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Genomic Biomarkers for Molecular Imaging: Predicting the future

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“Our nation always has been about the urge to dream and the will to accomplish them” N. Tyson.

A. Introduction

The theme of this issue of the seminars in Nuclear Medicine is “The hybrid imaging.” PET/CT, SPECT/CT, PET/MRI continue to contribute to daily health care issues and play a pivotal role in molecular imaging. Often, these tools provide physicians with excellent anatomic information; however, they fail to detect the subtle molecular changes that signal the imminent onset of serious illnesses. One of the most challenging and formidable illness of these is cancer. In 2006, 11 million new cases of cancer were diagnosed and 7.6 million deaths were attributed to cancer worldwide (1). In the USA alone, in the same year, 1.4 million new cases and 565,000 deaths were reported (2). This death toll is equal to more than one every minute of a day, through a year, every year.

Over the years great strides have been made to combat cancer. These have been able to prolong survival, but more often than not, at the expense of poor quality of life. If cancer is detected early, it can be treated effectively and the life of those victimized by it could be extended with quality.

How can cancer be detected early and accurately by those imaging tools? Cancer is a disease of the cell. Life begins with a single cell and it multiplies into an estimated 75 trillion cells by
adulthood. In order to sustain life, cells go through enormously complex chemistry all the time through out their life. Advances in genomics and proteomics unravel that complexity everyday and shed new light on many parameters that adversely influence the cell phenotype and genotype. One of the tasks they must perform is to deal effectively with many forms of different stress, toxins and carcinogens they encounter on a daily basis. Researchers believe that while encountering such constant threats, cells, suffer serious modulations in their chemical network. This leads to DNA damages that create chaotic chemical processes and initiate abnormal genomic and molecular pathways into the cell nucleus, cytoplasm, and their surface. In turn, these processes promote rapid and uncontrolled cell proliferation and are responsible for more than 80% of cancers. Molecular biologists have carefully identified these products, the biomarkers, as fingerprints that are specific and characteristic of a given cancer. Estrogen positive breast cancer, for example, more predominantly expresses CCND1, and estrogen negative cancer, the HER2 proto-oncogene (3.4). More than 100 such specific oncogenes have been identified for various cancer types. CCND1 encodes cyclin D1 protein and the HER2 oncogene which is also known as ERBB2/neu, Her2 (5).

One of the primary goals in cancer research during the molecular biology era remains to better understand the genetic basis of cancer and to explain its proliferative nature. Such investigations in years to come will unfold the deeper basis of oncogenesis and will continue to provide a greater array of biomolecules of cancer subsets. More importantly, these biomarkers make themselves available for targeting significantly sooner than the characteristic phenotypic changes that the cell undergoes later. Today, the histologic confirmation of cell morphology serves as a gold standard for cancer diagnosis. Unfortunately, by this time, the cancer is fully blown and even metastasized.
This article will briefly describe how investigators in academia and industry, in the near future can harness the power of this knowledge of specific biomarkers, for early diagnosis of cancer, its stratification for treatment and to monitor the effectiveness of that treatment, all noninvasively. Instead of blasting tumors with radiation, chemical agents or surgical excision, all of which destroy plenty of healthy tissue along with tumors, the future drugs will interface only with the molecular or genetic processes that initiate development cancer said Paul Workman; Director of the CRC center for cancer therapies at the Institute of Cancer Research, Sutton, United Kingdom. (6).

Some scholarly approaches on molecular level, such as treating the cancer with angiogenesis inhibitors (7), starving the cancer with anti-VGEF molecules (8) and to track metastatic lesions using microarray chips (9) have placed diagnostic and therapeutic modalities at major crossroads. However, relatively little has yet been done to explore the use of this knowledge in the management of this formidable disease using scintigraphic/molecular imaging.

**B. Diagnostic Applications**

**B1. Mutant, Surface Biomarkers**

Using the biomarkers overexpressed on malignant cell surface, we have made some progress in diagnosis and therapy. Examples of these are illustrated in Table I.

**Table 1. Mutant cell surface as Targets**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Product</th>
<th>Cancer Type</th>
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<tbody>
<tr>
<td>Somatostatin</td>
<td>Octreotide (In-111,Lu-177, Y-90, Ga-68)</td>
<td>Endocrine</td>
</tr>
<tr>
<td>CD20</td>
<td>Bexxar (I-131)</td>
<td>B-cell NHL</td>
</tr>
<tr>
<td>CD20</td>
<td>Xevalin (I-131, Y-90)</td>
<td>Indolent NHL</td>
</tr>
<tr>
<td>CEA</td>
<td>ProstaScint</td>
<td>Prostate</td>
</tr>
<tr>
<td>CEA</td>
<td>CEA Scan</td>
<td>Colorectal</td>
</tr>
<tr>
<td>CD15</td>
<td>NeutroSpec</td>
<td>Infection</td>
</tr>
</tbody>
</table>
The most familiar of these are the somatostatin receptors targeted with a variety of specific peptide analogues labeled with In-111 for planar or SPECT (single photo emission tomography) (10), with Ga-68 for PET (position emission tomography) (11), and with Lu-177 or Y-90 for therapeutic applications (12/13).

More ubiquitously, over expressed mutant malignant cell surface receptors of these are VPAC receptors. Designated as VPAC1, VPAC2, and PAC1, these receptors have been targeted with analogues of two primary peptides labeled either with Tc-99m for planar or SPECT imaging (14-19). First, VIP, a 28 amino acid, peptide, isolated from porcine intestine (16). VIP has three Lysine (Lys) residues (at positions 15, 20, and 21), two tyrosine (Tyr) residues (at positions 10 and 22), two Arginine (Arg) residues (at position 12 and 14), and an essential histidine (His) residue at the N terminus and (Asn) amidated C-terminus. All 28 amino acids are required for full biologic activity of VIP (17). Second, Pituitary Adenylate Cyclase Activating Polypeptide (PACAP). This 38-amino acid peptide was isolated from bovine hypothalamus (18). PACAP was found to stimulate accumulation of intracellular and extracellular cAMP in rat anterior pituitary cells (20). Like VIP<sub>28</sub>, PACAP<sub>27</sub> also has an amidated (leu) C-terminal and His at the N-terminal.

The biological actions of VIP and PACAP are mediated by a family of three G protein-coupled receptors, which are designated as VPAC1, VPAC2 and PAC1 (21-24). These gene receptors are located on the plasma membrane of tumor cells (25). Among the tumors on which VPAC1 receptors have been found in high density and high incidence, include cancers of breast, prostate and urinary bladder (100%), colon (96%), pancreas (65%), lung (58%), stomach (54%), and liver
We labeled VIP28 and PACAP27 with Tc-99m (t1/2 6 h, γ=140 keV, 94%) which required modification of the peptide to covalently accommodate a group of additional amino acids to chelate Tc-99m (29-30). The use of 99mTc analogues allowed efficient imaging of human breast cancer (15-19). We also labeled VIP analogues with Cu-64, a 12.7 hr half-lived positron emitting radionuclide. All breast tumors we examined, expressed VPAC receptors in high density (> 10^5 receptors/cell) (Fig. 1) and had several fold greater radioactivity uptake (Fig. 2) than the adjacent ‘normal” tissue. At least in four patients, out of sixteen, with abnormal mammography, and one patient with an occult lesion in the neck, possibly secondary to childhood glioblastoma, that were not detectable by Tc-99m sestaMIBI were imaged with Tc-99m VIP. Examples are given in Fig. 3 and 4. All expressed VPAC1 receptors in high density.

With the hypothesis that the better spatial resolution of PET will permit us to detect smaller lesions at an early stage with high sensitivity and specificity, the peptides were labeled with Cu-64. These analogues not only detected subcutaneously implanted human breast tumor in a athymic nude mice (Fig. 5) but also spontaneously grown mammary tumors, that were not detectable with F-18-FDG (F-18-fluorodeoxyglucose) in MMTV/nue (Mouse Mammary Tumor Virus) (Fig. 6). Similarly, spontaneous PIN IV, but not PIN II, hyperplasia of the prostate in TRAMP (Transgenic Adenocarcinoma of Mouse Prostate) mouse was correctly detectable by this technique but not by F-18-FDG (15) (Fig. 7). It is important to note that 85% of men with PIN-IV hyperplasia develop fully blown prostate cancer within 6 months and the remaining 15% within a few months there after (31/32).

Spontaneously grown tumors in transgenic MMTV mice over express CCND1 oncogene or TRAMP mice over express CCND1 oncogene (see later) and VPAC1 receptors. Depicted by just a few examples, the notion that tumors can be imaged at an early stage with high specificity by
targeting specific oncogene products expressed on malignant cells at the onset of oncogenesis, provides great possibilities and high significance for the enormous opportunities targeting of these biomarkers provide.

B.2. Intracellular Biomarkers

Since their discoveries 25 years ago, more than 100 oncogenes and more than 30 tumor suppressor genes have been identified. It has become increasingly apparent that oncogene expression in individual cells deviates substantially than their normal cohorts. Data are too extensive and are beyond the scope of this article. However, these data provide us with an array of novel biomarkers. A few of these biomarkers that can be targeted with specific probes for early detection of certain cancers are listed in Table 2 (33). Targeting mRNA copies in dividing cells in living animals is exciting as well as challenging.

Table 2: Examples of some oncogenes expressed predominantly on certain tumors.

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Chromosomal location</th>
<th>Tumor</th>
</tr>
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<tbody>
<tr>
<td>Cyclin D1</td>
<td>11q 13</td>
<td>Breast, other</td>
</tr>
<tr>
<td>KRAS</td>
<td>12p 12.1</td>
<td>Pancreas, colon, lung</td>
</tr>
<tr>
<td>ERBB2/Nue</td>
<td>17q 11.2-12</td>
<td>Breast, ovary</td>
</tr>
<tr>
<td>N-RAS</td>
<td>1 p 13.2</td>
<td>Thyroid, melanoma</td>
</tr>
<tr>
<td>C-myc</td>
<td>8 q 24</td>
<td>Carcinomas, lymphomas</td>
</tr>
<tr>
<td>N-myc</td>
<td></td>
<td>Neuroblatoona</td>
</tr>
</tbody>
</table>

Nevertheless, we among a few others, have designed, synthesized and evaluated Tc-99m and Cu-64 labeled specific probes that target specific oncogene mRNAs for SPECT or PET imaging of breast, pancreatic and colorectal cancers (34-42). Our probes consist of a short 12 mer peptide nucleic acid (PNA) to which is attached tetramer cyclic peptide (Cys-Ser-Lys-Cys-CNH2)
specific for IGFR-1 receptors at one end a chelating agent such as DOTA (1,4,7,10-tetraazaclodecane-N\(^1\),-N\(^{11}\), N\(^{111}\), N\(^{1111}\)-tetraacetic acid) at the other, for strong chelation of a radionuclide. The hypothesis is that the tetrapeptide will provide a specific IGFR1 receptor binding, facilitate internalization of the radiolabeled PNA probe, and hybridize with the ample copies of the oncogene mRNA. It is estimated that more than 10\(^6\) IGFR1 receptors are expressed on certain human breast cancer cells, as compared only to a few on normal cells. This provides high specificity for the probe to bind and internalize in malignant cells (34-42). Similarly, approximately 6 x 10\(^3\) cyclinD1 mRNA copies are expressed per MCF7 human breast cancer cell that provide up to 10\(^{12}\) targets per gram of a tumor. Mismatch PNAs were also synthesized to serve as controls. Such constructs, labeled with Cu-64, enabled us to image experimental human breast cancer (Fig 8), and experimental pancreatic cancer (Fig 9) grown in athymic nude mice. The probe also allowed us to image a non palpable, spontaneous breast tumor in MMTV mice (Fig 10) that was not detected with F-18-FDG (43). These examples render a wide scope of applications in humans for detection of cancer at an early stage, leading to better management of this disease.

C. Therapeutic Applications

Since cancer is the molecular process of genomic deregulation, the current therapeutic approaches have been designed to block the division of cells. However, these approaches do not discriminate between healthy and abnormal cells and lead to serious consequences that degrade the quality of patient life. One approach to provide better cell specificity will be to apply antisense technology as described below.

C.1. Background and Hypothesis for antisense Technology
Antisense technology has emerged as an exciting approach for cancer therapy. The approach is based upon the sequence specific binding of an antisense oligonucleotide to target RNA, preventing the oncogene mRNA translation. The specificity of hybridization by Watson-Crick base pairing makes antisense oligonucleotides an attractive therapeutic tool as to selectively modulate the expression of oncogenes involved in the pathogenesis of malignancies. In principle, this approach is also versatile as a variety of oncogenes are known to be overexpressed in different cancers, but not in normal cells.

As a result, a number of clinical trials have been initiated, the first of which being in 1996 (44-48). However, they have been met with some challenges. Three prominent of these are in vivo stability, sequence length, and poor uptake in malignant cells. In all clinical trials, antisense oligonucleotides were injected systemically when their in vivo stability was unknown or questionable.

In our approach, there are 4 important differences. First, we use PNA, which is resistant to nuclease attack. Second, we use a short, 12mer nucleotide sequence for optimum selectivity. Third, we use PNAs to which are covalently attached a cyclic tetrapeptide specific for receptor-mediated endocytosis by IGF1R. These growth factor receptors are overexpressed on the surface of many malignant cells including those of the breast and the prostate. The receptor ligand provides the PNA chimera with the specificity for the malignant cells. Fourth, for our therapy, we inject the PNA chimeras directly into the tumor. Intratumoral injection helps minimize the loss of PNA to the blood and other organs, as opposed to intravenous administration. The intratumoral approach is practically feasible under ultrasound guidance in localized, solid cancers, such as those of the prostate, breast, liver, and lungs. Furthermore, the PNA chimera is not expected to interact significantly with normal cells as these cells may not overexpress
IGF1R. Therefore, once injected they are expected to be highly specific for the malignancy, but not for normal tissue.

We therefore hypothesize that intratumoral administration of a PNA chimera, specific for an oncogene mRNA overexpressed in tumor cells, will down regulate the oncogene mRNA and arrest the growth of the cancer.

C.2. Intratumor Injections of WT4186 PNA specific for CCND1:

In three groups of 4 athymic nude mice, MCF7 human breast cancers were grown to approximately 5 mm in diameter. One group of mice received 2 µg of non-radioactive WT4186 PNA specific for cyclin D1, the oncogene mRNA overexpressed in MCF-7 tumors. Injections were made in 100 µL of normal saline. The mice in the other two groups each received 2 µg of mismatch controls, namely WT4171 or WTF4113. Twenty-four hours later, animals were sacrificed, tumors excised, RNA extracted, and cyclin D1 protein levels were determined by Western blot (Fig. 11 (41)).

Kruskal-Wallis ANOVA analysis determined that the 3 groups were different ($P=0.0308$). When each group was compared against another, the Dunn multiple-comparison method determined that the PNA mismatch chimera, WT4172, and the PNA antisense chimera, WT4185, groups were different ($P=0.02$). The peptide mismatch chimera, WT4113, and the PNA antisense chimera, WT4185, groups were different ($P=0.04$). The PNA mismatch chimera, WT4173, and the peptide mismatch chimera, WT4113, groups, however, were not different ($P=0.39$). The PNA antisense chimera, WT4185, significantly reduced cyclin D1 protein expression in MCF7:IGF1R xenografts by approximately 50%. These data are highly encouraging and provide a novel genetic approach for tumor specific therapy that may spare normal tissues. In prostate
cancer, such a treatment may eliminate incontinence and impotence that most prostate cancer patient suffer from the current mode of therapy.

C.3. Monitoring effectiveness of Therapy

In order to improve therapy regimens, and patient safety, various patient parameters are evaluated ex-vivo using body fluids or in-vivo by external imaging. Periodic estimation of plasma PSA (prostate specific antigen) in prostate cancer patient and the tumor volume or SUV (standard uptake value) assessment by PET imaging are nearly routine methods. However, they are not considered to be fully absolute and totally reliable. Possibilities are strong that periodic longitudinal patient imaging by targeting specific oncogene mRNA will enable physicians to calculate the aggressive upregulation or progressive downregulation of oncogene mRNA copies by a few mouse clicks and to reliably determine the proliferative status of a given tumor following chemo and/or gene therapy. To the referring physicians, such a number will be more meaningful to determine the effectiveness of therapy or to stratify cancer for improved therapeutic intervention. Such practice will not be limited only to oncologic therapy but will also be extended to other gene therapy approaches in which inherited or acquired bad genes are corrected to minimize their consequences. A bad gene will not control mankind’s destiny. Techniques have already been developed to detect a single mRNA molecule (49).

D. Other Possibilities

Intense and growing interest has continued to foster improved understanding of cancer pathogenesis, resulting into an array of biomarkers. Many of these can be applied for diagnosis and treatment of cancer. Such applications combined with Molecular imaging techniques can revolutionize the management of cancer and of cancer patients. Just a couple of examples are given below.
D.1. Tumor Suppressor Gene p-53

The p-53 gene plays a role in normal cell division, DNA repair, detecting inappropriate growth signals in cells and imposing a tumorigenesis barrier in precancerous cells. In cells under stress, its transcripts constantly shuttle between the nucleus and cytoplasm providing the protection to the cell. Under severe stress, p-53 gets phosphorylated and ceases its transcription ability, paving the way for cell malignancy and abnormal cell proliferation. In 50% of tumors, phosphorylated p-53 mutation has been found (50).

In the future, guided by specific p-53 probes, molecular imaging will be able to determine p-53 transcription in a cell and its threshold, and thereby be able to predict the susceptibility for cancer. This will warrant preventive therapy on which biologists are already working.

D.2. Ki-67 protein

Ki-67 protein is expressed in all phases of cell cycle except $G_0$ and is strongly associated with cell proliferation. Studies have shown that Ki-67 index has a significant correlation, high proliferation rates, and disease free survival (51). This proliferation index has become an important factor in breast cancer management (52). To determine the expression of Ki-67 histologic tissues has drawn considerable attention. Yet nothing has been done to target Ki-67 for noninvasive diagnostic imaging. It is highly likely that Ki-67 will play an important role in diagnostic molecular imaging of cancers. Molecular imaging is a rapidly progressing biomedical modality.

Molecular biology is a relatively young science. Targeting the biomarkers already provided by it, appears to extend the current knowledge to new meaningful dimensions to fight human diseases. However, this is only scratching the surface of the iceberg. The possibilities are endless and limited only by imagination. However, molecular imaging is not only about seeking key
biomarkers and smart probes to target them, but it is also about having the equipment to image them.

E. Future Possibilities

Science of understanding the genesis of neoplasm is changing rapidly. Molecular biologists will continue to provide investigators, both in academia and industry, exquisite data about a specific disease as well as about a specific patient. These data will provide selective biomarkers and will enable physicians to ensure better overall patient management strategies with stratifying cancer uniquely for personalized medicine. Personalized medicine will be a major tool in how the drugs will be developed in which specific biomarkers will provide major guidance and molecular imaging will play a pivotal role in their effective applications. In molecular imaging, although development in magnetic resonance and optical imaging, depict a bright future, it appears as if the scintigraphic imaging will continue to lead, particularly when quantification will be the key parameter that will control the decision making process.

What scintigraphic imaging will need is smarter instruments, with higher spatial resolution and greater sensitivity. If microscopes can permit us to see a single cell ex-vivo, a smart imaging tool should allow us to see a cluster of cells in-vivo. Phenomenal progress is being made by innovative scientists in the laboratory. Photo-acousting devices that detect a tumor seated several cm deep in the body with μm resolution (53) or a Raman molecular imaging equipment with a low background that uses quantum dots providing long-term photo stability, not available in fluorescence imaging (54), promise to contribute heavily. Such smart equipment will compliment a smart probe to target a specific biomarker. Together they will permit a true early diagnosis, and will enable the design of a promising therapy that will allow the monitoring of its effectiveness noninvasively.
Molecular imaging will change the practice of medicine in the next generation, more than it has ever been thought possible previously.

Acknowledgement

A part of the content was presented in the 2008 “Cassen Award” presentation at the SNM (The Society of Nuclear Medicine) annual meeting in June 2008 in New Orleans LA. The author is indebted to many of his colleagues, including Eric Wickstrom PhD, and Carol Marcus MD PhD., who have contributed in the work presented. The author also acknowledges the support of NIHCA 10923, CO27175, EB001809, ISIORR23709, and PAME03184.
VPAC1 protein levels in human breast tumor tissues were determined by Western blotting. Lane 9 represents the level of its expression in adjacent normal breast tissue of one patient. All patients (lanes 1-8) had abnormal Tc-99m-VIP analog scan.
Ten µm thick sections of human breast tumor tissues and adjacent “normal” tissues were exposed to Cu-64-TP4200 (a VIP analog) and digital autoradiography was performed. The average Cu-64 uptake in tumors was 5.7 times greater than in the “normal” tissue.
A 20-year-old woman with a history of neurofibroma of the brain in childhood presented with mass in left neck that was evident for one month. Tc-99m MIBI scan (center) was negative. Bone scan (right) showed faint blood pool. However, Tc-99m VIP scan (left) showed unequivocally positive uptake (arrow). Immunohistology of lesion showed that it was a high-grade spindle cell sarcoma.
A 52-year-old woman had left breast mastectomy and silicon implant. Five years later, she was presented with a palpable mass (also by mammography) of the right breast. Tc-99m MIBI scans (lower panel) was normal. The tumor was detectable by Tc-99m-VIP analog. (upper panel arrow).
MCF7 human breast cancer tumors were grown in the right thigh of several immuno suppressed, athymic nude mice. Tumors were clearly visualized by PET imaging at 4hr and 24hr post administration of three biologically active analogs of VIP, labeled with Cu-64.
$^{64}$Cu-TP3805 PET (Coronal)

CT and $^{64}$Cu-TP3805 PET Fusion (Coronal)

FDG PET (Coronal)

Fusion of surface rendered bone from CT, coronal CT slice, coronal PET slice and surface rendered PET

Tumor A
$^{64}$Cu-TP3805 PET (Transverse)

Tumor A
$^{64}$Cu-TP3805 PET – CT Fusion (Transverse)

Tumor B
$^{64}$Cu-TP3805 PET (Transverse)

Tumor B
$^{64}$Cu-TP3805 PET – CT Fusion (Transverse)
Spontaneously grown, non-palpable breast tumors in a MMTV/neu mouse were clearly detectable (A and B, arrows) by PET scanning with Cu-64 TP3805 (VIP analog) but not with F-18-FDG. RT-PCR performed using the excised tumor tissues demonstrated extensive quantity of VPAC1 receptor protein.
Fig. 7

Transaxial PET images of two separate TRAMP mice with prostate cancer, (PIN II, upper panel and PIN IV lower panel.) Images were obtained with F-18-FDG (vertical panel no.2) and Cu-64 TP3939 (vertical panel no.3), a biologically active VIP analog. PIN IV prostate cancer (arrow) was detectable with Cu-64-TP3939 but not with F-18-FDG. F-18-FDG had extensive bladder activity. PIN II tumor was not detectable with either agent.
At 4 hr and 24hr, PET images targeting CCND1 mRNA on MCF-7 human breast cancer grown in athymic nude mice. At both times, points tumor was detectable with specific Cu-64 antisense PNA WT4348 but not so clearly either with mismatch PNAs WT4273, or with ionic Cu-64 chloride.
KRAS oncogene mRNA was targeted in human pancreatic cancer grown in athymic nude mice. Cu-64 labeled specific antisense PNA WT4286 and several mismatch PNAs were used. Tumors (arrow) were clearly detectable by PET imaging with Cu-64-WT4286 and a single mismatch Cu-64-WT4213 but not with any other agent.

In last (vertical) panel, the KRAS specific Cu-64-WT4286 was injected to human breast tumor, BT474, in nude mice. BT474 tumor-cells do not express KRAS mRNA. The tumor was not visualized by PET imaging with Cu-64-WT4286.
Fig. 10

Spontaneously grown breast tumors in MMTV mice over express CCND1 oncogene mRNA. Tumors (arrow) were detectable by PET imaging with Cu-64 labeled CCND1 specific probe. (These tumors were not imaged with F-18-FDG).
Western blots of 100 µg of protein extracted from MCF7:IGF1R estrogen receptor-positive breast tumor cell xenografts at 24h after direct injection of the PNA mismatch chimera, WT4172, the peptide mismatch chimera, WT4113, and the PNA antisense chimera, WT4185. CD= cyclin D1; B-actin = β-actin, an experimental control. CCND1-mRNA protein is down regulated by nearly 50% in all mice receiving a specific PNA chimera but not the mismatch ones.
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