Immunochemistry for p16, but not Rb or p21, is an independent predictor of prognosis in conservatively treated, clinically localised prostate cancer

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Abstract

Treatment decisions are difficult in clinically localised prostate cancer and further biomarkers of aggressive behaviour are required. We investigated the hypothesis that the tissue expression of three cell cycle markers, Rb, p21 and p16, would provide helpful prognostic information in a well characterised series of prostate cancers which were clinically localised and treated conservatively. The immunohistochemical staining expression of these markers was assessed in tissue microarrays and correlated with 10 year prostate cancer survival and overall survival and then compared with pathological data including contemporary Gleason score, measures of tumour extent and initial PSA level.

Rb over-expression did not show any significant association with Gleason score or prostate cancer survival. P21 protein expression showed a significant association with Prostate cancer survival ($p = 0.02$) and overall survival ($p=0.01$) in a univariate model but not in a multivariate model with pathological and Prostate Specific Antigen data

There was a significant association between p16 cytoplasmic expression and Prostate cancer survival (HR=2.52, CI=1.79-3.55, $p<0.001$) and overall survival (HR=1.54, CI=1.20-1.98, $p=0.001$) in a univariate model. P16 expression remained an independent prognostic factor for Prostate cancer survival (HR=1.50, 95% CI=1.05-2.14, $p=0.03$).

We conclude that p16 cytoplasmic expression is a strong predictor of outcome in conservatively treated prostate cancer. Rb and p21 show no independent association with outcome and therefore further research is not warranted.

Key words: Conservatively treated, tissue microarray, immunohistochemistry, prostate cancer survival, overall survival, Gleason score, Prostate specific antigen, Transatlantic prostate group

Introduction

Prostate cancer (PCa) is the second most common malignancy after lung cancer among men worldwide [1]. The incidence of PCa has increased over the past 20 years but this has not been reflected in the mortality rate. This is mainly due to an increase in Prostate specific antigen (PSA) screening despite its lack of specificity[2]. However, in many countries, prostate cancer is now diagnosed at a clinically localised asymptomatic stage. Curative treatment options for localised PCa include radical surgery, external beam radiotherapy, cryoablation and brachytherapy. While radical treatments are often curative, many tumours are indolent and would not have progressed within a patient's lifetime. Gleason score and serum Prostate Specific Antigen (PSA) remain only moderate predictors for outcome and further biomarkers are required to refine risk of progression.

The Transatlantic Prostate Group (TAPG) was created to collect and study a large retrospective cohort of clinically localised patients with contemporary Gleason score and serum PSA [3]. Tissue collected from this study is available as a resource to identify potential biomarkers that can predict PCa progression for eventual adoption into clinical use. As nearly all biomarker studies on prostate cancer examine tumours treated by radical means, the TAPG study enables an insight into the 'natural history' of the disease,. These cell cycle proteins were chosen as they represent part of a well known pathway, widely studied in prostate cancer, and that they all have potential to be translated to clinical use, as they are used in routine on a regular basis.

Rb is a tumour suppressor gene which regulates transition between G1 and S phases of the cell cycle. Loss of heterozygosity of Rb in PCa has demonstrated by several studies [4] [5] [6]. P21 acts as a tumour suppressor protein and initiates cell cycle arrest by inhibiting the activity of Cyclin dependent kinases (Cdk) 2, 3, 4 and 6 which have a direct role in the G1-S transition. In a quiescent cell p21 expression is low but it increases in response to mitogenic signals during the G1 phase progression. P21 is activated via p53 independent as well as dependent pathways. Gao et al., (1995)

identified a mutation of the p21 gene in PCa but in majority of PCa, p21 seem to be over-expressed rather than mutated [7].

P16 is also a tumour suppressor protein which is linked to Rb pathway and is located on chromosome 9p21-22. P16 belongs to the INK4a family where the p16 regulatory protein binds to cyclin dependent kinase 4 or 6 and inhibit cyclin D-dependent kinase phosphorylation of Rb. This halts the progression of G1 to S phase leaving the cells to repair or senesce. Loss of function of p16 due to mutation, deletion and loss of heterozygosity is common in human tumours. Over-expression of p16 correlates with tumour recurrence in radical prostatectomy patients [8].

Most of the studies related to the above mentioned proteins and PCa are on radical prostatectomy cohorts, radiotherapy groups or hormonal therapy groups. The relationship between each of the protein expressions above and their prognostic value on PCa have not been investigated in a conservatively treated cohort. In this study the TAPG cohort was utilised to test the hypothesis that this protein expression information might add to the overall prognostic model and assist in patient risk assessment.

Material and methods

Detailed information on patient data collection was published previously [3]. In brief, the patient information and tissues were collected from six cancer registries in the UK. Patients were diagnosed with clinically localised PCa by trans-urethral resection of prostate (TURP) or needle biopsies between the years 1990 and 1996. The patients were included only if PSA level was available pre-diagnosis and less than 100ng/ml and were under the age of 76 years.The diagnosis and Gleason score was re-assessed by an expert GU pathologist. Patients were excluded if they had radical surgery or radiotherapy within 6 months of diagnosis or if there was objective evidence of metastasis or clinical indication of metastasis. There were 1656 patients in total. After 10 years

follow up a prognostic model including Gleason score, clinical stage, age, extent of disease and PSA level was created.

Tissue from the TURPs, which was 55% of the cohort, was tissue microarrayed (TMA) into 24 wax blocks. Multiple (usually 3) tissue cores with a 0.6mm diameter were taken from each formalin fixed donor block and was transferred into a new recipient wax block. Four μm thick sections were cut from the TMA blocks and were immunohistochemically (IHC) stained for Rb, p21 and p16 using the following clones: Retinoblastoma, (Novocastra, Newcastle Upon Tyne, U.K) at a dilution of 1/50, p21 (DakoCytomation, Cambridgeshire, U.K) at a dilution of 1/1000, and p16 (Neomarkers, California, U.S.A) at a dilution of 1/100. Antigen retrieval by pressure cooking in citrate buffer at pH=6.0 was performed for Rb and p21. Diaminobenzidine was used as a chromogen and the slides were counterstained by hematoxylin. Hyperplastic tonsil, normal placenta, and cervical intraepithelial neoplasia grade 3 were used as positive controls for Rb, p21, and p16 respectively. The TMA without primary antibody incubation was used as negative control.

The protein expression was analysed semi-quantitatively. The percentage expression (P) was assessed between 0-100% and the intensity of the staining (I) was assessed between 0= no staining, 1=weak, 2=medium and 3=strong. This allowed an immunostaining score (IS) between 0-300, where IS= I x P. For p21 and Rb, only nuclear staining was assessed. For P16 expression both nuclear and cytoplasmic expressions were scored.

The maximum stained core was considered as representation of tumour progression for p21 and p16 and the minimum stained core was considered for Rb as loss of immunoexpression was taken as a reflection of tumour progression. For each protein, the correlation of IS with demographics and tumour characteristics, including Gleason score, was examined (using the w2 test and Fisher's exact test for categorical variables and analysis of variance for numerical variables). The main end points were time to death from PCa and time to death from any cause. Univariate and multivariate analyses were performed by proportional hazard (Cox) regression analysis. The multivariate models

include Gleason score, age at diagnosis, baseline PSA level and extent of disease. All p values were two sided and 95% confidence intervals (CIs) were based on normal distribution.

Results

Rb

1809 tissue cores were identified as cancer representing 702 patients with Rb expression data. The Rb protein expression was compared with Gleason score, clinical stage and initial PSA value. There was no significant association between Rb protein expression and Gleason score (p=0.78). There was no association between Rb expression and PCa survival (HR=0.98, 95% CI=0.67-1.45, p=0.94) nor with overall survival (HR=0.88, 95% CI=0.68-1.14, p=0.34) in univariate analyses. In a multivariate model including Gleason score, age and extent of disease Rb expression did not show any significant association between PCa survival (HR=1.16, 95% CI=0.78-1.72, p=0.46) and overall survival (HR=0.98, 95% CI=0.76-1.27, p=0.88).

P21

Expression of p21 protein was analysed in 1789 cancer tissue cores which represented 694 patients. The p21 expression was significantly correlated with Gleason score ($p < 0.001$). Both PCa survival (HR=1.61, 95% CI=1.08-2.42, p=0.02) (Figure1) and overall survival (HR=1.42, 95% CI=1.08-1.86, p=0.01) showed significant correlation with p21 protein expression in a univariate model but failed to show any significance in a multivariate model.

P16

There was information of nuclear expression and cytoplasmic expression for 1287 cancer cores which was a representative of 534 patients. Both p16 nuclear expression (p=0.001) and cytoplasmic expression ($p<0.001$) were significantly associated with Gleason score. There was a significant association between PCa survival (HR=2.52, CI=1.79-3.55, $p<0.001$) and overall survival

(HR=1.54, CI=1.20-1.98, p=0.001) in a univariate model for p16 cytoplasmic expression. In a multivariate setting including Gleason score, age, extent of disease and PSA level, p16 cytoplasmic expression remained a independent prognostic factor for PCa survival (HR=1.50, 95% CI=1.05-2.14, p=0.03) (Figure2). P16 nuclear expression was significantly associated with PCa survival (HR=1.63, CI= 1.13-2.34, p=0.008) but not with overall survival in a univariate model and did not remain significant in a multivariate model.

Discussion

The immense size of the literature on biomarkers in prostate cancer has not translated into the use of any candidate tissue markers into current clinical practice. This is for many reasons, but the most important is that most biomarker assessments occur on radical prostatectomy specimens, or on biopsies of patients who undergo some other form of radical treatment such as radiotherapy. However, decisions on definitive treatment are made after diagnosis, but prior to treatment, either on TURP, or more usually in trans-rectal ultrasound biopsy specimens. The TAPG series is the only conservatively treated cohort with extended follow up, contemporary Gleason scoring and serum PSA in which translational material is available. It is therefore a tool to consider those biomarkers which may be of use in future or current prospective series which may yield translational tissue resources. Comparing this study with other comparable series with long term follow up, the study by Albertsen et al, has not yielded translational data while the study by Mucci et al on the cohort developed by Johannson et al has not yet yielded significant information [9], [10], [11]. Unfortunately, this latter series does not include pre-diagnostic serum PSA measurements. There is no current cohort of patients treated in a uniform fashion by either active surveillance or watchful waiting in which translational material is yet available. Although ideally, such a series would be conducted on biopsy material, conforming to modern methods of diagnosis, we here utilised TURP tissue which constituted 55% of our series. Our future aim is to translate promising biomarkers in the TURP material into the much more difficult biopsy series where tissue is extremely limited. It should be

noted that for our multivariate analyses we included disease extent as well as Gleason score and serum PSA level to ensure that as much prognostic information as possible could be extracted from standard parameters.

These data show that Rb is not a promising biomarker for disease outcome. Deletion of the Rb gene and loss of expression has been long reported in PCa [12]. Rb protein in tumour tissue can either be down-regulated due to loss of function or due to interactions of redundantly acting genes which mask the expression of Rb protein [13]. Previous studies of Rb protein expression unfortunately did not show significant associations with the clinical and pathological parameters but one paper did show a significant association with disease specific survival [14]. Our study showed reduced Rb expression with most of the cancer tissue showing weak staining intensity. This has been observed in quite a few studies [15], [16], [17] done on early stage PCa. If the Rb gene is mutated the tumour suppressor function will be altered and this may be another reason for reduced Rb protein expression. The anti Rb antibody that was used in the study only recognised the unphosphorylated Rb, which should have identified all the unphosphorylated Rb in the nucleus and if Rb has lost its function due to one inactive gene the other half of the gene which is active has the ability to express Rb.

P21 protein expression showed a significant association with Gleason score in addition to PCa survival and overall survival in a univariate model. Association between p21 protein expression with survival varies between cancer types. In colorectal cancer [18], bladder cancer [19] and hepatocellular carcinoma [20] reduced or absence of p21 expression was correlated with poor clinical outcome, but in breast cancer [21], carcinoma of the head and neck [22] and esophageal carcinoma [23] p21 over-expression was associated with worse prognosis. Nevertheless expression of p21 protein was shown to be correlated with the Gleason score in two previous studies on radically treated cancers [24], [25]. Although the results were significant in a univariate model, the

lack of significance in a multivariate model, suggest that this marker is not sufficiently powerful to progress to the future experimental stage of testing in a biopsy cohort.

The cytoplasmic expression of P16 was significantly correlated to PCa survival in both univariate and multivariate models. Jarrard et al (2002) showed an association with p16 protein expression and Gleason score as an independent variable, also Henshall et al, in a multivariate model showed association with both Gleason score and clinical stage [26]' [27] in radically treated disease. Development of a standardised IHC technique for p16 expression cut-off may increase the acceptability as a prognostic method so that it can be used in a clinical setting. A major disadvantage of IHC is that there is an absence of standardised techniques, either nationally or internationally. This reduces its acceptability as a robust technique for clinical diagnosis. It is possible, in common with a number of other studies, that cytoplasmic expression of p16 is representative of aberrant expression secondary to mutation, deletion or loss of heterozygosity.

In this unique series of conservatively treated prostate cancers p16 cytoplasmic expression proved an independent predictor of prostate cancer death, whereas Rb and p21 did not add to this prognostic model. We suggest that p16 but not Rb or p21 be investigated further for potential clinical use in determining risk of progression in clinically localised prostate cancer.

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