

p53 Immunostaining guided laser capture microdissection (p53-LCM) defines the presence of p53 gene mutations in focal regions of primary prostate cancer positive for p53 protein

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Objectives: A wide range of p53 mutations (5–65%), detected by various methods, has been reported in primary prostate cancers (CaP). IHC staining of radical prostatectomy specimens shows marked heterogeneity of focally distributed p53-positive cells. However, a significant relationship between the focal staining of p53 and cancer recurrence after radical prostatectomy has been noted. Increased frequency of p53 mutations has been generally observed in advanced stage CaP and metastatic prostate cancer cell lines. The significance of focal p53 immunostaining in primary CaP remains uncertain with respect to the p53 gene mutation or tumor progression. The goal of this study was to evaluate p53 gene mutations in focal regions of primary prostate cancers positive by p53 immunostaining.

Methods: Whole-mount prostates from men with clinically organ-confined prostate cancer were immunostained for p53 protein. Laser capture microdissection (LCM) was used to harvest p53 positive cells from areas of tumor and prostatic intraepithelial neoplasia and benign gland. DNA from microdissected cells were amplified for p53 exons 5–8 by polymerase chain reaction (PCR) and analyzed for mutations by single strand conformation polymorphism and DNA sequencing. Mutation analysis of the p53 gene exons 5–8 was performed in the p53 immunostaining positive focal regions (1+ to 4+) of whole-mount prostate sections from 16 patients.

Results: Of 16 patients with p53 IHC positive tumors, 11 (69%) had p53 gene mutations as determined by DNA sequence analysis. However, randomly microdissected tumor cells from 4 of 18 patients (22%) negative for p53 IHC also demonstrated mutations in the p53 gene. A significant fraction of prostate tumors with focally positive immunostaining for p53 have been confirmed to contain mutations in the p53 gene.

Conclusions: p53 immunostaining guided LCM combined with DNA-based analyses emphasizes the presence of focal p53 mutations in primary prostate cancers and underscores the significance of previous observations showing a correlation between focal p53 immunostaining in primary CaP and cancer recurrence after radical prostatectomy.

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Introduction

p53 tumor suppressor gene, the most frequently altered gene in human cancers,^{1–3} is a cellular gatekeeper and protects the genome against genotoxic and other types of stresses.^{4–7} Numerous studies have evaluated the status of the p53 alterations in CaP (reviewed in eight) and have reported a wide range of p53 mutations/p53 immunopositivity (5–60%) in primary CaP specimens. Due to the apparent discrepancies in the frequency of p53 alterations in primary CaP, it has been difficult to understand the biologic significance of p53 alterations in the genesis of primary CaP, as well as its prognostic value in assessing the disease progression. Previous studies from our group^{9,10} revealed a significant relationship between the 'focal p53 immunostaining' of the radical prostatectomy derived specimens and the cancer recurrence in patients after surgery despite the heterogeneous and focal patterns of the p53 immunostaining. Our initial observations have been supported by other studies.^{11–15} Of note, p53 gene mutation analysis in paired primary and metastatic prostate cancer tissues showed clonal expansion of the mutant p53 containing tumor cells in metastatic tumors in comparison to the primary tumors.¹² Studies from our group and others have also consistently reported a higher rate of p53 mutations in metastatic prostate cancer, especially in the hormone refractory disease.^{13–17} However, other investigators have not found p53 expression to correlate with disease progression after radical prostatectomy for localized prostate cancer or with the rate of progression after metastatic prostatic cancer.^{8,18}

DNA-based mutation detection assays generally find a low rate of p53 alterations (5–10%), while immunostaining methods show a much higher frequency of p53 positive cells in primary CaP. IHC methods therefore, are an indirect method of detecting p53 mutations. Therefore, p53 immunostaining results need to be interpreted with caution. Immunostaining of radical prostatectomy specimens shows marked heterogeneity of focally distributed p53 positive cells from tumor to tumor as well as within single tumors. The heterogeneous nature of CaP, differences in grading of p53 by immunostaining methods, focal p53 immunopositivity and technical difficulties with antibody cross-reactivities are likely reasons for the apparent discrepancy in the reported frequencies of p53 alterations in primary prostate cancer. Due to the focal and heterogeneous nature of p53 alterations,³ *in situ*-based methods such as immunostaining methods despite inherent limitations provide higher sensitivity of detection than DNA-based methods involving analysis from a mixed population of cells. Our goal in this report is to better define the status of p53 gene in p53 immunostaining positive focal regions of prostate cancers. We have used p53 immunostaining guided laser capture microdissected tumor cells to directly address this issue.

Materials and methods

Tissue specimens

Formalin-fixed, paraffin-embedded tissues from primary CaP specimens were obtained from the whole-mounted

prostate sections archived at the Armed Forces Institute of Pathology. The specimens were obtained from men who had undergone radical retropubic prostatectomy at Walter Reed Army Medical Center for clinically localized prostate cancer.

Immunohistochemical staining

Whole-mount prostate sections (4 μm) containing the greatest number of tumors and largest tumor volume per patient were stained with H&E and p53 antibody (Novocastra Laboratory, UK; catalog #NCL-p53-1801).

The slides were graded for p53 protein expression by a single pathologist as previously described by our group.^{9,10} The areas of focal staining with the highest percentage of nuclei positive for p53 were used to grade the tumor as follows: 1+ = 1–25%, 2+ = 26–50%, 3+ = 51–75%, 4+ = 76–100%, rare, and none.

Laser capture microdissection (LCM)

After grading, the cover slips were removed and the cell populations of interest were isolated using an Arcturus PXL-100 LCM instrument and procedures recommended by the manufacturer.¹⁹ With an average pulse diameter of 30 μm, epithelial cells of a prostate gland were selectively microdissected (Figure 1). On average, 40 pulses were used per cap, with an estimated recovery of four to five cells per pulse. A rough estimate of the number of positively stained cells per sample based on grading is as follows: 1+ = 15 cells, 2+ = 40 cells, 3+ = 90 cells, 4+ = 120 cells.

DNA extraction

Laser microdissected cells were captured on a thin film attached to a cap, which was then inserted into an Eppendorf tube containing 50–100 μl of cell lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Tween-20 and 40 μg/μl proteinase K incubated overnight at 37°C in inverted position at 95°C for 8 min to inactivate the proteinase K. A volume of 5 μl of cell lysates were used for polymerase chain reactions (PCRs).

p53 Gene amplification of exons by PCR

PCR amplifications of exons 5–8 were performed using the primer sequences in Table 1. PCR amplifications were performed in a final volume of 50 μl containing 1 × TBE, 2.5 mM MgCl₂, 10 ng of each primer, 0.2 mM dNTPs and 2.5 U of Amplitaq Gold DNA Polymerase (Perkin-Elmer, Branchburg, USA). PCR cycles were: 95°C for 10 min, 1 cycle; 95°C for 30 s, 60°C for 45 s, and 72°C for 90 s, 42 cycles; and 72°C for 5 min, 1 cycle using either an MJ Research PTC-200 or PTC-100 or a Perkin-Elmer Gene Amp System 9600 thermal cycler.

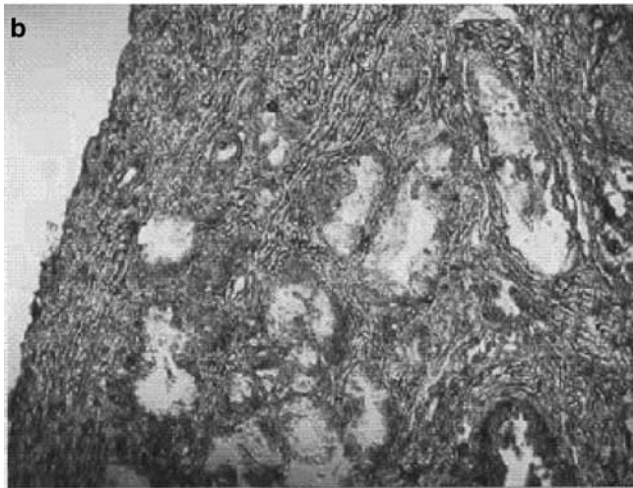
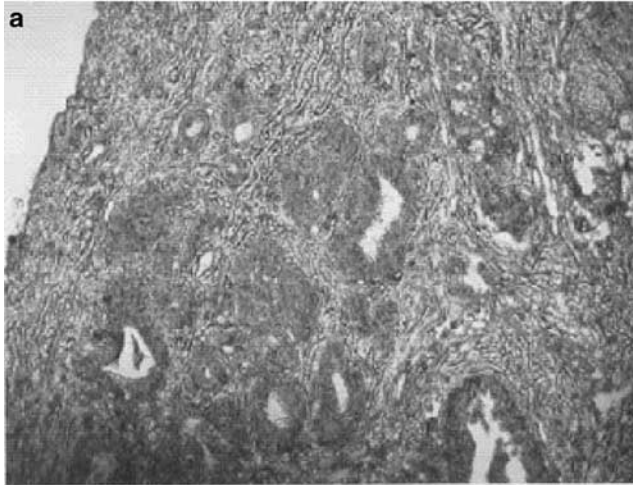


Figure 1 Laser capture microdissection of prostate tumor cells immunohistochemically stained for p53 protein expression. Whole mount prostatectomy specimens were immunohistochemically stained with anti-p53 antibody. (a) Image of prostate epithelial tumor cells graded high for p53 protein expression. (b) Image of the prostate tumor cells after laser capture microdissection. (c) Image of prostate tumor cells captured on the film.

Single-stranded conformational polymorphism analysis (SSCP)

SSCP analysis was performed on p53 exons 5–8 for each microdissected sample. A volume of 5 μ l aliquots of PCR product and 15 μ l of methyl mercury hydroxide (MMH)-dye (680 μ l 1 \times TBE, 100 μ l of 0.25% bromophenol blue and 0.25% xylene cyanol), 20 μ l of 1.0 M MMH) were heated to 95°C for 10 min, then chilled on ice for 10 min and electrophoresed on precast 20% polyacrylamide-TBE gels (Novex, San Diego, CA, catalog A#EC6315) at constant temperature in 1.0 \times TBE buffer (Table 1). Gels were stained with SYBR[®] Gold and all aberrant bands were excised and DNA was eluted into 50 ml of PCR grade water by multiple freeze/thaw cycles. The DNA from 5 μ l aliquot suspensions of each aberrant band were then amplified using the previously listed primers and PCR conditions.

DNA sequencing analysis

All suspected mutations identified by SSCP analysis were sequenced. Sequence analysis was accomplished using fluorescent-labeled dideoxy dNTPs as chain terminators. DNA sequencing using dRhodamine terminator mix was performed on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). All of the specimens displaying SSCP abnormalities were either sequenced in both directions or from PCR products obtained from two independent reactions.

Results

In total, 54 whole-mount prostatectomy specimens were examined by H&E as well as by p53 IHC staining. Of 54 specimens, 30 had positive focal areas of IHC staining. A wide range of samples were chosen based on p53 IHC staining (IHC scores: 1+ to 4+). p53 mutation analysis was performed on whole-mount specimens from 19 patients. Specimens from 16 of these 19 patients had positive IHC staining (1+ to 4+). All but one of the 19 specimens had tumor with negative IHC staining for p53. These negatively stained areas were also laser microdissected and subjected to mutation analysis. Mutation analysis was also performed from a laser microdissected benign portion of each prostate gland. Representative SSCP screening and DNA sequence analyses are shown in Figures 2 and 3. The four specimens with IHC scores of 3+ or 4+ had sequence-proven p53 mutation rates of 100%. Four of the seven (57%) specimens with a maximum IHC score of 1+ had sequence-proven mutations. Three of the five (60%) specimens with a maximum IHC score of 2+ had sequence-proven mutations. A summary of DNA sequence data is provided in Table 2. Portions of negatively stained tumors were found in each of the specimens, except 1. These were also microdissected and amplified for p53 exons 5–8. Mutation analysis was performed using SSCP. All suspected mutations were confirmed by sequence. The p53 mutation rate in these negatively staining tumors was 22%. By the same methods,

Table 1 Primers and cold-SSCP conditions for PCRs (5' to 3') for p53 exons 5–8

Exon	Primer	Nucleotide	Fragment Size	Cold SSCP Conditions
5	p577 p590	CTT CCT gCA gTA CTC CCC Tg CAg CTg CTC ACC ATC gCT ATC	204 bp	25°C, 160 min, 300 V, 20% TBE gel
6	p77 p78	TTg CTC TT A ggT CTg gCC CC Cag ACC TCA ggC ggC TCA T A	128 bp	13°C, 80 min, 300 V, 20% TBE gel
7	p729 p730 p131 p132	Tgg gCC TgT gTT ATC TCC TAg g AAg AAA TCg gTA AgA ggT ggg C AgTggTAATCTACTgggACgg ACC TCg CTT AgT gCT CCC Tg	223 bp 141 bp	20°C, 150 min, 300 V, 20% TBE gel 15°C, 180 min, 300 V, 20% TBE gel

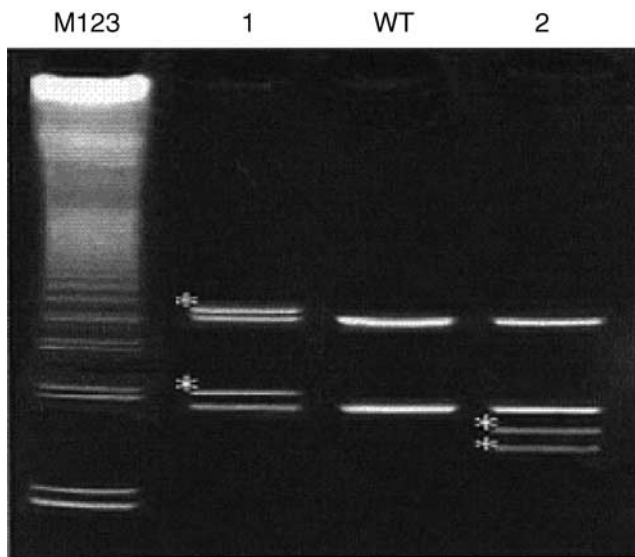


Figure 2 Representative of SSCP analysis of p53 gene. The PCR product was Electrophoresed on 20% TBE polyacrylamide gel. M123 is the molecular weight standard. WT indicates the wild type DNA. Samples 1 and 2 show mutated alleles.

Table 2 Comparison of immunohistochemical staining to sequenced mutation

Specimen	n	Mutation	No mutation
Tumor (+p53)	16	11 (69%)	5
Tumor (–p53)	18	4 (22%)	14
PIN (+p53)	2	1 (50%)	1
Benign gland	19	4 (21 %)	15

Key: +p53=positively staining for p53; –p53=negatively staining for p53; PIN=prostatic intraepithelial neoplasia.

LCM-derived cells from benign glands in these men with prostate cancer were found to have a mutation rate of 21%. A summary of these findings is in Table 2.

Discussion

There are numerous reports that have studied the role of p53 alterations in prostate cancers. While frequency of p53 alterations remain controversial in primary prostate cancer (CaP), generally a higher rate of p53 alterations

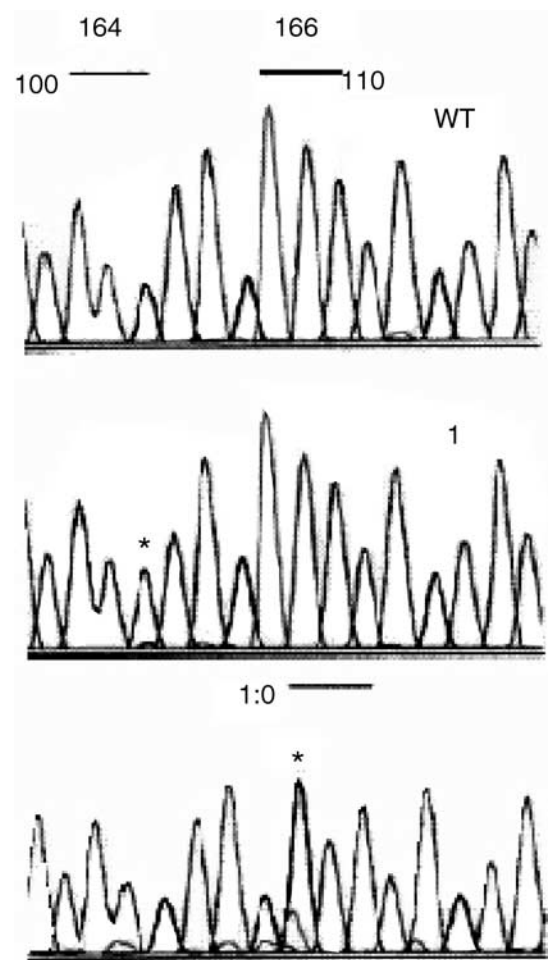


Figure 3 Representative DNA sequence analysis of p53 gene from prostate tumor cells. The figure indicates the mutation of p53 gene at codon 164 in Sample 1 and mutation of p53 gene at codon 166 in Sample 2. Wild-type (WT) sequence is provided as reference.

are reported in advanced CaP and metastatic CaP cell lines.^{13,21} Studies in mice also demonstrate that loss of p53 function leads to prostate cancer metastasis.²² However, there are concerns on the biologic significance of p53 mutations in CaP cells. Association of LOH and the presence of p53 mutation or complete functional inactivation of the p53 locus, has not been clearly established in human prostate cancer cells. In part, these results may arise due to technical challenges inherent in

the analysis of focal changes in tumor specimens. The use of LCM has allowed us for the first time to accurately sample focally positive immunostaining portions of prostate tumors and confirms that they do contain mutations by DNA analysis. The data here confirm that in general, IHC staining represents the presence of mutations in prostate cancer. Here we reported mutation as positive, not based on immunohistochemistry, or by aberrant bands on SSCP, but by proven DNA sequence data. We found that 69% of specimens staining positively for p53 had mutation at DNA level. Given the technical issues involved, these results are reasonable. It is worth noting that even with accurate microdissection afforded by LCM, there is still room for sampling error, especially when the quantity of harvested cells is small and only a fraction of those cells typically exhibit positive IHC staining. Recent improvements in LCM can further refine this analysis. It is intriguing that 22% of negatively stained tumors had mutations confirmed by sequence and suggests that these mutant p53 do not result in an increased half life of the p53 protein.

LCM coupled with DNA analysis confirms the marked heterogeneity of prostate cancer with respect to p53 mutations. Several groups^{14,15,23} have demonstrated this previously using other technical approaches. Also previously reported is the relatively high frequency of silent p53 mutations. Meyers *et al*²³ reported a p53 mutation frequency of 30% in men with benign prostatic hyperplasia. Our data confirms this finding. The significance of p53 mutations in benign prostate glands is controversial and needs further evaluation. Focal p53 alterations are associated with metastatic prostate cancer and with increased risk of recurrence following prostatectomy for clinically, locally, confined prostate cancer. A prospective evaluation of LCM-derived CaP cells from OCT-embedded frozen CaP specimens with or without IHC followed by p53 mutational analysis is needed to accurately understand the status and biologic significance of focal nature p53 in the genome of primary prostate cancers and its association with clinicopathologic features.

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