shown to be negative for disease by nested RT-PCR, and then transplanted back into the patient (point 16).

Trial patient C presented with very high amounts of V-type PML/RARα mRNA (RDC = 2342%; Fig. 1C, point 1). The patient received ATRA therapy and achieved hematologic remission in 12 weeks (point 5). At this time, PML/RARα transcripts were still detectable by nested RT-PCR, but subsequent analysis by DzyNA RT-PCR showed that expression was very low. The fusion transcript became undetectable by both protocols 12 weeks later (point 6) and remained undetectable for 6 months (points 7 and 8). DzyNA analysis detected very low amounts of fusion transcripts (point 9), which increased in the subsequent sample (point 10). These fusion transcripts had been detected prospectively by nested RT-PCR in this sample (point 10), and the patient was offered ATRA despite the absence of evidence of hematologic relapse. Despite ATRA therapy, fusion transcripts remained high for the next 17 weeks (points 11–14). This patient then received an allogeneic bone marrow transplant, which was successful in inducing hematologic and molecular remission (point 15).

In the three cases above, the relative PML/RARα amounts determined by DzyNA RT-PCR correlated well with the clinical history. The assay detected increases in transcript concentrations 3–6 months before morphologic or cytologic symptoms of relapse. Although the literature reports that most patients achieve clinical remission within 1–2 months of ATRA therapy (10), the rates of clearance of fusion transcripts appear more variable. Patient A took 6 weeks to achieve clinical remission, but it was >9 months before fusion transcripts were undetectable. In contrast, patient B demonstrated rapid clearance of PML/RARα transcripts in as little as 4.5 weeks after treatment. Patient C initially achieved hematologic remission after initial ATRA therapy despite the very high leukemic burden. However, despite prompt re-initiation of therapy on detection of fusion transcripts, the amounts of fusion transcript remained high, indicating resistance to ATRA. Persistence of fusion transcripts should aid the identification of “nonresponders” who would benefit from alternative therapy. In patient B, fusion transcripts were detected at very low amounts in a sample (point 5) between two remission samples, which were negative for fusion transcripts. Ongoing studies will determine whether these “interruptions” of molecular remission are of prognostic significance and/or identify patients who have not completely cleared the malignant clone and may be at greater risk of relapse.

Studies monitoring APL patients by DzyNA RT-PCR will continue to assess the prognostic significance of several factors highlighted by the examples in this study. Potential factors influencing outcome may include the fusion transcript isoform, the relative amounts at diagnosis, and rates of clearance and accumulation, as well as intermittent detection of transcripts during clinical remission. Continuing studies aim to further demonstrate the potential for use of quantitative molecular monitoring to facilitate early prediction of imminent relapse and to investigate the impact on disease-free survival of efforts to optimize the timing of administration of salvage therapy based on molecular information.

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References

Homogeneous Assay for Tyrosine Kinase: Use of Bacteriophage Antibody Conjugates in an Assay for p56
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Protein kinases play a critical role in almost every cellular regulatory process and have been identified as key players in diseases such as cancer and immune syndromes (1). For this reason, there has been substantial interest in the development of assays for protein kinases for use as both diagnostics and drug discovery tools (2). Although several assays exist for kinases, the most commonly used involve monitoring transfer of the γ-phosphoryl group from [γ-32P]ATP to a peptide substrate (3). These assays

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are cumbersome to implement because the unreacted [γ-32P]ATP needs to be separated from the phosphorylated peptide by use of separation techniques such as gel electrophoresis.

In drug discovery programs, fluorescence resonance energy transfer (FRET) kinase assays are becoming increasingly popular because they are homogeneous, making them relatively easy to automate (4). In typical FRET assays, the anti-phosphotyrosine antibody and the substrate peptide are labeled with “donor” and “acceptor” fluorophores. On phosphorylation of the peptide, the anti-phosphotyrosine antibody binds to the peptide, and the two fluorophores are brought in close proximity to each other. Excitation of the acceptor fluorophore leads to energy transfer to the donor molecule, which emits fluorescence. The principal disadvantage of FRET-based assays is that they are difficult to configure because the two fluorophores need to be within a closely defined distance of each other.

The Dual Phage technology is a new ultrasensitive biological amplification system of broad applicability that uses bacteriophages as biological amplification tools (5). The Dual Phage technology uses two types of bacteriophages that encode two selectable markers. The phages are labeled with an interacting pair, e.g., a receptor/agonist or enzyme/substrate combination. When the interaction takes place, the two phages become spatially linked in the complex and can then infect the same bacterial host cell (the “indicator organism”), thus conferring resistance to both selective agents. This dual infection event is markedly enhanced by the close proximity of the two phages in the complex. When the two selectable agents are added to the medium, only the doubly infected indicator organisms survive, and the signal monitored is the growth of these cells.

Several other biological amplification methods have also been devised for the study of molecular interactions. For example, immuno-PCR is a sensitive technique that detects antigens by binding a DNA-tagged antibody to the antigen, amplifying the DNA by PCR, and then detecting the DNA product (6). In immunodetection amplified by T7 RNA polymerase, a double-stranded oligonucleotide containing the T7 promoter is conjugated to an antibody, and then T7 RNA polymerase is used to amplify RNA from the double-stranded oligonucleotides coupled to the antibody in the antibody–antigen complex (7).

Using a version of the Dual Phage technology adapted for enzyme assays (Fig. 1A), we have developed a highly sensitive homogeneous assay for lck kinase (p56lck). p56lck kinase is a membrane-associated nonreceptor tyrosine kinase that is found exclusively in natural killer (TK) cells and T cells (8) that play a critical role in T-cell development and activation. The p56lck kinase is localized to a site on the genome that frequently contains chromosomal abnormalities in lymphomas and neuroblastomas (9). In light of these observations, inhibitors for p56lck kinase could have important applications in the treatment of autoimmune and cancer disease.

The kinase substrate peptide (RRLIEDAEYAARG-biotin; Pierce) was coupled to streptavidin-derivatized M13 (encoding for ampicillin resistance) by standard biotin–streptavidin conjugation techniques (10). Biotinylated anti-phosphotyrosine antibody (Sigma) was coupled to a streptavidin-derivatized M13 bacteriophage encoding for chloramphenicol resistance. These phage conjugates were purified on an affinity column containing anti-M13 antibody (Sigma) bound to agarose. Using the Sigma protein tyrosine kinase assay reagent set (nonradioactive), we determined that each phage carried ∼10–100 ligands.

As illustrated in Fig. 1B, the Dual Phage technology has been successfully applied to the development of a homo-

Fig. 1. Schematic of the dual phage kinase assay (A) and lck kinase dilution curve (B).

(A), synthetic peptide substrate is attached to a phage carrying resistance to chloramphenicol (phage C). The second phage is labeled with an anti-phosphotyrosine antibody and codes for ampicillin resistance (phage A). In the presence of kinase and ATP, the peptide substrate on phage C is phosphorylated, and the anti-phosphotyrosine antibody on phage A binds the newly generated phosphate group, forming the dual-phage complex. Addition of E. coli (the indicator organism) leads to rapid dual infection of the bacterial cells by phages A and C. The growth of viable E. coli cells (in the presence of chloramphenicol and ampicillin) is monitored in real time by use of a redox indicator. (B), serial dilutions of p56lck kinase (Upstate) were prepared in kinase buffer [10 g/L bovine serum albumin, 20 mmol/L HEPES (pH 7.4), 10 mmol/L MgCl₂, 100 μmol/L/L CaCl₂]. A 10-μL aliquot of each dilution was placed in a flat-bottomed black microtiter plate together with 10 μmol/L ATP; 10 μmol of phage C-peptide substrate (10⁵ virions) conjugate was then incubated with the kinase for 30 min at room temperature. Phage A–anti-phosphotyrosine antibody conjugate (10 μL, containing 10⁵ virions) was then added to the reaction and left to incubate for 30 min at room temperature. A 200-μL aliquot of a log-phase culture of E. coli (10⁷ cells) was added and incubated at 37 °C for 5 min; 5 μL (5 μmol/L) of C₁₂-resazurin (Molecular Probes) and 10 μL of ampicillin and chloramphenicol (10 μg of each) were added to the reactions. The plate was covered with a transparent “breathable” plate seal (Nalge Nunc), and the change in fluorescence (excitation/emission at 530/590 nm) per min was recorded over a period of 4 h (Vₘₚₚ) on a plate reader.
hogeneous microtiter plate-based assay for p56\textsuperscript{ck} kinase. In the assay incubation, the optimum number of each phage was 10\textsuperscript{5} virions. Previous optimization experiments had shown that use of a lower phage concentration decreased the signal and increased the detection time, whereas use of a higher concentration led to an increase in the background signal. At the optimum phage concentration, the signal-to-background ratio of the Dual Phage technology was >10:1. In replicate p56\textsuperscript{ck} kinase assays, the CV was 5.1\% (n = 10).

We conclude that the Dual Phage lck kinase assay has a lower detection limit (0.05 pmol/L lck kinase) lower than other homogeneous lck kinase assays. For example, the Packard HTRF lck assay has a lower detection limit of 2 pmol/L (11). The homogeneous nature of the Dual Phage assay makes it ideally suited to both automation and miniaturization. The labeled phages and the indicator organism are extremely robust (no loss of activity has been seen in phage conjugates and freeze-dried Escherichia coli stored at 4°C over a period of 6 months) and can be readily prepared by standard techniques. The flexibility of the Dual Phage technology suggests that the kinase assay can be implemented in many formats, such as microplate, magnetic particle, or microfluidics systems. The output signal from the indicator organism can be adapted to match existing instruments such as fluorometers, colorimeters, or luminometers. Potential uses for this kinase assay include discovery of kinase inhibitors in high-throughput screening campaigns and use in clinical diagnostic assays.

Detection of Circulating Thyroid Cancer Cells by Reverse Transcription-PCR for Thyroid-stimulating Hormone Receptor and Thyroglobulin: The Importance of Primer Selection, Manjula K. Gupta,,2 Leslie Taguba,,3 Rosemary Arciaga,4 Allen Siperstein,1 Charles Faiman,2 Adi Mehta,2 and S. Sethu K. Reddy2 (Departments of 1 Clinical Pathology, 2 Endocrinology, and 3 General Surgery, The Cleveland Clinic Foundation, Cleveland, OH 44195;4 address correspondence to this author at: Department of Clinical Pathology, L-30, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195; fax 216-445-9444, e-mail guptam@ccf.org)

Monitoring for thyroid cancer recurrence is routinely done through measurement of serum thyroglobulin (Tg) and 131I whole-body scanning (WBS) after total thyroidectomy and radioactive iodine ablation (1). Serum Tg has been a useful marker to detect residual or metastatic disease, but its limitations include interassay variability, insufficient sensitivity of some commercial assays, and the frequent presence of interfering anti-Tg antibodies in patient serum (2, 3). Although the ability of serum Tg to detect metastatic disease improves greatly after thyroid hormone withdrawal, hormone withdrawal produces symptomatic hypothyroidism and significant morbidity in many patients.

Sensitive detection of circulating cancer cells by reverse transcription-PCR (RT-PCR) of tumor-specific mRNA appears to be a useful adjunct in monitoring of some other malignancies (4, 5). RT-PCR has been used to detect thyroid cells in circulation by amplifying transcripts of the thyroid tissue-specific Tg gene (6–8), but Tg mRNA can be found normally in circulation (7, 8). Recently, real-time quantitative RT-PCR has been reported to detect small amounts of Tg mRNA in the blood of healthy individuals and to identify thyroid cancer patients with recurrent and residual disease (9–11). Furthermore, Savagner et al. (11) reported that the amount of a Tg mRNA alternative splicing variant closely correlated with the thyroid volume and thyroid-stimulating hormone (TSH) concentration.

Thyroid carcinomas contain functional TSH receptor (TSHR) (12, 13), and differentiated thyroid cancer micro-metastases have been detected by RT-PCR of TSHR and Tg mRNAs (14). TSHR has not been exploited to detect circulating cancer cells, and smaller TSHR transcripts have been detected in human lymphocytes (15).

We investigated the specificity of different PCR primer pairs in the amplification of TSHR and Tg mRNA signals in blood samples from healthy individuals and in thyroid cancer tissue. Selected primer pairs with specificity for thyroid tissue and no reactivity with normal peripheral blood mononuclear cells (PBMCs) were further tested to evaluate the potential for clinical utility on detection of circulating thyroid cells.

Four TSHR primer pairs were tested against a panel of normal PBMC RNA and thyroid cancer tissue RNA. One of these was designed to amplify a segment in exons 9 and 10, starting at nucleotide 873 and ending at nucleotide

References