element; 7-AAD, 7-amino-actinomycin D; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PE, phycoerythrin; nt, nucleotide(s); MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor.

under hypoxic conditions (22). Our previous study results have shown that survivin gene transcription is increased in hypoxic tumor cells (23). Due to the combined results of these studies, we speculated that the observed EGFR signaling- induced survivin gene expression might be mediated by transcriptional activity of HIF-1α in an oxygen-independent manner.

In this study, we examined the effects of EGFR activation on the apoptotic response and survivin gene expression in human breast cancer cells. We found that EGF stimulation increases survivin gene expression specifically in breast cancer but does not in normal breast cells. Up-regulation of survivin gene expression reduces apoptosis induced by the chemotherapeutic drug docetaxel. We also discovered that in breast cancer cells, EGF up-regulates the level of HIF-1α and that by down-regulation of HIF-1α using HIF-1α siRNA, we could significantly decrease those EGF-induced levels of survivin expression. Thus, cross-talk or a feedback loop between EGFR activation and HIF-1α expression is implied. Furthermore, we demonstrated direct binding of HIF-1α to the survivin promoter, which strongly suggests that EGF-activated survivin gene expression is indeed mediated by induction of transcriptional activity of HIF-1α under normoxic conditions.

MATERIALS AND METHODS

Cell Lines—Normal immortalized human mammary epithelial cell line MCF-10A and breast cancer cell lines SK-BR-3, MDA-MB-231, and MCF-7 were obtained from the ATCC (Manassas, VA). SK-BR-3 and MDA-MB-231 cells were maintained in RPMI 1640, and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium/F-12 medium (50:50; Mediatech) supplemented with 10 μg/ml of insulin. All of the above media were supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) as well as 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Mediatech Herndon, VA). MCF-10A cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 20 ng/ml EGF, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, 2 mM L-glutamine, and 5% FBS.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Cell Proliferation Assay—To measure the effects of EGF alone or in combination with docetaxel on breast cancer cells, 8×10^3 cells/well of MCF-7 and SK-BR-3 cells were plated in 96-well plates and cultured in the medium containing EGF and/or docetaxel (Aventis Pharma, Bridgewater, NJ) in various combinations of the drugs for 3 days. The percentage of viable cells in each well was examined by an MTT cell proliferation assay (Sigma), and the remaining viable cells were determined using Spectra Max Plus (Molecular Devices, Sunnyvale, CA).

Transfection—Plasmids expressing a full-length HIF-1α cDNA gene were provided by Dr. Hua Zhong (Emory University, Atlanta, GA). The control empty pcDNA3 or pHIF-1α plasmids were transfected into cultured tumor or normal cell lines in 6-well tissue culture plates using Lipofectamine 2000 (Invitrogen). Some groups of the plasmid-transfected cells were treated with docetaxel in the absence or presence of 100 ng/ml human EGF. Twenty-four hours after transfection, the cells were collected for Western blot analysis to determine the levels of HIF-1α and survivin proteins or FACScan analysis for the percentage of the apoptotic cells.

Apoptosis Assay—Cellular apoptosis was determined using Annexin V-phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD) (BD Biosciences). SK-BR-3 and MCF-7 cells were treated for 3 days with 25 or 50 nM of docetaxel in the absence or presence of 100 ng/ml EGF. Floating and adherent cells were labeled with Annexin V-PE and 7-AAD and then analyzed by FACScan (BD Biosciences) to determine the percentage of apoptotic cells.

Real Time Reverse Transcription-PCR—Total RNAs were isolated using the RNA Bee kit (Tel-test, Friendswood, TX). Each 2-μg sample of RNA was amplified with the Omniscript RT kit using an oligo(dT) primer (Qiagen Inc., Valencia, CA) to generate 20 μl of cDNAs. A 1–2-μl sample of the cDNA was then quantified by real time PCR using primer pairs for survivin or β-actin with SYBR Green PCR Master mix. Real time PCR was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). The primer pairs for detection of survivin gene expression were as follows: survivin forward, 5′-TCCACTGCCCCACTGAGAAC-3′; surviving reverse, 5′-TGGCTCCCAGCCTCCA-3′. These amplify a 77-nt PCR product located at nt 130–206 of the survivin mRNA. Amplification of the β-actin gene was used as an internal control for real time reverse transcription-PCR. The primer pair for the β-actin gene was as follows: forward, 5′-AAAGACCTGTACGCCAACACAGTGCTGTCTGG-3′; reverse, 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′, which generates a 219-nt PCR product from nt 870 to 1089 of the β-actin mRNA sequence. The quantity of PCR product generated from amplification of the survivin gene was standardized using the quantity of β-actin product for each sample to obtain a relative level of gene expression.

Western Blot Analysis—For the various treatments described, cells were cultured in medium containing 10% serum and then changed to medium containing 2% serum or no serum when treated with 100 ng/ml human EGF (Invitrogen) and/or docetaxel. Inhibitors for PI3K (LY294002), MAPK (PD98059), and EGFR (AG1478) were obtained from Calbiochem. At the end of the assay, cells were lysed in lysis buffer, and Western blot analysis was performed as previously described (18). The detection antibodies for phosphoserine 473 Akt, p44/p42 MAPK, XIAP, and cleaved caspase-3 were from Cell Signaling Technology Inc. (Beverly, MA). Goat anti-human survivin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mouse monoclonal anti-β-actin antibody (Sigma) were also used.

Caspase Activity Assay—Cells were treated with or without docetaxel, in the absence or presence of 100 ng/ml human EGF, for a period of 2 days. Cells were collected, and their lysates were examined for caspase-3-like activity using a specific substrate, Ac-DEVD-7-amino-4-trifluoromethylcoumarin, which detects the activities of caspase-3, caspase-7, caspase-10, or caspase-9 activity using Ac-LEHD-7-amino-4-trifluoromethylcoumarin, according to a standard protocol (Calbiochem). The results were measured using a Spectra Max fluorescence microplate reader (Molecular Devices). For each experiment, control groups with specific caspase inhibitors, including caspase-3 inhibitor (benzyloxycarbonyl-DEVD-aldehyde; BD Biosciences) and caspase-9

inhibitor (benzyloxycarbonyl-LEHD-aldehyde; Alexis Biochemicals, San Diego, CA), were done to ensure the specificity of the assay.

Chromatin Immunoprecipitation (ChIP) Assay—To demonstrate direct binding of HIF-1α protein to the survivin promoter region in EGF-stimulated cancer cells, the SK-BR-3 cells were treated with 100 ng/ml EGF for 4 h. After cross-linking chromatin with proteins by 1% of formaldehyde, the assay was performed using a ChIP assay kit from Upstate (Charlottesville, VA), according to the company's protocol. A monoclonal anti-HIF-1α antibody (BD Biosciences) was added to precipitate the protein-chromatin complexes. A PCR primer pair for amplification of a 230-nt survivin promoter fragment is as follows: forward primer, 5′-GCGTTCTTTGAAAGCAGT-3′; reverse primer, 5′-ATCTGGCGGTTAATGGCG-3′.

Modified McKay Assay—A core survivin promoter containing 269 nt of the 5′-flanking region of the survivin gene was cut out from a survivin promoter-luciferase reporter plasmid (pluc-cyc1.2) (23). A DNA fragment containing six repeats of the hypoxia-responsive element (HRE) of vascular endothelial growth factor (VEGF) was also cut from pBI-GL V6R plasmid (23) as a positive control. The purified promoter fragments were 5′-end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). The radiolabeled promoter fragments were then incubated with nuclear extracts obtained from breast cancer cells treated with or without 100 ng/ml EGF in a 1× binding buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 12.5% glycerol, and 0.2 mM dithiothreitol on ice for 1 h. Protein A-Sepharose beads conjugated with monoclonal anti-HIF-1α antibody were added to precipitate the protein-DNA complexes. After washing for three times with a 1× TE buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, the final pellet was resuspended in a 0.1 M $NaHCO_3$, 0.1% SDS, 1× sample buffer and heated to 65 °C for 2 min. Then the protein A-Sepharose beads were pelleted, and the supernatant was analyzed by electrophoresis on a 1.4% agarose gel followed by autoradiography to determine whether the EGF-induced HIF-1α protein had bound to the survivin promoter DNA fragments.

Luciferase Assay—The effect of EGF stimulation on survivin promoter activity was determined in breast cancer cells after transfecting pluc cyc-1.2 plasmid, a survivin promoter-luciferase reporter plasmid (23), for 24 h, followed by EGF treatment for 4 h. A pRL-SV-40 plasmid that expresses a *Renilla* luciferase gene (Promega, Madison, WI) was also cotransfected for all studies as an internal control. To determine whether direct overexpression of the HIF-1α gene activates the survivin promoter, breast cancer and normal cells were cotransfected with pluc cyc-1.2 and pHIF-1α plasmids for 24 h. The ability of direct activation of HRE by EGF-induced HIF-1α was demonstrated using a MDA-MB-231 cell line, stably transfected with pBI-GL V6R plasmid containing six copies of HRE fragments of the VEGF gene and a firefly luciferase reporter gene (24) (provided by Dr. Hyunsuk Shim at Emory University). The transfected cells or MDA-MB-231 stable cells were cultured in the absence or presence of 100 ng/ml of EGF for 45 min. After various treatments as described above, the promoter activity of the cell lysates was determined using a dual luciferase activity assay kit from Promega.

Production of Adenoviral Vectors Expressing HIF-1α siRNA—Plasmid vectors containing either HIF-1α siRNA sequence 5′-CAGTGGATTACCACAGCTGA-3′ or survivin siRNA 5′-GGCTGGCTTCATCCACTGCCC-3′ were generated by cloning the synthesized oligonucleotide into pSilencer 2.1-U6 Neo plasmid (Ambion Inc., Austin, TX). Control pSilencer 2.1-U6 Neo plasmid vector containing a scrambled siRNA sequence, 5′-ACTACCGTTGTTATAGGTGT-3′, was obtained from the company. Adenoviral vectors expressing siRNA to HIF-1α and control siRNA were produced by cloning a HindIII-EcoRI fragment from pSilencer 2.1-U6 Neo plasmid, which contains a U6-promoter-siRNA cassette (HIF-1α or control siRNA), to a pcDNA3 HindIII-EcoRI site and then transferring the NotI-HindIII fragment from the pcDNA3 to the pAdtrack plasmid (25). After performing homologous recombination with an AdEasy adenoviral DNA backbone, the viral vectors were produced by transfecting into the human embryonic kidney cell line 293 (ATCC). The AdEasy system was provided by Dr. Bert Vogelstein at The Johns Hopkins University. Additional vector amplification was also performed in the 293 cell line. High titer viral vectors were purified by centrifugation and CsCI banding.

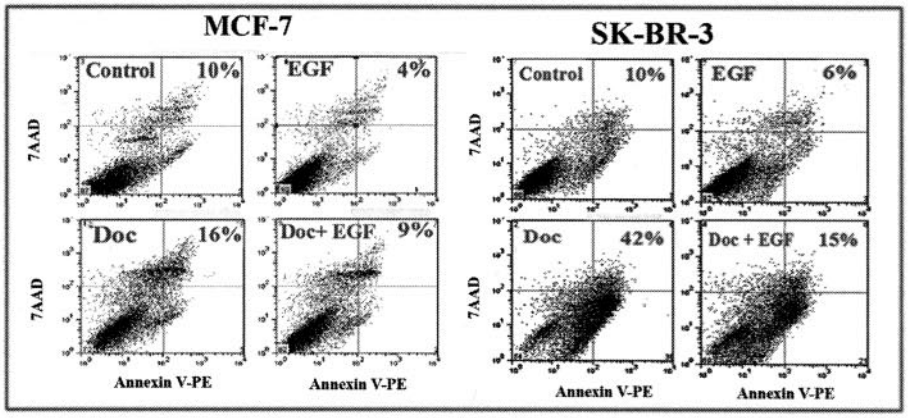
To determine the effect of down-regulation of HIF-1α on tumor cells, 2×10^5 tumor cells/well were first cultured in 6-well plates and then transduced with Ad HIF-1α siRNA or Ad Control siRNA vectors. 24 h after transduction, cells were cultured in serum-free medium overnight and treated with (or without) EGF for 45 min. Some groups received cotransfection of pSilencer HIF-1α siRNA and pHIF-1α plasmids to determine whether overexpression of HIF-1α gene could rescue HIF-1α siRNA-mediated down-regulation of survivin in EGF-treated cells. Last, collected cell lysates were examined for the levels of both HIF-1α and survivin by Western blot analysis.

To examine the effect of EGF-induced HIF-1α and survivin on apoptotic death in breast cancer cells after docetaxel treatment, 5×10^4/well of MCF-7, SK-BR-3, and MDA-MB-231 cells were plated in 12-well tissue culture plates. Cells were then transduced with AdControl siRNA or AdHIF-1α siRNA vector. Some treatment groups were transfected with psilencer 2.1 U6-survivin siRNA plasmids. Following a 2-day treatment with docetaxel, the percentage of remaining viable cells was examined using an MTT cell proliferation assay.

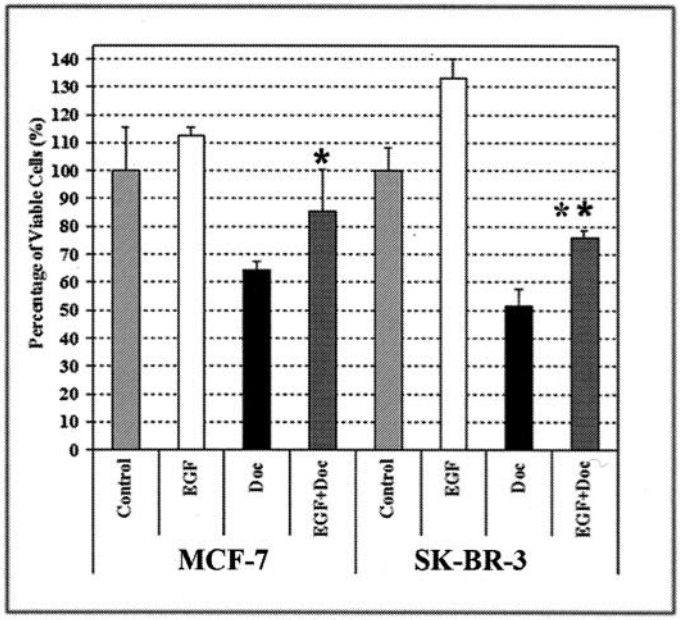
RESULTS

EGF Stimulation Decreased Docetaxel-induced Apoptotic Response in Breast Cancer Cells—In order to determine the effect of activation of the EGFR signaling pathway on the apoptotic response in human cancer cells, we treated the human breast cancer cell lines SK-BR-3 and MCF-7 with a chemotherapeutic drug, docetaxel, in the absence or presence of human recombinant EGF. Previous reports have shown that SK-BR-3 cells have high levels of EGFR and Her-2/neu expression, whereas MCF-7 cells express low levels of EGFR and Her-2/neu (26). We chose those two cell lines to determine whether the levels of the EGF receptors present affect the EGF-induced apoptotic response. After treating the cancer cells with docetaxel in

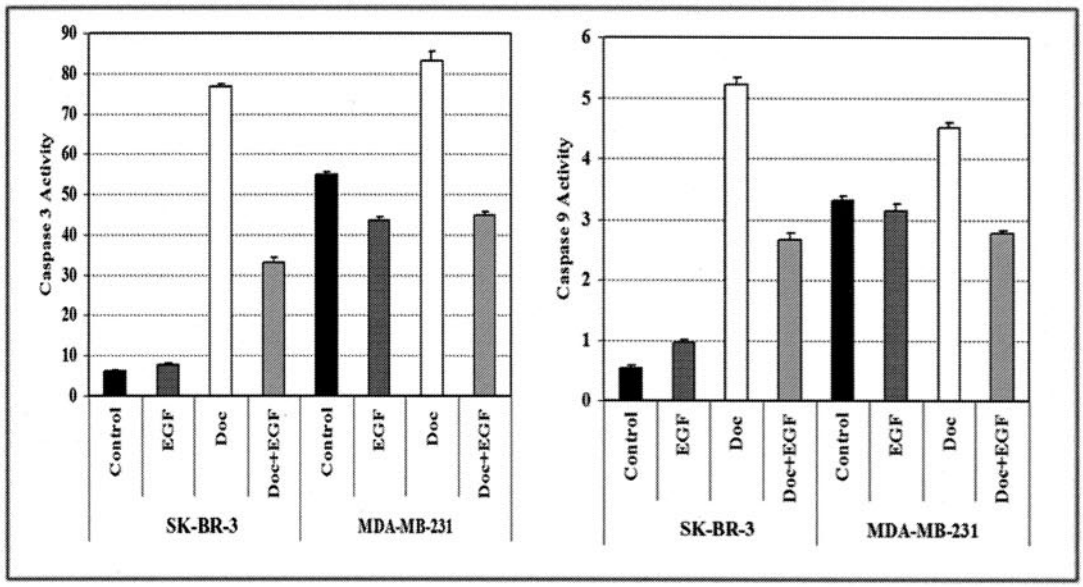
A. FACScan analysis of percentage of apoptotic cells



B. MTT cell proliferation assay



C. Caspase activity assay



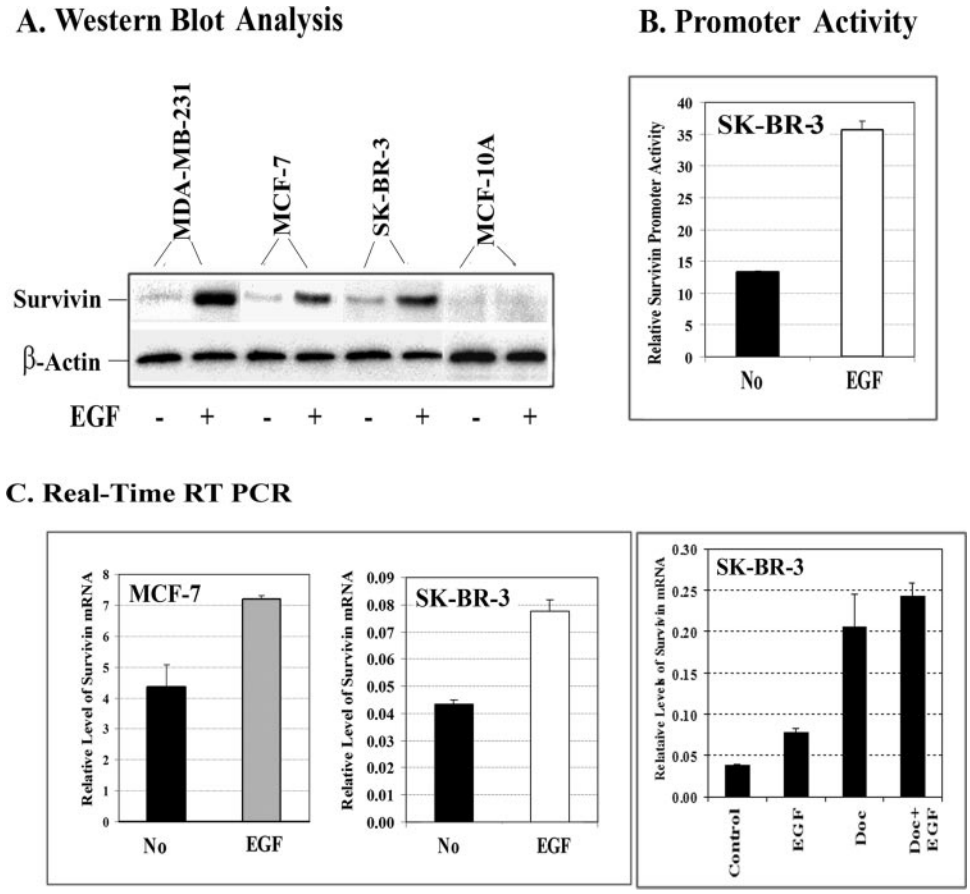


FIGURE 2. **EGF stimulation increases the levels of survivin expression in breast cancer cells.** Cells were cultured in 2% FBS medium overnight, followed by treatment with human EGF at 100 ng/ml for 45 min to 4 h. *A*, examination of the level of survivin protein (16.5 kDa) in breast cancer and normal cell lines following EGF stimulation for 45 min by Western blot analysis. *B*, EGF stimulation activates survivin promoter activity. SK-BR-3 cells were transfected with a survivin promoter luciferase reporter plasmid (pluc cyc1.2) for 24 h and then treated with EGF for 4 h. The cell lysates were collected for the luciferase assay. *C*, total RNAs were isolated and analyzed by real time reverse transcription-PCR, as described under "Experimental Procedures," to determine changes in the level of survivin mRNA after EGF treatment. EGF stimulation increases the levels of survivin mRNA in both MCF-7 and SK-BR-3 cell lines. Additionally, docetaxel treatment could also up-regulate survivin gene expression, and its expression level is further enhanced in the presence of EGF. The relative level of survivin mRNA is a ratio of the quantity of survivin to β-actin PCR products. A mean value of three repeat samples is shown.

the absence or presence of EGF for 2 days, the percentage of apoptotic cells was determined using Annexin V-PE and 7-AAD staining followed by FACScan analysis. We found that the presence of EGF markedly decreased the percentage of apoptotic cell death induced by docetaxel. EGF treatment reduced the percentage of apoptotic cells present from 16 to 9% for MCF-7 cells and from 42 to 15% for SK-BR-3 cells (Fig. 1*A*). Additionally, we found that EGF decreased the rate of spontaneous apoptosis in both cell lines (Fig. 1*A*). Consistent with the results from the apoptosis assay, we found that EGF stimulation significantly decreased sensitivity of SK-BR-3 and MCF-7 cells to docetaxel as detected by the MTT cell proliferation assay (Fig. 1*B*). Furthermore, we found that EGF-induced resistance to apoptosis was detected in breast cancer cell lines expressing both a high level of EGFR (SK-BR-3) and a low level of EGFR (MCF-7).

To elucidate the mechanism of apoptosis resistance, we examined changes in regulation of apoptotic signals in EGF-stimulated cancer cells. Since caspase activation is a critical step in induction of apoptotic cell death, we first examined caspase-3-like and caspase-9 activities in MDA-MB-231 and SK-BR-3 cell lines 2 days after docetaxel treatment. As expected, docetaxel increased these caspase activities in both tumor cell lines. However, in the presence of EGF, the docetaxel-induced caspase-3-like and caspase-9 activities were markedly inhibited (Fig. 1*C*), suggesting that the EGF-induced resistance to apoptosis by docetaxel is mediated through inhibition of caspase activity.

EGF Up-regulates Survivin Expression in Human Breast Cancer Cells—It is well known that the IAPs block apoptotic signaling through inhibition of caspase activity (27). To determine whether IAPs play a role in inhibition of caspases in tumor cells having an activated EGFR signal, we examined changes in the level of an important IAP family protein, survivin, in both breast cancer and normal cell lines following EGF treatment. Our results from Western blot analysis demonstrated that EGF stimulation did increase the level of survivin protein in three breast cancer cell lines, including MDA-MB-231, SK-BR-3, and MCF-7 (Fig. 2*A*). Interestingly, survivin was not detected in the immortalized normal human mammary epithelial cell line MCF-10A, and EGF treatment failed to induce any survivin expression (Fig. 2*A*). To

FIGURE 1. **Activation of the EGFR signaling pathway increases resistance to docetaxel-induced apoptosis in human breast cancer cells.** *A*, apoptosis assay using Annexin V staining and FACScan analysis. MCF-7 and SK-BR-3 cells were cultured for 2 days in medium containing 100 ng/ml EGF in the absence or presence of 25 nM (SK-BR-3) or 50 nM (MCF-7) docetaxel and 2% FBS. The cells were then stained with Annexin V-PE and 7-AAD and analyzed by FACScan. The percentages of apoptotic cells are shown in the *upper panel*, including both early (Annexin-V^+, 7-AAD^-) and late stage (Annexin$^+$, 7-AAD^+) apoptotic cells. *B*, EGF stimulation decreases the sensitivity of human breast cancer cells to docetaxel treatment. Cancer cells cultured in 96-well plates were treated for 3 days with the reagents as described above. The percentage of remaining viable cells was determined using a MTT cell proliferation assay. The results of this assay are expressed relative to the cell density of untreated cells. Each value in the graph represents the mean ± S.D. of five repeat samples. An *asterisk* indicates a significant difference when compared with the control value (*, $p < 0.05$; **, $p < 0.001$). *C*, activation of the EGFR signal inhibits docetaxel-induced caspase activity. The cells were treated with docetaxel in the absence or presence of EGF for 3 days. 25 μg of proteins from their total cell lysates were examined for caspase-3-like or caspase-9 activity using substrates specific for these caspases. Fluorescence intensity was measured by a fluorescence microplate reader. The *numbers* in the figure represent mean values ± S.D. from three repeat groups.

determine the mechanism of survivin up-regulation, we further examined survivin promoter activity and the level of survivin mRNA in EGF-treated cells. Using a luciferase reporter plasmid containing a 269-nt survivin core promoter fragment, we found a marked increase in survivin promoter activity in EGF-stimulated SK-BR-3 cells (Fig. 2*B*). Consistent with the level of survivin protein, survivin mRNA was elevated in the EGF-treated SK-BR-3 and MCF-7 cells (Fig. 2*C*) detected by real time reverse transcription-PCR. Additionally, we found that treatment of the cells by docetaxel increases the levels of survivin gene expression. A combined effect of docetaxel and EGF treatment further increased the level of survivin gene expression (Fig. 2*C*).

Determination of Signaling Pathways Responsible for EGF-induced Survivin Gene Up-regulation—It is well known that EGFR signaling leads to the activation of both PI3K/AKT and MAPK pathways. We examined the roles of these pathways in the EGF-induced survivin expression in breast cancer cells. As expected, we observed increases in the levels of p-AKT and p44/42 MAPK in EGF-treated cells. Although the level of survivin expression was also up-regulated in those cells, the level of another IAP protein, XIAP, remained unaffected by EGF treatment (Fig. 3).

After pretreatment of the cancer cells with either the PI3K inhibitor LY294002 or the MAPK inhibitor PD98059, we were able to achieve marked reductions in the levels of p-AKT and p44/42 MAPK present in EGF-treated SK-BR-3 and MCF-7 cells. Furthermore, we found that inhibition of p-AKT completely prevented EGF-up-regulated survivin expression in both the SK-BR-3 and MCF-7 cell lines (Fig. 3). However, we believe that activation of AKT is probably not required for maintaining a basal level of survivin expression in these cancer cells, since similar levels of survivin expression were detected in both control cells and cells treated only with PI3K inhibitor (Fig. 3).

EGF stimulation also increased the level of p44/42 MAPK in SK-BR-3 and MCF-7 cell lines, with SK-BR-3 cells showing a very high level of up-regulation (Fig. 3). Blocking the MAPK pathway with PD98059 inhibited the level of p44/42 MAPK in both cell lines. The fact that a significant reduction of survivin was seen in the MAPK-blocked, EGF-treated SK-BR-3 cells suggests that both the AKT and MAPK pathways are involved in EGF-induced survivin up-regulation (Fig. 3).

After treating the cancer cells with EGF in the absence or presence of the EGFR inhibitor AG1478, we found that AG1478 counteracted the effects of EGF on survivin expression in both SK-BR-3 and MCF-7 cells. However, AG1478 treatment alone had no significant effects on the basal levels of survivin protein in these cells (Fig. 3).

EGF-induced HIF-1α Up-regulates Survivin Expression in Breast Cancer Cells—Previous reports have shown that activation of EGFR signaling can induce HIF-1α in human tumor cells under normoxic conditions (20, 21). We previously found that survivin promoter activity is up-regulated in hypoxic tumor cells (23). To determine if the EGFR signaling-induced HIF-1α is a mediator for survivin gene expression, we first examined the changes in both HIF-1α and survivin levels in breast cancer cells after EGF treatment. Western blot analysis did show that

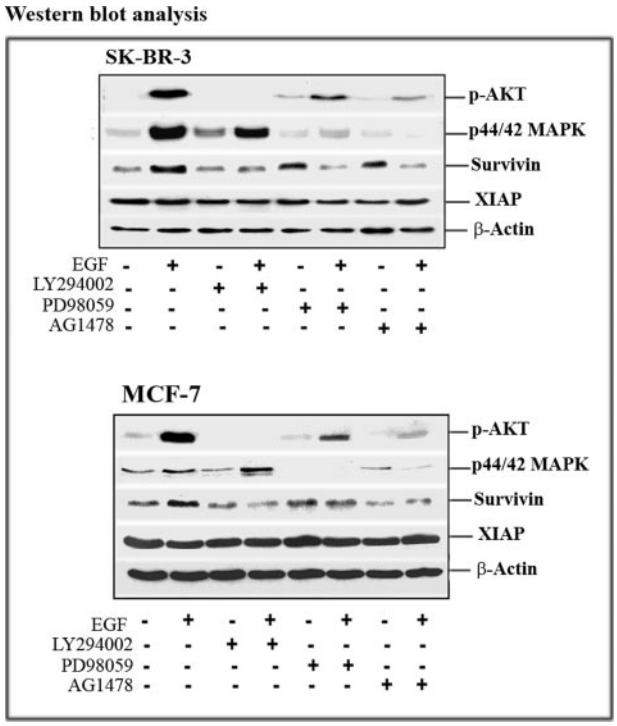


FIGURE 3. **Analysis of signal transduction pathways regulating EGF-induced survivin gene expression.** Cells were pretreated with 10 μM LY294002, PD98059, or AG1478 for 24 h in culture medium containing 2% FBS medium. Then 100 ng/ml EGF was added for 45 min. Total cell lysates (50 μg of protein) were examined by Western blot analysis to determine the levels of phosphorylated serine 473 Akt (*P-Ser473 Akt*), phosphorylated p44/p42 MAPK, survivin, and XIAP. In both breast cancer cell lines, EGF stimulation activated the AKT and MAPK signal pathways, and blocking the PI3K/AKT signal prevented EGF-induced survivin expression. On the other hand, inhibition of the EGFR signal pathway with AG1478 reduced the level of survivin protein. The level of XIAP was not affected by EGF stimulation or the PI3K/AKT, MAPK, and EGFR inhibitors.

EGF treatment induces high levels of both HIF-1α and survivin expression in breast cancer cells (Fig. 4*A*). We further found that transfection of the plasmids expressing a HIF-1α gene into cells increases the levels of HIF-1α in both breast cancer SK-BR-3 and normal breast MCF-10A cell lines. However, up-regulation of the level of survivin protein is only detected in SK-BR-3 cells. This result provides direct evidence supporting HIF-1α-mediated survivin up-regulation. Additionally, the absence of survivin expression in HIF-1α-transfected MCF-10 A cells suggests that intrinsic transcriptional inhibitory factors prevent the basal level as well as HIF-1α-induced survivin transcription in normal cells.

We then transduced the cells for 48 h with adenoviral vectors expressing either HIF-1α siRNA or control siRNA. Next, the transduced cells were treated with or without EGF for 45 min before they were collected. The resulting cell lysates were analyzed by Western blot for the levels of HIF-1α and survivin. We found that overexpression of HIF-1α siRNA markedly reduced EGF-induced HIF-1α. Meanwhile, EGF-induced up-regulation of survivin expression was completely blocked by the HIF-1α siRNA (Fig. 4*B*). To further demonstrate the critical role for HIF-1α in regulating survivin gene transcription, we co-expressed HIF-1α siRNA and HIF-1α gene plasmids in MCF-7 cells. 24 h after transfection, the cells were treated with EGF and collected for Western blot analysis. In the presence of excessive amounts of HIF-1α,

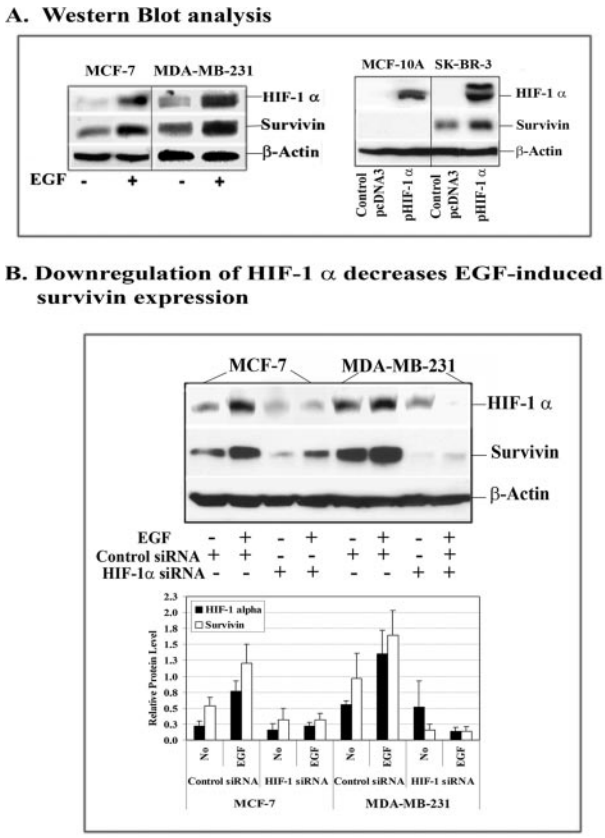


FIGURE 4. **Determination of the role of HIF-1α in survivin up-regulation in normoxic breast cancer cell lines after EGF treatment.** *A*, Western blot analysis showed that EGF stimulation induces high levels of both HIF-1α and survivin expression. Furthermore, transfection of HIF-1α gene-expressing plasmids increases the level of survivin in breast cancer SK-BR-3 cells but not in normal breast epithelial MCF-10A cells. *B*, overexpression of HIF-1α siRNA by transduction of the cells with a multiplicity of infection of 100 plaque-forming units/cell of adenoviral vectors inhibited EGF-induced as well as basal levels of HIF-1α expression in MCF-7 and MDA-MB-231 cells. The levels of survivin protein were also markedly reduced. The relative levels of HIF-1α and survivin proteins in the cells treated with control and HIF-1α siRNAs were quantified by measuring the density of protein bands using Scion Image (Scion Corp., Frederick, MD). HIF-1α or survivin protein level was calculated from a ratio of the density relative to β-actin for each sample. The *number* in the figure represents the mean value of three repeat Western blots. *C*, overexpression of the HIF-1α gene rescues HIF-1α siRNA-induced survivin down-regulation. Breast cancer MCF-7 cells were co-transfected with HIF-1α siRNA and pHIF-1α plasmids for 24 h, and the levels of HIF-1α and survivin proteins were examined by Western blot analysis. In the presence of an excessive amount of exogenous HIF-1α, expression of siRNA to HIF-1α could no longer inhibit the level of EGF-induced survivin expression. Although transfection of pSilencer HIF-1α siRNA plasmids showed less inhibitory effects on the levels of HIF-1α and survivin proteins, compared with the effect produced by using AdHIF-1α siRNA seen in this figure (*B*), it is appropriate for the design of this study, since the HIF-1α gene is expressed from a plasmid vector.

expression of HIF-1α siRNA could no longer block EGF-induced survivin down-regulation (Fig. 4*C*), supporting direct involvement of HIF-1α in EGF-activated survivin expression.

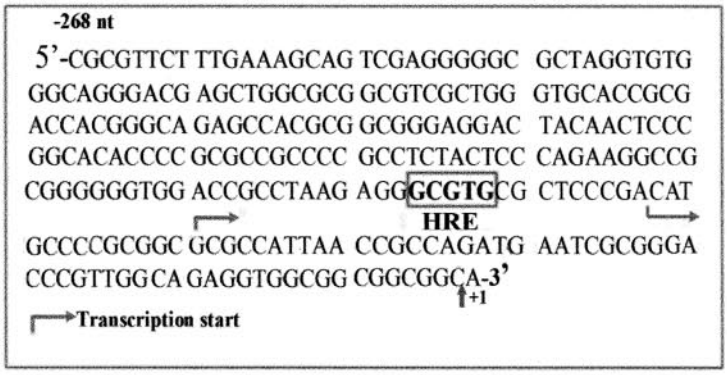
HIF-1α Activates Gene Transcription through Direct Interaction with the Survivin Promoter—At present, the mechanisms by which cellular factors may regulate survivin gene expression are not fully understood. Our previous study identifies a 269-nt DNA fragment located at the 5′-flanking region of the survivin gene that is able to activate tumor-specific gene transcription, exhibiting enhanced promoter activity under hypoxic conditions (23). To determine whether the EGF-activated survivin gene transcription is mediated by transcriptional activity of HIF-1α, we first analyzed the survivin core promoter sequences and found a putative HRE, 5′-GCGTG-3′, located at nt −81 to −85 of the 5′-flanking region of the survivin gene (Fig. 5*A*). To demonstrate the binding of HIF-1α to the survivin promoter in living cells, we performed a ChIP assay in MCF-7 cells with or without EGF treatment. In the chromatin fraction pulled down by an anti-HIF-1α antibody, we detected a higher level of the survivin promoter PCR fragments in EGF-treated cells than that in control cells (Fig. 5*B*). However, survivin promoter PCR fragments were not found in samples pulled down by a control IgG antibody.

To further confirm direct binding of HIF-1α to the survivin core promoter, we used a modified McKay assay (28) to pull down survivin promoter DNA fragments (Fig. 5*C*). Our results demonstrated that the survivin promoter fragments did bind to the HIF-1α protein and could be pulled down by HIF-1α antibody. EGF-treated cell lysates showed much higher levels of these survivin promoter fragments as compared with control groups in both breast cancer cell lines (Fig. 5*C*). Since there is a moderate basal level of HIF-1α found in those tumor cells, we also detected low to intermediate levels of survivin promoter fragments in our control groups (Fig. 5*C*).

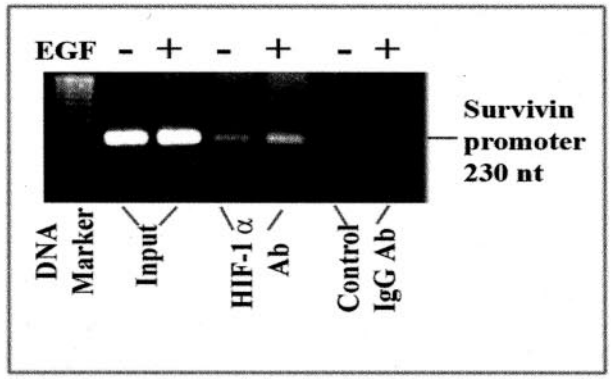
To determine whether the binding of HIF-1α to the survivin promoter actually could activate the promoter activity, we cotransfected pHIF-1α plasmid with survivin promoter reporter pluc cyc1.2 plasmid into breast normal and cancer cell lines. We found that overexpression of the HIF-1α gene markedly increased the survivin promoter activity in breast cancer MCF-7 cells but not in normal breast MCF-10A cells (Fig. 5*D*). Next, we wanted to determine if EGF-induced HIF-1α interacts with the HRE site under normoxic conditions to activate the gene transcription. We used the human breast cancer cell line MDA-MB-231, stably transfected with a luciferase reporter plasmid containing six copies of the HRE fragment of the VEGF gene. These cells were treated with EGF in normoxia, and the resultant luciferase activity was measured in cell lysates. We found that EGF-induced HIF-1α was able to activate the HRE-mediated transcription of the luciferase gene, suggesting that under normoxic conditions, HIF-1α can bind to the HRE site and activate HRE-mediated gene transcription (Fig. 5*D*).

Overexpression of the HIF-1α Gene Inhibits Docetaxel-induced Apoptosis under Normoxic Conditions—To establish a direct link between EGF-induced HIF-1α up-regulation and resistance to apoptosis, we transfected SK-BR-3 cells with pHIF-1α plasmids and then treated the cells with docetaxel in the absence or presence of EGF for 24 h. The cells were collected for Annexin V-PE staining and FACScan analysis of the

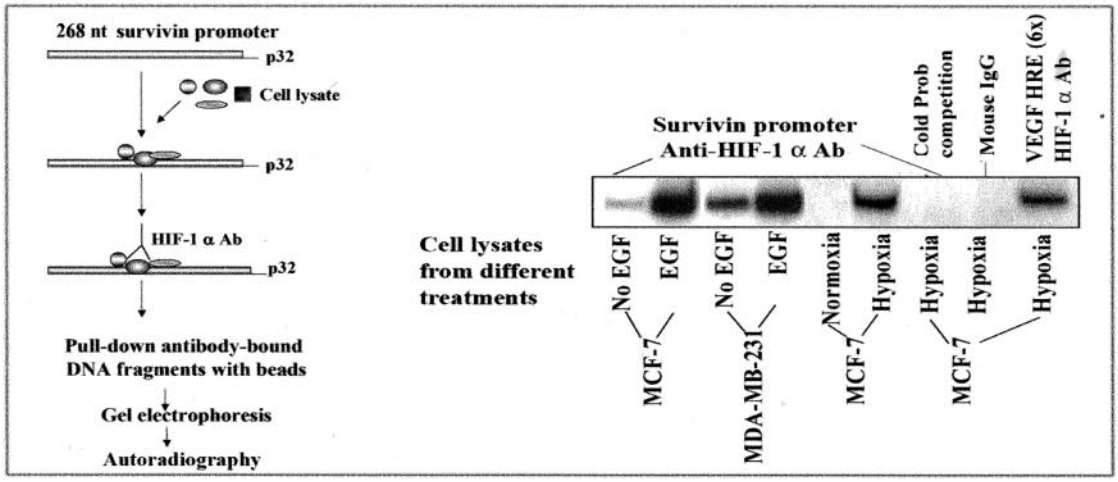
A. DNA sequence of the survivin core promoter



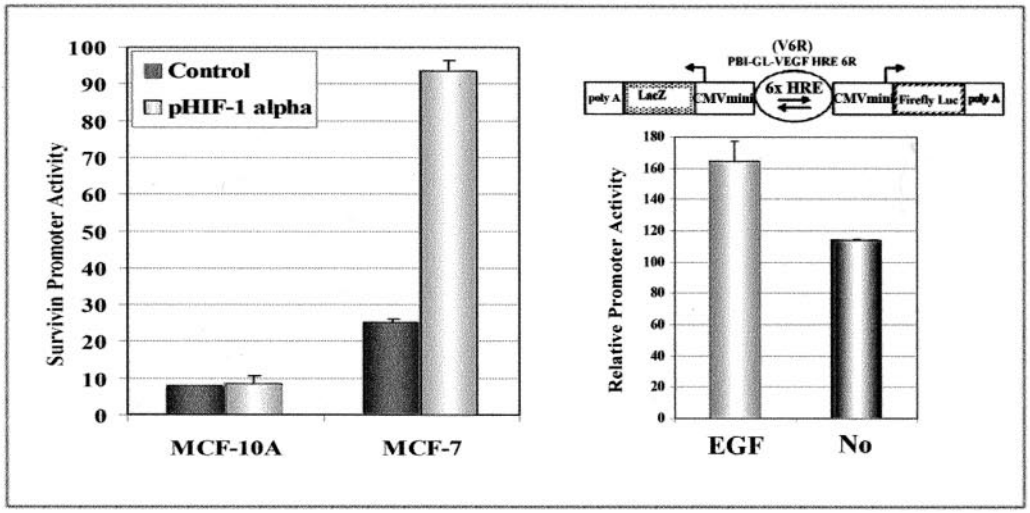
B. CHIP Assay



C. Modified McKay Assay



D. Luciferase activity assay



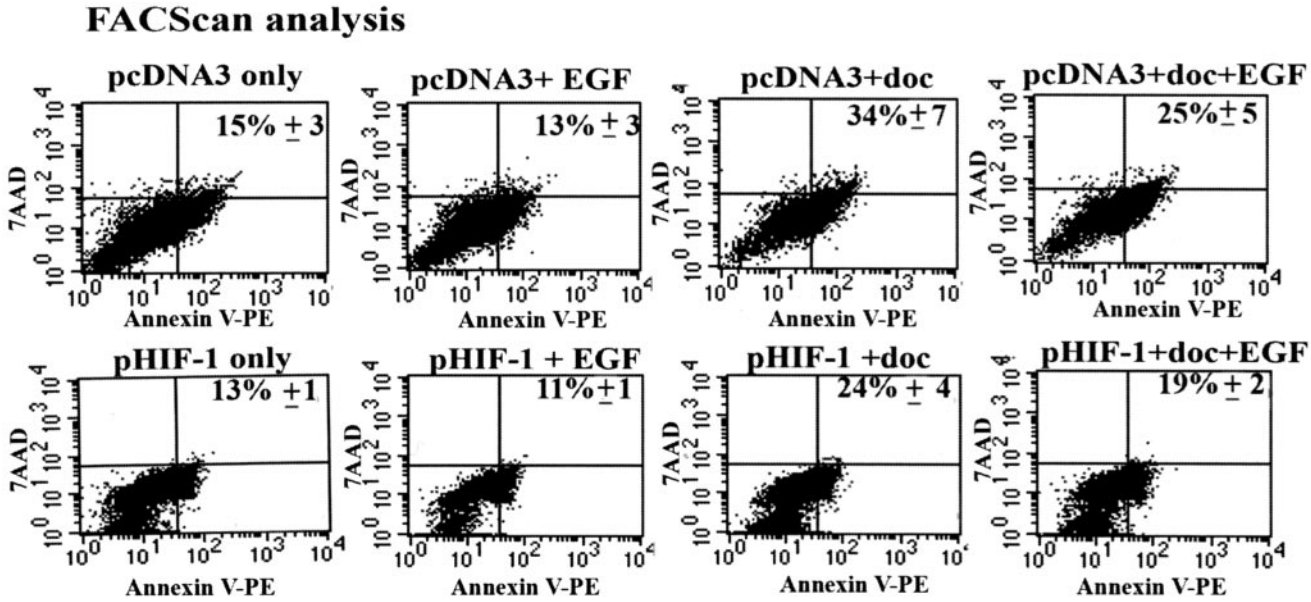


FIGURE 6. **Examination of the effect of overexpression of HIF-1α gene on docetaxel-induced apoptosis by FACScan analysis.** Breast cancer SK-BR-3 cells, transfected with pHIF-1α or control pcDNA3 plasmids for 24 h, were treated with 25 nM docetaxel in the absence or presence of 100 ng/ml of EGF for an additional 24 h. Both floating and adhesive cells were collected for Annexin V-PE and 7-AAD staining followed by FACScan analysis. Expression of the HIF-1α gene reduced the percentage of docetaxel-induced apoptosis. The combination of EGF activation with HIF-1α gene expression further increased the inhibition effects. Since the cells were cultured in the medium containing 2% of FBS due to EGF treatment, we observed a relatively high level of the basal level of apoptosis in pcDNA3 control plasmid-transfected cells. The *number* in the figure is the mean value ± S.D. from three repeat samples.

percentage of apoptotic cells. We found that overexpression of the HIF-1α gene reduced the percentage of docetaxel-induced apoptotic cells from 34 to 24%. The combination of overexpression of the HIF-1α gene with EGF treatment further enhanced the inhibitory effect on apoptosis (Fig. 6), suggesting that HIF-1α is a key mediator for EGF-induced resistance to apoptosis.

Down-regulation of the Levels of HIF-1α or Survivin Gene Expression Reverses EGF-induced Resistance to Apoptosis—To further demonstrate that EGF-induced resistance to apoptosis is indeed the result of up-regulation of HIF-1α, which consequently activates survivin gene expression, we examined the effects of HIF-1α down-regulation, using HIF-1α or survivin siRNA, on overall sensitivity to apoptosis induction. We found that expression of HIF-1α siRNA using adenoviral vectors in the no treatment control and the EGF-treated SK-BR-3 cells induced 8–10-fold increases in caspase-3-like activity as compared with control siRNA vector-containing cells (Fig. 7*A*). Furthermore, the presence of HIF-1α siRNA enhanced 2.5 times the level of active caspase-3 induced by docetaxel treatment. Although co-treatment with EGF reduced the docetaxel-induced caspase-3 activity, down-regulation of HIF-1α with siRNA completely reversed the ability of exogenous EGF to induce resistance to docetaxel (Fig. 7*A*). The changes in caspase-3 activity among the different treatment groups were also correlated with the percentage of apoptotic cell death in the cells. As shown in Fig. 7*B*, overexpression of HIF-1α siRNA significantly increased apoptotic cell death in docetaxel-treated cells by 18% ($p = 0.006$, Student's t test). EGF treatment protected the cells from docetaxel-induced apoptosis and increased the percentage of viable cells from 37% in the group without EGF to 57% in EGF-treated cells (Fig. 7*B*, $p = 0.01$). Importantly, overexpression of HIF-1α siRNA significantly reduced the EGF-induced resistance to docetaxel, since there was a 44% decrease in the percentage of viable cells in the AdHIF-1α siRNA-transduced cells when compared with control siRNA-transduced cells ($p < 0.0001$, Student's t test). We also examined the effect of direct inhibition of survivin gene expression on EGF-induced resistance to apoptosis. We found that expression of survivin siRNA significantly sensitized SK-BR-3 cells to docetaxel treatment (Fig. 7*C*, $p = 0.0004$, Student's t test). Down-regulation of survivin expression also increased docetaxel-induced cell death even in the presence of EGF stimulation (Fig. 7*C*, $p =$

FIGURE 5. **Determination of the mechanism of HIF-1α-activated survivin gene expression.** *A*, DNA sequence of the survivin core promoter. A putative HRE site, located at −81 to −85 nt, is marked in the promoter sequence. *B*, detection of the binding of HIF-1α to the survivin promoter in breast cancer cells using a ChIP assay. SK-BR-3 cells were treated with or without EGF for 4 h. A ChIP assay was then performed. 230-nt PCR products of the survivin promoter were only detectable in the samples pulled down by HIF-1α antibody and not in control IgG samples. A marked higher level of the survivin promoter PCR products was seen in the EGF-treated sample compared with the no treatment sample. *C*, modified McKay assay for detection of the binding of HIF-1α to the survivin promoter. Cancer cells were treated with or without EGF for 45 min, and nuclear extracts were incubated with 269-nt radiolabeled survivin promoter fragments, and the resulting HIF-1-DNA complexes were pulled down using anti-HIF-1α antibody-conjugated Protein A beads. As shown, a high level of HIF-1α-survivin promoter complexes was found following incubation with nuclear extracts from EGF-treated breast cancer cells. The binding specificity was further demonstrated by the absence of survivin promoter bands in samples with a 10 times excess of unlabeled (cold) survivin promoter fragments or using nonspecific mouse IgG-conjugated Protein A beads. A DNA fragment containing six repeats of the HRE fragments of the human VEGF promoter was used as a positive control. *D*, luciferase activity assay. Overexpression of the HIF-1α gene after co-transfection of pHIF-1α and pluc cyc1.2 increases survivin promoter activity in MCF-7 tumor cells but fails to induce the promoter activity in normal MCF-10A cells. EGF-induced HIF-1α was able to bind to the HRE and activate firefly luciferase gene expression under normoxic conditions, since luciferase activity was increased in the cell lysates from EGF-treated MDA-MB-231 cells stably transfected with pBI-GL-V6R plasmid, which has six repeats of VEGF HRE fragments.

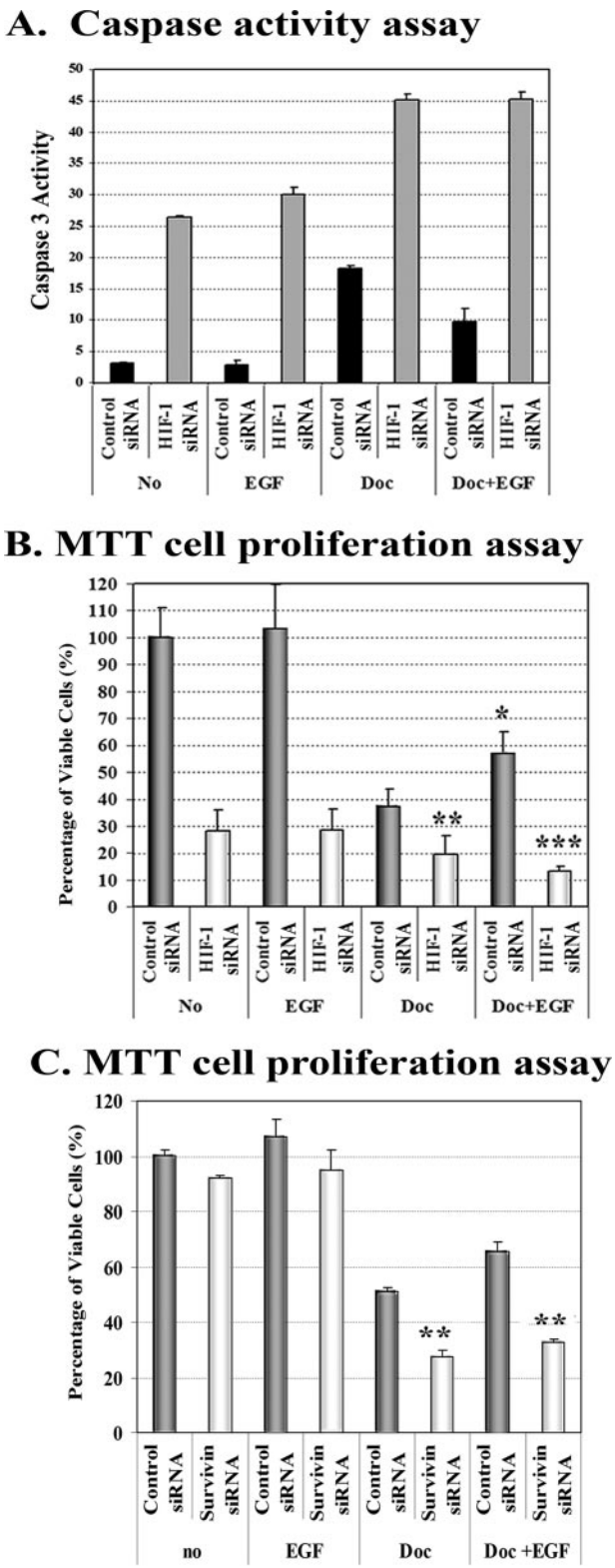


FIGURE 7. **Down-regulation of HIF-1α or survivin gene expression using siRNAs enhances docetaxel-induced apoptosis and reverses EGF signal-mediated resistance to apoptosis.** SK-BR-3 cells were transduced with AdControl siRNA or HIF-1α siRNA vectors for 24 h. The cells were then treated with 25 nM docetaxel, in the absence or presence of 100 ng/ml EGF for an additional 24 h. *A*, caspase activity was examined in the cell lysates using a caspase-3-like specific substrate. The *numbers* in the figure represent mean values of three repeat groups. Similar results were obtained from repeat experiments. As shown, inhibition of HIF-1α expression increases caspase-3 activity in tumor cells for all treatment groups. Additionally, EGF-induced inhibition of caspase-3 activity in docetaxel-treated cells was reversed by the down-regulation of HIF-1α with siRNA. *B*, results from the MTT cell proliferation assay show that down-regulation of HIF-1α significantly increased docetaxel-induced cell death and completely blocked EGF-induced resistance to docetaxel treatment in SK-BR-3 cells. On the other hand, a significant reduction of docetaxel-induced cell death is found in EGF-treated, control siRNA vector-transduced cells. *C*, transfection of plasmids expressing survivin siRNA significantly increases docetaxel-induced cell death and counteracts the protection effect of EGF stimulation. The absorbance value of cells transduced with AdControl siRNA vector or plasmids expressing control siRNA serves as a relative cell number of 100%. Each value in the *bar graph* represents a mean value ± S.D. of four repeat samples. *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$.

0.0003, Student's *t* test). Therefore, our results strongly supported a role for HIF-1α-mediated survivin up-regulation in EGF-induced resistance to apoptosis in breast cancer cells.

DISCUSSION

Overexpression of EGFR and activation of the EGFR signaling pathway are found in a high percentage of human breast cancer tissues and have been associated with poor prognosis for the patients (3, 5, 7, 29). Although EGFR signaling, including activation of EGFR and Her-2/neu, is known to confer resistance to apoptosis in cancer cells (30, 31), the exact mechanism by which EGFR signaling regulates the apoptotic pathway has yet to be elucidated.

In this study, we have examined the effects of the activation of EGFR signaling on the apoptotic response in human breast cancer cell lines. We have shown that EGF stimulation reduces the sensitivity of breast cancer cells to docetaxel, a chemotherapeutic drug that is commonly used for treatment of breast cancer. We have further demonstrated that decreased drug sensitivity is the result of inhibition of the activities of caspase-3 and caspase-9, which consequently causes the observed resistance to apoptosis in EGF-treated breast cancer cells.

IAP proteins, including survivin and XIAP, are known to act as caspase inhibitors, blocking caspase activation and further inhibiting activities of the activated caspases (27, 32, 33). Recent studies, including ours, have shown that activation of EGFR leads to up-regulation of survivin expression (11–14). Additionally, it has been demonstrated that overexpression of Her-2/neu and EGFR genes increases survivin expression and apoptosis resistance in breast cancer cell lines (11). Results from examination of 195 cases of human invasive breast cancer tissues indicated that up-regulation of survivin by EGFR signaling is not just a phenomenon seen in cancer cell lines *in vitro*. In fact, about 80% of the tumors were also found to be positive for survivin, and its expression level was correlated with co-expression of Her-2/neu and EGFR (11).

Our current study has further demonstrated that EGF stimulation induces survivin expression at both the mRNA and protein levels in breast cancer cells but not in normal mammary epithelial cells. It is possible that the much higher levels of EGFR seen in many breast cancer cell lines as compared with normal cells contribute in part to the difference in the response to EGF treatment. However, lower levels of EGFR may not be the only reason for these differences, since the MCF-7 cell line expresses a low level of EGFR but exhibits a similar effect as two other tumor cell lines expressing high levels of EGFR and/or Her-2/neu (34). The intrinsic properties of breast cancer cells may determine the responsiveness to activation of EGFR and up-regulation of survivin gene

expression, since survivin is not detected in normal breast tissues, and cultured normal mammary epithelial cells either express a very low level or lack survivin gene expression (23).

It has been shown that the XIAP, a strong caspase inhibitor, prevents activation of caspase-3 by blocking caspase-9 activity as well as binding to activated caspase-3 (35). Interestingly, we found that EGFR activation did not affect the level of XIAP. Thus, up-regulated survivin expression in EGF-treated cells may play a key role in inhibiting caspase activity and creating resistance to apoptosis. Although the role of survivin in blocking caspase-9 activity has been elucidated, there is no structural evidence showing a direct interaction between survivin and caspase-3 (33). It is possible that survivin may interact with XIAP or other proteins, such as p21, to enhance the inhibitory effect on caspase-3 (36, 37).

A recent study also showed that treatment of human ovarian cells with paclitaxel transiently induced EGFR phosphorylation and PI3K activation, resulting in an increase in the level of survivin expression. Inhibition of either the EGFR or PI3K pathway was found to enhance the apoptotic cell death induced by paclitaxel (13). Up-regulation of survivin gene expression in human cancer cells after paclitaxel or docetaxel treatment has been reported by several laboratories, including ours (13, 14, 38). It was also demonstrated that treatment of human cancer cells by way of survivin siRNA down-regulated survivin expression and was able to sensitize cells to paclitaxel-induced cell death (38). Establishment of a clear link between EGFR signaling and survivin up-regulation in the apoptotic response to chemotherapy drugs will provide us with new information and a justifiable rationale for targeting this signaling pathway in the development of novel therapeutic approaches.

At present, how EGFR signaling leads to the expression of the survivin gene is still unclear. It has been shown that activation of the PI3K pathway by EGFR signaling leads to survivin up-regulation (12, 13). Results of our study also showed that inhibition of the PI3K pathway blocked EGF-induced survivin up-regulation in human breast cancer cell lines. However, the basal level of survivin expression was not affected even when the level of p-AKT was completely inhibited by a PI3K inhibitor, suggesting that the mechanism for constitutive expression of survivin may be different from EGF-induced survivin gene transcription. Although the role of PI3K/AKT in up-regulation of survivin expression has been established, we found that the effect of MAPK on survivin expression differs among breast cancer cell lines. Inhibition of the MAPK pathway blocked survivin gene up-regulation in SK-BR-3 but not in MCF-7 cells.

The PI3K/AKT pathway has been associated with important cellular pathways controlling cell proliferation and survival (39). To develop therapeutic approaches targeting EGFR signaling-induced apoptosis-resistant cancer cells, it is crucial to determine how activation of PI3K/AKT activity leads to survivin gene transcription. Previous studies have revealed another link between EGFR signaling and up-regulation of HIF-1α protein synthesis mediated by activation of PI3K/AKT (19, 40). Our laboratory recently demonstrated that survivin promoter activity was up-regulated in hypoxic tumor cells (23). By further analysis of the survivin core promoter sequences, we have now identified a putative HRE consensus 5′-GCGTG-3′ region located in −81 to −85 nt of the 5′-flanking region of the

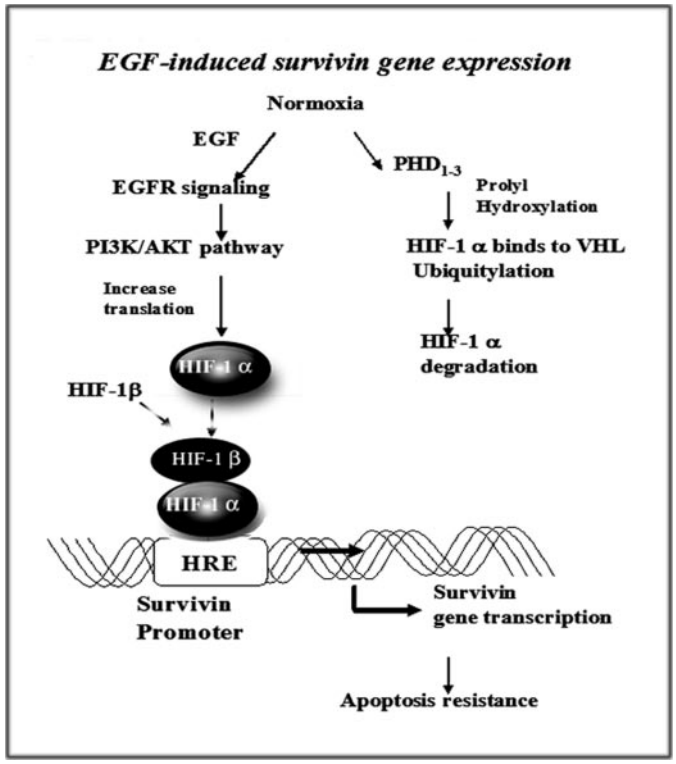


FIGURE 8. **Schematic illustration of a cross-talk between the EGFR and HIF-1α signal pathways showing up-regulation of survivin gene expression and induction of resistance to apoptosis.** Activation of EGFR signaling increases HIF-1α protein synthesis under normoxic conditions through the PI3K/AKT signal pathway. HIF-1α then directly interacts with the survivin promoter, up-regulating the level of survivin gene expression, which results in resistance to apoptosis in tumor cells.

survivin gene. Therefore, we believed it possible that transcriptional activation of survivin gene expression by EGF is mediated by HIF-1α. Our present study results demonstrated that the levels of both HIF-1α and survivin are significantly increased in EGF-treated, normoxic tumor cells. We further determined that HIF-1α is indeed a key transcription factor for EGFR signaling-activated survivin gene expression, since down-regulation of HIF-1α using HIF-1α siRNA significantly reduced the level of survivin expression in human cancer cells. Maintaining the level of HIF-1α-activated survivin seems to be very important for survival of normoxic tumor cells, since overexpression of HIF-1α siRNA alone resulted in activation of caspase activity and apoptosis in about 70% of the breast cancer cells. In addition, HIF-1α siRNA also markedly enhanced apoptotic cell death in docetaxel-treated tumor cells, even after EGF stimulation.

The results of our study have demonstrated, for the first time, that there is cross-talk between EGFR and HIF-1α signaling pathways that can up-regulate survivin gene expression and increase resistance to apoptosis. First, we showed that overexpression of the HIF-1α gene activates survivin promoter activity and the level of the protein. Using ChIP and modified McKay assays, we determined that there is a direct interaction between HIF-1α and the survivin promoter, thus finding that the transcriptional activity of HIF-1α is highly likely to activate survivin gene transcription. HIF-1α has been previously defined as a hypoxia-inducible transcriptional factor, and its role in normoxic tumor cells remained largely unclear. Our results provide

direct evidence that HIF-1α is able to bind to HRE sites and activate HRE-mediated gene transcription under normoxic conditions in human tumor cells. In another study, we found that hypoxia-induced HIF-1α up-regulation could also activate survivin gene expression using a similar mechanism.[3] Currently, the precise mechanism for HIF-1α-mediated transcriptional activation of the survivin gene is under investigation in our laboratory.

In this study, we found that EGF stimulation does not induce survivin expression in normal mammary epithelial cells. Moreover, overexpression of the HIF-1α gene activated survivin promoter activity and increased the level of survivin protein in breast cancer cell lines but not in normal cells, suggesting the presence of transcriptional inhibitory factor(s) in normal cells, preventing activation of the survivin promoter by HIF-1α. At present, we are conducting studies to identify the transcriptional factors involved in the tumor cell-specific activation of survivin gene expression.

In conclusion, we have identified a novel pathway that mediates resistance to apoptosis in EGFR signal-activated human tumor cells. Our results have demonstrated that activation of the EGFR signaling pathway leads to the up-regulation of HIF-1α through the PI3K/AKT pathway. We revealed that HIF-1α directly binds to the survivin promoter to activate gene transcription, resulting in resistance to apoptosis in normoxic, EGFR signal-activated tumor cells (Fig. 8). Since activation of the EGFR signaling pathway and the presence of hypoxic tumor regions are commonly found in solid tumors, it is possible that coexistence of activation of those two conditions makes tumor cells highly resistant to apoptosis through HIF-1α-mediated up-regulation of survivin as well as other factors. Results from our study further highlight the importance of HIF-1α-mediated expression of survivin in tumor progression and resistance to therapy. Additionally, our results provide a strong rationale for the development of novel approaches targeting HIF-1α for prevention of tumor progression as well as for cancer therapy, alone or in combination with other chemotherapeutic agents. Thus, the benefits from elucidation of this most likely common pathway that can function in both normoxia and hypoxia should eventually lead to much improved control and treatment of breast and other cancers.

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Real-time Detection of Gene Expression in Cancer Cells Using Molecular Beacon Imaging: New Strategies for Cancer Research

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Abstract

Development of novel approaches for quantitative analysis of gene expression in intact tumor cells should provide new means for cancer detection and for studying the response of cancer cells to biological and therapeutic reagents. We developed procedures for detecting the levels of expression of multiple genes in fixed as well as viable cells using molecular beacon imaging technology. We found that simultaneous delivery of molecular beacons targeting survivin and cyclin D1 mRNAs produced strong fluorescence in breast cancer but not in normal breast cells. Importantly, fluorescence intensity correlated well with the level of gene expression in the cells detected by real-time reverse transcription–PCR or Western blot analysis. We further show that molecular beacons can detect changes of survivin gene expression in viable cancer cells following epidermal growth factor stimulation, docetaxel treatment, and overexpression of *p53* gene. Thus, molecular beacon imaging is a simple and specific method for detecting gene expression in cancer cells. It has great potential for cancer detection and drug development. (Cancer Res 2005; 65(5): 1909-17)

Introduction

Development of new approaches for detecting cancer cells and determining the responses of the cells to therapeutic reagents holds great promise to increase the survival of cancer patients. It is well known that human cancer cells develop due to abnormalities in gene expression that provide growth advantages, metastatic potential, and apoptosis resistance to the cells (1–3). Methods for specific detection of abnormal gene expression in intact single cancer cells should provide new tools for identifying cancer cells in clinical samples, studying biological effects, and evaluating the effects of therapeutic reagents on specific molecular targets in cancer cells.

In this study, we developed a molecular beacon fluorescence imaging approach to detect the levels of expression of multiple genes simultaneously in single cells. Molecular beacons are stem-loop type oligonucleotide probes dual-labeled with a fluorophore and a quencher. In the absence of the target, the stem brings the fluorophore and quencher molecules together, which prevents the production of a fluorescent signal. When the molecular beacon hybrids to its specific target sequence, the stem is forced to break apart, which enables it to generate a fluorescent signal (4–6). Because binding conditions between the loop and complementary target sequences are very stringent, only a target with perfectly matching sequences is able to hybridize to the molecular beacon (5). During the last several years, molecular beacon technology has been used in various applications to detect oligonucleotides in solution, including DNA mutation detection and real-time quantification of PCR products and protein-DNA interaction (6–8).

The ability of molecular beacon probes to detect specific target molecules without separation of unbound probes also provides an opportunity to detect intracellular mRNA molecules in intact cells. The feasibility of detecting intracellular mRNA has been examined in several laboratories (9–13). It has been shown that molecular beacons were able to visualize mRNA molecules in several human and animal cell lines after introducing into cells through microinjection or liposome delivery (9–11, 14). It has also been shown that the detection limit of preformed molecular beacon/β-actin mRNA duplexes microinjected into the cells is 10 mRNA molecules, suggesting that molecular beacon technology is a very sensitive method for detecting mRNAs in cells (9).

Although previous studies suggested that detection of intracellular mRNA using molecular beacons is a feasible approach, the question remains of how to develop this novel technology into a simple procedure that can be used broadly in basic research and clinical laboratories. To address this issue, we developed procedures that enable us to detect gene expression in fixed as well as viable cells. We designed molecular beacons targeting survivin and cyclin D1 mRNAs, which are highly expressed in breast cancer cells (15, 16). Survivin is a member of the inhibitor of apoptosis protein family that plays a crucial role in the apoptosis resistance of tumor cells (17). Increasing evidence indicates that survivin is also a promising tumor marker because it is normally expressed during fetal development but is not expressed in most normal adult tissues (18). However, high levels of survivin are detected in many human cancer types including 70% of breast cancers (16, 19). Also, cyclin D1, an important regulator of cell cycle, is overexpressed in 50% to 80% of breast cancer tissues, whereas it is low or absent in normal breast tissues (15). In this study, we examined the feasibility of detecting expression of survivin and cyclin D1 genes in human breast cancer cells using the molecular beacon–imaging technology.

Materials and Methods

Human Breast Cancer or Normal Cell Lines and Tissues

Breast cancer cell lines SKBr-3, MDA-MB-231, and MCF-7 and normal immortalized human mammary epithelial cell line MCF-10A were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-435 cell line was provided by Dr. Zhen Fan (MD Anderson Cancer Center, Houston, TX).

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Frozen human breast cancer and normal tissues were obtained according to an approved institutional review board protocol at Emory University from breast cancer patients during surgery to remove the tumors. Tissues were frozen immediately in liquid nitrogen and kept at −80°C.

Design and Synthesis of Molecular Beacons

The sequences of molecular beacons targeting survivin or cyclin D1 mRNAs were unique for each gene. These include (*a*) survivin MB-FITC: 5′-FITC-TGGTCCTTGAGAAAGGGC*GACCA*-Dabcyl-3′, (*b*) survivin MB-Cy3: 5′-Cy3-*C*TGAGAAAGGGCTGCCAG*TCTCAG*-Dabcyl-3′, and (*c*) Cyclin D1 MB-Texas Red: 5′-Texas-Red-TGGAGTTGTCGGTGTAGA*CTCCA*-Dabcyl-3′. Control molecular beacons for targeting human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GAPDH MB-Cy 3 or GAPDH MB-6-FAM, were also synthesized as the following: 5′-Cy3 or 6-FAM-*C*GAGTCCTTCCACGATAC-CC*ACTCG*-Dabcyl-3′. The underlined bases were those added to form a stem with an optimal Tm condition. All molecular beacons were synthesized by MWG-Biotech Inc. (High Point, NC).

The specificity of the molecular beacons in solution was determined using synthesized oligonucleotide targets (Sigma Genosys, Woodlands, TX). These include (*a*) survivin target: 5′-CCTGCCTGGCAGCCCTTTCTCAAG-GACCACCGCATCTCTACATTCAAGAAC-3′, (*b*) cyclin D1 target: 5′-AGAAGCTGTGCATCTACACCGACAACTCCATCCGGC-3′, (*c*) HER-2/*neu* gene target: 5′-AGTGTGCACCGGCACAGACATGAAGCTGCGGCTCCCT-3′, and (*d*) K-*ras* gene: 5′-GTAGTTGGAGCTGGTGGCGTAGGCAA-GAGTGCCTTGACGATACAGCTAATT CAG-3′. Survivin or cyclin D1 molecular beacon (200 nmol/L) was mixed with 1 μmol/L of various DNA targets in 100 μL of Opti-MEM (Invitrogen, Carlsbad, CA). After incubating at 37°C for 60 minutes, fluorescence intensity was measured by a fluorescence microplate reader (Bioteck FL600 fluorometer, Winooski, VT).

Real-time Reverse Transcription–PCR

Total RNAs were isolated and amplified with an Omniscript reverse transcription kit (Qiagen Inc, Valencia, CA). Real-time PCR was done on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). The primer pairs for detecting the expression of survivin gene were survivin forward 5′-TCCACTGCCCCACTGAGAAC-3′ and survivin reverse 5′-TGGCTCCCAGCCTTCCA-3′. PCR primers for cyclin D1 were forward 5′-AGAAGCTGTGCATCTACACCGACAACTC-CATCCGGC-3′ and reverse 5′-GGTTCCACTTGAGCTTGTTCACAA-3′. The primer pairs for β-actin gene were β-actin forward, 5′-AAAGACCTGTA CGCCAACACAGTGCTGTCTGG-3′, and β-actin reverse, 5′-CGTCA-TACTCCTGCTTGCTGATCCACATCTGC-3′, and for GAPDH were forward 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse 5′-CATGTGGGC-CATGAGGTCCACCAC-3′.

Western Blot Analyses

Cell lysates were collected after different treatments and total cellular protein was resolved on polyacrylamide SDS gels. Western blot analysis for the level of survivin protein was done according to a standard protocol as described (19). The membranes were incubated for 1 hour with goat anti-human survivin (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal antibodies to β-actin (Sigma Chemical Co, St. Louis, MO). The levels of specific proteins in each lysate were detected by enhanced chemiluminescence using ECL plus (Amersham International, Buckingham, United Kingdom) followed by autoradiography.

Detection of Gene Expression in Fixed Cells

Cells were plated on chamber slides for 24 hours and then fixed with ice-cold acetone for 5 to 10 minutes. The slides were stained with a mixture of 200 nmol/L of survivin MB-FITC and cyclin D1 MB-Texas Red in Opti-MEM at 37°C for 60 minutes and then examined using a confocal microscope (LSM 510 Meta, Carl Zeiss Microimaging, Inc., Thornwood, NY).

For detecting survivin gene expression on tissue sections, 5-μm frozen sections of breast normal and cancer tissues fixed with ice-cold acetone were incubated with 200 nmol/L survivin MB-Cy3 for 60 minutes and then counterstained with 10 μg/mL Hoechst 33342 (Molecular Probes, Inc., Eugene, OR). For immunofluorescence labeling, acetone-fixed frozen sections were incubated with a goat anti-human survivin antibody and then with FITC-conjugated anti-goat antibody. For double-labeling survivin MB and human endothelial cell marker CD31, tissue sections were incubated with survivin MB-Cy3 and then with an anti-CD31 antibody followed by a FITC-conjugated secondary anti-mouse antibody. One tissue section was double-labeled with goat anti-human survivin and mouse anti-human CD31 antibodies followed by FITC-labeled donkey anti-goat antibody or biotinylated horse anti-mouse antibody and Texas Red avidin. The tissue slides were observed under a Nikon fluorescence microscope (Nikon Eclipse E800, Nikon Instruments Inc. Melville, NY). Fluorescence images were taken using an Optronics Magnafire digital imaging system (Meyer Instruments, Houston, TX).

Quantification of the Level of Gene Expression in Viable Cells Using Molecular Beacons

FACScan Analysis. Cells were plated in six-well plates and cultured in medium containing 2% fetal bovine serum overnight. The cells were transfected with 400 nmol/L of either survivin MB-FITC or GAPDH MB-6FAM using LipofectAMINE 2000 in Opti-MEM (Invitrogen). Three hours after transfection, 100 ng of human recombinant epidermal growth factor (EGF; Invitrogen) were added to the EGF-treated group for 1 hour and the cells were collected for FACScan analysis (Becton Dickinson, Mansfield, MA).

Cells transduced with an adenoviral vector expressing a wild-type *p53* gene (Adp53, Qbiogene, Carlsbad, CA) or control adenoviral vector (Adcmv) at a multiplicity of infection of 50 plaque-forming units for 24 hours were collected and divided into two groups. One group of the cells was transfected with 400 nmol/L of survivin MB-FITC and the other was transfected with 400 nmol/L of GAPDH MB-6-FAM using LipofectAMINE 2000 in Opti-MEM (Invitrogen). Fluorescence intensity of the cells from all groups was examined using FACScan analysis.

Fluorescence Microplate Reader. Cells were plated in 96-well culture plates at 80% confluence for 24 hours. EGF-treated and nontreated groups were cultured in the medium with 2% fetal bovine serum. The cells were then transfected with a mixture of 400 nmol/L of survivin MB-FITC and internal control GAPDH MB-Cy 3. At 3 hours after transfection, 100 ng/ml of EGF was then added to the wells in the EGF-treated group and 10 or 50 nmol/L of docetaxel (Aventis Pharma, Bridgewater, NJ) were added to the docetaxel-treated group. The culture plates were immediately placed in the microplate reader and fluorescence units in each well were measured at different time points.

Results

Survivin and Cyclin D1 Molecular Beacons Specifically Bind to DNA Targets. The design of the survivin and cyclin D1 molecular beacons and illustration of the mechanism of binding molecular beacons to specific oligonucleotide targets are shown in Fig. 1*A*. We showed that survivin or cyclin D1 molecular beacon specifically bound to its DNA target and generated 5- to 8-fold higher fluorescent signal when mixed with specific DNA target compared with other targets (Fig. 1*B*).

Detection of Human Breast Cancer Cells Using Molecular Beacons Targeting Tumor Marker mRNAs. We examined whether molecular beacons targeting different tumor marker mRNAs can be labeled with different fluorophores and expression of the tumor marker genes can be determined simultaneously in single cells. We found that a combination of survivin and cyclin D1 molecular beacons detected the expression of both survivin and cyclin D1 genes simultaneously and generated fluorescent signals corresponding to either survivin (green) or cyclin D1 (red) mRNA in the cancer cells (Fig. 2*A*). Importantly, the fluorescent signal was very low for both molecular beacons in a normal immortalized human mammary epithelial cell line (MCF-10A), indicating that survivin and/or cyclin D1 molecular beacons can be used as fluorescence probes for the detection of breast cancer cells (Fig. 2*A*). The results of examination of fluorescence

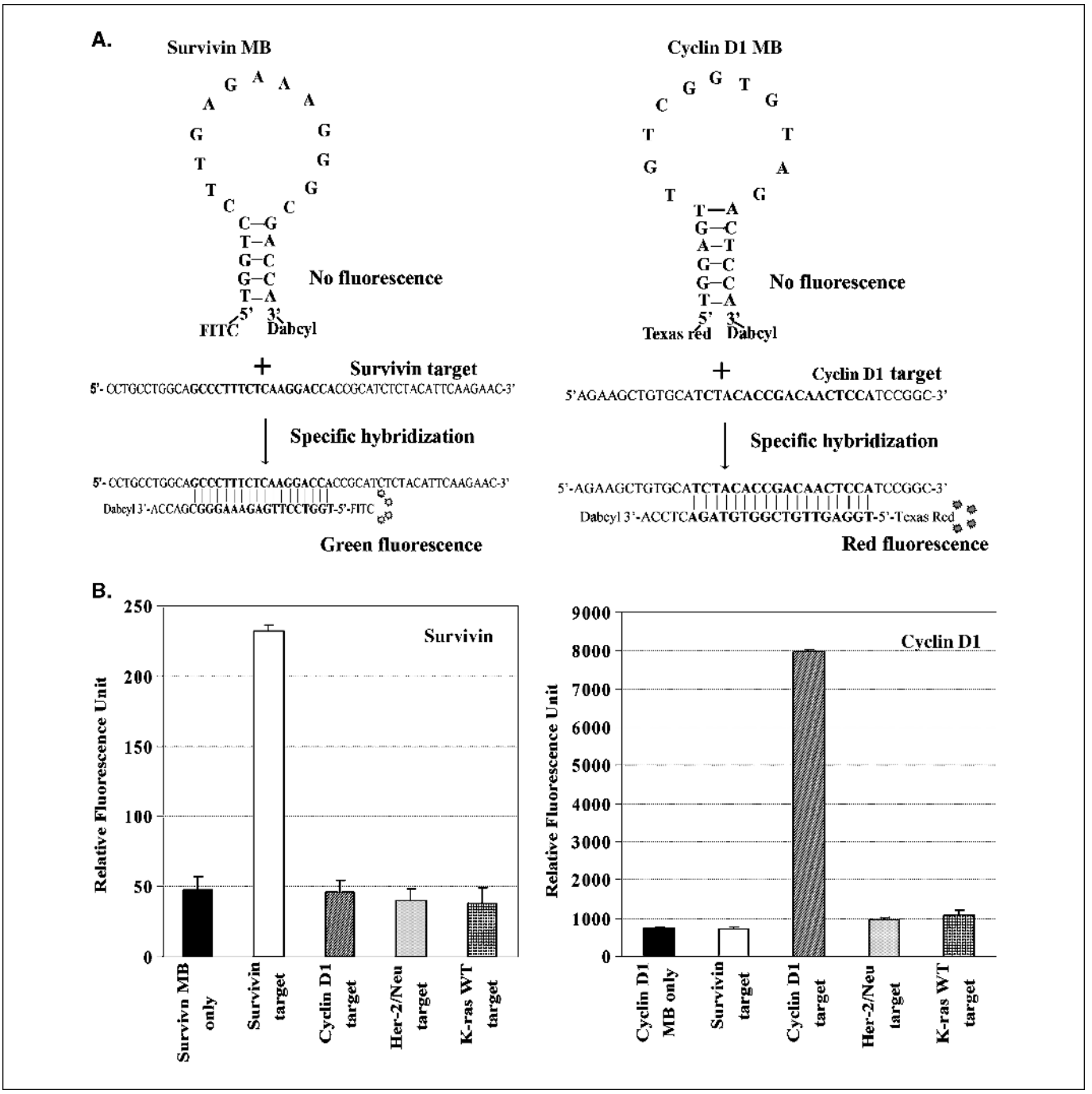

Figure 1. Schematic illustration of molecular beacon design and examination of specific binding of the molecular beacons to their oligonucleotide targets. *A,* both survivin and cyclin D1 molecular beacons have 23 nucleotides with 5′ stem and loop sequences complementary to survivin or cyclin D1 gene. The stem length for survivin molecular beacon is 5 nucleotides with the 5′ end labeled with FITC and the 3′ end labeled with a quencher (*Dabcyl*). Cyclin D1 molecular beacon has a stem containing 6 nucleotides with the 5′ end labeled with Texas Red and the 3′ end with Dabcyl. Survivin and cyclin D1 molecular beacons only generate fluorescent signals when hybridized to their specific DNA target. *B,* examination of specificity of the molecular beacons *in vitro.* Survivin or cyclin D1 molecular beacon was mixed with various synthesized DNA targets. The fluorescence units were measured using a fluorescence microplate reader. Survivin or cyclin D1 molecular beacon only bound and generated strong fluorescent signal when mixed with its specific DNA target. *WT,* wild-type.

intensity and the level of survivin or cyclin D1 gene expression in tumor and normal cell lines further showed that the fluorescent signals detected by the molecular beacons correlated very well with the levels of survivin or cyclin D1 gene expression, both in mRNA and protein levels (Fig. 2*B-D*). For example, MDA-MB-435 and SKBr-3 expressed very high levels of survivin gene, and the strongest fluorescent signal was detected in these cell lines. Conversely, these cell lines expressed low levels of cyclin D1 gene and showed a weak red fluorescence staining (Fig. 2*A-D*). MCF-7 cells expressed a moderate level of survivin gene but had a very high level of cyclin D1 gene expression. Delivery of survivin and cyclin D1 molecular beacons into this cell line produced a strong red fluorescent signal (cyclin D1) and an intermediate level of green fluorescent signal (survivin; Fig. 2*A-D*). Our results show

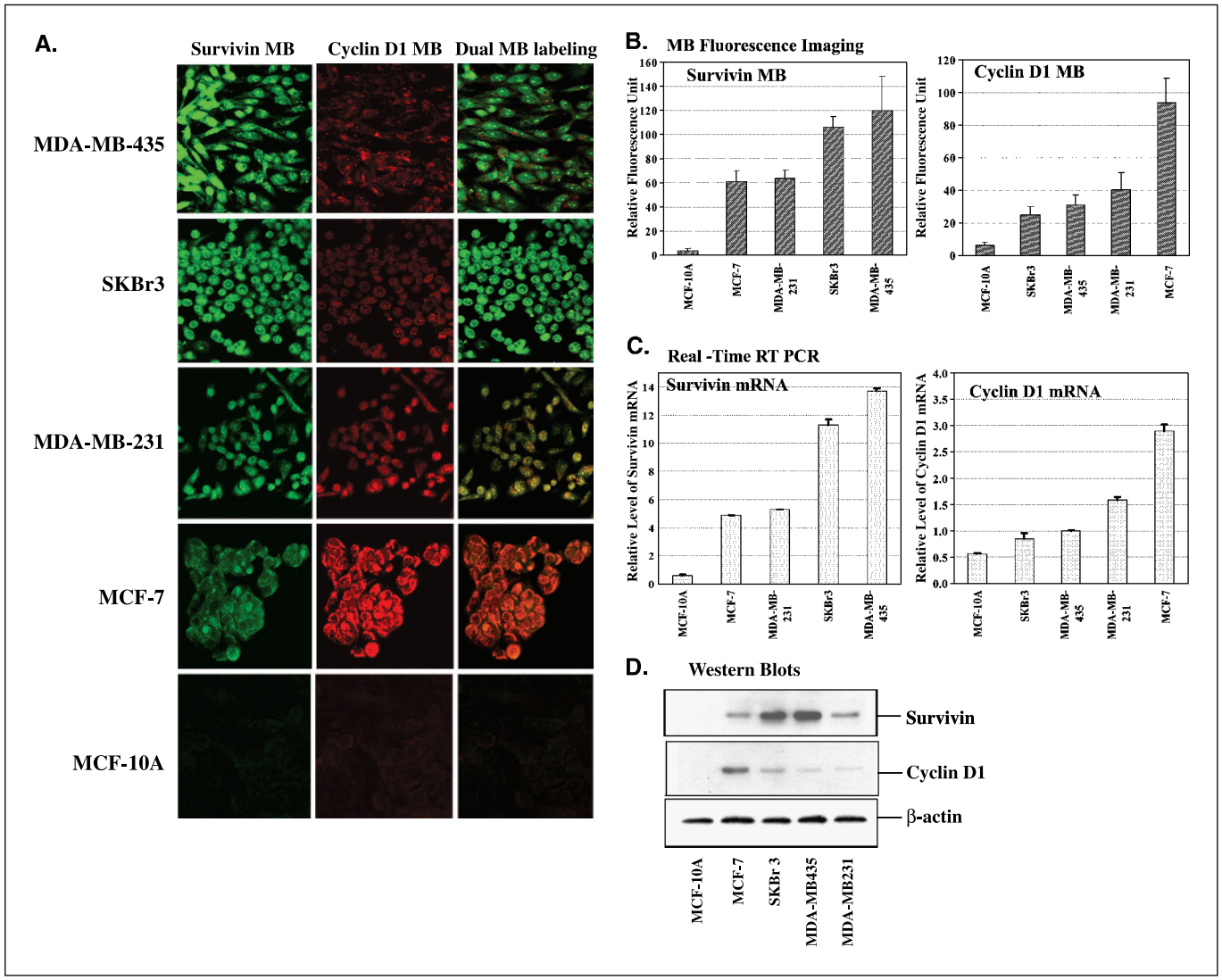

Figure 2. Simultaneous detection of the levels of survivin and cyclin D1 mRNAs in breast cancer cells. *A,* dual molecular beacon (*MB*) imaging of breast cancer cells. A mixture of survivin and cyclin D1 molecular beacons was incubated with the fixed cells and then examined under a confocal microscope. *B,* quantitative analysis of the level of fluorescence intensity produced in breast cancer and normal cells. Fluorescence intensity was determined by measuring the mean fluorescence units from four randomly selected areas for each image taken under a confocal microscope. The mean fluorescence unit from four areas of each cell line is shown in the figure. Similar results were observed in repeat experiments. *C,* detection of the levels of survivin and cyclin D1 mRNAs by real-time RT-PCR. Relative level of survivin or cyclin D1 mRNA was calculated from the quantity of survivin or cyclin D1 PCR products and the quantity of β-actin PCR products. *D,* examination of the levels of survivin protein in tumor and normal cell lines by Western blotting. The levels of survivin or cyclin D1 protein correlated well with the levels of survivin or cyclin D1 mRNA detected *in situ* in fixed tumor cells using molecular beacon detection or with the real-time RT-PCR results.

that a combination of molecular beacon technology with fluorescence imaging is a novel approach to simultaneously detect the levels of multiple gene expressions in intact single cells.

Molecular Beacons Detect Cancer Cells on Frozen Sections of Breast Cancer Tissues. We further developed a simple and fast procedure that allows us to detect survivin gene expression *in situ* on frozen tissue sections. Our previous study showed that survivin is expressed in 72% of breast cancer tissues, including 34 invasive breast ductal carcinoma and 2 lymph node metastases, using Western blot analysis of tissue lysates obtained from frozen tissue samples of the patients with cancer (19). In this study, we examined survivin gene expression on frozen tissue sections of those cancer tissues using survivin MB. We found that survivin MB-Cy3 was able to produce strong red fluorescent signals in breast cancer cells on frozen tissue sections (Fig. 3*A*). A high level of survivin gene expression was consistently detected in the breast cancer cells in nine of nine invasive ductal carcinoma tissues and one lymph node with metastastic lesions that were previously found positive for survivin protein by Western blot analysis. Two breast cancer tissues that were negative for survivin protein expression also lacked survivin MB positive cells (data not shown). Moreover, the survivin MB positive cells were not found in frozen tissue sections of all five paired normal breast tissues (Fig. 3*A* and *B*, representative results of survivin molecular beacon imaging and immunofluorescence labeling with a survivin antibody).

We have also examined the expression of survivin proteins in ductal carcinoma *in situ* (DCIS) tissues by immunohistochemical staining on frozen or paraffin sections using a polyclonal anti-survivin antibody. Eleven of 17 DCIS tissues displayed various levels of survivin protein expression (data not shown). We further

examined frozen tissue sections from two DCIS tissues and found that breast cancer cells in those DCIS tissues were positive for survivin molecular beacon, suggesting that survivin gene expression is an early event in the tumorigenesis of breast cancer (Fig. 3*A*).

In addition, we found that survivin gene–expressing cells in breast cancer tissues included cancer cells as well as cells in the vascular structures (Fig. 3*C*). When the same section was double-labeled with an antibody specific for a CD31 human endothelial cell marker (20), those survivin-expressing cells in the vascular structures were shown to be endothelial cells (Fig. 3*C*). Establishment of this molecular beacon detection method for measuring gene expression *in situ* should provide pathologists with a new tool to identify cancer cells in clinical samples.

Monitoring the Level of Real-time Gene Expression Using Survivin Molecular Beacon. We used three model systems to determine whether survivin molecular beacon was able to detect changes of survivin gene expression in viable cells, including EGF or docetaxel induced up-regulation and tumor suppressor gene *p53*–induced down-regulation of survivin gene expression (21, 22). Breast cancer cells were transfected with a mixture of survivin and GAPDH molecular beacons and observed under a fluorescence microscope after treatment with EGF for 1 hour or docetaxel for 24 hours. Our results showed that treatment of the cells with EGF or docetaxel increased the level of survivin gene expression. Under a fluorescence microscope, the green fluorescence intensity (survivin MB-FITC) was stronger in the cells treated with either EGF or docetaxel compared with untreated control whereas the fluorescent signal for GAPDH molecular beacon (Cy3, red) was relatively consistent (Fig. 4*A*). We further used FACScan analysis to determine the mean fluorescence intensity in each cell population. Consistent with our observation with the fluorescence microscopy, we detected higher levels of fluorescent signal in EGF-treated cells compared with the untreated group in breast cancer cells (Fig. 4*B*). The relative level of survivin mRNA could be quantified from the FACScan data using the fluorescence unit of *GAPDH* gene as an internal control. We found that EGF treatment induced ~1.5-fold increases in the level of survivin gene expression in breast cancer cells.

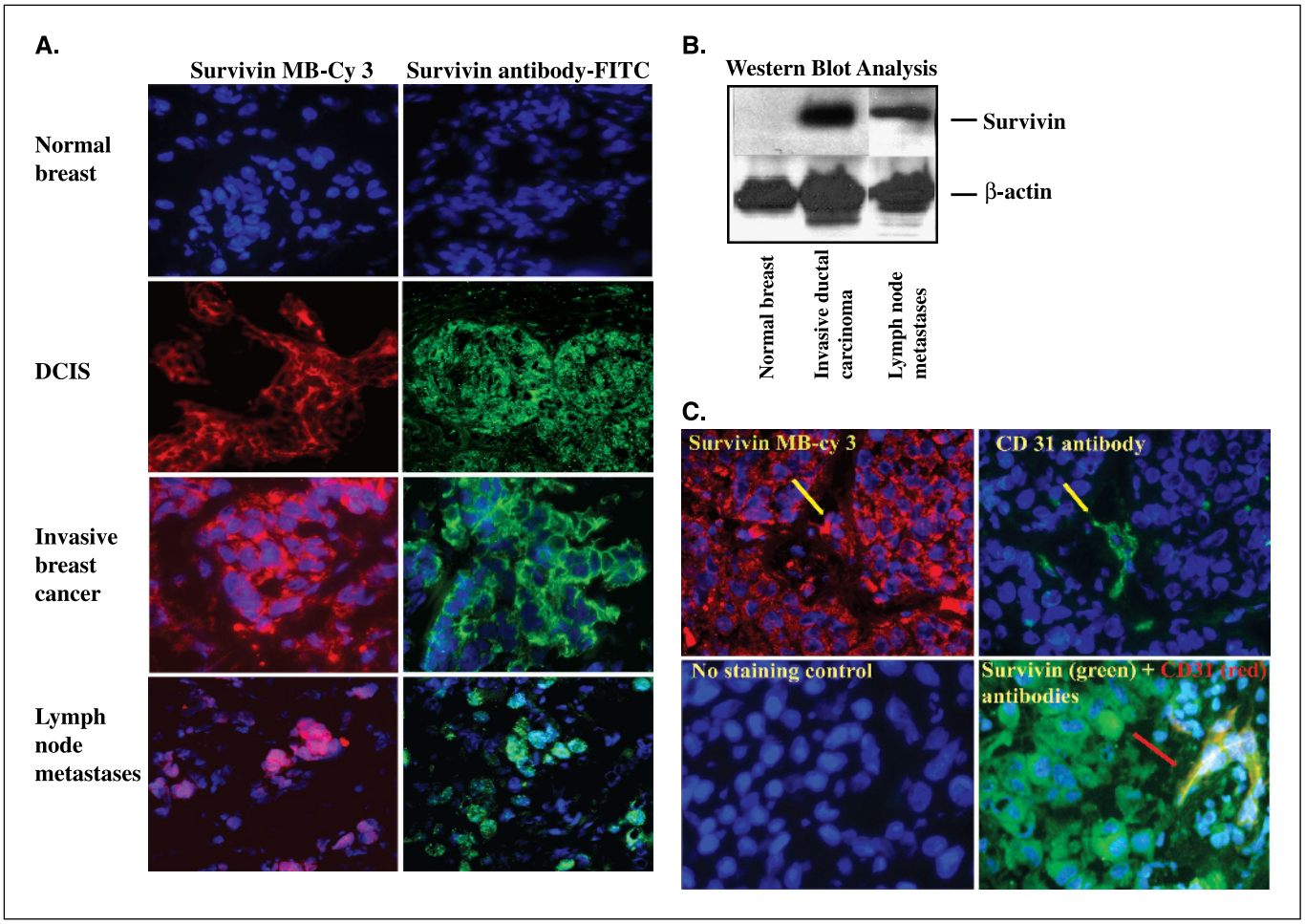

Figure 3. Detection of survivin gene expression on frozen tissue sections obtained from patients with breast cancer. Expression of survivin gene was detected in different stages of breast cancer tissues. Frozen tissue sections were fixed with acetone and incubated with survivin MB-Cy3. The sections were counterstained with Hoechst 33342 (*blue nuclei*). Survivin-expressing cells (*red*) were found in all stages of breast cancer tissues including DCIS, invasive carcinoma, and lymph node metastases, but not found in normal breast tissues. Different sections from the same tissues were also stained with a survivin antibody to confirm the presence of survivin positive cells (*green*). *B,* Western blot analysis showed a high level of survivin protein (16.5 kDa) in primary breast cancer and lymph node with metastases but not in normal breast tissues. *C,* detection of survivin gene expression in breast cancer and tumor endothelial cells in breast cancer tissues using double-labeling survivin MB-Cy3 with an antibody to CD31. Expression of survivin mRNA was labeled by survivin MB-Cy3 (*red*) and tumor endothelial cells were labeled with an anti-CD31 antibody (*green, yellow arrow*). Another tissue section was double-labeled with goat anti-human survivin (FITC, *green*) and mouse anti-human CD31 antibodies (*red*). All sections were counterstained with Hoechst 33342 (*blue*). *Red arrow,* tumor endothelial cells expressed both survivin and CD31 (*orange*).

In addition to the detection of levels of up-regulated genes, we examined the feasibility of quantifying the relative level of down-regulated gene expression. It has been shown that overexpression of *p53* gene decreases the expression of survivin gene (22). We transduced the tumor cells with Adp53 vector or control vector Adcmv for 24 hours and then delivered survivin or GAPDH molecular beacons into the transduced cells. Using FACScan analysis, we found that the relative fluorescence was decreased ~2-fold in Adp53 vector–transduced cells compared with the untreated or empty Adcmv vector control group (Fig. 4*C*). The ability of molecular beacons to detect a decreased level of gene expression suggests that the fluorescent signals detected intracellularly after molecular beacon transfection are not from nonspecific degradation of the molecular beacons because the same amount of survivin and GAPDH molecular beacons is delivered into Adp53 and control vector–transduced cells. The results from real-time reverse transcription-PCR (RT-PCR) further confirmed that EGF increased the transcription of survivin gene and overexpression of the *p53* gene decreased the level of survivin mRNA (Fig. 4*D*).

Although detection of the level of gene expression by FACScan could accurately measure the fluorescence intensity in individual cells as well as in cell populations, the procedure for FACScan is time-consuming and does not easily detect changes of gene expression in real time in the same cell population. To develop a high-throughput method for monitoring the changes of gene expression in real time in viable cells, we examined the feasibility of detecting levels of gene expression in cells cultured in 96-well plates using the molecular beacon-transfection approach. Breast cancer cells were plated in 96-well plates and transfected with a mixture of survivin and GAPDH molecular beacons for 3 hours. After adding EGF or docetaxel, the fluorescence units were

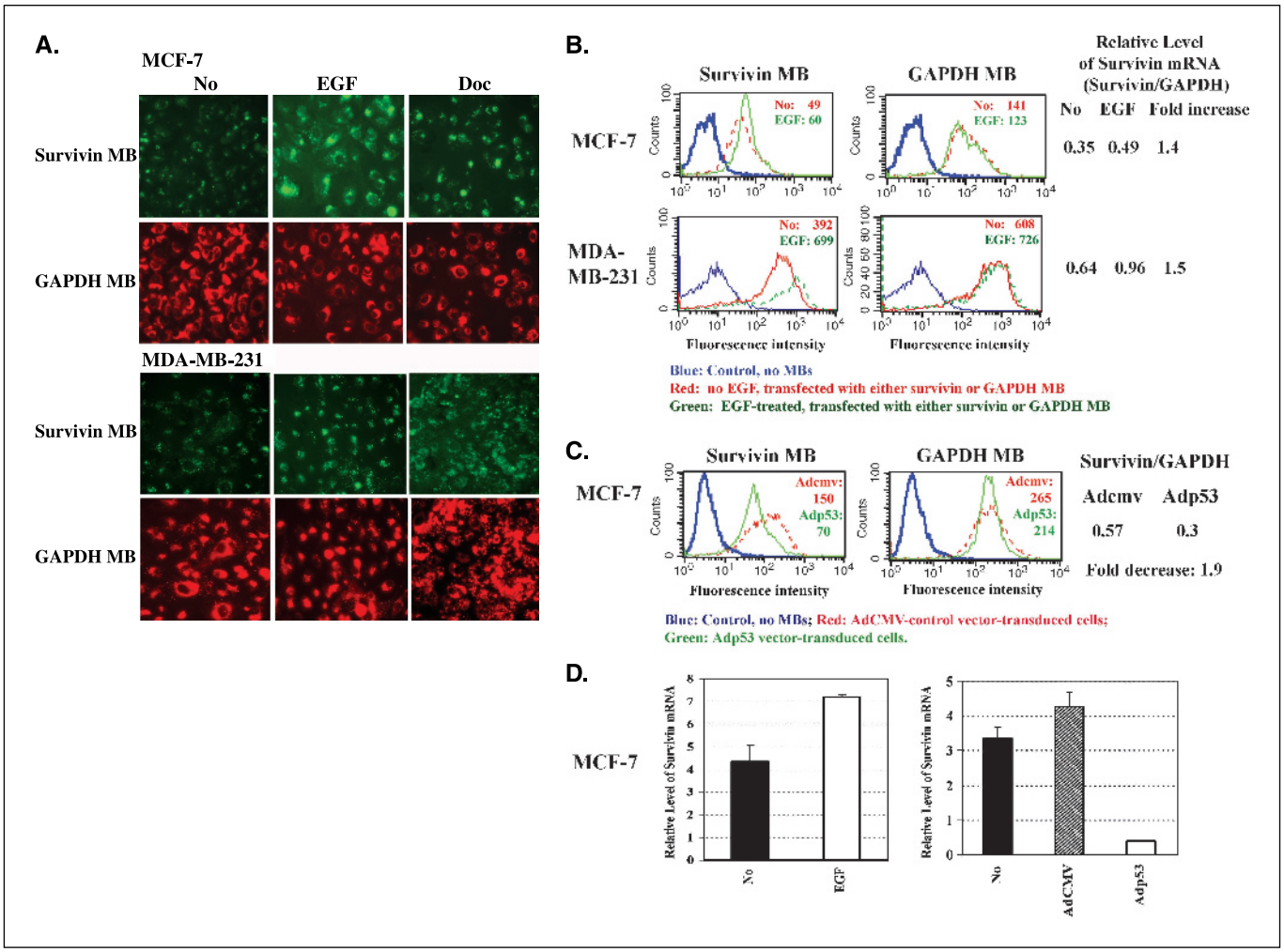

Figure 4. Detection of the levels of survivin gene expression in viable cells using survivin molecular beacon. *A,* survivin MB-FITC produced green fluorescence in cytoplasm of breast cancer cells after transfecting into viable cells. Treatment of the cancer cells with EGF for 1 hour or docetaxel (Doc) for 24 hours increased the fluorescence intensity in the cells. The fluorescence intensity generated by GAPDH MB-Cy3, which was cotransfected with the survivin molecular beacon, was relatively consistent in the cells. *B* and *C*, The level of survivin or GAPDH mRNA in molecular beacon-transfected cells could be measured by FACScan analysis to determine the mean fluorescence unit for each sample. The level of survivin mRNA was increased in EGF-stimulated cancer cell lines (survivin molecular beacon, *green line*), whereas there was no change in the fluorescence units detected in GAPDH molecular beacon–transfected cells (GAPDH molecular beacon, *green line*). On the other hand, the level of survivin mRNA decreased in Adp53 vector- (*green line*) but not in Adcmv vector–transduced cells (*red dotted line*). Numbers in the figure represent the mean fluorescence intensity for each group. Relative levels of survivin mRNA were calculated from the ratio of mean fluorescence intensities of survivin molecular beacon and GAPDH molecular beacon. Real-time RT-PCR analysis showed that the level of survivin mRNA was increased by EGF treatment but decreased after overexpression of *p53*. The numbers in the figure represent the mean numbers from three repeat samples. The relative level of survivin gene expression was calculated as a ratio of the quantity of survivin and GAPDH PCR products.

measured at different time points using a fluorescence microplate reader. We found that EGF-induced up-regulation of survivin gene expression occurred as early as 15 minutes after the treatment and lasted for >3 hours (Fig. 5*A*). There were 2.3 (MCF-7)- to 2.8 (MDA-MB-231)-fold increases in the relative levels of survivin mRNA after EGF treatment. We also examined the level of survivin protein using Western blot analysis and further confirmed that the level of survivin protein increased after EGF treatment (Fig. 5*A*).

For real-time detection of the level of gene expression in viable cells, it is important to determine how long the molecular beacon probes will stay in the cells and still be able to produce fluorescent signals that reflect the relative level of the gene expression. It has been shown that the chemotherapy drug docetaxel increases in the level of survivin gene expression as early as 4 hours after the treatment (21). We examined the level of real-time survivin gene expression in molecular beacon–transfected cells after docetaxel treatment from 0 to 48 hours. We found that the level of survivin mRNA was increased at 5 hours and reached higher levels 24 and 48 hours after treatment (Fig. 5*B*). The relative level of survivin mRNA is ~1.5-fold higher in docetaxel-treated cells than control cells and the difference detected 48 hours after docetaxel treatment is statistically significant (Student's t test, $P < 0.05$ for both MCF-7 and MDA-MB-231 cell lines). We also found a similar increase in the level of survivin mRNA detected by real-time RT-PCR compared with survivin molecular beacon detection, and the level of increase in survivin protein after docetaxel treatment (Fig. 5*B*, *inset* is real-time RT-PCR result).

One of the important issues to be addressed in developing an oligo-based approach for detecting gene expression in viable cells is whether the binding of the molecular beacon probes to their target RNA leads to degradation of the mRNA by RNase H, which may affect the level of target mRNA (23). To answer this question, we transfected breast cancer cells with either survivin molecular beacon or control GAPDH molecular beacon for 24 hours and then examined the level of survivin protein by Western blot analysis. We found that compared with cells transfected with a nonspecific GAPDH molecular beacon, the presence of the survivin molecular beacon in the cells did not reduce the level of survivin protein (Fig. 5*C*).

Discussion

We have developed a novel molecular beacon-based molecular imaging approach that allows identification of tumor cells expressing specific marker genes. Because molecular beacon is highly specific in detecting target mRNAs, and molecular beacons targeting various genes can be labeled with different fluorescent dye molecules and delivered into single cells, expression of several tumor marker genes in a single cell can be analyzed at the same time. Human cancers contain heterogeneous cell populations with various genetic changes (24). Simultaneous detection of overexpression of several tumor marker genes, especially when a single cell expresses more than one marker gene, may have a high predicative value for identifying cancer cells and therefore increase the sensitivity and specificity of cancer detection. Using molecular beacons targeting survivin and cyclin D1 mRNAs, we showed that delivery of a mixture of survivin and cyclin D1 molecular beacons into fixed cells produced fluorescent signals in breast cancer cells but not in normal breast cells. Interestingly, the fluorescence intensities in the cells correlated well with the level of the gene expression in different tumor cell lines. Previous methods for detecting gene expression *in situ* were not quantitative because the signals were amplified by either the presence of multiple fluorescent dye labeled nucleotides in an oligonucleotide probe or amplification of the signals with secondary antibodies to labeled nucleotides. Because each molecular beacon has only one fluorophore and unbound molecular beacons do not fluoresce, the fluorescence intensity generated by hybridization of the molecular beacon with a specific mRNA should reflect more accurately the level of the mRNA expressed in the cells.

At present, molecular beacon technology has been mainly used in various applications *in vitro*, which were done in solutions with defined molecular beacon-target conditions. Although previous studies showed the feasibility of detecting mRNAs and monitoring the transportation of RNAs in cells, the procedure for delivery of the molecular beacons through microinjection or by liposome delivery has made it difficult to apply this technology into broad research areas or into a routine clinical procedure (9–12). A recent study showed that it is feasible to transfect a molecular beacon into living cells to detect doxorubicin-induced activation of p21 gene expression (13).

We developed this molecular beacon-based procedure for the detection of gene expression in viable cells. We showed that transfecting survivin molecular beacon into cells produces a strong fluorescent signal in survivin-expressing tumor cells and the level of survivin gene expression can be monitored real time in cells either by FACScan or by using a fluorescence microplate reader. Using these methods, we detected an increase in the level of survivin gene expression following EGF and docetaxel treatment. Although we used GAPDH molecular beacon as an internal control for our experiments, simultaneous detection of survivin and *GAPDH* gene expression real time in viable cells indicates that it is feasible to monitor the levels of expression of several genes in the same cell population using molecular beacons labeled with different fluorophores.

Quantitative measurement of mRNA levels by molecular beacons is very important for the future use of this technology for cancer cell detection because many tumor marker genes are not unique to cancer cells and the difference between normal and cancer cells can be only the level of gene expression. Although we used two molecular beacons to detect the expression of tumor marker genes, a proof of principle from this study will lead to the use of more molecular beacons with multiple dye molecules to analyze the expression of several tumor genes. In addition, because only a small amount of abnormal cells are present in a large amount of normal cell background in clinical samples, there is a clear advantage of direct fluorescence imaging of individual cells expressing tumor marker genes for early detection of cancer cells compared with conventional RT-PCR to amplify the expression of tumor marker genes from isolated total RNA, which may be difficult to detect the differences in the level of gene expression in a few cancer cells over the normal background.

Current methods for the identification and classification of cancer cells from clinical samples rely on examining the morphology of the cells or immunostaining with antibodies for tumor-related protein markers. Although the *in situ* hybridization using labeled linear probes has been used to detect gene expression in tissue sections, it is very time-consuming and

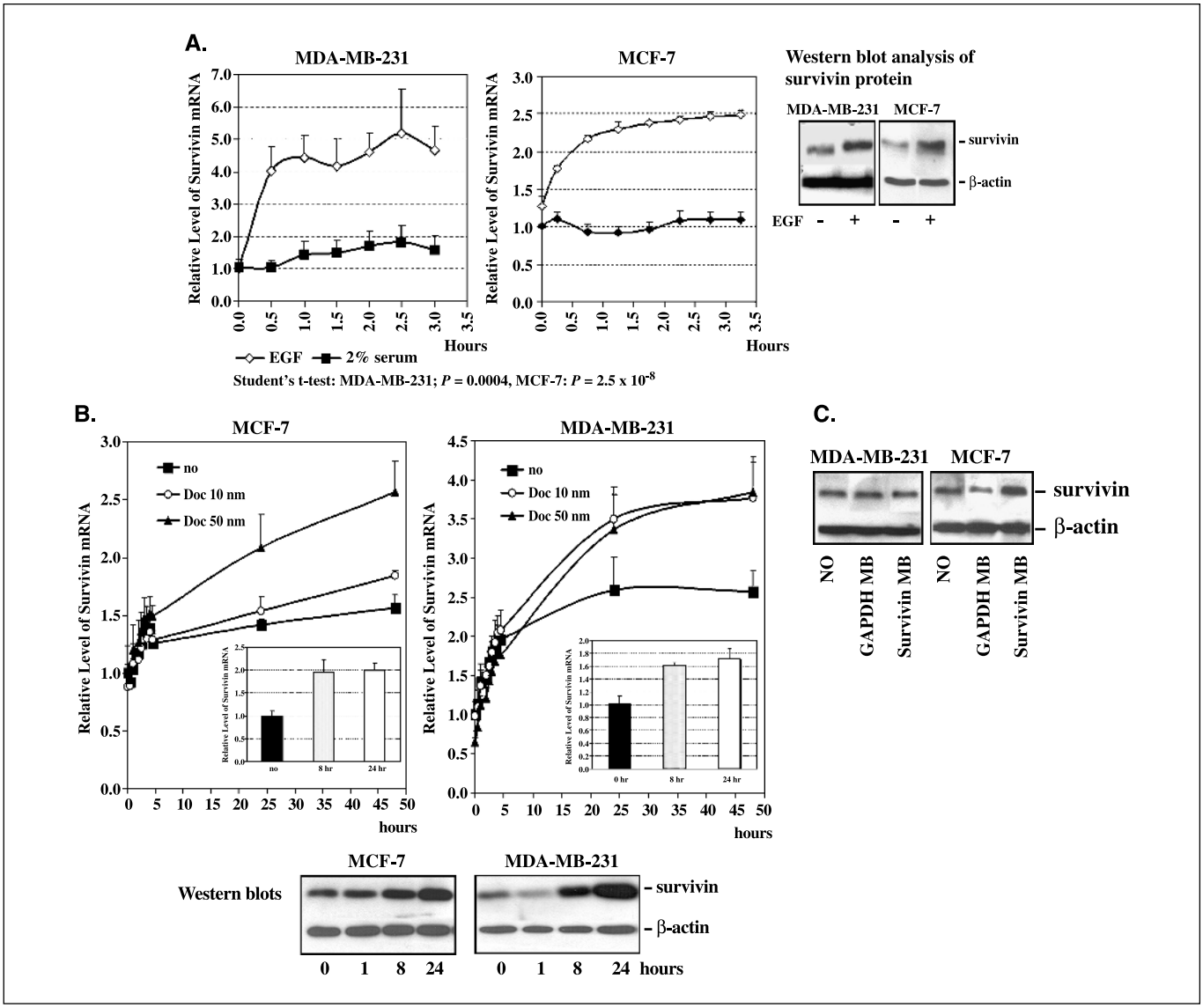

Figure 5. Real-time monitoring the level of survivin gene expression in breast cancer cells. Cells cultured in 96-well plates were transfected with a mixture of survivin MB-FITC and GAPDH MB-Cy3 and then added human EGF or docetaxel. The fluorescence intensity was measured at different time points following treatment using a fluorescence microplate reader. Each point in the curve is a ratio of the mean fluorescence unit of survivin molecular beacon (FITC, Ex/Em 480/530) and mean fluorescence unit of GAPDH molecular beacon (Cy3, Ex/Em 530/590) from four repeat samples. Similar results were obtained from three independent studies. *A,* EGF treatment significantly increased the level of survivin mRNA (Student's *t* test for all time points, $P < 0.0005$). Western blotting further showed that EGF increased the level of survivin protein. *B,* docetaxel treatment increased the level of survivin gene expression. Significant increases in the level of survivin mRNA were seen 24 to 48 hours following the treatment (Student's *t* test, $P < 0.05$). The levels of survivin gene expression after treatment were also examined by real-time RT-PCR (*inset*). Western blot analysis showed up-regulation of survivin protein by docetaxel. *C,* transfection of survivin or control GAPDH molecular beacon into viable cells did not significantly decrease the level of survivin protein as determined by Western blot analysis of cell lysates after transfected with either survivin molecular beacon or GAPDH molecular beacon for 24 hours.

usually accompanied by a high background because unbound probes also produce fluorescent signals. In our study, we found that molecular beacons could be used to detect the expression of genes on frozen tissue sections. The procedure is very simple and results can be examined within 30 to 60 minutes without the extensive staining and washing steps. Demonstration of the feasibility of combining the molecular beacon and immunofluorescence approaches to detect the expression of tumor marker genes and proteins *in situ* in the same cell population makes its potential application in pathologic diagnosis of human cancers more appealing. It is possible that the level of gene expression detected by molecular beacon-fluorescence imaging in clinical samples with intact tumor cells, such as fine-needle aspirates and exfoliated cells in body fluids is more quantitative than that detected in cancer cells on frozen tissue sections because most cells in tissue sections have been cut through and lost part of their cellular components.

One concern in the delivery of unmodified molecular beacons to viable cells is that the molecular beacons may be digested by nucleases in the cells or nonspecific interaction between molecular beacons, and cellular proteins may open up the stem of the molecular beacons, resulting in nonspecific fluorescence. However, our results showed that the fluorescence intensity detected by either FACScan or microplate reader correlated well

with the level of survivin mRNA in the tumor cells. Because a similar level of the molecular beacons was delivered into the tumor cells, it seemed that increases in the fluorescence intensity in EGF- and docetaxel-treated cells or a decrease in *p53*-expressing cells were not due to nonspecific degradation of the molecular beacons.

In this study, we showed that molecular beacon imaging of tumor cells is a simple and specific approach for the detection of breast cancer cells. This study is the first to apply state-of-the art molecular beacon–based methodology for cancer cell detection and for real-time monitoring the level of expression of tumor marker genes in viable cells. Based on this study, high-throughput assays for measuring the expression of multiple genes critical for drug response can be developed for screening cancer drugs that target specific molecules or pathways in cancer cells. To increase the specificity of molecular beacon detection, the molecular beacons can be further modified to make them resistant to nuclease or RNase H, such as by using 2′-*O*-methyl molecular beacon probes (12).

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Protocol title:
Early Detection of Breast Cancer Using Molecular Beacons

Subtitle:
Examination of the Sensitivity of Using Molecular Beacons Targeting Survivin, Cyclin D1 and Her-2/neu mRNAs to Detect Breast Cancer Cells in Ductal Lavage and Aspirates of Fine Needle Biopsy in Breast Cancer Patients

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Introduction

Breast cancer is the most common type of cancer and one of the leading causes of death among women. A crucial factor to increase survival is to diagnose it early. Although early screening with mammography decreases the mortality of the disease, nearly 20% of breast cancer patients are still missed by mammography. Furthermore, of all patients with abnormal mammograms, only 10 to 20% were confirmed to be breast cancer by biopsy (1). At present, there is no reliable serum tumor marker for diagnosis of breast cancer. Therefore, development of novel approaches for early diagnosis of breast cancer is of critical importance for the successful treatment and for increasing survival of the patients. It is well known that over 90% of breast cancers arise in the epithelial cells lining the ductal system. Most breast cancers developed over a period of 8 to 10 years before detected by standard methods. In order to detect early stage breast cancer or ductal carcinoma *in situ* (DCIS), it is important to develop novel and non-invasive approaches with high sensitivity and specificity.

It is well established that cancer cells develop due to genetic alterations in oncogenes and tumor suppressor genes and abnormalities in gene expression that provide growth advantage and metastatic potential to the cells. A novel way of achieving early detection of cancer is to identify the cancer cells through detection of mRNA transcripts that are expressed in the cancer cells but at low levels or not expressed in normal cells. We have developed a molecular beacon (MB)-based approach for direct examination of gene expression in viable and fixed cells(2, 3). The proposed study focuses on the evaluation of the feasibility of detection of breast cancer cells in ductal lavage and/or fine needle aspiration using MBs targeting survivin, cyclin D 1 and Her-2/neu mRNAs, which are tumor markers found in over 70 to 80% of DCIS tissue samples.

MBs are oligonucleotides with a stem-loop hairpin structure, dual-labeled with a fluorophore at one end and a quencher at the other. Delivering MBs into cells produce fluorescence signal if the MBs hybridize to target mRNAs. Thus, when the target mRNAs correspond to the molecular markers of a cancer, cancer cells (bright) can be distinguished from normal cells (dark)(2). We have established the structure-function relationships of MBs and experimental conditions that enable us to identify breast cancer cells using MBs.

Recently, a class of new fluorescent emitting particles, semiconductor quantum dots, has been developed for using as sensitive probes for biomolecular and cellular imaging (4,5). These quantum dots (QDs) are tiny, nanometer-scale light-emitting particles that have unique optical and electronic properties such as size-tunable light emission, improved signal brightness, resistance against photobleaching, and ability to simultaneous excite multiple fluorescence colors (4,5). These properties are most promising for improving the sensitivity of molecular imaging and quantitative cellular analysis by 1-2 orders of magnitude. Another advantage is that multicolor QD probes can be used to image and track multiple tumor markers simultaneous in single cells. It has been shown that antibodies conjugated to QDs are able to detect several protein markers in cancer cells. Therefore, we plan to detect the levels of mRNA with MBs and protein levels using QD-labeled antibodies simultaneous in clinical samples.

Working hypothesis: Simultaneous detection of mRNA and protein levels of tumor markers, such as survivin, Her-2/Neu and cyclin D1, using MBs and QD-labeled antibodies, has a high specificity and sensitivity in identifying cancer cells. The ultimate goal is to develop a simple, sensitive and efficient clinical procedure for early detection of breast cancers.

Significance

The proposed study will be the first to apply state-of–the art nanotechnology, including both MB-based and QD methodologies for detection of breast cancer. Survivin is a newly discovered protein in the inhibitor of apoptosis protein family (IAP). This investigation will also be the first such study to evaluate the feasibility of using survivin as an early breast cancer marker. Since MB is highly specific in detecting target mRNAs, and MBs targeting various genes can be labeled with different fluorescent-dye molecules and delivered into single cells, expression of several tumor marker genes in a single cell can be analyzed at the same time. QD-labeled antibodies are able to detect the protein levels of several tumor markers in caner cells. Human breast cancers contain heterogeneous cell populations with various genetic changes. Simultaneous detection of overexpression of several tumor marker genes and proteins, especially when a single cell expresses more than one marker gene, may have a high predictive value for identifying cancer cells, and therefore increase the sensitivity and specificity of cancer detection. For example, although survivin is detected in over 70% of breast cancer tissues, about 30% of patients don't express survivin and will be missed if only survivin will be used. About 20 to 30% of DCIS tissues are also negative for cyclin D or Her-2/neu. We plan to use MBs targeting survivin, cyclin D1 or Her-2/neu mRNAs that are labeled with different fluorescence dye molecules to determine the relationship between the detection of the cells expressing none, one, two or three markers and the feasibility of early detection of breast cancers in ductal lavage or fine needle aspirates. Additionally, we will use the sample to detect cells expressing high levels of survivin, cyclin D1 and Her-2/neu proteins using fluorescent QD-dot labeled antibodies. Since fluorescent emission wavelength of three QDs are different from each other and are also distinguishable from fluorescent dyes in MBs, we should be to determine the mRNA and protein levels of each gene.

The results of our study *in vitro* in human breast normal and cancer cell lines have shown that delivery of a mixture of survivin and cyclin D1 MBs into fixed cells produced fluorescent signals in breast cancer cells but not in normal breast cells. Interestingly, the fluorescence intensities in the cells correlated well with the level of the gene expression in different tumor cell lines. Previous methods for detecting gene expression *in situ* were not quantitative since the signals were amplified by either the presence of multiple fluorescent-dye labeled nucleotides in an oligonucleotide probe or amplification of the signals with secondary antibodies to labeled nucleotides. Since each MB has only one fluorophore and unbound MBs do not fluoresce, the fluorescence intensity generated by hybridization of the MB with a specific mRNA should reflect more accurately the level of the mRNA expressed in the cells. Additionally, since only a small amount of abnormal cells are present in a large amount of normal cell background in clinical samples, there is a clear advantage of direct fluorescence imaging of individual cells expressing tumor marker genes for early detection of cancer cells compared to conventional RT-PCR to amplify the expression of tumor marker genes from isolated total RNA, which may be difficult to detect the differences in the level of gene expression in a few cancer cells over the normal background.

Therefore, the MB–based cancer cell detection has the potential to become a simple clinical procedure for early detection of breast cancer with a high sensitivity and specificity. Additionally, determination of protein levels of those tumor markers in the cells should further enhance the specificity and sensitivity of the detection.

Experimental procedures:

Examination of the sensitivity of using molecular beacons targeting survivin, cyclin D 1 and Her-2/neu mRNAs and QD-labeled antibodies to above three markers to detect breast cancer cells in ductal lavage and aspirates of fine needle biopsy in breast cancer patients.

First, we will examine the specificity and sensitivity of MB- and QD- imaging cancer cells in ductal lavage or fine needle aspiration from breast cancer patients. This study will allow us to determine whether the MB and QD detections are more specific and sensitive than cytological method in detecting breast cancer cells. The proposed study will also provide us with new information regarding using survivin, cyclin D 1 and Her-2/neu as tumor markers for identifying the breast cancer cells at DCIS stage.

Study subject selection:

Eligibility Criteria:

Patients of all ages and races diagnosed with breast cancers or with possibility of breast cancer, who come to Emory Clinic or Emory Hospital and are able to read and speak English.

Ineligibility Criteria:

Breast cancer patients who have received chemotherapy or radiation therapy for breast cancer or other cancers within last three months, or are currently pregnant or nursing.

Recruitment methods and the informed consent process:

Breast cancer patients, who come to Emory Clinic or Emory Hospital and are cared for by Co-PIs, Drs. Wood and Carlson, in the Departments of Surgery and the Winship Cancer Institute, will be informed by their physicians about the clinical trial. If the patients are interested in participating in the study, the informed consent forms will be given to the patients. The patients and their family members will allow time to read and ask questions about the study. If the patients are unable to provide their own consent to participate the study, their legally authorized representative will be contacted. The informed consent form will be given to the representative and allow time to read and ask questions. The patients will only be enrolled in the study after obtaining signed informed consent form from their legally authorized representatives.

If subjects are willing to participate in the study and the consent form is signed, extra samples of fine needle aspiration from a scheduled diagnosis procedure will be collected for the MB study. Additionally, ductal lavage will be performed right before the scheduled operation for removing the breast tumor under general anesthesia according to the procedures described in Experimental Design and Methods.

All study procedures will be conducted in compliance with the protocol, GCP and the applicable regulatory requirements.

Study protocol:

The purpose of this study is to determine whether detection of high levels of survivin, cyclin D 1 and Her-2/neu mRNAs and proteins are able to identify breast cancer cells in fine needle aspirate (FNA) and ductal lavage samples which have a high probability of the presence of breast cancer cells. We will also determine the percentage of the cells that express one, two or three tumor marker genes at different stages of the disease, especially in the samples from patients diagnosed with DCIS.

Fine needle aspiration:

Fine needle aspiration is the easiest and fastest method of obtaining a breast biopsy. It is an FDA approved biopsy procedure and has been used in outpatient clinics routinely as a diagnosis procedure for breast cancer (4). It uses a thin needle on a syringe to draw fluid and/or cellular material from breast tissues.

FNA samples will be collected during a scheduled diagnostic procedure by Dr. Lewis in the outpatient exam rooms in the Winship Cancer Institute at Emory University, Surgical Oncology Suite,

Clinic C, 1365 C Clifton Road, NE, Atlanta, GA. FNA is performed under local anesthesia. Under sterile conditions, a fine hollow needle that is attached to a syringe to extract fluid from a solid lesion is inserted into the breast mass of breast cancer patients. The needle used in this procedure is very small (smaller than those used to draw blood). The procedure takes a few minutes. The FNA samples will be sent to Dr. Yang's research laboratory immediately to determine whether tumor marker-gene expressing cells are present in the FNA samples.

Recovery after the FNA procedure is generally quick and uncomplicated. Most patients are able to resume normal activity almost immediately afterwards. Pain is minimal and can usually be managed with an over-the-counter pain reliever. Complications for procedures are rare, but excessive swelling, redness, and bleeding or other drainage can indicate an infection or abnormal bleeding. The PI and CO-PIs, who usually are the physicians for these patients, should be notified immediately.

Ductal lavage:

Ductal lavage is an FDA-approved and minimally invasive procedure to collect breast ductal epithelial cells for cytopathological analysis(5, 6). This procedure is typically performed in an outpatient exam room in a clinic and takes about 30 minutes. However, for patients undergoing lumpectomy or more extensive surgery, ductal lavage will be performed by Dr. Wood or Dr. Carlson in the operating room of the Emory University Hospital after administration of general anesthesia and before surgery.

In the operating room and under sterile conditions and general anesthesia, a microcatheter (Firstcyte ultraslim dilator, an FDA-approved Class II device from Cytyc Health Corp., MA), will be inserted 0.5 to 1.0 cm into a nipple orifice and 10 ml of sterile saline will be slowly infused. For each patient, two ducts will be lavaged. The effluent fluid from each duct will be collected and placed in separate vials that are labeled with a specific study ID number for each patient. <u>The samples will be placed on ice and sent to Dr. Yang's research laboratory immediately to determine mRNA and protein levels of three tumor marker genes in the cellular fraction of the duct lavage using a mixture of survivin, cyclin D1 and Her-2/neu MBs, and QD-labeled antibodies.</u> About 13,500 cells per duct can be collected for analysis of the presence of normal, atypical, or malignant breast ductal cells. The procedure for ductal lavage under general anesthesia can be finished within 10 minutes, which will prolong the general anesthesia time for 10 minutes and may slightly increase the risk for the patients.

For each breast cancer patient, we plan to collect three samples for this study: 1) FNA samples from a scheduled diagnostic procedure (if there are available), 2) ductal lavage before surgery, and 3) breast cancer tissues after surgery. After examination of the FNA and ductal lavage samples from breast cancer patients, we should be able to determine which method is more sensitivity for detection of breast cancer cells.

<u>Cellular analysis of tumor marker gene expression using molecular beacons and QD-labeled antibody.</u>

The ductal lavage and FNA samples will be processed immediately after collection to ensure the quality of RNA in the cells. After a brief centrifugation, the cell pellets will be placed on pretreated glass slides using a cytospin. About 10 to 15 cytospin slides will be obtained from one ductal lavage. After fixing the cells in ice-cold acetone, the slides will be incubated with a mixture of survivin, cyclin D1 and Her-2/neu MBs at optimized incubation conditions for 1 hour. <u>After a brief washing, a mixture of QD-labeled anti- survivin, cyclin D1 and Her-2/Neu will be added onto the slides and incubated for 1 hr. The slides will then be counterstained with Hoechst 33342 and then examined under a confocal microscope.</u>

Breast cancer cell lines with known levels of expression of survivin, cyclin D1 and Her-2/neu genes will be used as positive controls. Normal human mammary epithelial cell lines and human primary fibroblasts will also be used as negative controls. Images of cells from patient sample, positive and negative control will be taken using the identical instrumental sittings for confocal mincroscope. The levels of fluorescent intensity for each fluorescent dye will be analyzed on all of the cells observed on the slides. The cells displaying a fluorescent intensity that is two fold-higher than negative control cells will be labeled as positive cells. In comparison with the fluorescence intensity in breast cancer cell lines, we should be able to score the fluorescent intensity in patient's samples from -, +, ++, +++ to ++++. The number of cells showing positive labeling for each MB and QD-antibody, and the number of cells have one, two or three gene expression will also be recorded. We will further compare results obtained from FNA and ductal lavage from the same patients to determine the specificity and sensitivity of detecting cancer cells.

Since the levels of mRNA and protein for three tumor markers are labeled with different fluorescent dyes, the number of the cells overexpressing survivin, cyclin D 1, Her-2/neu, two of the genes or all three genes in ductal lavage and FNA samples will be determined. We will also compare results obtained from FNA and ductal lavage to determine the specificity and sensitivity of detecting cancer cells. Since FNA samples may contain more cancer cells, this will also provide with us an additional source to evaluate the specificity of the MB-detection. Upon finishing the examination, the same slides will be stained with H&E and analyzed by a cytopathologist (Dr. Lewis) for the presence of benign, atypical or malignant cells. We will then compare the results of the MB detection with cytological findings and pathological diagnosis after surgery to determine whether MB method is more sensitive and specific for the detection of breast cancer cells than current cytology methods. We will also determine if the MB-detection of the cells expressing survivin, cyclin D 1 and Her-2/neu genes is able to identify cancer cells at DCIS stage. For each patient, we will obtain breast cancer and surrounding normal tissues after surgery and examine for the expression of survivin, cyclin D 1 and Her-2/neu on frozen tissue sections by immuostaining with specific antibodies.

Current method for identification of different cell types in ductal lavage and FNA samples is by morphological classification. Development of molecular approaches for the detection of cells at different stages of the tumorigenesis will enhance the specificity and sensitivity for early detection of breast cancer cells. Since each MB targeting a specific mRNA or QD-labeled antibody detecting a specific protein is labeled with a specific emission wavelength, a major advantage of the our approach as compared with RT-PCR and immunohistochemistry for early cancer detection is that a mixture of MBs targeting multiple tumor specific mRNAs and QD-antibodies can be delivered to a single cell at the same time, and the expression of all these markers can be observed in a single assay using a fluorescence microscope.

Adverse effect /IND safety report:

Fine needle biopsy is a minimally invasive procedure that has been used in clinic routinely for diagnosis of breast cancer. Ductal lavage is also a minimally invasive procedure and has been used on over 500 high risk women and no serious adverse effects were found (6).

For breast cancer patients, ductal lavage will be collected during the surgery and fine needle aspirates will be obtained from scheduled biopsy. Participation in the study will not add to significant risks or discomforts to the patients. It is possible that ductal lavage prolongs anesthesia time of the patients for 10 minutes, which may potentially increase risk for the patients. Close monitoring of the

patients during general anesthesia, as it done routinely, should reduce the risk of any adverse effects related to ductal lavage.

Any adverse effects related to participation in the study will be documented whether or not considered to be related to the study. This definition includes inter-current illnesses and injuries and exacerbation of pre-existing conditions. The following information will be included in the IND safety reports: subject identification number and initials; investigator's name and name of the medical treatment facility/hospital or research facility; subject's date of birth, gender, and ethnicity; date of procedure performed; signs/symptoms and severity; date of onset; date of resolution; relationship to the study; action taken; concomitant medications including dose, route and duration, and date of the last dose.

Unanticipated problems involving risk to volunteers or others, serious adverse events related to participation in the study and all volunteer deaths should be promptly reported by phone (301-619-2165), by e-mail (hsrrb@det.amedd.army.mil), or by facsimile (301-619-7803) to the Army Surgeon General's Human Subjects Research Review Board. A complete written report should follow the initial telephone call. In addition to the methods above, the complete report can be sent to the U.S. Army Medical Research and Material Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

In case of adverse effects related to the study, the PI and/or CO-PI will be notified immediately. The PI will refer the patients to Army Hospitals in Georgia, to receive treatment free of charge for injuries directly caused by the study. The primary physician and the PI of the study will follow up the patients until they are fully recovered. The Amy will not pay for transportation to and from the hospital or clinic If the patients paid our-of pocket expenses for medical care elsewhere for injuries caused by this study, they will need to contact the PI. All questions concerning the medical care will be addressed by the PI and CO-PIs. If there are issues cannot be resolved, the U. S. Army Medical Research and Material Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at 301-619-7663/2221 will be contacted.

Proposed patient sample size:

The purpose of this protocol is to carry out a pilot study for the determination of sensitivity and specificity of detection of breast cancer cells using the molecular beacon approach. We plan to use a minimum sample size that will give us information on the feasibility of detection of breast cancer cells in clinical samples. We plan to analyze samples from 50 breast cancer patients, which include at least 25 patients with DCIS. However, we will use statistical analysis such as power calculations based on results from our pilot study, which will be obtained after we start the trial, to determine the final sample size. The informed consent forms will be signed by the cancer patients before performing any procedures.

A total of 50 breast cancer patients will be enrolled in this study.

Statistical methods for data analysis

Statistical Power Assessment:

The statistical power analysis will be conducted using the Cox regression analysis to determine whether one or the combination of three tumor markers is better diagnostic markers for detecting breast cancer cells.

Sensitivity and specificity of detection of cancer cells using MBs and QD-labeled antibodies in the patient's sample will be analyzed using standard statistical analyses such as student's t-test or ANOVA analysis. Differences in the prevalence of detecting cancer cells in ductal lavage or FNA from breast cancer patients by the MB detection, QD-detection or the combination of MB and QD, and by cytology will be calculated by multi-variance ANOVA test. The prevalence of identifying tumor cells by MB- and QDS-labeled antibody detection of the cells expressing each marker alone, or more than one marker will be calculated by Chi-square test or Kruskal-Wallis one-way analysis of variance on ranks. A P-value of < 0.05 will be considered statistically significant.

Data Handling:

All patients and normal control subjects enrolled in the study will be assigned an ID number by the PI. The clinical samples will be collected by Dr. Wood, Dr. Carlson, Dr. Styblo and Dr. Lewis, who are co-investigators of the protocol. All samples will be placed in sterile tubes pre-labeled with the patient's ID number and sent to Dr. Yang's research laboratory immediately. Only the PI and CO-PIs will have an access to the names and hospital record numbers of the participating breast cancer patients. The research staff in the lab will only be given the ID number of each sample. All research results will be recorded using the ID numbers. The original experimental records and CDs for storing the image files will be kept in Dr. Yang's laboratory and office in locked cabinets located in the Clinic C Building, Room C-4088 and C-4038, 1365 C Clifton Road NE, Atlanta Georgia. Information on study ID numbers, patient's names and hospital numbers will only be kept in Dr. Yang's computer in her office and with a copy of CD disk locked in the cabinet. All research results will be stored and kept in Dr. Yang's office for three years or until the study and all follow-up studies are finished. The paper records concerning the patient's information, hospital number and study ID number will be shredded using a paper shredder. The files in computer hard driver and on CDs will be deleted.

The PI will inform the CO-PIs about the experimental results from all participants. Since the results are non-CLIA approved research results, the participants will not be informed about the test results.

People other than those doing the study may look at both medical charts and study records. Agencies that make rules and policy about how research is done have the right to review these records. These include the Department of Defense, the National Cancer Institute, the Emory Clinic, Emory University Hospital and the Emory University Institutional Review Board. Records can also be opened by court order. We will use a study ID number rather than patient's name on study records where we can. Patients' name and other facts that might point to the patient or normal subjects will not appear when the results of this study are presented or published.

Modification of the study protocol:

Any change in the protocol, personal, and numbers of enrollment will be submitted to Emory IRB and the HSRRB of the DOD for an approval.

Requests for termination or extension of the protocol will also be submitted to Emory IRB and the the HSRRB of the DOD for an approval.

In the events of a departure from the protocol, a subject withdraws from the protocol and

termination of a participant by the PI and/or CO-PIs, the PI will be notified and reasons for subject withdraw or termination will be recorded. The PI will report all information on the continuation application form to Emory IRB each year and in the annual report to DOD Breast Cancer Research Program.

The subject's participation may be terminated by the PI and Co-PIs if she becomes pregnant before the procedure, has received chemotherapy or radiotherapy, or have developed other illnesses that are inappropriate for conducting ductal lavage or FNA procedure.

Timeline for the study

We plan to start this sub-project in October 2006 and finish targeted enrollment by July 31 2007.

Appendix A

Qualification of the PI and Co-PIs for the study

Principal investigator: **Lily Yang**, MD. PhD, is an Assistant Professor in the Department of Surgery and the Winship Cancer Institute. Dr. Yang has been working in the field of cancer research since 1986 and is trained in both cellular and molecular biology. Her PhD. study involved the cellular origin of liver cancer and identification of liver stem cells. She worked on projects for the development of new approaches for cancer gene therapy including preclinical studies on the delivery of angiogenic inhibitors by adenoviral vectors for the treatment of primary and metastatic breast cancers. Dr Yang has been working on targeting survivin as a therapeutic approache for breast cancer since 1999. In May of 2001, she started working on molecular imaging of breast and pancreatic cancer cells using MBs detecting mutant K-ras, survivin, cyclin D1 and Her-2/neu. Many assays and methods for evaluation of the specificity of MBs have been established in her laboratory. She will direct all aspects of the project and is responsible for experimental design, protocol, data analysis and manuscript preparation.

William C. Wood, MD, is the Joseph Brown Whitehead Professor and the Chairman of the Department of Surgery at Emory University. Dr. Wood is a world-leading expert in breast cancer treatment. He will perform the procedures for ductal lavage on breast cancer patients and will also provide us with surgically resected human breast cancer and adjacent normal tissues. He will participate in study design for the detection of cancer cells and perform ductal lavage of breast cancer patients, the high risk population and normal volunteers.

Grant W. Carlson, MD, Dr. Carlson is Professor of Surgical Oncology in the Department of Surgery at Emory University. Dr. Carlson's has expertise in both breast cancer surgery and plastic surgery for breast remolding. Dr. Carlson participated in a multi-center research project on examination of cell types in ductal lavage of high risk women as an indication for tamoxifen risk-reduction therapy and has performed ductal lavage procedure. He will be responsible for performing ductal lavage in breast cancer patients, normal volunteers and women with high risk of developing breast cancer.

Toncred Styblo, MD. Dr. Styblo is a Professor in the Department of Surgery**.** Dr Styblo is a leading expert in treating breast cancer. She has many years of experience operating on breast cancer patients and performed ductal lavage. Dr. Styblo will collect ductal lavage samples from the breast cancer patients.

Melinda M. Lewis, MD., FCAP: Dr. Lewis is an Associate Professor, Director of Cytopathology in the Department of Anatomic Pathology and Director of Fellowship for Cytopathology. She is an expert on breast cytology and is a board-certified cytopathologist for performing FNA and characterization of cell types in FNA and ductal lavage samples. She has been responsible for cytopathology in Breast Cancer Clinic in the Winship Cancer Institute at Emory University and performed FNA daily on the breast cancer patients since 1988. Her research interests focus on the application of immunohistochemical and molecular techniques to cytologic specimens to enhance diagnostic and prognostic information. One of her research projects is on the comparison of immunohistochemistry and fluorescence *in situ* hybridization in the evaluation of Her-2/neu in image-guided breast fine needle aspiration. Dr. Lewis is also involved in a research project on the examination of cell types in ductal lavage of women with high risk of breast cancer for preventive tamoxifen treatment. In the proposed study, Dr. Lewis will be responsible for performing FNA and cytological analysis of cell types in ductal lavage and FNA samples.

Xiang-Hong Peng, MD., Postdoctoral Fellow: Dr. Peng is a medical oncologist with research training in molecular and cellular biology. He will be responsible for characterization of MBs, cellular assays, analysis of ductal lavage and FNA samples and molecular imaging of cancer cells.

Dr. Yang (PI) has filled a patent application, U.S. patent, 60/439,771, on January 13, 2004, entitled "Methods of detecting gene expression in normal and cancerous cells". The application is pending and under reviewing by the patent office.
Co-PIs don't have a conflict of interest for this study.

Appendix B

Medical Mediator

Ruth O'Regan, MB, MRCPI, MB FRK

Dr. Ruth O'Regan will be the medical monitor for this study. Dr. O'Regan is an Assistant Professor of Hematology and Oncology; Director, Translational Breast Cancer Research Program. Dr. O'Regan joins the WCI from Northwestern University in Chicago where she was an Assistant Professor of medicine at Northwestern Hospital specializing in breast cancer. Her areas of research include the evolution of tamoxifen therapy in breast cancer. Dr. O'Regan is studying novel selective estrogen receptor modulators-SERM, an area in which she has studied under one of the world's leading authorities, Dr. V. Craig Jordan at Northwestern. She has received numerous awards including the compassionate care award from the Women's Board of Northwestern Hospital and has several seminal publications on mechanisms of resistance of SERM's among her more than 30 peer-reviewed publications. Dr O'Regan is an Assistant Professor in the Department of Hematology and Oncology in the Winship Cancer Institute at Emory University. She is not under the supervision of the PI and Co-PIs. She doesn't have a conflict of interest for this study. Dr. Ruth O'Regan's biosketch is enclosed.

Appendix C

Case Report Form

Study Title: Early Detection of Breast Cancer Using Molecular Beacon

PI: Dr. Lily Yang,

Co-PI: Dr. William C Wood, Grant W. Carlson and Melinda M. Lewis

Study ID number						
Enrollment groups	**Breast cancer patients**					
Age:	**Race:**					
Physician's name						
Consent date	**Obtained by:**					
FNA date	**Performed by:**					
Ductal lavage date	**Performed by**					
Adverse effects during the procedure						
Post procedure care						
Reported adverse effects after the procedure						
Number of slides obtained						
Results of MB-detection	**Survivin**		**Cyclin D1**		**Her-2/neu**	
Total number of positive cells						
Results of QD-detection	**Survivin**		**Cyclin D1**		**Her-2/neu**	
Total number of positive cells						
Semi-quantitative analysis of levels of mRNA and protein	**MB**	**QD**	**MB**	**QD**	**MB**	**QD**
Number of cells in each level of fluorescence intensity Total number of cells presented	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )	++++( ) +++( ) ++ ( ) + ( ) ( )
Number of cells expressing more than one marker genes Survivin + Cyclin D1 Survivin + Her-2/neu Cyclin D1 + Her-2/neu Survivin + Her-2+ cyclin D1						
Frozen breast cancer and normal tissues collected: yes ( ), No ( )	Immunostaining results for survivin, cyclin D1 and Her-2/neu expression in frozen tissue sections.					

	Survivin ( ), cyclin D1 ( ), Her-2/neu ( )
Pathological diagnosis • Cytopathology from ductal lavage • Cytopathology from FNA • Pathology evaluation of breast cancer tissues	
Treatments received after surgery	
Follow up information ***Breast cancer patients*** Recurrence Prognosis Contact by PI or Co-PIs by telephone Yes ( ), No ( ) If yes, any information.	

BIOGRAPHICAL SKETCH

Give the following information for all ***new*** key personnel.
Copy this page for each person.

NAME	POSITION TITLE
Ruth M. O'Regan	Assistant Professor in Hematology/Oncology

EDUCATION/TRAINING *(Beginning with baccalaureate or other initial professional education, such as nursing, and*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University College, Dublin, Ireland	MB, BcH, BAO (MD)	1982-1988	Medicine
Royal College Physicians, Ireland	MRCPI	1992	Medicine
University College, Dublin	Medical Doctorate	2000	Oncology

A. Professional Experience

7/03-present	**Assistant Professor**	**Emory University, Atlanta, GA**
9/02-6/03	**Assistant Professor**	**Northwestern University, Chicago, IL**
7/99-8/02	**Clinical Instructor**	**Northwestern University, Chicago, IL**
7/98-6/99	**Resident (Internal Medicine PGY3)**	**Northwestern University, Chicago, IL**
7/95-6/98	**Fellow (Hematology/Oncology)**	**Northwestern University, Chicago, IL**
7/94-6/95	**Resident (Internal Medicine PGY3)**	**Medical College Wisconsin, Milwaukee, WI**
7/92-6/94	**Fellow (Oncology)**	**Mater Hospital, Dublin, Ireland**
7/91-6/92	**Resident (Internal Medicine)**	**Meath Hospital, Dublin, Ireland**
7/88-6/91	**Resident (Internal Medicine)**	**Mater Hospital, Dublin, Ireland**

Honors, Awards, and Membership

Compassionate Care Award , Womens' Board of Northwestern Hospital 1997
Coakley Medal from UCD North American Alumni 1999
NSABP Young Clinical Investigator Award 2001
American Society of Clinical Oncology
American Society of Cancer Research
University College Dublin Medical Graduates Association of North America

B. Selected Peer Reviewed Publications

1. **Shah AP, Parmar S,** O'Regan R. **Right atrial and ventricular thrombus infiltrated with osteoblastic osteosarcoma. J Cardiovasc Pharmacol Ther 8;307-311, 2003**
2. **Dardes RC**, O'Regan RM, **Gajdos C, Robinson SP, Bentrem D De Los Reyes A, Jordan VC. Effects of a new clinically relevant antiestrogen (GW5638) related to tamoxifen on breast and endometrial cancer growth in vivo. Clin Cancer Res 6;1995-2001, 2002**
3. O'Regan RM, **Gajdos C, Dardes RC, De los Reyes A, Park WC, Jordan VC. Effects of raloxifene after tamoxifen on breast and endometrial cancer growth.** J Natl Cancer Inst **20;274-83, 2002**

4. **Dardes RC, Bentrem D,** O'Regan RM**, MacGregor-Schafer J, Jordan VC. Effects of the new selective estrogen receptor modulator LY353381.HCL (Arzoxifene) on human endometrial cancer growth in athymic mice**. Clin Cancer Res **7;4149-4155, 2001**
5. MacGregor-Schafer J, Lee ES, Dardes R, **O'Regan RM**, Jordan VC. Analysis of cross-resistance of the selective estrogen receptor modulators arzoxifene (LY353381) and LY 117018 in tamoxifen-stimulated breast cancer xenografts. **Clin Cancer Res** 7;2505-12, 2001
6. **Lee, ES, MacGregor-Schafer, J, Yao, K, England G,** O'Regan RM, **De Los Reyes, A, Jordan VC**. **Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice.** Clin Cancer Res **6:4893-4899, 2000**
7. **MacGregor-Schafer JI, Lee ES,** O'Regan RM**, Jordan VC. Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice.** Clin Cancer Res **6:4373-4380, 2000**
8. **Yao K, Lee ES, Bentrem D, England G, MacGregor-Schafer JI,** O'Regan RM**, Jordan VC. Antitumor action of physiologic estradiol on tamoxifen-stimulated tumors in athymic mice.** Clin Cancer **Res 6:2028-2036, 2000**
9. O'Regan RM**, Cisneros A, England GM, MacGregor JI, Muenzner HD, Assikis V, Bilimoria MM, Piette M, Dragan Y, Pitot HC, Chatterton R, Jordan VC. Growth characteristics of human endometrial cancer transplanted in athymic mice and treated with new antiestrogens, toremifene and ICI 182,780.** J Natl Cancer Inst 90:1552-1558, 1998.
10. Tonetti, DA, **O'Regan RM**, Tanjore S., England G., and Jordan, VC. Antiestrogen-stimulated human endometrial cancer growth; Laboratory and clinical considerations**. J. Ster. Biochem. Mol. Biol. 65:181-189, 1998**.
11. Eustace S, **O'Regan R,** Graham D, Carney D. Primary multifocal Hodgkin's disease confined to the bone. **Skeletal Radiology 24:61-63, 1995**
12. Kaklamani V, **O'Regan RM.** New targeted therapies in breast cancer. Sems in Onc (in press)
13. Gradishar WJ, **O'Regan RM**. Progress in systemic adjuvant therapy of early stage breast cancer. Int J Clin Oncol 8;239-247, 2003
14. Kaklamani VG**, O'Regan RM.** Breast Cancer Prevention: the risks and benefits of drug therapy. Am J Cancer 1:173-178, 2002
15. **O'Regan RM**, Khuri FR. Farnesyl Transferase Inhibitors: The Next Targeted Therapies for Breast Cancer? Endocrine Related Cancer (accepted)
16. **O'Regan RM,** Jordan VC. The evolution of tamoxifen therapy in breast cancer: selective estrogen receptor modulators and down-regulators. **Lancet Oncology** 3;207-14, 2002
17. O'Regan RM, **Jordan VC. Tamoxifen to raloxifene and beyond** Sems in Oncol **28;260-273, 2001**
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C. Research Support

Ongoing Research Support

None

Completed Research Support

Avon Cosmetics Foundation 2000-2001
Avon Cosmetics Foundation 2001-2002

EMORY HEALTHCARE
EMORY HOSPITALS

Consent to Surgical or Medical Treatment

Date: ____/____/____ Time: _____ Room Number _____

Diagnosis: _____ Procedure: _____

1. I understand the following about the procedure described above:
   a. **Nature and purpose of procedure** (*Describe in laymen's terms*): _____
   b. **Material risks of procedure: DEATH, RESPIRATORY ARREST, CARDIAC ARREST, BRAIN DAMAGE, DISFIGURING SCAR, PARAPLEGIA OR QUADRIPLEGIA, PARALYSIS OR PARTIAL PARALYSIS, LOSS OR FUNCTION OF ANY LIMB OR ORGAN, SEVERE LOSS OF BLOOD, ALLERGIC REACTION AND INFECTION.** These are material risks of any surgical procedure.
      Other risks of this procedure are: _____
   c. **Likelihood of success:** ❑ Good ❑ Fair ❑ Poor
      ❑ Unknown because: _____
   d. **Practical alternatives to procedure:** ❑ None
      ❑ Other: _____
   e. **Prognosis if procedure rejected:** ❑ Good ❑ Fair ❑ Poor
      ❑ Unknown because: _____
   f. ❑ **If applicable, DNR Order or DNI/Special Code status suspended unless indicated otherwise:**

2. **Consent:** The procedure identified above has been explained to me and all of my questions have been answered. I acknowledge that no guarantees have been made concerning the outcome of the surgical or medical treatment, and I realize that the practice of medicine and surgery are not an exact science. I hereby consent to the procedure by Dr. _____ and/or any assistants who may be present. I also consent to the administration of anesthesia to be applied by or under the direction and supervision of the Section of Anesthesiology of The Emory Clinic.

3. I realize that, during the procedure, the physician/surgeon may become aware of conditions which were not apparent before the start of the procedure, or may determine that additional or different operations or procedures are necessary or appropriate. I therefore authorize and request that the above named physician/surgeon and/or any assistants who may be present to perform additional or different operations or procedures the physician/surgeon deems necessary or advisable; so long as these additional procedures do not conflict with my stated DNR or DNI/Special Code preferences as indicated above.

4. Any tissue, organ, specimen, member or implant, removed or severed in any operation or procedure, may be retained, preserved, used for scientific or teaching purposes, or disposed of by the Hospital or by the Section of Pathology of The Emory Clinic at the discretion of the Hospital or Section, except for the following:

5. If acceptable to the physician/surgeon, I authorize observers to be present during the surgery or procedure. (❑ Yes ❑ No). I further authorize the physician/surgeon, or his designee, to photograph/videotape me before, during and/or after my surgery or procedure, for the purpose related to my care and treatment and/or for the purpose of medical education (❑ Yes ❑ No).

Witness	Patient Signature
Signature of person obtaining consent	Signature of person authorized to consent for patient
❑ Check if telephone consent given	Relationship to patient

10699 **Over for Blood Transfusion Consent** 34 Consent 009 10/01

For Medical Device Recipients Only

Under Federal law, a hospital must report patient information to a company which manufactures medical devices, under some circumstances, including product recalls. However, the hospital can report your social security number to a manufacturer only with your permission. Your social security number may help the manufacturer identify you in the rare case of a product recall. I ❑ do ❑ do not authorize the hospital to report my social security number to the manufacturer of the medical device I receive.

Additional Authorizations for Surgical or Medical Treatment

The purpose of this section is to authorize repeat identical operations/procedures which have already been explained on the reverse side. If there is any change in the operation or other procedure to be done or in the associated risks, another "Consent to Surgical or Medical Treatment" form is to be completed.

Date	Surgical or Medical Treatment	Patient/Person authorized to consent for patient

Consent for Administration of Blood or Blood Products

Date: ____/____/____ Time: _____

The use of blood and blood products to treat my condition has been explained to me and I have been given an opportunity to ask questions.

I understand those precautions and procedures in selecting donors and in collecting, processing, preserving and administering blood and blood products developed by the American Red Cross, American Association of Blood Banks or the Food and Drug Administration will be followed. This will include testing for hepatitis B surface antigen, hepatitis C, HTLV I and II, and antibodies to HIV (AIDS virus). I further understand that in an emergency situation it may be necessary to administer blood or blood products before all tests have been completed.

I realize that despite all precautions and procedures referred to above, adverse reactions may occur. These reactions include, but are not limited to: fever, chills, allergic reactions, shock and transmission of infection (including, among others, hepatitis infection and AIDS virus infection).

I understand there are no artificial or natural substances which can perform all functions of blood. Failure to transfuse blood or blood products when needed could cause additional medical problems or complicate existing ones. These medical problems or complications could cause serious illness or death.

Most of the time, use of blood from random donors is necessary, but in some cases it may be possible to use one of the following: directed donation (blood from donors I select); autologous donation (blood collected from me before or during surgery); fluid replacement (administration of non-blood volume maintenance fluids). These alternatives have been discussed with me and I hereby consent to receive transfusions of blood or blood products from random donors as deemed advisable by any physician involved in the management of my condition or any complications that may occur.

_____ _____

Witness Patient Signature

_____ _____

Signature of person obtaining consent Signature of person authorized to consent for patient

_____

Relationship to patient

❑ Check if telephone consent given

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EMORY UNIVERSITY AFFILIATED HOSPITALS

ONCOLOGY RESEARCH COOPERATIVE
INFORMED CONSENT FOR CLINICAL RESEARCH

STUDY TITLE:

Early Detection of Breast Cancer Using Molecular Beacons

Subtitle: Examination of the Sensitivity of Using Molecular Beacons Targeting Survivin, Cyclin D1 and Her-2/neu mRNAs to Detect Breast Cancer Cells in Ductal Lavage and Aspirates of Fine Needle Biopsy in Breast Cancer Patients

PRINCIPAL INVESTIGATOR: LILY YANG, MD/PHD
CO-INVESTIGATORS: WILLIAM C. WOOD, MD., GRANT W.CARLSON, MD., MELINDA M. LEWIS, MD , TONCRED M. STYBLO.

You are invited to participate in a research study conducted at Emory University by the above investigators. Your participation in this study is voluntary. You should read the information below, and ask questions about anything you do not understand, before deciding whether or not to participate.

PURPOSE OF THE STUDY

Breast cancer is the most common type of cancer and is one of the leading causes of death among women. However, if breast cancer is detected early, the chances of survival are much better. Mammograms (a special kind of x-ray of the breast) can detect breast cancer early. However, about 20% of women who have mammograms and will not have their early breast cancer detected. Of all patients who have abnormal mammograms, only 10 to 20% are confirmed to be breast cancer by biopsy. Therefore, it is important to develop new ways to diagnose breast cancer at its early stage.

Recently, a new technique has been developed to detect breast cancer cells. This technique uses very small, circular "probes" (called molecular beacons) that specifically look for tumor cells. When these probes are in the cells, they cause a fluorescent signal in breast cancer cells. Thus, cancer cells become "labeled" with bright fluorescent colors that can be distinguished from normal cells. We can identify these labeled cells by looking with a special microscope. Since above probes are used for the determination of the levels of message RNA for tumor marker genes, we

will also use fluorescent semi-conductor nanoparticle (quantum dot)-labeled antibodies to detect the levels of corresponding proteins in the same cellular fraction.

WHAT IS INVOLVED IN THE STUDY?

We are examining the possibility of detecting breast cancer cells using special techniques called ductal lavage or fine needle aspiration.

Ductal lavage is a method to collect cells of breast milk ducts. This procedure involves three steps. First, an anesthetic numbing cream is applied to the nipple area and gentle suction is used to locate the openings of the milk duct on the nipple. Then, a hair-thin catheter is inserted into a milk duct opening and two teaspoons of saline are injected slowly into the duct. Next, the ductal cell fluid is withdrawn through the catheter and collected for cellular analysis with the molecular beacon probes.

Fine needle aspiration is a routine biopsy procedure performed in the outpatient clinic that uses a thin needle on a syringe to draw fluid and/or cellular material from breast tissues. A long, thin needle is inserted through the breast into the area of breast abnormality. The cells collected from this procedure will be sent to research lab.

About Fifty (50) of women with breast cancers will be enrolled in this study to determine the feasibility of detecting breast cancer cells in ductal lavage and fine needle aspiration samples using the molecular beacon technology.

You are invited to take part in this study. Please take your time to make your decision.

During a scheduled fine needle aspiration diagnostic procedure, excess aspirates that are not needed for diagnosis, and otherwise would be discarded, will be saved and used for research**.** No extra tissue will be removed for this research.

Ductal lavage will be performed during scheduled surgery for removing the breast cancer. In the operating room and after administration of general anesthesia, ductal lavage is performed as described above. This procedure will extend your anesthesia time for about 10 minutes.

After removing the breast cancer, if there is excess breast cancer tissue that is not needed for pathological diagnosis, it will be collected and frozen in liquid nitrogen for the examination of the expression of tumor marker genes.

Yes, I am willing to have above procedures performed and specimen donated.
Please initial _____.

HOW LONG WILL I BE IN THE STUDY?

For you, the study will last as long as your scheduled examination, diagnostic and surgical procedures for the treatment of your diseases. Your medical records may be reviewed during the study to determine the sensitivity and specificity of the detection method. Additionally, your prognosis will be followed for three years by examining your medical records and phone calls from the PI or CO-PIs.

WHAT ARE THE RISKS OF THE STUDY?

A recent study on over 500 high risk women conducted at 19 breast cancer centers showed that ductal lavage is a well-tolerated procedure. The majority patients said that the procedure was no more uncomfortable than a mammogram and some felt the sensation as breast fullness. There was no serious side effect found in this study.

Ductal lavage will be collected during the surgery and fine needle aspirates will be obtained from scheduled biopsy. Participation in the study will not add to any discomforts or significant risk to the patients. It is possible that prolongation of general anesthesia for 10 minutes during ductal lavage may have a minimal healthy risk. However, you will be carefully monitored by surgical and anesthetic staff during the ductal lavage procedure to reduce any healthy risk.

WHAT THE PRECAUTIONS TO BE OBERVED BY THE PARTICIPATE BEFORE AND AFTER STUDY PROCEDURES

Please inform your physician if you have fever or feel pain in your breast before or after the fine needle biopsy. Please also contact your physician if you feel swelling in you breast or notice any discharges from your breast nipple after the procedure.
Your ductal lavage procedure will be performed before your surgery to remove the tumor. Your physician will inform you about all precautions for preparation of the surgery. All routine post operation care procedures will be followed and your physician will follow-up on your recovery after the surgery.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

You should not expect your condition to improve as a result of participating in this research. However, your participation in this study may help us learn more about the diagnosis of breast cancers and help doctors better identify and care for breast cancer in the future.

WHAT OTHER OPTIONS ARE THERE?

You have the right to refuse to participate in this study. Choosing not to take part will not result in any penalty or loss of benefits to which you are entitled.

Your participation may be terminated by your physician or principal investigator if you are no longer meet the inclusive criteria for the study, such as becoming pregnant or starting chemotherapy before the procedures are performed.

WHAT ABOUT CONFIDENTIALITY?

All participants of this study will be assigned a Study ID number. We will use the study number rather than your name on study records where we can. Your name and other facts that might point to you will not appear when we present this study or publish its results.
People other than those doing the study may look at both medical charts and study records. Agencies that make rules and policy about how research is done have the right to review these records. The Department of Defense has the Congressionally Directed Medical Research Programs (CDMRP) funding research on the diagnosis and treatment of breast cancer. This study is sponsored by this program. Those with the right to look at your study records are, the Department of Defense, the National Cancer Institute, the Emory Clinic, Emory University Hospital and the

Emory University Institutional Review Board. Records can also be opened by court order. We will keep your records private to the extent allowed by law. We will do this even if outside review occurs.

WHAT ARE THE COSTS?

There is no added cost to you for taking part in this study.

Other than medical care that may be provided specially stated in the consent form, there is no other compensation available for your participation in this research.

If you are hurt or get sick because of this research study, you need to notify your physician immediately. The PI and CO-PIs will make appropriate arrangement for you to receive medical care at an Army hospital or clinic free of charge. You will only be treated for injuries that are directly caused by the research study. The Army will not pay for your transportation to and from the hospital or clinic.

If you have questions about this medical care, talk to the principal investigator for this study, (Lily Yang, 404-778-4269). If you pay out-of-pocket for medical care elsewhere for injuries caused by this research study, contact the principal investigator. If the issue cannot be resolved, contact the U. S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at (301) 619-7663/2221.

Emory University has not set aside funds to pay for your care or to compensate you if you are injured.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled.

This is a feasibility study for the detection of breast cancer cells using molecular beacon. Since these results have not been validated and accepted as reportable results, the test results will not be provided to you.

During this study you will be asked to provide fine needle biopsy, ductal lavage and breast cancer tissue samples. There samples will be used for detection of breast cancer cells using molecular beacon and examination of tumor marker gene expression in breast cancer cells. They may be used for purpose that currently unknown. There is a chance that the samples that you are donating under this study may be used in other research studies and may have some commercial value. Should your donated samples lead to the development of a commercial product, Emory University will own it and may take action to patent and license the product. Emory University does not intend to provide you with any compensation for your participation in this study nor for any future value that the sample you have given may be found to have. You will not receive any notice of future uses of your samples.

WHAT IF NEW INFORMATION ABOUT THIS TREATMENT IS LEARNED?

We may learn new things during the study that you may need to know. If so, you will be notified about any new information.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, contact your physician or Dr. Lily Yang, Principal Investigator, at 404-778-4269.

For questions about your rights as a research participant, contact Dr. James W. Keller, Chairman of the Emory University Institutional Review Board (IRB) at (404) 727-5646. The IRB is a group of people who review the research to protect your rights.

WHO CAN BE INCLUDED IN THIS STUDY?

Patients of all ages and races diagnosed with breast cancers or with possibility of breast cancer, who come to Emory Clinic or Emory Hospital.

WHO SHOULD BE EXCLUSED FROM THIS STUDY?

Breast cancer patients who have received chemotherapy or radiation therapy within last three months.

Breast cancer patients who are currently pregnant or nursing.

Brest cancer patients who can not speak and read English.

CONSENT

If you agree to participate in this study, please sign below.
You are entitled to have a copy of the consent, regardless if you sign the document.

Please type your name_____

Signature _____ _____ _____
Participant Date Time

Or

Please type your name_____

Signature _____ _____ _____
Legally authorized representative Date Time

Permanent Address of the Participate

Street_____, Apt_____

City_____, State_____, Zip_____

Please type your name_____

_____ _____ _____
Person obtaining consent Date Time

_____ _____ _____
Principal Investigator (if different from above) Date Time

SIGNATURE OF WITNESS:

My signature as witness certifies that the subject signed this consent from in my presence as her voluntary act and deed.

_____
Name of Witness

_____ _____
Signature of Witness Date (Same as subject's)

Date of Approval:

Expiration Date:

INFORMED CONSENT ADDENDUM

FOR

TISSUE STORAGE/FUTURE RESEARCH

As a participant in the study on "Early Detection of Breast Cancer Using Molecular Beacons", I voluntarily donate ductal lavage, fine needle aspirates and breast cancer tissue samples to Emory University. These samples will be used for examination of the presence of tumor markers using molecular beacons and quantum dot-labeled antibodies, types of tumor marker expression in breast cancer cells and studies on early detection of breast cancer. There is a possibility that these samples that I am donating under this study may be used in other research studies and may have some commercial value. Should my donated sample(s) lead to the development of a commercial product, Emory University will own it and it is possible that it will be patented and licensed by Emory University. Emory University does not intend to provide me with any compensation for my participation in this study and will not give me any notice of future uses of my sample(s).

I am informed that my identity will be confidential and a specific ID number will be used to identify my tissue. The link between that number and my name will be carefully guarded. My tissue samples will be used only for research in Dr. Lily Yang's lab, and will not be sold. If I decide now that my tissue can be kept for research, I can change your mind at any time. Just contact Dr. Lily Yang at 404-778-4269 or Lyang02@emory.edu, and let her know that I do not want her to use my tissue. She can link my name to my specimen. My donated samples can be destroyed at anytime. I may agree to participate in the research protocol, but refuse to provide the additional samples discussed above.

Am I willing to donate my left over tissue for possible future research of early detection of breast cancer?

Yes_____ No_____

After making your choice, please sign below.

_____ _____ _____

Participant Date Time

_____ _____ _____
Person obtaining consent Date Time

_____ _____
Principal Investigator (if different from above) Date