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13. ABSTRACT ***(Maximum 200 Words)***

In a case-control study, nested within a cohort of 4,888 women with BBD, we demonstrated that p53 protein accumulation detected by immunohistochemistry was associated with a 2.5-fold increase in the risk of subsequent breast. However, by using immunohistochemistry alone, we may have underestimated the true risk of developing breast cancer. We hypothesized that p53 mutations in benign breast tissue are associated with increased risk of subsequent breast cancer. We are testing our hypothesis by: (1) analyzing benign breast tissue from 138 cases and 556 controls for the presence of p53 mutations using PCR-SSCP and PCR-direct DNA sequencing; and 2) estimating the risk of breast cancer in relation to: (a) the presence of p53 mutations in BBD; and (b) the presence of both p53 mutations and p53 protein accumulation in BBD. We also propose to compare mutations detected in the cancers with those detected in their preceeding benign breast tissue samples.

In the second year of the grant, we have continued the analysis of the p53 gene in DNA extracted from paraffin-embedded breast tissue. Tissue sections were cut from paraffin blocks, epithelium enriched tissue microdissected out, and DNA extracted. The DNA has undergone PCR-SSCP under two conditions for exons 2 to 11and those with abnormal gel patterns have undergone repeat PCR-SSCP. Abnormal migrating bands have been cut from the gel. These are being sequenced. We have determined that manual sequencing rather than automated sequencing is more appropriate to analyze the p53 gene in these small benign lesions in the paraffin embedded tissue. Manual sequencing is more sensitive than the p53 gene chip in detecting gene alterations.

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INTRODUCTION

Our work is directed towards identifying genetic and protein changes in benign breast disease (BBD) which might be involved in the pathogenesis of breast cancer, and which might serve as markers of risk. We have recently completed a case-control study, nested within a cohort of 4,888 women with BBD, in which we demonstrated that p53 protein accumulation detected by immunohistochemistry was associated with a 2.5-fold increase in the risk of subsequent breast cancer (1). However, by using immunohistochemistry alone, we may have underestimated the true risk of developing breast cancer in association with p53 changes since approximately 33% of p53 mutations do not alter the protein in such a way that there is positive immunostaining (2-5). Therefore, a more complete assessment of the role of the association between p53 and breast cancer risk will come from studies combining both immunohistochemistry and p53 gene sequencing. We hypothesized that p53 mutations in benign breast tissue are associated with increased risk of subsequent breast cancer.

We are testing our hypothesis by:

(1) analyzing benign breast tissue from 138 cases and 556 controls for the presence of p53 mutations using PCR-SSCP and PCR-direct DNA sequencing; and

2) estimating the risk of breast cancer in relation to: (a) the presence of p53 mutations in BBD; and (b) the presence of both p53 mutations and p53 protein accumulation in BBD. We will localize the mutations to determine whether they occur preferentially in specific sites of the DNA and to compare them to known mutations listed in p53 mutation banks (6-9). We also propose to compare mutations detected in the cancers with those detected in their preceeding benign breast tissue samples.

BODY

Task 1: Extracting DNA from paraffin blocks (Months 1-24):

A) Cut histological sections from paraffin embedded tissue.
B) Extract DNA

Task 2: PCR-SSCP analysis (Months 4-34):

A) PCR for exons 2-11
B) SSCP gels for each exon
C) Autoradiography

We currently doing both task 1 and 2. Primers for exons 2 to 11 of the p53 gene have been generated. We have developed the optimal PCR conditions, e.g. temperature, cycle number, primer concentration, and magnesium concentration, for each exon. We have cut histological sections from paraffin blocks (which contain the tissue to be analyzed), when appropriate microdissected out the appropriate area in the tissue, and extracted the DNA using proteinase K. We have examined the PCR products for each

exon. Each sample is run under two conditions (2 and 10% glycerol in the loading buffer). Samples that show abnormal band migration in either one or both gels (please see a representative SSCP gel in the appendix 1) undergo repeat PCR. The new PCR product then undergoes repeat SSCP analysis. Those samples that show reproducible abnormal band migration are identified as samples that require DNA sequencing to confirm the presence of a p53 gene alteration. The bands are excised from the gels and stored at 4^0C until they are further analyzed.

Task 3: Sequencing DNA with altered mobility on SSCP gels (Months 5-30):

A) Excise band with altered mobility
B) Elute DNA
C) PCR appropriate exon(s)
D) Sequence reaction and separation on acrylamide gels
E) Autoradiography
F) Automated sequencing

We are currently also working on task 3. Those samples that show reproducible abnormal band migration in the SSCP gels are identified as samples that require DNA sequencing to confirm the presence of a p53 mutation or polymorphism. The bands are excised from the SSCP gels and stored at 4^0C until they are analyzed further. The DNA is eluted into water and then undergoes PCR using the same primers as the in the initial PCR reaction. The product is run on a 2% agarose gel. The band is extracted using QIAquick gel extraction kit (Qiagen Inc, Mississauga, ON). The purified DNA will be sequenced using ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. Negative controls include DNA which contains no p53 mutation. Gene alterations are being compared to those listed for breast cancer in a p53 database (http://www.iarc.fr/p53). This work is nearing completion.

In addition we have compared the efficiency and reproducibility of Affymetrix p53 microarray technology to manual sequencing. Previous work indicated that DNA obtained form paraffin-embedded tissue is only suitable for automated p53 sequencing if sufficient DNA can be extracted. However in this study we have only small amounts of DNA because unlike breast cancer, benign breast disease lesions are much smaller and thus we have much less starting tissue. Thus automated sequencing could not be used so we investigated an alternative method that would require less DNA. To do this study 5 um sections were cut from the 62 paraffin blocks, dewaxed and stained briefly in hematoxylin. The cancer tissue was then microdissected out, collected in a microfuge tube and digested with proteinase K (GIBCO BRL, Burlington, ON, 0.5 mg/mL in 50 mM Tris HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hours at 55°C. The proteinase K was inactivated by heating to 95°C for 15 minutes. The DNA was then divided in half and analysed by direct manual sequencing and the p53 Genechip (Affymetrix, Santa Clara, CA) as follows.

p53 Genechip Assay

Aliquots of DNA were purified using a MiniElute Agarose Gel Purification kit (Qiagen, Mississauga, ON) using the manufacturer's protocol. The sampled was eluted using 10 to 15 ul depending on the amount of tissue microdissected. The DNA was amplified in a multiplex PCR reaction. Each 100 μL PCR reaction included 1X PCR buffer (PE Biosystems, Boston, MA) 2.5 mM $MgCl_2$, (PE Biosystems, Boston, MA) 0.2 mM each of dATP, dCTP, dGTP and dTTP (Eppendorf, Westbury, NY), 1X p53 Genechip primer set (Affymetrix, Santa Clara, CA) and 0.8 U of Amplitaq Gold (PE Biosystems, Boston, MA). The PCR was performed using a PE 9600 thermal cycler according to the manufacturer's directions. A 5uL aliquot of the multiplex PCR was visualized on a 10% polyacrylamide gel at 200 V for 1.5 hours to confirm amplification of 10 PCR products of the correct size. The 10 PCR products were fragmented using DNase I, and then labelled using Fluorescein-ddCMP. Each 50μL fragmentation reaction included 45μL of the multiplex PCR reaction, 0.005U fragmentation reagent (DNase I in 10 mM Tris HCl (pH 7.5), 10mM $CaCl_2$, 10 mM $MgCl_2$, 50% glycerol)(Affymetrix, Santa Clara, CA), 0.03mM EDTA, 0.05 U Calf Intestinal Alkaline Phosphatase (Roche, Laval, QB) and 0.5 mM Tris Acetate pH 8.2. Each reaction was incubated for 15 minutes at 25°C followed by heat-inactivation of the enzyme at 95°C for 10 minutes. To confirm the fragmentation a 5uL aliquot of the sample was visualised on 2% agarose gel to reveal a collapse of the 10 PCR products to fragments of approximately 50 base pairs.

Each terminal labelling reaction contained 50μL of the amplified and fragmented target, 1X reaction buffer (Enzo Diagnostics, Farmingdale, NY), 1X $CoCl_2$ (Enzo Diagnostics, Farmingdale, NY), 1X Flourescein ddCTP (Enzo Diagnostics, Farmingdale, NY), and 1X Terminal Deoxynucleotide Transferase (Enzo Diagnostics, Farmingdale, NY). The reaction was incubated in a 37°C waterbath for 45 minutes followed by the addition of 5μL of 0.2M EDTA to stop each reaction. To confirm the labelling of the multiplex PCR product a 3uL aliquot of the sample was visualised on 2% agarose gel using the UVP Gel DocSystem (UVP, Upland, CA). If adequate the DNA was then hybridized to the gene chip, washed and scanned (GeneChip Microarray Facility, at the Albert Einstein College of Medicine). The data analysis was performed using the Affymetrix Microarray Suite to generate a score for each sample that indicated the presence or absence of mutations. A score of ≥ 12 was considered indicative of a gene alteration. When an alteration was identified and not confirmed by direct sequencing, the DNA underwent repeat PCR and processing to repeat the evaluation by gene chip.

Direct Manual Sequencing

DNA was amplified using PCR and primers designed to contain each coding exon of p53 and the splice sites flanking each exon (Table 1). The 15 μL PCR reaction included 1.5 X PCR buffer, 1.5mM $MgCl_2$ (Qiagen, Mississauga, ON), 1X Q solution (Qiagen, Mississauga, ON), 0.1 mM of dATP, dCTP, dGTP and dTTP (Eppendorf, New York, NY), 0.3 μM of the appropriate forward and reverse primers (Gibco BRL, Burlington, ON), 0.09 μCi [α^{33}P] dATP (Dupont NEN, Boston, MA) and 0.07 U Hotstar Taq (Qiagen, Mississauga, ON). Thermal cycling (Eppendorf, Westbury, NY) was performed as described in Table 1. Aliquots of the PCR product were analysed by manual sequencing as described previously The remaining PCR product was visualised via 1% agarose gel electrophoresis (30-35 minutes at 150 V). The quantity of the DNA was

determined by comparing a low DNA mass ladder (GIBCO BRL, Burlington, ON) with the PCR product for each exon. Products that were greater than 50 ng were excised and purified using QiaQuick Agarose Gel Extraction Kit (Qiagen, Mississauga, ON) according to the manufacturers directions, eluting with 25-30μL of elution buffer. The purified PCR product was then sequenced using ThermoSequenase Radiolabelled terminator cycle sequencing kit according to the manufacturer's protocol(Amersham Life Sciences, Cleveland, OH). Following amplification, 4μL of stop/loading buffer was added to each reaction, then denatured for 3 minutes at 95°C and 2.5 μL was loaded onto a 6%, 8.3-M urea, denaturing polyacrylamide gel. The gel was processed for autoradiography and exposed to Biomax MR film (Kodak, Westhaven, CT) for 24-48 hours. For 22 samples the exon 4 portion of exon 3-4 partial PCR product was unreadable. For these samples a PCR reaction and sequencing was performed with primers that were specific for exon 4 only in order to read the 3' portion of exon 4. When an alteration was detected than the DNA underwent repeat PCR and sequencing (Figures 1, 2)

To determine whether gene chip microarray analysis or direct sequencing is better at identifying p53 mutations the sensitivities of these methods were determined. The changes detected by direct sequencing that are outside of the 2 base pairs beyond the splice site of each exon were not included as these are beyond the intronic boundaries of the p53 Genechip and thus could not be identified. The sensitivity of each method for identifying changes in each exon was calculated relative to the combined number of confirmed alterations detected by direct sequencing and the p53 Genechip as each method appeared to identify some different alterations. The sensitivity of direct sequencing was 78% (42/54) and was 50% (27/54) for the p53 Genechip.

The ability to detect different types of mutations varied between the two sequencing methods. The p53 Genechip showed a higher mutation detection rate for missense mutations 100% (19/19) than direct sequencing analysis (52%, 10/19). Direct sequencing was unable to identify 3 single base pair deletions that the p53 Genechip identified. Direct sequencing identified an unreadable change that the p53 Genechip was unable to detect. However, although the numbers are small, the two sequencing methods could identify other types of mutations, equally well. This included nonsense, splice junction mutations and intronic changes with mutation detection rates of 100% (2/2), 100% (2/2) and 100% (1/1) respectively.

Table 1: PCR Conditions for Exons 2-11

Exon	Primers	Exon Size (bp)	PCR product Size	Conditions	Number of Cycles
2	F-CCAGGGTTGGAAGCGTCT R-GACAAGAGCAGAAAGTCAGTCC	102	260	95°C 50sec; 56°C 50 sec;72°C 60 sec	35
3 Partial 4	F-ATGGGACTGACTTTCTGC R-CGGCATCTGGACCTGGT	Exon 3-22 Partial Exon4 - 80	238	95°C 50sec; 56°C 50 sec;72°C 60 sec	35
Partial 4	F-GGACCATATTCAACAATGGT R-ATGGAACCCAGCCCCTCAG	209	258	95°C 50sec; 56°C 50 sec;72°C 60 sec	35
4	F-ATCTACAGTCCCCCTTGCCG R-TGACTTGCACGGTCAGTTGC	289	345	95°C 50sec; 56°C 50 sec;72°C 60 sec	35
5	F-GCTGCCGTGTTCCAGTTGCT R-CCAGCCCTGTCGTCTCTCCA	188	295	95°C 50sec; 60°C 8 min;72°C 60 sec	35
6	F-GGCCTCTGATTCCTCACTGA R-GCCAGGACAACCACCCTTA	112	202	95°C 50sec; 56°C 50 sec;72°C 60 sec	35
7	F-TGCCACAGGTCTCCCCAAGG R-AGTGTGCAGGGTGGCAAGTG	200	196	95°C 50sec; 56°C 50 sec;72°C 60 sec	35
8	F-CCTTACTGCCTCTTGCTTCT R-ATAACTGCACCTTGGTCTC	137	225	95°C 50sec; 56°C 50 sec;72°C 60 sec	35
9	F-GCCTCAGATTCACTTTTATCACC R-CTTTCCACTTGATAAGAGGTCCC	74	152	95°C 50sec; 56°C 50 sec;72°C 60 sec	30
10	F-TGATCCGTCATAAAGTCAAACAA R-CCCTTACTGGCCCTACTCC	118	237	95°C 50sec; 60°C 8 sec;72°C 60 sec	38
11	F-GCACAGACCCTCTCACTCAT R-TGCTTCTGACGCACACCTATTG	119	272	95°C 50sec; 56°C 50 sec;72°C 60 sec	35

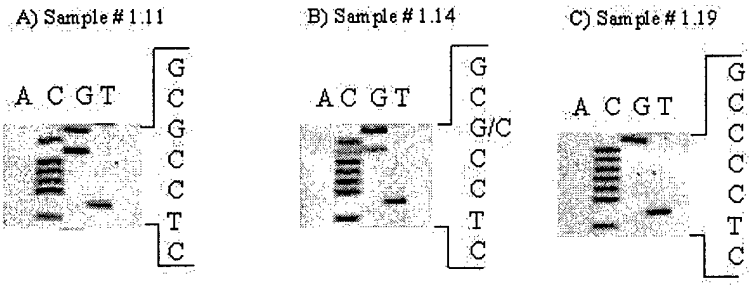

Figure 1: The polymorphism in codon 72 of exon 4 was undetected by the p53 Genechip. Shown here are three samples a) wildtype b) heterozygous c) homozygous at codon 72 of exon four as detected by direct sequencing.

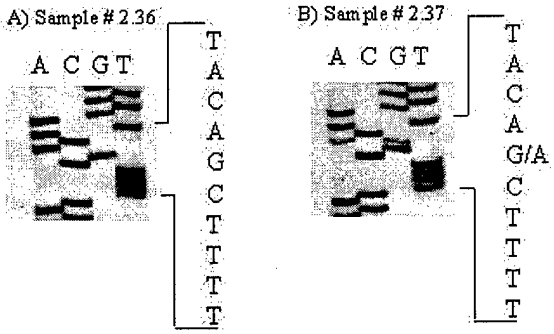

Figure 2: The polymorphism at codon 213 in exon 6 was undetected by the p53 Genechip. Shown here are two samples a) wildtype b) heterozygous at codon 213 of exon 6 as detected by direct sequencing.

KEY RESEARCH ACCOMPLISHMENTS

1) The results of the study are dependent on analysis of data from the entire case-control series and this not yet completed.

2) DNA extracted from paraffin embedded tissue is suitable to use for p53 gene analysis using the microarray technology (p53 gene chip, Affymetrix).

REPORTABLE OUTCOMES

1)We have received a grant from the Department of Health and Human Services, Public Health Service for grant support for a proposal entitled "p53 in benign breast disease and breast cancer risk: A multicenter Cohort". Tom Rohan is the principal investigator and Rita Kandel is one of the co-investigators. The grant will support the creation of a cohort of over 25,000 women from Portland, Detroit, London (England) and Toronto (Canada) to expand this study of p53 and breast disease and breast cancer risk.

2) We have published a paper in the journal Modern Pathology entitled "Correlation of p53 mutations in thin prep processed fine needle aspirates with surgically resected breast cancer." This is appended.

Individuals who have been employed or paid in whole or part from this grant include:
Melissa Cooper: MSc student
ShuQiu Li: Technician
Tajinder Bhardwaj: Technician
Hanje Chen: Technician
Hangjun Wang: Postdoctoral fellow/research assistant

CONCLUSIONS

1) We are able to extract DNA from the paraffin embedded tissue samples and the DNA obtained is suitable for PCR-SSCP, sequencing and analysis using microarray technology.

2) Gene abnormalities detected by PCR-SSCP are being confirmed using manual sequencing.

3) Microarray technology is less sensitive than direct sequencing for paraffin embedded tissue using the Affymetrix gene chip.

REFERENCES

1. Rohan TE, Hartwick W, Miller AB, Kandel R. Immunohistochemical-detection of c-erbB-2 and p53 in benign breast disease and breast cancer risk. J Nat'l Cancer Institute 1998;90:1262-1269.
2. Dunn JM, Hastrich DJ, Newcomb P, Webb JCJ, Maitland NJ, Farndon JR. Correlation between p53 mutations and antibody staining in breast carcinoma. Br J Surg 1993;80:1410-12.
3. Jacquemier J, Molès JP, Penault-Llorca F, Adélaide J, Torrente M, Vineis P, Birnbaum D, Theillet C. p53 immunohistochemical analysis in breast cancer with four monoclonal antibodies: comparison of staining and PCR-SSCP results. Br J Cancer 1994;69:846-52.
4. Sjögren S, Inganäs M, Norberg T, Lindgren A, Nordgren H, Holmberg L, Bergh J. The p53 gene in breast cancer: Prognostic value of complementary DNA sequencing versus immunohistochemistry. . J Nat'l Cancer Institute 1996;88:173-82.
5. Visscher DW, Sarkar FH, Shimoyama RK, Crissman JD. Correlation between p53 immuno- staining patterns and gene sequence mutations in breast carcinoma. Diagn Mol Pathol 1996; 5:187-93.
6. Beroud C, Verdier F, Soussi T. p53 gene mutation: software and database. Nucleic Acids Res 1996; 24:147-50.
7. De Vries EMG, Ricke DO, De Vries TN, Hartmann A, Blaszyk H, Liao D, Sousi T, Kovach JS, Sommer SS. Database of mutations in the p53 and APC tumor suppressor genes designed to facilitate molecular epidemiological analyses. Hum Mut 1996;7:202-13.
8. Hainaut P, Soussi T, Shomer B, Hollstein M, Greenblatt M, Hovig E, Harris CC, Montesano R. Database of p53 gene somatic mutations in human tumors and cell lines: updated compilation and future prospects. Nucleic Acids Res 1997;25:151-7.
9. Hollstein M, Shomer B, Greenblatt M, Soussi T, Hovig E, Montesano R, Harris CC. Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. Nucleic Acids Res 1996;24:141-6.

Correlation of p53 Mutations in ThinPrep-Processed Fine Needle Breast Aspirates with Surgically Resected Breast Cancers

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Mutations of the p53 gene are one of the most common genetic changes found in cancer; their presence may be prognostic and even influence treatment for breast cancer. In this study, we investigated whether DNA could be extracted from the residual cells left in ThinPrep-processed breast fine-needle aspirates and whether p53 gene changes could be detected in the DNA. The results were then correlated with DNA extracted from the matched formalin-fixed, paraffin-embedded, surgically resected breast cancer when available. DNA was successfully extracted from 54 of 62 aspirates and all 31 surgical specimens. p53 gene mutations were detected in 10 of the 54 cytology specimens (18.5%) and consisted of base pair substitutions or deletions. Silent or intronic p53 changes were found in five additional aspirates. One of the aspirates had two gene alterations, resulting in a total of six gene changes. Five of these changes were located in introns 6 or 9 and the sixth was a silent (no amino acid change) change in exon 6. p53 Polymorphisms were detected in nine aspirates (16.3%) and were located in codon 47 (one aspirate), codon 72 (six aspirates), and codon 213 (two aspirates). All cases with surgical material available showed identical p53 mutations, alterations, and polymorphisms in the resected tumors compared with those detected in the corresponding aspirates. The results of this study show that DNA suitable for analysis of p53 gene sequence changes can be successfully extracted from ThinPrep-processed breast fine-needle aspirates, and that identical alterations are detected in both the cytology and surgical specimens.

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KEY WORDS: Fine needle aspiration; breast cancer; p53 mutation; ThinPrep

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Mutations of the p53 gene are among the most common molecular changes detected in human cancers (1). Experimental studies have shown that functional p53 is required for the *in vitro* cytotoxic action of some chemotherapeutic agents (2) The presence of p53 mutations is associated with an increased chemoresistance to doxorubicin in breast cancer patients (3) and may be involved in the development of multidrug resistance (4). Clinical studies have shown that breast cancers that contain p53 gene mutations are associated with decreased disease-free and overall survival (3, 5–9). These results suggest that the presence of p53 mutations might provide prognostic information and influence the treatment of the breast cancer.

Fine-needle aspiration (FNA) of the breast is a safe, effective method for diagnosing breast cancer with minimal intervention and complications (10, 11). As reviewed by Bédard *et al.*, for the detection of carcinoma, it has a sensitivity ranging from 74 to 97% and a specificity ranging from 82 to 100% (12). ThinPrep-processed and conventionally processed breast FNA have been shown to have similar diagnostic accuracy (12). In addition, immunohistochemistry (13, 14) and molecular analysis (15–17) have been successfully applied to ThinPrep-processed specimens.

Because FNA is often the initial sampling of the tumor, it could be a source of cells for the early detection of p53 mutations. In this study, we examined whether p53 mutations could be detected in the cells present in the residual fluid from ThinPrep-processed breast FNAs. When available, the corresponding paraffin-embedded surgically resected tissue was also analyzed for p53 mutations and the results were correlated.

MATERIALS AND METHODS

Specimen Acquisition, Clinical History, and Pathology Review

Cytology reports from November 1997 to April 1999 in the files of Mount Sinai Hospital were reviewed. Of the cases diagnosed as positive or suspicious for malignancy, DNA could be extracted from 54 of 62 specimens of ThinPrep processed breast FNA obtained from 62 different women. In cases in which DNA was successfully extracted from the cytology fluid, the surgical pathology records were reviewed to determine whether there was a corresponding breast tumor specimen. Formalin-fixed, paraffin-embedded tissue was available for 31 women. Clinical details and tumor characteristics were obtained from surgical reports. The breast cancers were graded according to the Elston's modified Bloom and Richardson criteria (18). In 30 of the 31 surgical specimens, the tumor was removed after the cytology specimen. On average, the specimen was removed 33 days after the FNA (range, 8 to 72 days). In one case, the FNA was from a tumor recurrence in the scar 6 weeks after the mastectomy.

p53 Molecular Analysis

DNA Extraction: Cytology

After completing the cytological examination the residual preservative fluid (PreservCyt solution, Cytyc Corporation, Boxborough, MA) was stored at 4°C for up to 3 months. The fluid was centrifuged at 4000 *g* and the supernatant was removed. DNA was extracted from the remaining cells using TriZol (Gibco-BRL, Rockville, MD). DNA extraction was performed according to the manufacturer's instructions for cells grown in suspension. The DNA was stored at 4°C until used for analysis.

DNA Extraction: Surgical Specimens

Sections (5 μm) were cut from the paraffin blocks and stored for up to 2 weeks. Before microdissection, the sections were dewaxed and stained briefly with hematoxylin. A representative portion of the tumor containing minimal numbers of stromal and inflammatory cells was microdissected and placed in a microfuge tube. The tissue was digested with proteinase K (0.5 mg/mL in 50 mM Tris·HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hours at 55°C (19). The proteinase K was inactivated by heating at 95°C for 15 minutes. The DNA was stored at −20°C for up to 3 wk until further analyzed.

Polymerase Chain Reaction (PCR)—Single Strand Conformational Polymorphism Analysis (SSCP)

A 1-μL aliquot from each sample was added to 14 μL of PCR solution containing 1.5 mM $CaCl_2$, 20 mM Tris·HCl, pH 8.0, 50 mM KCl, 0.25 μM concentrations of each primer, 0.1 mM concentrations of each dNTP, 1 U *Taq* DNA polymerase (GibcoBRL, Rockville, MD), and 2 μCi [α-^{33}P]dATP. The primers and the cycling conditions for each exon are listed in Table 1. The reaction product was run on an 8% nondenaturing polyacrylamide gel and the gel was processed for autoradiography (20, 21). Potential mutations were detected by shifts in band mobility. If there was no band shift, the tissue was considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis was repeated. In cases in which different band shifts were detected in the cytology and corresponding paraffin-embedded samples, an additional paraffin block was selected, cut, microdissected, and processed as above. Negative controls, paraffin-embedded cells that contained no p53 mutation in the exon examined and a water control to replace the DNA, were included in each analysis. Positive controls for exons 5 to 9 (exon 5, SKBr3; exon6, T47D; exon 7, colo 320DM; exon 8, MDA-MB468; exon 9, SW480) were also included where appropriate.

p53 Sequencing

The abnormally shifted band was excised from the SSCP gel and the DNA was eluted into water. The DNA was reamplified by PCR using the same

TABLE 1. p53 PCR Primers and Cycling Conditions

Exon	Primer-sense (5′–3′) -antisense (5′–3′)	Product Size (bp)	Cycling Parameters
4	ATCTACAGTCCCCCTTGCCG GCAACTGACCGTGCAAGTCA	296	30 cycles; 50 s at 95°C, 50 s at 55°C, 60 s at 72°C
5	GCTGCCGTGTTCCAGTTGCT CCAGCCCTGTCGTCTCTCCA	294	30 cycles; 50 s at 95°C, 50 s at 58°C, 60 s at 72°C
6	GGCCTCTGATTCCTCAGTGA GCCACTGACAACCACCCTTA	199	30 cycles; 50 s at 95°C, 50 s at 55°C, 60 s at 72°C
7	TGCCACAGGTCTCCCCAAGG AGTGTGCAGGGTGGCAAGTG	196	30 cycles; 50 s at 95°C, 50 s at 56°C, 60 s at 72°C
8	CCTTACTGCCTCTTGCTTCT ATAACTGCACCCTTGGTCTC	225	30 cycles; 50 s at 95°C, 50 s at 55°C, 60 s at 72°C
9	GCCTCAGATTCACTTTTATCACC CTTTCCACTTGATAAGAGGTCCC	152	30 cycles; 50 s at 95°C, 50 s at 56°C, 60 s at 72°C

primers and the product was run on a 2% agarose gel. The band was extracted using a QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA). The purified DNA was sequenced using a ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product was resequenced using the antisense primer. Negative controls were included in each analysis. Cell lines with known mutations in exons 5 to 9 were also included where appropriate. Mutations were compared with those mutations listed for breast cancer in a known p53 database (http://www.iarc.fr/p53) (22).

Statistical Analysis

The associations between p53 gene alterations and clinical/tumor variables were examined using the χ^2 or, where appropriate, Fisher's exact test (23). Two-sided *P*-values below 0.05 were considered to be statistically significant.

RESULTS

Histological review of the 31 surgically resected breast tumors showed that they consisted of 29 infiltrating ductal carcinomas not otherwise specified, one invasive ductal carcinoma with lobular features, and one mucinous carcinoma. DNA was successfully extracted from all paraffin-embedded tumors.

Of 62 cytology samples, DNA suitable for p53 sequencing was extracted from 54, yielding an evaluable specimen in 87% of the cases. p53 Gene mutations were detected in 10 of the 54 cytology specimens (18.5%). As shown in Table 2, these consisted of base pair substitutions and deletions. For eight of these 10 aspirates, surgically resected breast tumor tissue was available for gene analysis. All eight cases showed identical p53 mutations in both the aspirate and the surgically resected tumor. A representative SSCP gel is shown in Figure 1 and the associated sequencing gel is shown in Figure 1B.

Other types of p53 gene changes were found in five other aspirates. One aspirate had two gene alterations resulting in a total of six gene changes. As shown in table 3, five changes were located in introns 6 or 9 and one was a silent change (no amino acid change) in exon 6. For two of these five aspirates, surgically resected breast tumor tissue was available for gene analysis and both of the cases showed identical p53 gene changes in the aspirate and the surgically resected tumor.

p53 Polymorphisms were detected in nine aspirates (16.3%) and as shown in Table 4 were located in codon 47 (one aspirate), codon 72 (six aspirates), and codon 213 (two aspirates). For seven of these nine aspirates, surgically resected breast tumor tissue was available for gene analysis and all seven cases showed identical p53 polymorphisms in both the aspirate and the surgically resected tumor.

The clinical features and tumor characteristics were correlated with the p53 gene status and are summarized in Table 5. DNA suitable for p53 sequencing could be obtained from aspirates of tumors of all three grades. The women whose tumors had a p53 mutation or an intronic change or a silent change were grouped together for these analyses because of the small numbers. There was a significant correlation between a younger age ($P = .038$) or larger tumor size ($P = .046$) with the presence of p53 gene alterations. There was no correlation between the presence of estrogen ($P = .449$) or progesterone ($P = 0.066$) receptors or tumor grade ($P = .227$) and the presence of p53 gene alterations.

DISCUSSION

This study demonstrated that DNA can be extracted from ThinPrep processed breast FNAs. This

TABLE 2. Summary of p53 Mutations

Case Number		Exon	Codon	Sequence Change	Amino-Acid Change
Surgical	Cytology				
20	13	5	*	del 23 bases	
9	7	5	130	C→T	Leu→Phe
10	3	5	175	G→A	Arg→His
36	61	5	183	C→G	Ser→STOP
38	29	6	209	del 2 bases	
13	19	6	220	A→C	Tyr→Ser
17	38	7	232	T→G	Ile→Ser
34	60	7	248	G→A	Arg→Gln
NA	59	8	270	T→C	Phe→Leu
NA	62	9	331	C→T	Gln→STOP

*, deletion (del) starting at nucleotide residue 13041 in intron 4 and involving codons in exon 5.
NA, tissue not available.

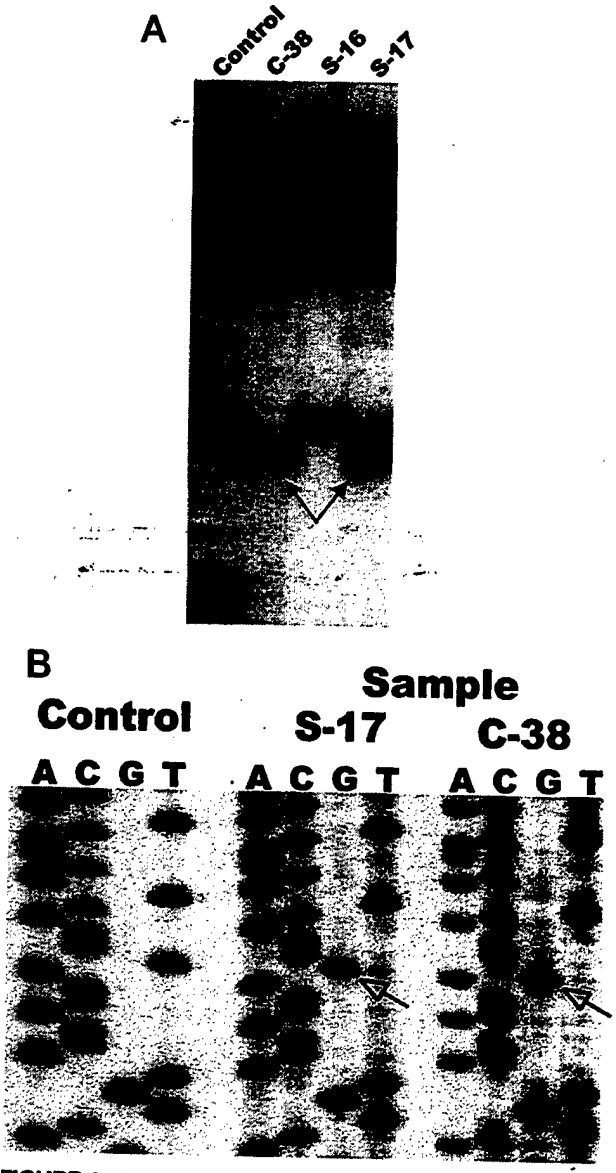

FIGURE 1. A, a representative SSCP gel of p53 exon 7 PCR product from three cases and a negative control (Control). S-16 (surgically resected breast cancer) shows no abnormality. The cytology sample (C-38) and the corresponding paraffin-embedded surgical sample (S-17) show similar band shifts (→). B, the sequencing gel for samples C-38 and S-17 shows a T-to-G base substitution (→). The wild type sequencing pattern (control) in the same region is also shown.

is in keeping with the findings of other groups that have reported successful extraction of RNA or DNA from ThinPrep-processed cytology specimens of breast and cervix (15–17). In addition, the current study showed that the extracted DNA was suitable for p53 gene analysis by PCR-SSCP and sequencing. Using the protocol described above, the mutations detected in exons 4 to 9 were identical to those found in the formalin-fixed, paraffin-embedded, surgically resected breast cancer when this tissue was available for analysis. In contrast, studies assessing p53 immunoreactivity in FNAs and formalin-fixed, paraffin-embedded tumors have shown variable correlations ranging from 73.5 to 93.3% (24–26).

Recent studies have shown that gene alterations detected in paraffin-embedded tissue may be artifacts induced by fixation or processing of surgical specimens (27, 28). Several precautionary steps were undertaken to minimize this possibility. The fidelity of the PCR amplification of DNA extracted from paraffin can be markedly improved by prolonged proteinase K digestion and using small DNA templates (29), so in this study the paraffin-extracted DNA was digested by proteinase K for at least 48 h and the primers were chosen to provide gene sequences of less than 300 base pairs in length. To ensure that the gene alterations were not caused by nucleotide substitutions as a result of *Taq* DNA polymerase misincorporation, all specimens with abnormal SSCP underwent repeat PCR-SSCP to confirm that the change was reproducible. Only those samples that showed similar changes on the repeat PCR-SSCP were considered to have a sequence alteration, which was then confirmed by sequencing. Furthermore, identical alterations were seen in the methanol fixed aspirates and in the corresponding formalin-fixed, paraffin-embedded, surgically resected tumors. This suggests that the p53 alterations identified in this study were genuine.

p53 Mutations were found in 18.5% of patients. This is within the frequency reported for breast carcinoma in other series (8, 9, 30–34). The majority of changes reported for breast cancer have been point mutations (22), and in our series, eight of the 10 mutations (80%) involved base pair substitutions. All mutations, except two (cytology specimens 7 and 13) have been previously reported to occur in breast cancer as listed in a p53 database (22). Silent gene changes were detected in 1.9% of patients, which is similar to the frequency (1.8%) reported by Burns *et al.* (6). In the database examined, there was no report of the silent change observed at codon 224 (cytology specimen 56). No similar comparison could be done for the intronic alterations because the nucleotide position of these types of gene changes is not provided in the database. Codon 47 in exon 4, codon 72 in exon 4 and codon 213 in exon 6 contained known polymorphisms in one, six, and two patients, respectively (1.8, 11.1, and 3.7% of the patients). This is within the range determined for the normal population (35–37). Because the frequencies of mutations and polymorphisms are similar to those shown by others, this suggests that our methodology to detect p53 gene changes is appropriate.

The presence of p53 alterations showed statistically significant associations with larger tumors and younger patient age. No significant association was seen between p53 alterations and tumor grade or the presence or absence of estrogen and progesterone receptors. Other studies examining the associ-

TABLE 3. Summary of p53 Silent and Intronic Changes

Case Number		Location	Site	Sequence Change	Amino-Acid Change
Surgical	Cytology				
NA	56	Exon 6	Codon 224	G→A	Glu→Glu
NA	18	Intron 6	nr 13449	G→C	
NA	55	Intron 6	nr 13964	Del 1 base	
8	35	Intron 6	nr 13964	Del 1 base	
8	35	Intron 9	nr 14755	G→T	
15	5	Intron 9	nr 14766	T→C	

nr, nucleotide residue; NA, tissue not available.

TABLE 4. Summary of p53 Polymorphisms

Case Number		Exon	Codon	Sequence Change	Amino-Acid Change
Surgical	Cytology				
2	36	Exon 4	47	C→T	Pro→Ser
15	5	Exon 4	72	G→C	Arg→Pro
NA	18	Exon 4	72	G→C	Arg→Pro
38	29	Exon 4	72	G→C	Arg→Pro
4	33	Exon 4	72	G→C	Arg→Pro
2	36	Exon 4	72	G→C	Arg→Pro
34	60	Exon 4	72	G→C	Arg→Pro
NA	37	Exon 6	213	A→G	Arg→Arg
31	39	Exon 6	213	A→G	Arg→Arg

NA, tissue not available.

TABLE 5. Patient and Tumor Features

Features	p53 Status		*P*-value
	Wild-Type	Altered[a]	
Age			
<40	4	2	
40–55	3	7	**0.038**
56–70	6	1	
>70	7	1	
Tumor Size			
≤2 cm	6	3	
2–5 cm	14	5	**0.046**
>5 cm	0	3	
Estrogen receptor			
+	13	5	0.449
−	7	6	
Progesterone receptor			
+	13	3	0.066
−	7	8	
Grade			
1	3	0	
2	8	3	0.227
3	9	8	

[a] Altered p53 status includes mutations, silent and intronic changes for surgically resected tumors.

ation between these clinical variables and p53 protein accumulation and/or mutations have yielded inconsistent and often conflicting results. For example, Caleffi *et al.* found that p53 mutations occurred in younger patients (38) but other studies have not found an association between age and p53 status (5, 39, 40). The number of patients in the current report is small and may have compromised the statistical power of the study to detect associations.

The use of residual cells from ThinPrep-processed samples has several advantages. First, the fluid from ThinPrep processing can be stored at 4°C for up to 3 months, before extracting the DNA, as observed in the present study. Second, because only the residual fluid is needed for analysis, the original diagnostic slides do not have to be used. Third, in contrast to paraffin-embedded tissue, which has to undergo proteinase K digestion for at least 48 h before DNA extraction, ThinPrep-processed aspirates can undergo DNA extraction the same day they are obtained. However, there may also be disadvantages to using the residual material from ThinPrep-processing. Not all cases have tumor cells remaining in the residual fluid and thus DNA may not be available for analysis. In addition, if the aspirate contains numerous benign cells admixed with the malignant cells, mutations may be missed (20, 21).

Immunohistochemical staining can be used to detect p53 protein accumulation in either cytological or surgical specimens (24–26) but the immunohistochemical results do not always reflect the presence of underlying genetic changes (33, 34, 41, 42). For example, nonsense mutations will not cause protein accumulation, so these cells will be negative by immunohistochemical staining. In keeping with this, the presence of p53 mutations in the breast cancer was shown to be associated with decreased disease free survival as well as overall survival (5–9, 31), but the presence of p53 protein detected immunohistochemically in the tumor has not consistently been associated with a worse prognosis (7, 8, 42). As molecular analysis of p53 may provide prognostic and treatment information for patients with breast cancer, ThinPrep aspirate is a

suitable alternative to the paraffin-embedded tissue as a source of cells for this type of analysis in patients who will receive neoadjuvant chemotherapy or have unresectable tumors.

In summary, ThinPrep-processed breast FNAs provide DNA suitable for molecular analysis more rapidly than paraffin-embedded tissue. FNAs seem to be a reliable source of cells to determine the p53 gene status, given that identical alterations were detected in both the cytology and surgical specimens examined in this study.

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REFERENCES

1. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature 1991;351:453–6.
2. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957–67.
3. Ogretmen B, Safa AR. Expression of the mutated p53 tumor suppressor protein and its molecular and biochemical characterization in multidrug resistant MCF- 7/Adr human breast cancer cells. Oncogene 1997;14:499–506.
4. Aas T, Borresen AL, Geisler S, Smith-Sorensen B, Johnsen H, Varhaug JE, et al. Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. Nat Med 1996;2:811–4.
5. Berns EMJJ, Klijn JG, Smid M, van Staveren IL, Look MP, van Putten WLJ, *et al.* TP53 and MYC gene alterations independently predict poor prognosis in breast cancer patients. Genes Chromosomes Cancer 1996;16:170–9.
6. Berns EMJJ, van Staveren IL, Look MP, Smid M, Klijn JG, Foekens JA. Mutations in residues of TP53 that directly contact DNA predict poor outcome in human primary breast cancer. Br J Cancer 1998;77:1130–6.
7. Sjogren S, Inganas M, Norberg T, Lindgren A, Nordgren H, Holmberg L, *et al.* The p53 gene in breast cancer: prognostic value of complementary DNA sequencing versus immunohistochemistry. J Natl Cancer Inst 1996;88:173–82.
8. Elledge RM, Allred DC. Prognostic and predictive value of p53 and p21 in breast cancer. Breast Cancer Res Treat 1998; 52:79–98.
9. Pharoah PD, Day NE, Caldas C. Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis. Br J Cancer 1999;80:1968–73.
10. The uniform approach to breast fine-needle aspiration biopsy. National Cancer Institute Fine-Needle Aspiration of Breast Workshop Subcommittees. Diagn Cytopathol 1997; 16:295–311.
11. Wilkinson EJ, Bland KI. Techniques and results of aspiration cytology for diagnosis of benign and malignant diseases of the breast. Surg Clin North Am 1990;70:801–13.
12. Bedard YC, Pollett AF. Breast fine-needle aspiration. A comparison of ThinPrep and conventional smears. Am J Clin Pathol 1999;111:523–7.
13. Leung SW, Bedard YC. Estrogen and progesterone receptor contents in ThinPrep-processed fine-needle aspirates of breast. Am J Clin Pathol 1999;112:50–6.
14. Leung SW, Bedard YC. Immunocytochemical staining on ThinPrep processed smears. Mod Pathol 1996;9:304–6.
15. Magda JL, Minger BA, Rimm DL. Polymerase chain reaction-based detection of clonality as a non-morphologic diagnostic tool for fine-needle aspiration of the breast. Cancer 1998; 84:262–7.
16. Dimulescu I, Unger ER, Lee DR, Reeves WC, Vernon SD. Characterization of RNA in cytologic samples preserved in a methanol-based collection solution. Mol Diagn 1998;3:67–71.
17. Sherman ME, Schiffman MH, Lorincz AT, Herrero R, Hutchinson ML, Bratti C, *et al.* Cervical specimens collected in liquid buffer are suitable for both cytologic screening and ancillary human papillomavirus testing. Cancer 1997;81:89–97.
18. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403–10.
19. Zhu XL, Hartwick W, Rohan T, Kandel R. Cyclin D1 gene amplification and protein expression in benign breast disease and breast carcinoma. Mod Pathol 1998;11:1082–8.
20. Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 1989;5:874–21.
21. Murakami Y, Hayashi K, Sekiya T. Detection of aberrations of the p53 alleles and the gene transcript in human tumor cell lines by single-strand conformation polymorphism analysis. Cancer Res 1991;51:3356–61.
22. Hainaut P, Hernandez T, Robinson A, Rodriguez-Tome P, Flores T, Hollstein M, *et al.* IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. Nucleic Acids Res 1998;26:205–13.
23. Dawson-Saunders B, Trapp RG. Basic and clinical biostatistics. Norwalk, CT: Appleton and Lange; 1994.
24. Tiniakos DG, Robinson KB, Greenwood H, Cullen P, Cook AIM, Horne CHW, *et al.* c-erb B-2 and p53 expression in breast cancer fine needle aspirates. Cytopathology 1996;7: 178–86.
25. Colecchia M, Frigo B, Zucchi A, Leopardi O. p53 protein expression in fine-needle aspirates of breast cancer: an immunocytochemical assay for identifying high-grade ductal carcinomas. Diagn Cytopathol 1995;13:128–33.
26. Makris A, Allred DC, Powles TJ, Dowsett M, Fernando IN, Trott PA, *et al.* Cytological evaluation of biological prognostic markers from primary breast carcinomas. Breast Cancer Res Treat 1997;44:65–74.
27. Williams C, Ponten F, Moberg C, Soderkvist P, Uhlen M, Ponten J, *et al.* A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol 1999;155:1467–71.
28. Wong C, DiCioccio RA, Allen HJ, Werness BA, Piver MS. Mutations in BRCA1 from fixed, paraffin-embedded tissue can be artifacts of preservation. Cancer Genet Cytogenet 1998;107:21–7.
29. Shiao YH, Buzard GS, Weghorst CM, Rice JM. DNA template as a source of artifact in the detection of p53 gene mutations using archived tissue. Biotechniques 1997;22:608–10,612.
30. Dunn JM, Hastrich DJ, Newcomb P, Webb JC, Maitland NJ, Farndon JR. Correlation between p53 mutations and antibody staining in breast carcinoma. Br J Surg 1993;80:1410–2.
31. Saitoh S, Cunningham J, De Vries EM, McGovern RM, Schroeder JJ, Hartmann A, *et al.* p53 gene mutations in breast cancers in Midwestern US women: null as well as missense-type mutations are associated with poor prognosis. Oncogene 1994;9:2869–75.
32. Osborne RJ, Merlo GR, Mitsudomi T, Venesio T, Liscia DS, Cappa AP, *et al.* Mutations in the p53 gene in primary human breast cancers. Cancer Res 1991;51:6194–8.
33. Sato T, Yuyama Y, Watabe K, Okazaki A, Toda K, Okazaki M, et al. Detection of p53 gene mutations in fine-needle aspiration biopsied breast cancer specimens: correlations with

nuclear p53 accumulation and tumor DNA aneuploidy patterns. Cancer Lett 1997;115:47–55.
34. Lavarino C, Corletto V, Mezzelani A, Della Torre G, Bartolli C, Riva C, et al. Detection of TP53 mutation, loss of heterozygosity and DNA content in fine-needle aspirates of breast carcinoma. Br J Cancer 1998;77:125–30.
35. Felley-Bosco E, Weston A, Cawley HM, Bennett WP, Harris CC. Functional studies of a germ-line polymorphism at codon 47 within the p53 gene. Am J Hum Genet 1993;53: 752–59.
36. Sjalander A, Birgander R, Hallmans G, Cajander S, Lenner P, Athlin L, *et al.* p53 polymorphisms and haplotypes in breast cancer. Carcinogenesis 1996;17:1313–6.
37. Carbone D, Chiba I, Mitsudomi T. Polymorphism at codon 213 within the p53 gene. Oncogene 1991;6:1691–2.
38. Caleffi M, Teague MW, Jensen RA, Vnencak-Jones CL, Dupont WD, Parl FF. p53 gene mutations and steroid receptor status in breast cancer. Clinicopathologic correlations and prognostic assessment. Cancer 1994;73:2147–56.
39. Soong R, Iacopetta BJ, Harvey JM, Sterrett GF, Dawkins HJ, Hahnel R, *et al.* Detection of p53 gene mutation by rapid PCR-SSCP and its association with poor survival in breast cancer. Int J Cancer 1997;74:642–7.
40. Pelosi G, Bresaola E, Rodella S, Manfrin E, Piubello Q, Schiavon I, et al. Expression of proliferating cell nuclear antigen, Ki-67 antigen, estrogen receptor protein, and tumor suppressor p53 gene in cytologic samples of breast cancer: an immunochemical study with clinical, pathobiological, and histologic correlations. Diagn Cytopathol 1994;11:131–40.
41. MacGeoch C, Barnes DM, Newton JA, Mohammed S, Hodgson SV, Ng M, *et al.* p53 protein detected by immunohistochemical staining is not always mutant. Dis Markers 1993; 11:239–50.
42. Jacquemier J, Moles JP, Penault-Llorca F, Adelaide J, Torrente M, Viens P, *et al.* p53 immunohistochemical analysis in breast cancer with four monoclonal antibodies: comparison of staining and PCR-SSCP results. Br J Cancer 1994;69:846–52.