CANCER
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ThinPrep®-Processed Fine-Needle Samples of Breast Are Effective Material for RNA- and DNA-Based Molecular Diagnosis

Application to p53 *Mutation Analysis*

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BACKGROUND. Fine-needle sampling is the least invasive method of in vivo breast carcinoma sampling and can provide material for breast carcinoma diagnosis. The aim of the current study was to assess the accuracy of molecular diagnosis techniques using fine-needle sample (FNS) material stored in PreservCyt® (Cytyc Corp., Boxborough, MA).

METHODS. The *p53* tumor suppressor gene was chosen as a model because it can be used for DNA, RNA, and protein analysis. Molecular analysis was performed using a yeast functional assay and DNA sequencing. p53 accumulation was evaluated by immunocytochemistry.

RESULTS. DNA and protein analysis indicated that samples stored for periods of several months, either at room temperature, 4 °C, or −20 °C, can be processed reliably. For RNA-based diagnosis, samples were still intact after 5 months of storage in PreservCyt® at 4 °C. In addition, using FNS material that was stored for 16 months at 4 °C, the authors detected *p53* mutations with either the functional assay for separating alleles in yeast (an RNA-based functional assay) or direct cDNA sequencing.

CONCLUSIONS. Fine-needle samples stored in PreservCyt® at 4 °C are very good material for molecular diagnosis techniques. In addition, it is feasible to adopt a strategy of storing excess FNS material to create cellular banks that will be invaluable for future gene studies. ***Cancer (Cancer Cytopathol)*** **2003;99:223–32.**

KEYWORDS: fine-needle sampling, cytology, *p53* tumor suppressor gene, ThinPrep®, molecular diagnosis, immunocytochemistry, cellular banking.

Because of the limited ability to extract sufficient quantities of high-quality RNA or protein from fixed tissue, application of most current molecular techniques requires access to fresh or frozen tissue specimens.[1] Progress in the clinical application of potentially useful molecular technologies also is hampered by fixation methods that fail to conserve the structure of nucleic acids and proteins in tissues. One critical issue is the conservation of RNA, which is essential for expression analysis using array technology and for mutation analysis of genes whose size and large number of exons prevent routine analysis. It also is possible for splicing defects that cause aberrant RNA processing to be underestimated, and only RNA analysis can detect the consequences of this type of alteration.[2] Although frozen tissue is suitable for RNA extraction, this is not the case for paraffin-embedded tissue. The establishment of DNA, RNA, and protein banks from clinical material is important for the purposes of

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large-scale studies using previously archived material and also for the organization of the processing of pathologic samples. Improved methods that allow better DNA, RNA, and protein extraction have been described and are commercially available. Nonetheless, these methods still can be improved in terms of effectiveness and versatility. Research aimed at developing new fixative procedures that ensure better preservation of macromolecules also is important, but it will take a long time for any new procedure to be tested and adopted as a part of routine clinical practice.

Of the various types of pathology procedures, fine-needle sampling, originally described more than 70 years ago, has become a very popular method for diagnosis of breast and thyroid lesions.[3] For detection of breast carcinoma, it has a sensitivity of 74–97% and a specificity of 82–100%.[4] Recently, liquid-based ThinPrep® (Cytyc Corp., Boxborough, MA) methods have been introduced as an alternative to conventional smears. These methods also allow unprocessed samples to be stored in a preservative solution.[4] ThinPrep® techniques, described widely in the literature, have been approved by the Food and Drug Administration. Several studies have demonstrated that molecular diagnosis can be performed on ThinPrep®-processed fine-needle breast aspirates, but most of these studies have addressed only DNA-based methods performed on samples that were stored for relatively short periods.[5–8] Because such cytologic samples stored in preservative could be a good source of genetic material for various types of analyses, we examined the way in which these samples should be stored and processed to ensure efficient nucleic acid and protein recovery. As a model for molecular analysis, we chose the *p53* gene, which is mutated in 20% of breast malignancies. DNA-, RNA-, and protein-based detection methods are available for mutation analysis of *p53*, making it a very convenient model for comparisons.

MATERIALS AND METHODS

Cell Lines

The 2 cell lines used in the current study, SKBR-3 (mutant *p53;* codon 175, CGC→CAC) and MCF7 (wild-type *p53*), were purchased from the American Type Culture Collection. Cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. For stability experiments, 4.5×10^7 cells were washed once in phosphate buffered saline and resuspended in 50 mL CytoLyt® solution (Cytyc Corp.). After 30 minutes at room temperature, cells were centrifuged and resuspended in 45 mL PreservCyt® solution (Cytyc Corp.). Twenty-seven 1-mL aliquots were prepared and stored at room temperature, 4 °C, or −20 °C. Each month, 100 μL was taken for immunocytochemistry, and the remainder was processed for DNA and RNA extraction.

Human Specimens and Sample Management

Fine-needle sampling was performed with a 23-gauge needle without aspiration as previously described.[9,10] The needle was moved back and forth very slightly while being positioned at various angles and different depths within the tumor before being withdrawn. Cellular material was transferred directly into the CytoLyt® solution and processed according to the procedure recommended by the supplier. A major problem associated with this procedure concerns the final volume of PreservCyt® solution (15 mL) used for storage of the sample; such a large, cumbersome volume could rapidly become inconvenient, especially if samples require storage at −20 °C. A convenient solution, after fixation of the cells, involves centrifuging and transferring the cells to a screw-cap Eppendorf tube (Eppendorf, Inc., Hamburg, Germany) containing 1 mL of PreservCyt® solution. Fine-needle samples (FNS) from patients with breast carcinoma were obtained during routine diagnosis under protocols approved by the review board of the Institut Curie. Written informed consent was obtained from each patient.

Immunocytochemistry

Cells were cytospun, fixed overnight in 95% ethanol, and postfixed (4 minutes at room temperature) in absolute ethanol and glacial acetic acid (volume:volume ratio, 3:1). The fixation step was optional, because the PreservCyt® solution acts as a fixative. Endogenous peroxidases were inhibited with 0.6% H_2O_2 (10 minutes at room temperature). Antigen retrieval was performed in citrate buffer pH 6.0 (20 minutes at room temperature), followed by incubation with primary antibody (DO7, 1:2700 dilution; Dako Co., Carpinteria, CA) for 30 minutes at room temperature. Detection was performed using the Vectastain ABC Elite peroxidase kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine as the substrate. Cytospins then were counterstained with Harris hematoxylin, dehydrated, and mounted.

DNA and RNA Extraction

DNA and RNA extraction were performed simultaneously using the DNA/RNA Mini Kit (Qiagen, Chatsworth, CA). DNA was resuspended in a final volume of 20 μL TE (10 mM Tris, pH 8.0; 1 mM ethylenediamine tetraacetic acid), and RNA was resuspended in a final volume of 25 μL water.

Analysis of RNA Quality

RNA quality was analyzed with the 2100 Bioanalyzer using the RNA 6000 LabChip assay kit (Agilent Technologies, Palo Alto, CA). The 2100 Bioanalyzer is a small benchtop system that uses LabChip to integrate sample separation, detection, quantification, and data analysis. Each disposable RNA chip is used to determine the concentration, purity, and integrity of 12 RNA samples in 25 minutes. This technique is better suited to routine clinical analysis than is conventional agarose gel electrophoresis, as it uses less material (1% of what is used in gel electrophoresis) and is faster and more precise.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) and PCR Analysis

RT-PCR on an RNA template was performed using 2 μL RNA (equivalent to the product of 9×10^5 cells), random primers (Invitrogen, Carlsbad, CA), 100 units (U) Superscript II Reverse Transcriptase (Invitrogen), 10 mM concentration of dithiothreitol, 40 U RNAse inhibitor (RNAseOUT; Invitrogen), and 1.25 mM concentration of dNTPs (Amersham Biosciences, Buckinghamshire, UK) for 1 hour at 45 °C in a final volume of 20 μL. After inactivation at 72 °C for 3 minutes, 2 μL of the cDNA preparation (the product of 9×10^4 cells) was used for the polymerase chain reaction (PCR) in a final volume of 20 μL (1.25 U error-free *Pfu* polymerase [Stratagene, La Jolla, CA], 0.5 μM concentration of primers, 50 μM concentration of each dNTP, and 10% dimethylsulfoxide). The amplification conditions were as follows: 5 minutes at 94 °C; followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 62 °C, and 2 minutes at 74 °C; and a final extension step of 10 minutes at 74 °C. Five μL of the PCR product was used for agarose gel analysis. For the yeast assay, the 5′ and 3′ ends of the *p53* cDNA were amplified separately. For the 5′ end, phosphorothioate-modified primers P3 (5′-ATT TGA TGC TGT CCC CGG ACG ATA TTG AAsC-3′, where *s* represents a phosphorothioate linkage) and P17 (5′-GCC GCC CAT GCA GGA ACT GTT ACA CAsT-3′) were used. For the 3′ end, P16 (5′-GCG ATG GTC TGG CCC CTC CTC AGC ATC TTsA-3′) and P4 (5′-ACC CTT TTT GGA CTT CAG GTG GCT GGA GTsG-3′) were used. The size of the 2 RT-PCR products was 611 and 569 base pairs (bp), respectively. For genomic DNA analysis, PCR was performed in a final volume of 25 μL (0.625 U *Taq* Gold polymerase [Applied Biosystems, Foster City, CA], 0.2 μM concentration of primers, and 200 μM concentration of each dNTP). The amplification conditions were as follows: 10 minutes at 95 °C; followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C, and 60 seconds at 72 °C; and a final extension step of 10 minutes at 72 °C. Five μL of the PCR product was used for agarose gel analysis.

Functional Assay for Separating Alleles in Yeast (FASAY)

Transcriptional activation is the critical biochemical function of the p53 protein, and this function is the basis for the tumor suppressor activity of p53. Mutations can lead to the failure of p53 to activate transcription, and p53 mutants that are inactive in humans also are inactive in yeast. A yeast strain (yIG397) defective in adenine synthesis because of a mutation in its endogenous *ADE2* gene but containing a second copy of the *ADE2* open reading frame, controlled by a *p53* response promoter, has been developed. Because *ADE2* mutant strains that are grown on low-adenine plates are red, yIG397 colonies containing mutant *p53* can be distinguished by their red color, whereas colonies containing wild-type *p53* are white. For the assay of p53 function in yeast, the strain yIG397 is cotransformed with RT-PCR–amplified *p53* and a linearized expression vector, and the *p53* cDNA is cloned in vivo by homologous recombination. To minimize mutations introduced during PCR, the high-fidelity *Pfu* DNA polymerase (Stratagene) is used. In the original assay, described by Flaman et al.,[11] only one RT-PCR product was amplified and used for yeast transformation. More recently, Waridel et al.[12] introduced an improvement to this assay involving an internal control in each transformation experiment. In the split FASAY of Waridel et al., 2 overlapping fragments of the *p53* cDNA are amplified by PCR and then independently transformed into the recipient yeast strain with one of two vectors, either pFW35 or pFW34. The first fragment (P3–P17) corresponds to residues 52–236, whereas the second fragment (P4–P16) corresponds to residues 195–364. Because there is only 1 mutation per p53 cDNA, the main advantage of the method of Waridel et al. is that for each sample, 1 PCR fragment will lead to background colonies, whereas the other fragment will lead to red colonies if a mutation is present. Using the split FASAY, which is much more sensitive than direct genomic DNA sequencing, we demonstrated that wild-type *p53* gave a consistent background of 4% red colonies due to reverse transcriptase and *Pfu* DNA polymerase errors. In addition, we found that all tumor specimens that generated $>$ 15% red colonies bore a *p53* mutation[13] (Fouquet C, Soussi T. Unpublished data).

For yeast transformation, the competent yeast strain yIG397 was cotransformed with 1 μL of the RT-PCR product and a recipient plasmid, either pFW35 or pFW34. After cells grew for 3 days at 30 °C, red colonies were scored. For patients with a suspected *p53* mutation, the RT-PCR product was pro-

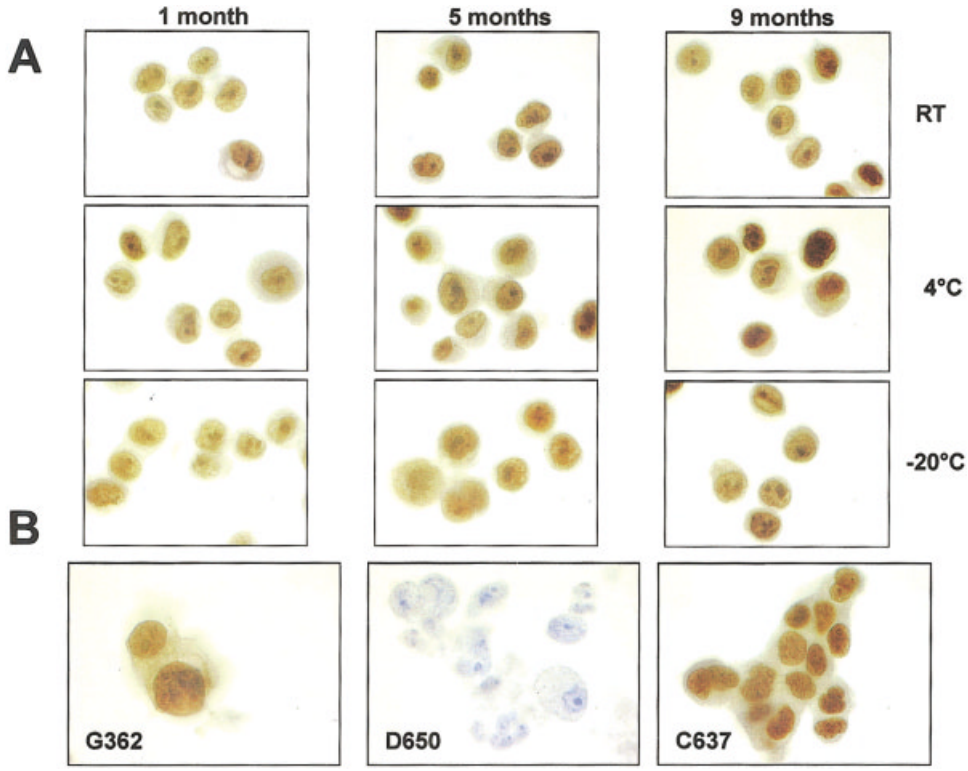

FIGURE 1. Detection of p53 accumulation. (A) Staining of SKBR-7 cells with DO7 antibodies; cells were stored at different temperatures for different periods of time. (B) Staining of fine-needle aspirates from 3 patients with breast carcinoma. RT: room temperature.

cessed directly for sequencing with the Big Dye Terminator kit (Applied Biosystems) and an ABI 3100 genetic analyzer (Amersham Biosciences).

RESULTS

Immunocytochemical Analysis

Accumulation of nuclear p53 was readily detectable in the SKBR-3 cell line, and staining intensity was constant over a period of 9 months of storage in PreservCyt® (Fig. 1A). No difference in cell morphology was observed, regardless of the storage temperature. Three FNS, stored for 16 months at 4 °C, from patients with breast carcinoma were stained with the p53 monoclonal antibody (Fig. 1B). These samples contained > 95% tumor cells, based on analysis with Papanicolaou staining (data not shown). In 2 samples, > 95% of cells showed marked accumulation of nuclear p53, whereas the third sample was completely negative for the presence of nuclear p53. Cells displayed an obvious malignant pattern, indicating that their appearance was not altered by 16 months of storage.

Nucleic Acid Analysis

Figure 2A shows the PCR analysis performed on DNA extracted from SKBR-3 cells that were stored in PreservCyt® at various temperatures. Similar results were obtained with MCF7 cells (data not shown). PCR was highly efficient, regardless of the temperature or duration of storage. Dilution experiments indicated that a PCR product could be obtained routinely using an amount of DNA equivalent to what is produced by 50 cells (data not shown). In addition, extraction of diluted cells stored in PreservCyt® indicated that a PCR product could be obtained routinely from 600 cells (data not shown). A similar situation was observed for breast carcinoma FNS that were stored in PreservCyt® for 1 year (data not shown).

RNA analysis yielded results that differed from those of DNA analysis. The quality of extracted RNA was analyzed with the Agilent 2100 Bioanalyzer and the RNA 6000 LabChip kit. The primary indicator of RNA integrity is the presence of the 18S and 28S ribosomal peaks (Figs. 3, 4). As shown in Figure 3, the RNA extracted from 900 SKBR-3 cells combined (about 10 ng RNA) gave rise to a clear electrophoretic pattern that could be interpreted easily. Storage of cells in PreservCyt® at room temperature led to marked RNA degradation that was detectable after 1 month (Fig. 4; some data not shown). Although the 18S and 28S ribosomal peaks still were visible after 1 month of cell storage, they could not be distinguished from the background after 2 months (Fig. 4; some data not shown). It is noteworthy that total degradation of RNA was not observed, as we never failed to obtain a smear ranging from 200 to 800 bp, regardless of storage time

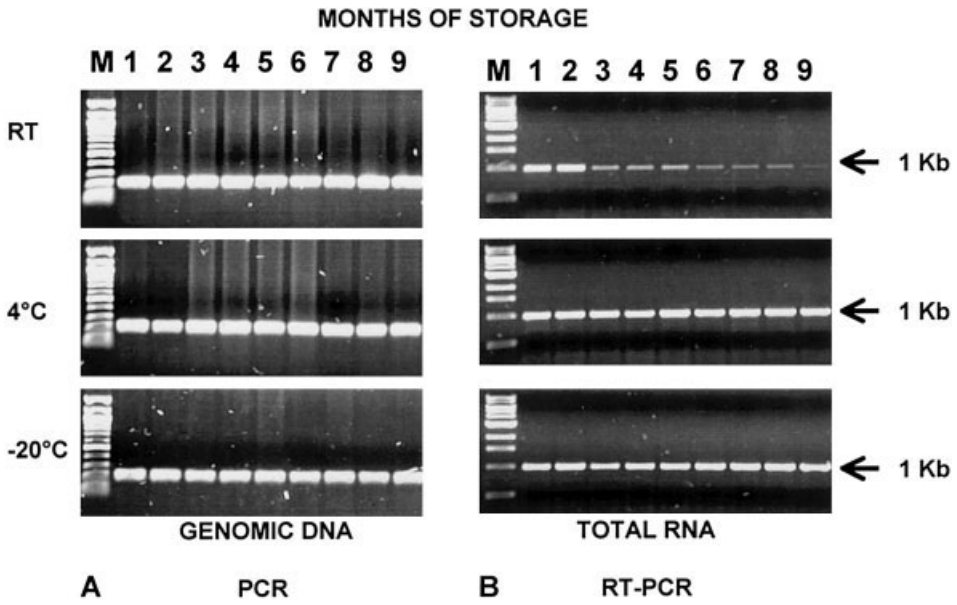

FIGURE 2. DNA and RNA analysis by polymerase chain reaction (PCR) amplification. (A) PCR using a primer with specificity for exon 9 of the *p53* gene was performed on DNA extracted from SKBR-3 cells after various storage times (range, 1–9 months) at different temperatures. (B) Reverse transcriptase–PCR was performed with primers that had specificity for most of the *p53* open reading frame (P3–P4) and RNA from MCF-7 cells. M: molecular weight marker.

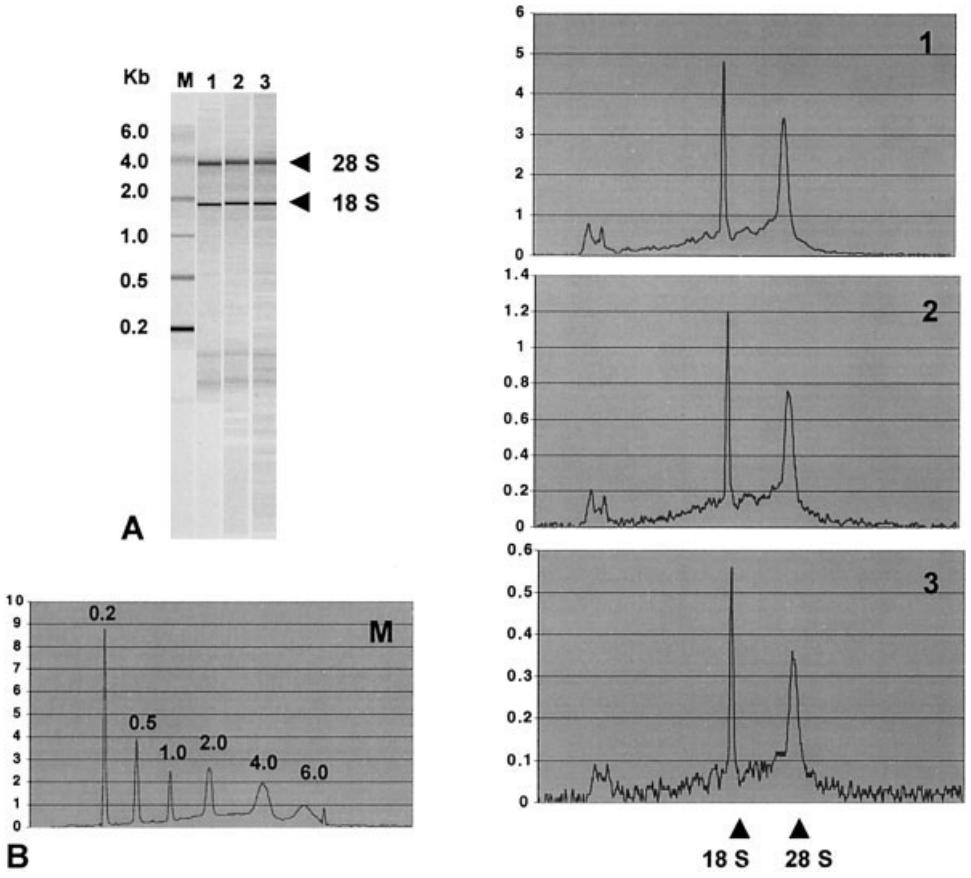

FIGURE 3. RNA analysis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). (A) A gel-like representation of RNA after migration. Band intensity was adjusted automatically by the computer. M: molecular weight marker; Lane 1: total RNA from 18,000 MCF7 cells; Lane 2: total RNA from 3600 MCF7 cells; Lane 3: total RNA from 1800 MCF7 cells. (B) Electropherotogram of the gel depicted in (A).

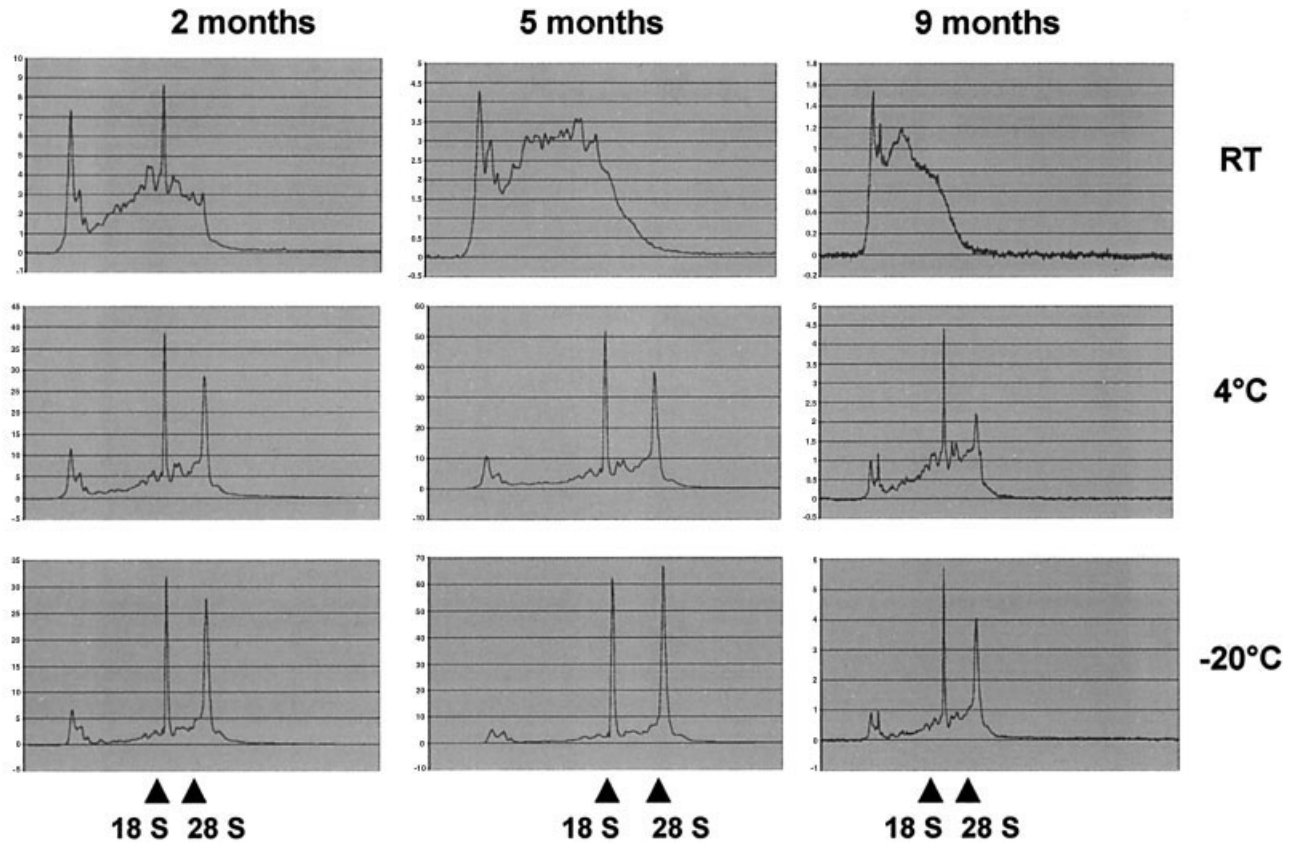

FIGURE 4. Quality of RNA analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). SKBR-3 cells were stored in PreservCyt® (Cytyc Corp., Boxborough, MA) at the indicated temperatures for various lengths of time. After extraction, RNA (the combined output of 18,000 cells) was analyzed using RNA chips (Agilent Technologies). Loss of 18S and 28S ribosomal RNA indicated degradation of RNA. Similar results were obtained with MCF7 cells (data not shown). RT: room temperature.

(Fig. 4). RNA stability was more pronounced at 4 °C; intact 18S and 28S peaks were observed after 5 months of storage (Fig. 4). Beyond 5 months, some signs of degradation were observed, and at 9 months, only the 28S peak was detectable. When cells were stored at −20 °C, no RNA degradation was evident, as the electrophoretic profile remained identical over a period of 9 months. RNA was analyzed with RT-PCR using primers that allowed amplification of most of the *p53* coding sequence (1100 bp). We were surprised to observe that a PCR product could be amplified from each sample. RNA from cells stored at room temperature for 1 or 2 months led to good amplification, but RT-PCR yield decreased dramatically over time, reaching a value of $< 5\%$ after 9 months of storage. When cells were stored at 4 °C or −20 °C, the yield of RT-PCR product was identical regardless of temperature or duration of storage. RNA from breast carcinoma FNS stored at 4 °C for 2 and 16 months also was analyzed using the Agilent Bioanalyzer or RT-PCR (Fig. 5). RNA from FNS stored for 2 months was moderately degraded, as the 18S ribosomal peak still was visible. This RNA allowed efficient RT-PCR with primers for the entire *p53* cDNA (1000 bp each). RNA extracted from FNS that were stored for 16 months showed signs of severe degradation. RT-PCR analysis with primers for the full-length *p53* cDNA failed in 4 of 5 instances. RT-PCR of shorter fragments, such as those used in the FASAY (611 and 569 bp), was successful in 4 of 5 instances, although the yield was very low for Patient C637.

FASAY for Detection of *p53* Mutations

FASAY analysis for detection of *p53* mutations was performed using RNA from the 9 patients (Table 1) The assay involved RT-PCR followed by introduction of the products into indicator yeast cells. *p53* cDNA was amplified and analyzed in 2 separate pieces, as described earlier in the current article. The assay was successful for each sample (Fig. 6; Table 1). *p53* mutations were found in 4 of the 9 patients who were

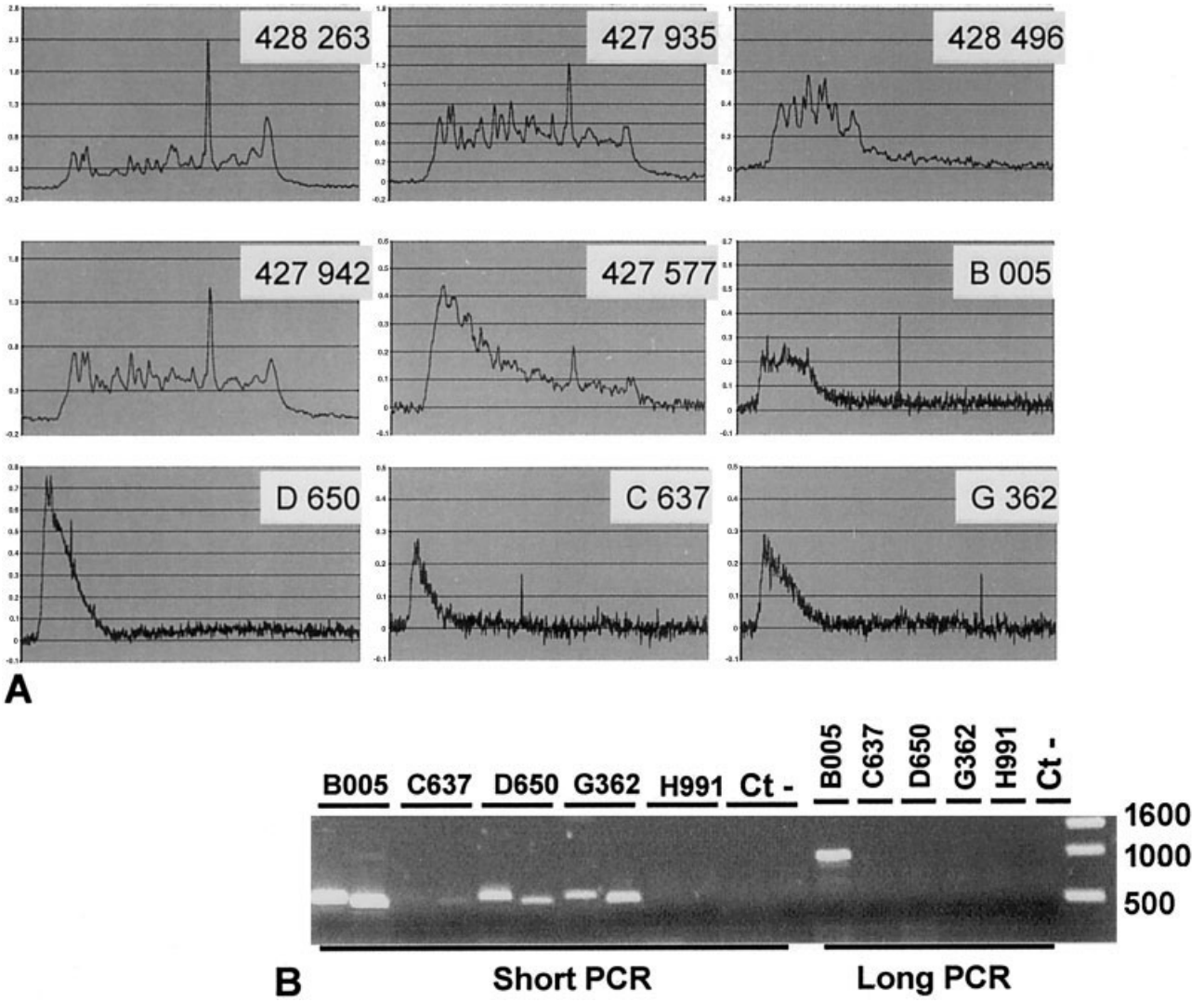

FIGURE 5. Quality of the RNA analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA was extracted from fine-needle samples (FNS) stored at 4 °C for 2 or 16 months (Table 1). (A) After extraction, RNA (the combined output of 18,000 cells) was analyzed on RNA chips (Agilent Technologies). Loss of 18S and 28S ribosomal RNA indicated degradation of RNA. (B) Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of FNS RNA using primers that generate either short (611 and 569 base pairs [bp]) or long (1000 bp) products.

analyzed. Of the 3 patients for whom immunohistochemical analysis was available, a good correlation between protein analysis and molecular analysis results was observed in 2 (G362 and C637), whereas discordant results were obtained for Patient D650. Direct sequencing of the RT-PCR product from Patient D650 revealed a C→T transition that made codon 213 a stop codon (CGA→TGA). This stop mutation precluded the production of a stable protein that could be detected by immunocytochemistry. Sequencing of the RT-PCR product from Patient G362 identified a G→A transition that led to an amino acid substitution at codon 141 (TGC→TAC).

DISCUSSION

In the current study, we evaluated the potential for using cytologic samples stored in PreservCyt® preservative solution as starting material for the formation of a nucleic acid or protein bank. Fine-needle sampling may provide a large number of cells that will not be processed for cytologic examination. Because of the large number of tumor cells in this type of sample, FNS can function as good starting material for studies in which contamination with normal cells is a possible problem. Cells from FNS should be stored in a way that allows them to be used in various molecular techniques that use DNA or RNA as starting material. It

TABLE 1
Summary of Fine-Needle Sample Analysis

Sample	Duration of storage at 4 °C (mos)	p53 ICC	RNA quality	RT-PCR 0.4 kb	RT-PCR 1.0 kb	FASAY (5′/3′)[a]	Sequencing	No. of cells
427 577	2	ND	Mediocre	Yes	Yes	12.5/13.2	ND	9.9×10^4
427 935	2	ND	Mediocre	Yes	Yes	5.2/12.6	ND	1.4×10^5
427 942	2	ND	Mediocre	Yes	Yes	42.7[b]/3.8	ND	9.1×10^4
428 263	2	ND	Mediocre	Yes	Yes	4.2/4.4	ND	1.77×10^6
428 496	2	ND	Mediocre	Yes	Yes	5.0/13.2[b]	ND	9.4×10^4
H991	16	ND	No RNA extracted	No	No	ND	ND	1.3×10^5
B005	16	ND	Poor	Yes	Yes	5.2/4.5	ND	3.8×10^4
C637	16	Positive	Poor	Yes (low yield)	No	53.3[b]/8.2	ND	2×10^5
D650	16	Negative	Poor	Yes	No	57.4[b]/79.2[b]	Codon 213, CGA→TGA	2×10^5
G362	16	Positive	Poor	Yes	No	93.5[b]/2.2	Codon 141, TGC→TAC	2×10^5

ICC: immunocytochemistry; RT-PCR: reverse transcriptase–polymerase chain reaction; FASAY: functional assay for the separation of alleles in yeast; ND: not done.
[a] Values indicate percentages of red colonies obtained using the 5′ and 3′ ends of the *p53* cDNA, respectively, for yeast transformation. For Patient D650, a large number of red colonies were obtained with both sets of primers because the *p53* mutation was located in a region that was common to both RT-PCR products.
[b] Mutant *p53*.

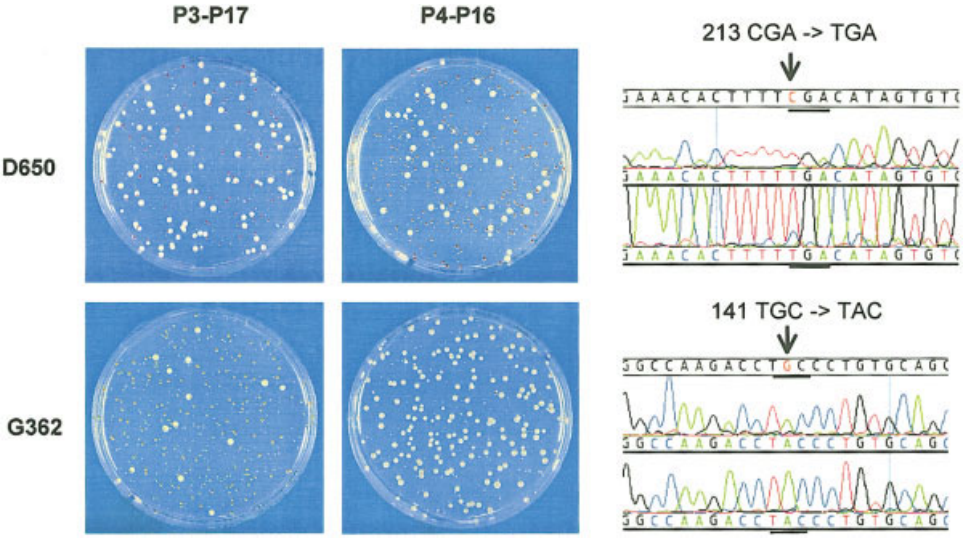

FIGURE 6. Detection of loss of *p53* function using the functional assay for separating alleles in yeast. Reverse transcriptase–polymerase chain reaction (RT-PCR) products that included either the 5′ (amino acids 52–236) or 3′ (amino acids 195–364) region of the *p53* gene were introduced into the indicator yeast strain with the appropriate recombination plasmid. After 3 days of growth at 30 °C, white (wild-type *p53*) and red (mutant *p53*) colonies were counted. For Patient D650, red clones were observed with the two PCR products, as the mutation is located at position 2/3, a region commonly amplified in both PCR products. For Patient G362, mutation was found only in the 5′ PCR product.

also is essential to ensure that specimen handling and storage methods are compatible with routine clinical practice. Procedures that require prechilled buffer, snap freezing, or even storage at −80 °C are not suitable, because they will not be feasible in every pathology laboratory. ThinPrep® preparation calls for cells to be washed in a transport/washing solution and then resuspended in preservative solution for storage before processing for cytologic analysis. No additional manipulation is necessary for sample handling. We used *p53* as a study model because it frequently is mutated in breast carcinoma and because diagnosis of *p53* alterations can be performed on DNA, RNA, or protein. Accumulation of p53 protein in the nuclei of tumor cells generally is explained by its prolonged stability due to an atypically low rate of degradation.

Although several studies have addressed the detection of nuclear p53 accumulation in FNS,[6,7] to our knowledge, none have addressed its feasibility in ThinPrep®-processed fine-needle sample (FNS). Immunocytochemical staining of cells stored for up to 9 months at various temperatures and patient FNS stored for more than 1 year at 4 °C showed that cell morphology and the ability of cells to exhibit p53

accumulation were not altered during storage. Similar results were observed for FNS stored for up to 16 months from patients with breast carcinoma. Of the 3 tumors that were evaluated by immunocytochemistry, 2 displayed a strong signal, whereas the third was negative. Although these results could have suggested that only the 2 positive tumors displayed mutated p53, this hypothesis was not supported by molecular analysis, which revealed p53 alterations in all 3 samples. The subsequent identification in the third tumor of a frame-shift mutation that prevented p53 accumulation emphasizes the need to use molecular techniques for the diagnosis of *p53* mutations.

DNA extraction and PCR amplification have been successful in all samples (cell line samples and FNS from patients with breast carcinoma) processed to date. Although the experiments presented in Figure 2 were performed with 10^6 cells, we have obtained similar results with samples containing fewer than 1000 cells (data not shown). We have not explored this finding in detail, as several studies already have demonstrated that DNA can be extracted from FNS. Lavarino et al.[7] described the detection of *p53* mutations in breast FNS. DNA was extracted from cells that were processed immediately after aspiration. Although the number of starting cells was not specified, nested PCR was used for analysis of *p53* mutations. Pollet et al.[8] and Dillon et al.[14] successfully analyzed *p53* mutations in samples stored in PreservCyt® for up to 5 years. Other molecular analyses using DNA that was extracted from FNS stored in PreservCyt® have been described for the detection of human papillomavirus, oncogene rearrangement, and oncogene mutation.[15–17] In the current study, we used a procedure that allowed simultaneous extraction of DNA and RNA. Although convenient, this method was not optimal with respect to yield and was used as a compromise to gain greater specificity in exchange for yield. A more specific extraction procedure should make it possible to improve both the yield and sensitivity of detection. One of the major objectives of the current study was to evaluate the quality of RNA obtained from cells stored in PreservCyt®. Dimulescu et al.[18] already have addressed this issue, but their study was limited to cells stored for 24 hours at room temperature or 4 °C; in addition, Dimulescu et al. evaluated the success of only the RT-PCR assay. In the current study, we used 3 assays to evaluate RNA quality: 1) analysis with the Agilent 2100 Bioanalyzer; 2) RT-PCR of a 1-kilobase (kb) product; and 3) a yeast assay for p53 activity. Because *p53* expression typically is very low ($<$ 100 copies per cell), it is a good target for the development of a sensitive assay. Characterization of RNA quality using the 2100 Bioanalyzer is one of the most sensitive and specific methods available. This method depends on the integrity of the 18S and 28S ribosomal RNA. Our data clearly demonstrated that storing samples in PreservCyt® at room temperature led to rapid RNA degradation, which was clearly visible after 1 month. At 4 °C, degradation is delayed and is detectable after 5 months of storage. When cells were stored at −20 °C, no degradation was observed for up to 9 months. Similar results were observed for FNS, with more marked RNA degradation observed in samples stored at 4 °C for 16 months compared with samples stored for 1 month. In the majority of RNA-based assays, RNA is used for RT-PCR that can be analyzed using the protein truncation test; direct sequencing; or indirect mutation screening techniques, such as single-stranded conformation polymorphism, denaturant gradient gel electrophoresis, or denaturing high-performance liquid chromatography. These assays typically use PCR products that range in size from 300 to 600 bp. In the current study, a 1-kb RT-PCR product was obtained from every sample, including those that exhibited degradation profiles on the RNA 6000 LabChip. It is clear that for samples stored at room temperature, PCR efficiency rapidly declines with increasing storage time. Nevertheless, RT-PCR products from samples stored at either 4 °C or −20 °C were detectable after 9 months of storage. The discrepancy between Bioanalyzer and RT-PCR results suggests that RT-PCR does not require fully intact RNA to generate an observable product. RNA obtained from FNS was analyzed successfully by FASAY and direct DNA sequencing.

In two recent reports, FNS were used in gene expression microarray analyses of breast carcinoma.[19,20] Ellis et al. used material that was frozen in liquid nitrogen and later used for RNA extraction. Although this procedure preserved RNA integrity as analyzed by the Bioanalyzer, it did not allow morphologic analysis and required extra manipulation. Sotiriou et al. also used snap freezing immediately after aspiration.

The current study clearly demonstrates that ThinPrep®-processed FNS are a viable source of material for various types of DNA, RNA, and protein analysis. Storage of cells at 4 °C allowed the recovery of quantities of DNA and RNA that were suitable for most routine diagnostic molecular procedures. In addition, for studies requiring high-quality RNA (e.g., expression arrays), storage of samples at −20 °C allowed efficient RNA recovery for up to 5 months after sampling. It therefore is feasible to adopt the storage strategies described in the current study for building genetic material banks that will be invaluable for future studies.

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