INTRODUCTION
Pancreatic cancer is the fourth leading cause of cancer death in the US because of its extremely poor prognosis [1]. A recent American Cancer Society report has predicted that in year 2002 about 29,200 Americans will be diagnosed with pancreatic cancer and 28,900 of them will die of this disease [2]. Current clinical diagnostic procedures such as CT-scan and endoscopic retrograde cholangiopancreatography (ERCP) have a low sensitivity in detecting pancreatic tumors less than 2 cm in size [3]. Over 90% of the patients with pancreatic cancer have already undergone metastasis at the time of diagnosis; the 5-year overall survival rate is only 3-5% [4]. Therefore, it is extremely important to have early detection of pancreatic cancer, possibly based on molecular markers rather than the size of the tumor.

Cancer cells are developed due to genetic alterations in oncogenes and tumor suppressor genes that provide growth advantage and metastatic potential to cells. A novel way of achieving early detection of cancer is to detect mRNA transcripts of cancer-causing genes in living cells. Here we report the development of a molecular beacon-based approach for the early detection of pancreatic cancer. We demonstrated that molecular beacons could detect cancer cells with higher sensitivity and specificity. Our long-term goal is to develop a simple clinical procedure for early cancer detection.

MATERIALS AND METHODS
Molecular Beacons
As shown schematically in Figure 1, molecular beacons are dual labeled antisense oligonucleotides (ODNs) with a fluorophore at one end and a quencher at the other; they are designed to form a stem-loop hairpin structure in the absence of a complementary target so that fluorescence of the fluorophore is quenched (dark) [5]. Hybridization with the target mRNA changes the hairpin structure to an open conformation (Fig. 1), leading to a detectable fluorescence signal (bright) when the beacon is properly excited by light. When molecular beacons designed to target tumor markers are delivered into cells in a sample, cancer cells can be identified using fluorescence imaging.

Molecular Markers of Pancreatic Cancer
Two tumor markers of pancreatic cancer were targeted: K-ras mutations and survivin. A member of the G-protein family, K-ras is involved in transducing growth-promoting signals. Point mutations of K-ras have been found in 80-100% of pancreatic adenocarcinomas, suggesting that mutant K-ras is a sensitive marker for pancreatic cancer detection [6-8]. Further, K-ras mutations occur almost exclusively in three hot spots (codons 12, 13 and 61) [6], most of them are concentrated at codon 12. Recently, there is increasing evidence to suggest that survivin, one of the inhibitor of apoptosis proteins (IAPs), is a good tumor marker. Survivin is normally expressed during fetal development but not in normal adult tissues. However, high levels of survivin expression have been detected in many human cancers [9]. In particular, a recent study has demonstrated the presence of survivin in 77% of pancreatic duct cell adenocarcinomas [10], suggesting that survivin is a good molecular marker for detecting pancreatic cancer.

Cancer Cell Detection
Pancreatic cancer cell lines Panc-1, MiaPaCa-2 and BXPC-3 were used for studying cancer cell detection, and a normal human dermal fibroblast cell line (HDF) was used as a control. These cells were cultured in chamber slides and fixed with ice-cold acetone for 5 minutes. The fixed cells were then incubated with 200 nM of molecular beacons designed to target K-ras condon 12 GGT to GAT mutation or survivin mRNA (Table 1). All the delivery and hybridization assays were carried out at 37 °C in a PBS buffer.

EARLY DETECTION OF PANCREATIC CANCER USING MOLECULAR BEACONS
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expressing Miapaca-2 cells after mixing Miapaca-2 cells and survivin beacons targeting survivin were able to detect single survivin large number of survivin negative cells. We found that molecular survivin expressing cells in a mixed cell population that contained a double positive for both K

demonstrated by the purple color, indicating that they are Panc-1 cells nuclei were found to also show fluorescence emitted by Cy3 (red), as targeting K
identified by the blue nuclei. After incubation with molecular beacons cells that did not have nuclear labeling. Panc-1 cells could thus be

proteins in different cell lines quantified by Western blotting.

RESULTS
Detection of K-ras Mutations
To determine whether molecular beacons can detect a specific mutation (e.g., Codon 12 from GGT to GAT) of K-ras mRNA, we designed and synthesized molecular beacons specific for targeting GGT to GAT transition at codon 12 of K-ras. The specificity of the molecular beacons in detecting this mutation was evaluated using three different cell lines: (1) pancreatic cancer cell line Panc-1, which contains the same K-ras mutation, GGT to GAT; (2) Miapaca-2 cells containing a different K-ras codon 12 mutation, GGT to TGT; and (3) BXPC-3 cells expressing the wild type K-ras gene. We found that after incubation with molecular beacons, strong red fluorescence was observed only in Panc-1 cells that have a GGT to GAT mutation, but not in Miapaca-2 or BXPC cells. This demonstrated that the molecular beacons have a high specificity in detecting point mutations and in distinguishing different point mutations.

Detection of Survivin
We further examined the specificity of molecular beacons for detecting survivin mRNA in pancreatic cancer cell lines Miapaca-2 and BXPC-3 expressing different levels of survivin gene and in normal human fibroblast cells as a control. The probe sequence of the survivin-targeting molecular beacon is shown in Table 1. After incubation of survivin-targeting molecular beacons with fixed cells at 37°C for 30 to 120 minutes, the cells were washed with PBS and observed under a fluorescence microscope. We found that tumor cells displayed from intermediate (in BXPC-3 cells) to strong (in Miapaca-2 cells) red fluorescence signal whereas in normal cell line HDF, only background fluorescence was observed. Furthermore, the intensity of the fluorescence signal correlated well with the amount of survivin proteins in different cell lines quantified by Western blotting.

Detection Sensitivity
To determine whether the molecular beacon based methodology is sensitive enough to identity a small number of tumor cells in a pool of a large amount of normal cells, we mixed Panc-1 cells that contained K-ras codon 12 GGT to GAT mutation and BXPC-3 cells that have no K-ras mutation with a 1:10,000 ratio. The nuclei of Panc-1 cells were labeled with Hoechst 33342 before mixing with BXPC-3 cells that did not have nuclear labeling. Panc-1 cells could thus be identified by the blue nuclei. After incubation with molecular beacons targeting K-ras codon 12 mutation GGT to GAT, cells with blue nuclei were found to also show fluorescence emitted by Cy3 (red), as demonstrated by the purple color, indicating that they are Panc-1 cells (double positive for both K-ras mutation and Hoechst 33342). In contrast, no expression of mutant K-ras mRNA was detected in BXPC-3 cells. We further examined the feasibility of detecting survivin expressing cells in a mixed cell population that contained a large number of survivin negative cells. We found that molecular beacons targeting survivin were able to detect single survivin expressing Miapaca-2 cells after mixing Miapaca-2 cells and survivin –negative HDF cells with 1:100,000 ratio. The Miapaca-2 cell showed purple color owing to the staining with Hoechst 33342 (Blue) and the fluorescence (red) of the survivin-targeting molecular beacons.

CONCLUSIONS
In summary, our results have demonstrated that: 1) molecular beacons can be delivered into viable or fixed cells with a very high efficiency; 2) survivin expressing tumor cells can be detected by molecular beacons with high specificity, and the level of the fluorescence intensity in these cells correlated well with the amount of survivin proteins in cells; 3) tumor cells expressing specific mutant mRNA of K-ras can be detected by molecular beacons; and 4) a small number of survivin-expressing or mutant K-ras containing cells can be identified from a pool of a large number of negative control cells by the corresponding molecular beacons. These results have shown compelling evidence that with proper design and synthesis, molecular beacons have the potential to detect cancer cells with high specificity and sensitivity. We are currently conducting studies to detect pancreatic cancer cells in clinical samples using molecular beacons and to compare the results with that of cytology-based cancer diagnosis.

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REFERENCES