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, doctaxel 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 hybridizes 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).
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 beacon targeting survivin or cyclin D1 mRNA were unique for each gene. These include (a) survivin MB-FITC: 5'-FITC-TGGCTCTTGAGAAAGGCGAGCCA-Dabcyl-3', (b) survivin MB-Cy3: 5'-Cy3-CTGGAGAAAGGCCACGCTCGACTG-Dabcyl-3', and (c) Cyclin D1 MB-Texas Red: 5'-Texas-Red-TGGAGTTGCTCGAGAGACATCGACACTCA-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-CGAGTCCTCCACGACATCACCTGAC-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'-CTCTGCTGGACGAGCTTTCTCAAGGACACCGCATCTCATTACAAAGACTAC-3', (b) cyclin D1 target: 5'-AGACGCTGCTGATCCTACACGACGCAATCCATCCGGC-3', (c) HER-2/neu gene target: 5'-AGTGGTAGCAGGACAGCATAGAAGCTGCGGCTCC-3', and (d) K-ras gene: 5'-GTAAGGTGCTGGTAGGCTAGGCAAGATTGTCCAGGATACGTCATTAG-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 FL6000 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 mRNA were designed as follows: forward 5'-TCCAAGCTGGCAACTGAGAC-3' and reverse 5'-TGGCTCTCTGAGCTAC-3'. The specificity of the molecular beacons in solution was determined using synthesized oligonucleotide targets (Sigma Genosys, Woodlands, TX). These include (a) survivin target: 5'-CTCTGCTGGACGAGCTTTCTCAAGGACACCGCATCTCATTACAAAGACTAC-3', (b) cyclin D1 target: 5'-AGACGCTGCTGATCCTACACGACGCAATCCATCCGGC-3', (c) HER-2/neu gene target: 5'-AGTGGTAGCAGGACAGCATAGAAGCTGCGGCTCC-3', and (d) K-ras gene: 5'-GTAAGGTGCTGGTAGGCTAGGCAAGATTGTCCAGGATACGTCATTAG-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 FL6000 fluorometer, Winooski, VT).

**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-6-FAM 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 transfected with an adenosine vector expressing a wild-type p53 gene (Adp53, Qbiogene, Carlsbad, CA) or control adenosine 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 control adenovirus (Adcmv) for 6 hours. The cell cultures 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. 1A. 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. 1B).

**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. 2A). 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. 2A). The results of examination of fluorescence...
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. 2A-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. 2A-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. 2A-D). Our results show...
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. 3A). 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 metastatic 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. 3A 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

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.
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. 3A).

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. 3C). 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. 3C). 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. 4A). We further used FACScan analysis to determine the mean fluorescence intensity in each cell population. Consistent with our observation with the fluorescence microscope, we detected higher levels of fluorescent signal in EGF-treated cells compared with the untreated group in breast cancer cells (Fig. 4B). 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 metastases (red 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. 4C). 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. 4D).

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](https://cancerres.aacrjournals.org/article/65/5/1914/)

**A.** Survivin MB

**B.** Survivin MB

**C.** Survivin MB

**D.** Survivin MB

**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 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–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. 5A). 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. 5A).

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. 5B). 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. 5B, 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. SC).

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 transfact 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
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

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.
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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