Clustered p53 Immunostaining: A Novel Pattern Associated with Prostate Cancer Progression


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ABSTRACT

Abnormal p53 protein accumulation is typically defined as present when greater than 5 or 10% of cancer cells stain positively. We present a novel approach whereby immunopositivity is defined when 15 or more cells within a 300 × 400-μm² field exhibit p53 protein accumulation; a feature that we have called “clustered” staining. We assessed p53 immunostaining of moderately differentiated, clinically localized prostate cancers derived from two patient groups: those without cancer recurrence 5 years after radical prostatectomy, and those in whom cancer had recurred following radical prostatectomy. Clustered p53 immunopositivity was present in 10 (63%) of 16 patients in the recurrent group and in only 7 (21%) of 33 in the nonrecurrent group.

INTRODUCTION

A dilemma in the diagnosis and treatment of prostate cancer is that only a small fraction of these cancers progress to be clinically significant. New or revised molecular markers may potentially offer an improved predictive index of the malignant potential. The aberrant expression of the tumor suppressor gene p53, caused mainly by gene mutations (1), has been implicated in the initiation and progression of numerous human malignancies. Immunohistochemical staining of nuclear p53 is typically indicative of an accumulation of mutant p53 protein (2) and can differentiate patients at high risk of cancer recurrence for a spectrum of human carcinomas (3, 4).

In the human prostate, abnormal p53 protein accumulation has been identified primarily in poorly differentiated, metastatic, and late clinical stage cancers, indicating an association with poor prognosis (5–7). Typically, studies have defined abnormal nuclear accumulation of p53 protein only when more than 5 or 10% of the total number of cancer cells were immunolabeled. Such criteria have not provided a tool of discriminant prognostic value for well or moderately differentiated cancers at an early clinical stage (6, 8). More recently, there is evidence that indeed low levels of p53 immunopositivity in primary prostate cancer may have prognostic significance (9, 10).

There is also experimental evidence that p53 abnormalities in the primary tumor have the potential to influence the clinical course. Using the well-described mouse prostate reconstitution model, p53 gene dysfunction has been clearly linked to metastatic progression (11). This study also demonstrated that metastases can seed from genetically abnormal cells which comprise only a small percentage of the total cell population within primary tumor. In a more recent study using paired human primary and metastatic tissue harvested from patients prior to any endocrine manipulation, we have demonstrated that p53 immunoreactivity is more frequently found at the metastatic site (7). Almost one-quarter of the pelvic lymph node metastases had positive p53 immunostaining compared with 10% of locally advanced cancers and none of those cancers that were organ confined. In this study, the often quoted limit to signify p53 immunopositivity of >5% of tumor cell nuclei staining was used. This may be an artificial limit that restricts the full stratification of primary cancers and thereby reduces the potential usefulness of p53 immunoreactivity as a prognostic marker.

Cancer cells with abnormal p53 accumulation may exist and potentiate the risk of metastatic progression but be in such small numbers as to be missed by a global scan of the tissue section when assessing p53 immunoreactivity. These cells with a bias for biological aggressiveness may stem from a small “focal” event and therefore be “clustered” together in a recognizable pattern prior to their potential influence over the natural history of that cancer. In fact, the presence of a small number of p53-positive cells heterogeneously distributed in early prostate cancer has been previously described (12, 13). Therefore, we redefined the criteria for positivity of p53 immunostaining to encompass these views that there may be clustering of p53 positive cells, a feature which may better serve as a predictive marker for progression in moderately differentiated cancers.

PATIENTS AND METHODS

Radical prostatectomy specimens were evaluated from 49 patients with moderately differentiated prostate adenocarcinomas (Gleason sum, 5–7) clinically localized to the prostate (stage T1/T2, N×, M0) as determined by physical examination, transrectal ultrasound, a serum prostate-specific antigen of <20 ng/ml, and a negative whole-body bone scan. Cases were accrued in a consecutive manner to give a reasonable sample size. No patient received cancer-specific treatment before or after their surgery and prior to recurrence, and they were subsequently categorized into two groups: those without cancer recurrence after a follow-up period of at least 5 years (nonrecurrence, n = 33), and those with cancer recurrence...
determined either clinically or by an elevated prostate-specific antigen level (>0.4 ng/ml on repeated analysis) within 5 years (recurrent group, n = 16). The groups were comparable for age (P = 0.34, Student’s t test) and Gleason sum (P = 0.41, Student’s t test). Of importance, the pathological stage determinations showed no differences between the two groups (ratio T2:T3a of 20:13 in the nonrecurrent group, and 9:7 in the recurrent group; P > 0.99, Fisher’s exact test).

Immunohistochemistry was performed on formalin-fixed paraffin-embedded 5-μm sections derived from punch blocks of cancer specimens. The mouse monoclonal anti-p53 antibody DO-7 (DAKO Corporation, Carpinteria, CA) and an ABC kit (Vector Laboratories, Burlingame, CA) were used. In brief, after tissue sections were deparaffinized and rehydrated through graded alcohol, they were heated in 0.01 M citrate buffer at pH 6.0 by microwave for 7 min to enhance antigen retrieval. The sections were then treated with 2% H2O2 for 10 min to inactivate endogenous peroxidase activity. Following a 20-min blocking step with 1.5% normal horse serum, the sections were sequentially incubated in the DO-7 antibody diluted 1:80 for 90 min, then in biotinylated horse antimouse IgG and the avidin-peroxidase complex for 30 min each. The immunoreaction was developed using 3',3'-diaminobenzidine to produce a brown precipitate. Included in each experiment was a negative control for which the primary antibody DO7 was substituted with nonspecific mouse IgG and a positive control consisting of human colon cancer tissue with a known p53 mutation that manifests p53 nuclear accumulation.

Immunostaining was assessed using a Zeiss light microscope equipped with a video camera that projected an image (one field) onto a Trinitron monitor (Sony) that corresponded to a tissue area of 300 × 400 μm². Each complete section was viewed systematically field by field, and p53 immunopositivity was recorded when >15 cancer cell nuclei were stained in one or more fields.

RESULTS
Abnormal accumulation of p53 protein was evident only in the nuclei of cancer cells, and the p53-immunopositive cells were heterogeneously distributed throughout many of the sections examined. Based on our criteria for clustered p53 immunostaining detailed above, 17 (35%) of the total number of 49 patients were p53 immunopositive. Ten (63%) of 16 p53-immunopositive cancers were from the patient group with recurrent cancer and 7 (21%) of 33 were from the nonrecurrent group, indicating that p53 immunopositivity is associated with cancer recurrence (P < 0.01, Fisher’s exact test; see Table 1). An example of “clustered” p53 immunopositive staining with a

Fig. 1. Left panel, photomicrograph of a hematoxylin and eosin-stained section of primary prostate cancer; right panel, section from the same tissue block immunohistochemically labeled with DO7, an anti-p53 primary antibody. The immunoreaction was developed with 3',3'-diaminobenzidine to produce a brown precipitate against the counterstain of methyl green. Clustered p53 immunostaining is noted. ×200.
nearby hematoxylin and eosin-stained section for comparison is seen in Fig. 1. Two authors (T. M. W., L. D. T.), blinded to the patients' details, independently evaluated the sections, and concordance between these observers was 97%. Each analysis was performed quickly, typically taking <5 min/tissue section. When the conventional criteria of >5% total tumor cells needing to be positive was used, only six immunopositive cancers were identified; four of which came from the recurrent group. This low rate of p53 positivity with conventional criteria is consistent with other reports in the literature and is of little prognostic value in this group of moderately differentiated prostate cancers.

**DISCUSSION**

In this study, we have demonstrated a statistically significant correlation between the recurrence of prostate cancer following radical prostatectomy for localized disease and a new measurement of p53 immunopositivity within the primary tumor. The stimulus to reexamine p53 immunoreactivity in primary prostate cancer was borne after data linked metastasis in the mouse prostate reconstitution animal model to the loss of normal p53 function in the primary cancer, and, in addition to this, increased p53 immunostaining noted in metastatic lesions compared to primary cancers in humans prior to any specific cancer therapy. Small discrete areas of p53 immunostaining had been previously observed and suggested rigidity in the conventional determination of p53 positivity. The requirement that 5 or 10% of all tumor cells need to be p53 positive has failed to predict biological aggression in the largest category of grade of prostate cancer: those that are moderately differentiated (Gleason sum, 5–7). Our findings suggest that by determining the presence of the foci of p53-positive cancer cells useful prognostic information can be obtained. However, this is a relatively small study, and additional data will need to be collected to further evaluate these hypotheses. Analyses need not be confined to prostate cancer—cancers originating from other organs may equally be assessed in a retrospective fashion to test the usefulness of these new immunohistological criteria. We are not the first to suggest that conventional immunohistological criteria for p53 lack discriminatory power. Shurbaji et al. (9) have recently presented data indicating that any degree of p53 immunostaining was prognostically important in primary prostate cancer specimens, although the initial survival advantage conferred by a lack of p53 immunoreactivity was lost after 6 years of follow-up in their series. By lowering the cutoff point to zero to determine p53 immunopositivity, increased discriminatory power was gained. A conceivable advantage with the definition of positivity in this study is its ability to more closely define the tumor biology. Others have found no statistically significant association between p53 immunopositivity and patient survival when conventional criteria were used (8).

Our modifications to the detection limits of p53 immunopositivity still encompassed those cases defined as positive by conventional means while increasing the positive case numbers nearly 3-fold. This method has improved the sensitivity of p53 immunostaining as a predictor of cancer progression. Additional refinements to this type of immunohistochemical analysis may yet improve the positive and negative predictive values (59 and 81%, respectively) found in this study. The ease of analysis using a video camera and monitor is simple, quick, and relatively inexpensive. Additional studies are warranted to evaluate the potential of this modification of an established technique to act as a useful clinical marker of cancer progression.

**REFERENCES**

Clustered p53 immunostaining: a novel pattern associated with prostate cancer progression.


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