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Cancer Diagnostics: Review

Real-Time PCR Technology for Cancer Diagnostics

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Background: **Advances in the biological sciences and technology are providing molecular targets for diagnosing and treating cancer. Current classifications in surgical pathology for staging malignancies are based primarily on anatomic features (e.g., tumor-node-metastasis) and histopathology (e.g., grade). Microarrays together with clustering algorithms are revealing a molecular diversity among cancers that promises to form a new taxonomy with prognostic and, more importantly, therapeutic significance. The challenge for pathology will be the development and implementation of these molecular classifications for routine clinical practice.**

Approach: **This article discusses the benefits, challenges, and possibilities for solid-tumor profiling in the clinical laboratory with an emphasis on DNA-based PCR techniques.**

Content: **Molecular markers can be used to provide accurate prognosis and to predict response, resistance, or toxicity to therapy. The diversity of genomic alterations involved in malignancy necessitates a variety of assays for complete tumor profiling. Some new molecular classifications of tumors are based on gene expression, requiring a paradigm shift in specimen processing to preserve the integrity of RNA for analysis. More stable markers (i.e., DNA and protein) are readily handled in the clinical laboratory. Quantitative real-time PCR can determine gene duplications or deletions. Furthermore, melting curve analysis immediately after PCR can identify small mutations, down to single base changes. These techniques are becoming easier and faster and can be multiplexed. Real-time PCR methods are a favorable option for the analysis of cancer markers.**

Summary: **There is a need to translate recent discoveries in oncology research into clinical practice. This requires objective, robust, and cost-effective molecular techniques for clinical trials and, eventually, routine use. Real-time PCR has attractive features for tumor profiling in the clinical laboratory.**

The sequence for most of the human genome is now publicly available and can be applied to understand, characterize, and treat complex diseases such as cancer. The biological differences between tumors that account for variations in morphology and clinical behavior can be analyzed using gene expression microarrays *(1–5)*, comparative genomic hybridization (CGH)[1] *(6–9)*, fluorescence in situ hybridization *(10, 11)*, quantitative PCR *(12)*, and mutation analysis *(13, 14)*. Normal cell regulation can be disrupted by many factors, including viral infections *(15, 16)*, DNA methylation *(17)*, and sequence alterations *(18, 19)*. Cancer develops when these insults affect the function of genes controlling cell division, cell repair, apoptosis, and angiogenesis. Current molecular techniques are providing the tools needed to investigate tumor biology and to discover the genetic and epigenetic causes of cancer.

Microarrays together with clustering analysis have allowed genome-wide expression patterns in biological systems to be deciphered and compared. Hierarchical clustering of microarray data groups together genes that are coordinately expressed under different conditions *(20–22)*. Using microarrays, investigators have developed gene expression-based classifications for many malignancies, including lymphoma *(4)*, leukemia *(3)*, lung carcinoma *(5)*, and both hereditary *(23)* and sporadic *(1, 2)* breast tumors. A unique signature can be found within the genetic programming of each tumor, revealing its molecular history. This allows tumor histology to be molecularly dissected based on the unique expression

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[1] Nonstandard abbreviations: CGH, comparative genome hybridization; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; Cp, crossing point; and topo, topoisomerase.

profile of each cell type in the sample. For example, in breast tumors, the unique gene expression of mammary tumor cells can be distinguished from other cell types within the sample, such as lymphocytes and stromal cells *(1, 24)*. In addition, molecular subtypes within a given histologic classification can often be identified. For example, there are two classes of B-cell chronic lymphocytic leukemia *(3)*, two classes of diffuse large B-cell lymphoma *(4)*, five classes of non-small cell lung tumors (including three types of adenocarcinoma) *(5)*, and at least four molecular classes of invasive ductal cell breast carcinoma *(1, 25)*. Finally, these molecular subtypes are clinically significant because they predict patient outcome and explain variability seen in the natural course of certain tumors with the same anatomic diagnosis *(2–4, 25)*.

Additional information about the nature of a tumor can be gleaned from its associated mutations. A mutation within a particular gene is sometimes reflected by the gene expression profile *(3, 23)*. By far the most common mutations in human malignancy are sporadic mutations in tumor suppressor genes such as *p53*. A *p53* gene mutation is found in >50% of all malignancies and is particularly common in carcinomas of the head and neck, lung, skin, bladder, and colon *(19)*. Mutations in *p53* correlate with aggressive histologic features *(26)*, early invasive potential, and resistance to therapy *(13, 27–29)*. In breast cancer, where the frequency of a *p53* mutation is estimated at 20–30% *(13, 27)*, the detection of a *p53* mutation by nucleic acid analysis is an independent predictor of poor response to tamoxifen, based on multivariate analysis *(13)*. Nucleic acid methods of mutation detection are preferred over immunohistochemistry (IHC) for determining *p53* status. Although *p53* IHC is associated with mutation status in some tumors (e.g., colorectal), the concordance is generally poor *(30, 31)*. IHC is a convenient method to score for molecular markers in surgical pathology, but it is subject to variability from differences in antibody specificity, scoring criteria, and storage *(32, 33)*. Additional advantages of nucleic acid analysis include correlating specific mutations to treatment response and providing a marker for monitoring residual disease *(34, 35)*.

The need for solid-tumor molecular markers in pathology is clear. However, how these new tests will be best implemented is less certain. For example, specimens received in surgical pathology are routinely formalin fixed and paraffin embedded to preserve the architecture of the tissue. This processing makes recovery of mRNA unreliable; thus, expression analysis by microarray or reverse transcription-PCR is not feasible unless there is a paradigm shift in sample procurement for solid tumors. Markers in the form of DNA and protein are typically more stable than RNA, allowing them to be used within the current framework of surgical pathology for determining mutation status and gene expression.

Quantitative Analysis

Lessons learned from HER-2/neu testing

IHC is a semiquantitative technique for protein expression that allows convenient interpretation by an anatomic pathologist using light microscopy. Although IHC is cost-effective and allows histology to be simultaneously evaluated with the marker of interest, it is subjective and prone to error. This has been particularly apparent in the evaluation of breast cancer for HER-2 protein overexpression *(36, 37)*. A positive HER-2 status indicates aggressive tumor transformation and a poor prognosis *(38–40)*. The IHC-based "HercepTest" for HER-2 was approved to predict response to trastuzumab (Herceptin). However, it is difficult to differentiate between weak positive and background staining. In addition, different commercially available antibodies can give different results *(41)*. The difficulties with HER-2 IHC has led many laboratories to offer fluorescence in situ hybridization (FISH) as a method to determine *HER-2* gene amplification. There are now two Food and Drug Administration-approved FISH-based tests for *HER-2* status *(42)*. Although there is good concordance between the FISH methods (98%), there is generally poor agreement between FISH and IHC *(43, 44)*. Experience with HER-2 clinical testing underscores the need for consistency and reliability in solid-tumor molecular assays, a need that will become even more acute as more targets are discovered and used.

Real-time quantitative PCR

In pace with the development of microarray technologies have been advances in real-time quantitative PCR. Real-time quantitative PCR is a homogeneous method that includes both amplification and analysis with no need for slab gels, radioactivity, or sample manipulation. There are now several platforms commercially available for combining thermal cycling with fluorescence acquisition *(45)*. The fluorescence of DNA dyes or probes is monitored each cycle during PCR. At a certain point during cycling, the product accumulates enough to increase fluorescence above background. The point where fluorescence rises above background noise is best quantified as the second derivative maximum [crossing point (Cp)] of the curve and correlates to the amount of starting copies within a PCR reaction *(46)*.

As the number of initial template copies increases, fluorescence appears sooner and the Cp is lower. The relative copy number between two samples (experimental and control) can be determined by the difference in their Cp values (Fig. 1A). Because PCR is an exponential process, the relative copy number is equal to the PCR efficiency raised to the power, ΔCp. Because it may be difficult to know the total amount of DNA present in different samples, results of the test gene are often normalized to a reference gene presumed to be invariant (Fig. 1B). Regions of genomic stability (i.e., not altered) for a given tumor type can be identified by CGH studies and can be used for DNA controls. An inexpensive and

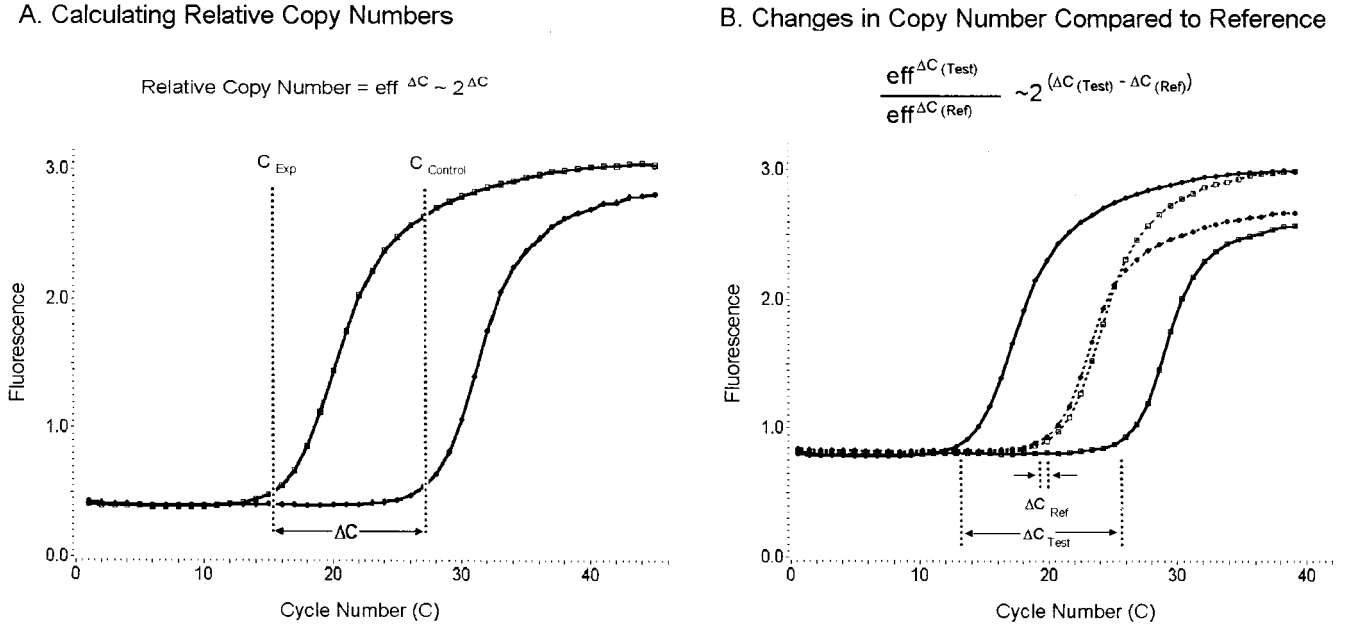

Fig. 1. Relative quantification by real-time PCR.

(*A*), the amounts of target in an experimental sample and a control sample are compared after PCR amplification and fluorescence monitoring each cycle. For example, genomic DNA may be analyzed to assess gene amplification or deletion. Expression of mRNA may also be studied after reverse transcription. The sample with the greater amount of DNA (or cDNA) will show an earlier increase in fluorescence. The second derivative maxima of the curves (*vertical dotted lines*) are determined as fractional cycle numbers. The relative copy number between samples is the PCR efficiency (*eff*) raised to the difference between fractional cycle numbers (ΔC). The calculation assumes that the PCR efficiency is the same between samples. The PCR efficiency is usually between 1.7 and 2.0. As a first approximation, an efficiency of 2 is often assumed. This analysis assumes that the starting amount of material (DNA or cDNA) in each sample is the same. (*B*), another option is to use a test target normalized to a reference target. The amount of starting material in each sample is normalized to a reference (*Ref*) or housekeeping gene. Both experimental and control samples are amplified for both the test and reference targets. Any difference in the amount of starting material is normalized by the results of the reference target amplification. This method assumes that the reference target is invariant between samples and that the PCR efficiency for each target does not vary between samples. As a first approximation, an efficiency of 2 is often assumed for both targets and has become known as the $\Delta\Delta C$ method.

common method to validate microarray experiments is relative quantification by real-time PCR using SYBR Green I as a fluorescent indicator of double stranded DNA production *(47)*.

The correlation between Cp and starting copy and the use of these values to calculate PCR amplification efficiency are shown in panels A and B, respectively, of Fig. 2. The PCR amplification curves were generated with hybridization probes specific for the serum *albumin* gene [primer/probe sequences and amplification conditions can be found as an online supplement at LightCycler University (http://www.idahotech.com/lightcycler_u/index.html)]. Genomic leukocyte DNA was used at decreasing concentrations in a series of 10-fold dilutions. The fluorescence was acquired every cycle and then plotted against cycle number to construct an amplification curve at each dilution (Fig. 2A). Linear regression through a plot of the Cps at each dilution against the log of genomic DNA gives the average efficiency of the PCR reaction (Fig. 2B). This is calculated using the formula:

$$\text{Efficiency} = 10^{\frac{-1}{\text{slope}}}$$

The *y*-intercept of the calibration curve is a function of the minimum detectable amplicon *(48)*.

REAL-TIME MULTIPLEX PCR FOR DIAGNOSTICS

Real-time quantitative PCR can analyze multiple genes simultaneously within a single reaction. The main advantages of multiplexing over single-target analysis are the ability to provide internal controls, lower reagent costs, and preservation of precious samples. Multiplexing can be particularly important when there is a need to analyze several targets from microdissected tissue. Microdissection of solid tumor samples is usually necessary when determining gene copy numbers because the presence of DNA from normal diploid cells will interfere. Protocols for obtaining nucleic acids from microdissected tissue are well established *(49)*. Two genes in which quantitative DNA analysis may be important for prognosis and treatment of breast cancer are *HER-2* and *topoisomerase IIα* (*topo IIα*). In ~20% of breast cancers, the HER-2 gene becomes amplified at the DNA level, leading to an increase in message and overexpression of the protein *(37, 44)*. The *topo IIα* gene is physically located near *HER-2* within the chromosome band region 17q12-q21, an area that is frequently mutated in breast tumors *(6, 50, 51)*. DNA amplification of *HER-2* can occur concomitantly with *topo IIα* alterations (amplification or deletion), and changes in *topo IIα* copy number may dictate response to chemotherapy with topo II inhibitor drugs *(52, 53)*.

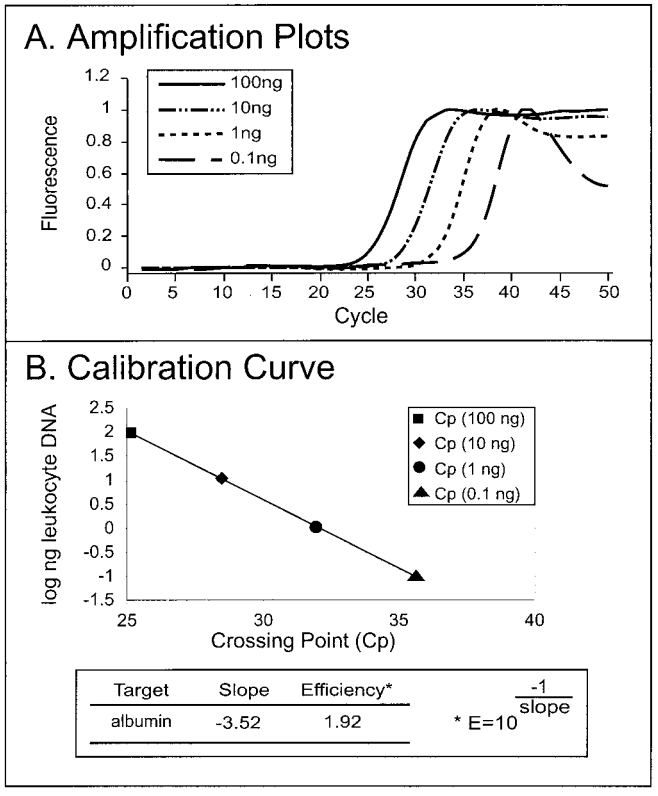

Fig. 2. Establishing PCR efficiency.

(*A*), the overall efficiency of PCR within a run can be found by use of a dilution series of DNA (e.g., leukocyte) to generate amplification curves at different concentrations of template. (*B*), the overall efficiency of PCR for a given primer and probe set can be calculated from the linear regression through a plot of Cp vs log ng DNA. For *albumin*, the slope of the trend line was −3.52, yielding an efficiency of 1.92.

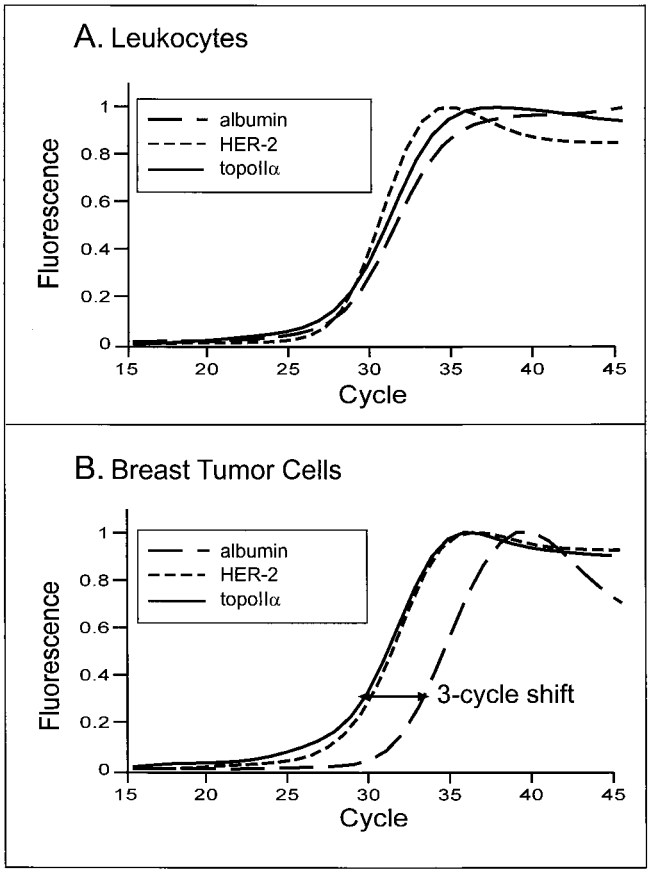

Fig. 3. Determining efficiency and gene copy numbers using three-color multiplexing.

There is greater variation in PCR efficiency between runs than within a run. We established the efficiency of amplification for three targets within a run using color multiplexing and used the calculated efficiencies to determine gene amplification in breast tumors. After color compensation and correction for differences in PCR efficiency, Cps of *HER-2*, *topo IIα*, and *albumin* were compared in healthy leukocytes and breast tumor cells. All three genes had nearly the same Cp for wild-type leukocyte DNA (*A*). However, the DNA from the breast tumor cells showed an early three-cycle shift for both *HER-2* and *topo IIα*, corresponding to an eightfold (2^3) amplification over *albumin* (*B*).

We used a three-color multiplex PCR reaction to simultaneously determine starting copy numbers of *HER-2* and *topo IIα* relative to the control gene *albumin*. Acceptor dyes LCRed640 (*HER-2*), Cy5 (*albumin*), and LCRed705 (*topo IIα*) were paired with fluorescein in three sets of adjacent hybridization probes. Each target was identified by the different emissions of the acceptor-labeled hybridization probes. Overlap between the emission spectra of these dyes was compensated in software to isolate the unique signal from each probe *(54)*. Multiplexed reactions within the run were used to establish the amplification efficiency of each gene. If all three targets are present in same number of copies and each target amplifies with the same efficiency, then all three targets should have the same Cp. Fig. 3 compares the amplification of wild-type genomic leukocyte DNA (Fig. 3A) and DNA isolated from a breast tumor (Fig. 3B). As expected, the Cps for all three targets in the wild-type genomic DNA are the same, but in the tumor sample, both *HER-2* and *topo IIα* have Cps shifted three cycles earlier than *albumin*. This corresponds to an eightfold amplification of these genes. This tumor sample was independently determined to have DNA amplification with mRNA overexpression for both genes by CGH and cDNA microarray analysis *(1, 50)*.

Mutation Analysis

REAL-TIME PCR FOR MUTATION DETECTION IN SOLID TUMORS

Mutations in DNA can include large rearrangements, such as translocations, inversions, and gene amplifications/deletions, as well as small alterations, such as point mutations and base insertions/deletions. Large alterations frequently occur in hematologic malignancies and are routinely analyzed by DNA techniques in cytogenetics (e.g., chromosomal spread and FISH) *(10, 11)*. In contrast, small alterations frequently occur in carcinomas, and there are few, if any, routine clinical assays for identifying small somatic alterations in solid tumors.

Somatic mutations are acquired during the evolution of many tumors and may significantly impact the course of malignancy. The *p53* tumor suppressor gene is the most frequently mutated gene in human cancers. Approximately 90% of all mutations in *p53* occur within the

DNA-binding domain (exons 5–8), and most (87%) are single-base changes *(19)*. Mutations in exons 5–8 are the best indicators of outcome *(13, 27, 29, 55)*. Studies suggest that particular hotspot mutations in *p53* (e.g., codon 273) may confer resistance to the anthracyclines *(55, 56)*, which are the first-line therapy in metastatic breast cancer. The presence of a *p53* mutation may be further exploited for monitoring recurrence or therapy response. For example, tumor cell death releases mutated *p53* nucleic acids into the bloodstream that can be detected in the serum of patients *(34, 35)*. The detection (and even quantification) of this mutated DNA can be used for periodic monitoring. Despite the demonstrated value of *p53*, routine clinical testing of *p53* continues to be debated. The reasons for this include conflicting results between testing methodologies and finding a technique that is amenable to use in the clinical laboratory.

A variety of scanning methods and direct sequencing are used to establish associations between genotype and disease *(57)*. Conventional scanning methodologies use slab gels to resolve mobility shifts attributable to mutations that cause single-stranded conformational changes or changes in heteroduplex melting. Gel techniques that differentiate homo- and heteroduplexes by exploiting differences in duplex stability include denaturant gradient gel electrophoresis *(58)*, constant denaturant gel electrophoresis *(59)*, and temporal temperature gradient gel electrophoresis *(60)*. Alternatively, heteroduplexes may be detected by chemical or enzymatic cleavage followed by gel fragment sizing *(61)*. Advances in scanning methodologies include the incorporation of fluorescence with single-strand conformational polymorphism analysis and the use of denaturing HPLC *(62, 63)*. Methods capable of scanning for somatic mutations in a rapid, sensitive, and cost-effective manner will have the greatest utility in the clinical laboratory.

Fluorescent hybridization probes are often used in the clinical laboratory for homogeneous genotyping. For example, melting curve analysis with hybridization probes detects small germline mutations/polymorphisms in the genes that cause common inherited diseases, such as cystic fibrosis *(64)*, venous thrombosis *(65, 66)*, emphysema *(67)*, hemochromatosis *(68)*, and hypercholesterolemia *(54, 69)*. Melting curve analysis with the double-stranded DNA-binding dye SYBR Green I has been used to detect DNA methylation *(70)*, which can produce transcriptional silencing. In addition, melting curve analysis of single-labeled probes can be used to scan for somatic mutations. Fig. 4 shows the detection of *p53* mutations in exons 6 (Fig. 4A) and exon 8 (Fig. 4B) from two different colon cancers by use of a series of overlapping fluorescein-labeled oligonucleotides complementary to a wild-type *p53* sequence. The probes are designed so that fluorescence decreases on target annealing because of deoxyguanosine quenching *(71)*. After amplification in a LightCycler, the instrument begins a melting program where the reactions are cooled to anneal the probes and then slowly heated (0.1 °C/s) while fluorescence is continuously monitored. Somatic mutations are identified by changes from a characteristic wild-type melting curve profile. When melting curves from nonmutated and mutated colon cancer samples are compared, additional melting peaks (Fig. 4A) or changes in peak-area ratios (Fig. 4B) indicate a sequence alteration under the probe. As many as three probes have been placed in one tube for melting temperature multiplexing, scanning a region of ~100 bp.

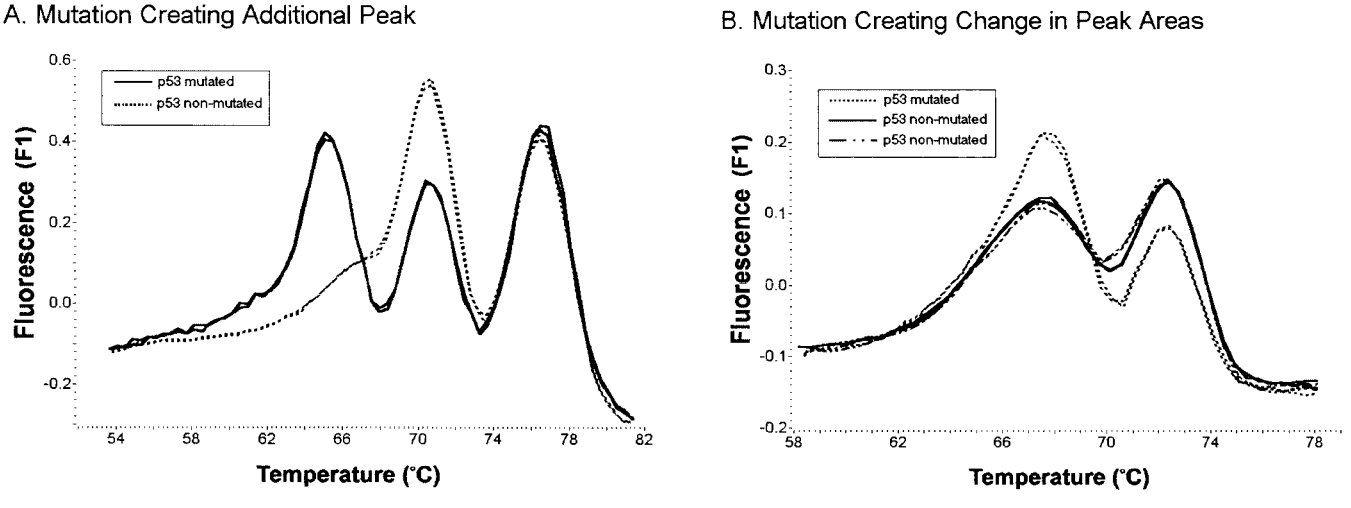

Fig. 4. Homogeneous scanning of *p53* in colorectal tumors.

Overlapping fluorescein-labeled probes were used to scan colon cancers for mutations in exons encoding the DNA-binding domain of *p53*. Multiple probes complementary to the wild-type sequences were placed within the same reaction, and the different sites were identified by their specific probe/target duplex melting temperatures. The position of each probe/target melting temperature and the relative ratio of the melting peak areas determined wild-type profiles. For example, an aberrant profile in exon 6 (*A*) was detected from an additional melting peak, and a mutation in exon 8 (*B*) was detected from a change in peak areas. Both mutations were confirmed by sequencing, which showed a G-to-T base change (C:T mismatch) under the lower melting probe/target duplex in exon 6 and a G-to-A base change (C:A mismatch) under the higher melting probe site in exon 8.

Summary

Cancer research is focusing on the importance of new molecular markers *(72)*. The integration of molecular markers into existing histomorphologic classifications in surgical pathology will provide additional stratification for a more accurate prognosis. Furthermore, a molecular definition of cancer may often allow the guidance of therapy and the ability to monitor residual disease.

The technology used today in cancer research, such as expression microarrays and CGH, allow genome-wide scanning and the discovery of altered genes involved in cancer. Expression microarrays can identify coordinately regulated genes, defining pathways that explain the different clinical behaviors observed between tumors. CGH can provide genome-wide coverage for the identification of regions that are DNA amplified or deleted during tumor evolution. By combining these technologies, important biological pathways can be identified on multiple molecular levels. For example, CGH has identified a region of DNA amplification on chromosome 17q that is has been shown by expression microarray to involve an amplicon containing *HER-2*, *Grb-7*, and *topo IIα (1, 50)*. Identification of targets at the DNA level allows the option of target validation retrospectively on formalin-fixed, paraffin-embedded tissue blocks. It also allows these targets to be easily integrated into the current flow of sample processing in pathology. Microarrays and CGH are powerful tools that can be used to find differences between tumors and to find common mechanisms leading to malignancy. In the near future, tumors will be defined and treated based on biological pathways that drive malignant cell proliferation and metastasis *(73–76)*, rather than solely on histologic appearance.

The platforms for providing clinical molecular assays are still developing. Expression microarrays and CGH are invaluable to understanding the biology of cancer. However, once cancer genes and control genes have been identified, these methods may be superfluous for clinical diagnostics. Real-time PCR will play an increasingly important role in clinical testing because it can provide information about gene expression, gene amplification or loss, and small alterations (e.g., point mutations). In addition, it can be applied to detect and quantify viral causes of cancer, such as Epstein–Barr virus *(16)* and human papillomavirus *(77)*. The use of real-time PCR for molecular diagnostics is attractive because it is objective, rapid, versatile, and cost-effective and can be performed on small tissue samples.

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