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RESEARCH

p53 and Cyclooxygenase-2 Expression are Directly Associated with Cyclin D1 Expression in Radical Prostatectomy Specimens of Patients with Hormone-Naïve Prostate Cancer

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Abstract Prostate cancer (PCa) is a potentially curable disease when diagnosed in early stages and subsequently treated with radical prostatectomy (RP). However, a significant proportion of patients tend to relapse early, with the emergence of biochemical failure (BF) as an established precursor of progression to metastatic disease. Several candidate molecular markers have been studied in an effort to enhance the accuracy of existing predictive tools regarding the risk of BF after RP. We studied the immunohistochemical expression of p53, cyclooxygenase-2 (COX-2) and cyclin D1 in a cohort of 70 patients that underwent RP for early stage, hormone naïve PCa, with the aim of prospectively identifying any possible interrelations as well as correlations with known prognostic parameters such as Gleason score, pathological stage and time to prostate-specific antigen (PSA) relapse. We observed a significant ($p=0.003$) prognostic role of p53, with high protein expression correlating with shorter time to BF (TTBF) in univariate analysis. Both p53 and COX-2 expression were directly associated with cyclin D1 expression ($p=0.055$ and $p=0.050$ respectively). High p53 expression was also found to be an independent prognostic factor ($p=0.023$). Based on previous data and results provided by this study, p53 expression exerts an independent negative prognostic role in localized prostate cancer and could therefore be evaluated as a useful new molecular marker to be added in the set of known prognostic indicators of the disease. With respect to COX-2 and cyclin D1, further studies are required to elucidate their role in early prediction of PCa relapse after RP.

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Introduction

The dramatic increase in the diagnosis of PCa since the widespread introduction of serum PSA screening has caused a significant rise in the use of RP as monotherapy with a curative intent. Currently, prognosis after RP is based on pre-operative PSA and pathological findings such as Gleason score, stage and positive margins [1]. However, the wide variability in the biological behavior of PCa limits the prognostic value of these parameters and necessitates the identification of other prognostic markers. The use of molecular markers to supplement clinical information concerning the biological aggressiveness of prostate cancer may allow for more optimal selection of treatment by identifying a subset of patients who would benefit from more intensive post-operative surveillance and/or adjuvant therapy, perhaps providing an advantage of improved disease-specific survival. Numerous attempts have been made to use tissue biomarkers to enhance the prediction of outcome after RP. Although many potentially prognostic markers have been studied, none has been incorporated into prognostic models or therapeutic decision making.

The clinical significance of the tumor suppressor p53 has been a common subject of investigation through the last two decades. Alterations in the TP53 gene may be evidenced indirectly by increased immunoreactivity of the respective aberrant gene product due to its prolonged half-life, thus rendering p53 staining a measure of gene inactivation [2], although immunohistochemical results are not always concordant with mutational analysis results [3]. Studies dealing with p53 overexpression in PCa have yielded conflicting results, although the association between such p53 alterations and clinicopathological parameters of poor outcome was a general finding.

COX-2 is the inducible form of cyclooxygenase that is frequently elevated in cancer tissues [4]. In recent years there has been considerable interest in the expression of the inducible isoform cyclooxygenase (COX-2) in PCa. COX-2 expression is induced by pro-inflammatory stimuli and growth factors [5]. COX-2 was consistently found to be overexpressed in PCa in contrast to benign prostate tissues. Typically, most studies reported low or no expression in normal prostate tissue, benign prostate hyperplasia (BPH) or low-grade PCa but increased levels in prostate intra-epithelial neoplasia (PIN) and PCa [6–12]. Notably, increased COX-2 expression is not a typical abnormality in PCa in general, but occurs in high grade tumors [13].

Cyclin D1 is a G1 checkpoint regulatory protein and a candidate proto-oncogene whose aberrant expression has been implicated in the pathogenesis of several types of neoplasia [14], including PCa. It has been suggested that amplification and/or overexpression of cyclin D1 is not a common event in both primary and tumor derived prostate cell lines, however it is present in more aggressive PCa phenotypes, defining a different molecular biology [15, 16].

To determine the roles and interrelations of p53, COX-2 and cyclin D1 status as well as their potential prognostic value after RP, we performed p53, COX-2 and cyclin D1 immunostaining on prostatectomy specimens from hormone-naïve patients with early stage PCa and recorded clinical data prospectively, particularly focusing on TTBF. To our knowledge, there is no previous study examining these potential biomarkers together, with the exception of a study of p53 and cyclin D1 in a smaller group of patients, disclosing no apparent correlations with clinicopathologic factors, except from a p53-high Gleason grade association [17].

Materials and Methods

Patient Characteristics and Tissues

Following Institutional Review Board approval, we retrospectively reviewed all relevant clinical information of prostate cancer patients that had undergone radical prostatectomy. Patient demographics, tumor grade, pathological stage, PSA recurrence and survival data were abstracted into a study-specific database. 70 representative H&E-stained tissue sections were examined to evaluate the histopathological characteristics of each case. Patient selection was based solely on the availability of both adequate follow-up data and representative pathology specimens for immunohistochemical analysis (IHC). Clinico-pathological parameters included pathological stage, and Gleason score, and were re-scored by a single histopathologist. Cases were grouped as either low Gleason score ($\leq$7, 7=3+4, n=50) or as high Gleason score ($\geq$7, 7=4+3, n=20). Cases were also grouped according to pathological stage into either organ confined disease (TNM $\leq$2; n=42) or advanced tumors extending beyond the prostatic capsule (TNM $\geq$3; n=28). All of the patients were hormone naïve at the time of surgery.

Immunohistochemical Analysis

p53, COX-2 and cyclin D1 expression were assessed by immunohistochemistry, using a p53 mouse monoclonal antibody (DAKO, D07) in a dilution of 1:50, a COX-2 mouse monoclonal antibody (NOVOCASTRA, 4H12) in a

dilution of 1:100 and a cyclin D1 rabbit monoclonal antibody (NEOMARKERS, SP4), in a dilution of 1:50 respectively. Prostate cancer tissues were classified according to their level of p53 expression by evaluating the percentage of positive nuclear staining: ≥5% was considered positive whereas <5% was evaluated as negative p53 expression. For cyclin D1, the same mode of assessment was used, with >10% positive cells being used as a cut-off point. For evaluation of COX-2 we assessed cytoplasmic immunostaining, by combining the percentage of positive cells (<25%, 25–50%, 50–75%, >75%) with intensity of expression (0, 1, 2, 3) to produce a final score of ≥3 (positive expression) or <3 (negative expression).

Statistical Analyses

The response variable, time to PSA relapse, was defined as the time from RP to the time of the first detectable (non-zero) PSA measurement. To confirm PSA relapse, three consecutive increases of PSA were required; however, the time of relapse was defined as the time of the first detectable PSA measurement [18]. All of the patients were hormone naïve at the time of PSA relapse. The Fisher's and χ^2 tests were used to explore associations between p53, COX-2, cyclin D1 expression patterns and Gleason score, tumor stage. The Cox proportional hazards model was used to assess the relationship between p53, COX-2, cyclin D1 expression and time to PSA failure after controlling for pathological stage and Gleason score. All of the p values were two-sided.

Results

p53 Expression is Inversely Associated with TTBF and is an Independent Predictor of PSA Recurrence

Patients were classified in two groups of either negative p53 staining (n=58, 82.86%) or positive p53 immunohistochemical expression n=12, 17.14%). Thirty-seven (52.86%) patients developed PSA recurrence during follow up, thirty-three (47.14%) did not have a PSA relapse and 2 patients (2.86%) expired. The estimated median follow up time, as calculated by the reverse Kaplan-Meier method was 30 months while the median time to biochemical failure was 56 months. In univariate analysis, the expression of p53 was found to be inversely associated with TTBF, with a median time to biochemical progression of 56 months for the group of negative p53 expression, whereas only 9 months for patients with tumors expressing p53 (p=0.003) (Fig. 1). As expected, pathological stage and Gleason score were directly interrelated (p<0.001) and both

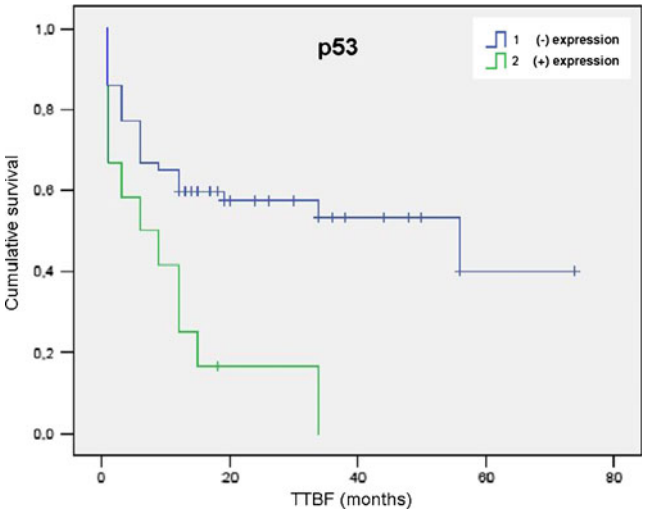

Fig. 1 Cumulative survival in 70 patients with hormone naïve PCa after RP, according to p53 expression

were inversely associated with TTBF (p<0.001 and p< 0.001 respectively), although there was no significant correlation observed between p53 and grade (p=0.749) or p53 and stage (p=1.000) (Table 1). In multivariate analysis, we found that p53 was significantly associated with TTBF after controlling for tumor stage and Gleason score (p= 0.023; hazard ratio, 2.364; 95% confidence interval, 1.123–4.976).

p53 Is Directly Associated with Cyclin D1 Expression

Patients were also classified according to cyclin D1 expression in groups of either low (n=37, 52.86%) or high (n=33, 47.14%) immunoreactivity. Elevated p53 expression was directly correlated with increased cyclin D1 expression in univariate analysis, as the majority of p53-expressing tumors (9/12) also displayed cyclin D1 expression (Table 2, Fig. 2a and b). In contrast, 34 out of 58 of p53-negative PCa tissues were characterized by absence of cyclin D1 expression. This relation was marginally significant (p=0.055) (Table 2). There were no associations between cyclin D1 expression and TTBF (p=0.810), tumor stage (p=0.629), or grade (p=0.628) respectively (Table 1).

COX-2 Expression is Directly Related to Cyclin D1 Expression

A total of 59 patients (~84.28%) featured positive COX-2 expression in their tumors, whereas only 11 samples (~15.72%) were COX-2 negative. In univariate analysis, 31/59 of COX-2 expressing tumors were positive for cyclin D1 expression profile (Table 2, Fig. 3a and b), whereas in 9/11 of COX-2 negative cases, cyclin D1 expression was

Table 1 Correlations between levels of p53, COX-2, Cyclin D1 expression and clinicopathological characteristics

Parameters		p53				COX-2				Cyclin D1				All
		−		+		−		+		−		+		
		n	%	n	%	n	%	n	%	n	%	n	%	n
Gleason score	≤7 (3+4)	25	86.2	4	13.8	4	13.8	25	86.2	14	48.3	15	51.7	29
	≥7 (4+3)	33	80.5	8	19.5	7	17.1	34	82.9	23	56.1	18	43.9	41
	P value	0.749				1.000				0.628				
pT stage	≤2	35	83.3	7	16.7	5	11.9	37	88.1	21	50	21	50	42
	≥3	23	82.1	5	17.9	6	21.4	22	78.6	16	57.1	12	42.9	28
	P value	1.000				0.328				0.629				
TTBF	Non-relapsed	32	97	1	3	4	12.1	29	87.9	19	57.6	14	42.4	33
	relapsed	26	70.3	11	29.7	7	18.9	30	81.1	19	51.4	18	48.6	37
	P value	0.003				0.527				0.810				

pT stage pathologic TNM stage, *n* number of patients, *TTBF* time to biochemical failure

absent too, revealing a significant direct correlation between COX-2 and cyclin D1 expression (p=0.05) (Table 2). There were no associations between COX-2 expression and TTBF (p=0.527), tumor stage (p=0.328), or grade (p=1.000) respectively (Table 1).

Discussion

The concurrent examination of immunohistochemical expression of p53, COX-2 and cyclin D1 proteins in PCa, which was the aim of this study, may be justified by the existence of gross data supporting their multiple interconnections at the preclinical level. Firstly, in vitro studies indicate an indirect functional connection between p53 and cyclin D1 in G1 phase transition or arrest, as p53 acting via its target gene, p21, inhibits different complexes of cyclin/cyclin-dependent kinases (Cdks) including Cyclin D-Cdk4/6 complex [19, 20]. Secondly, p53 has been shown to suppress cyclin D1 transcription through inverse regulation of NF-κB/IκB family member proteins [21]. In a xenograft model, elevated cyclin D1 expression was evidenced in androgen-independent sublines, the androgen-withdrawal manipulation of which resulted in a progressive and sustained decrease of the former [22]. On the other hand, there is also a significant relationship between COX-2 and cyclin D1 as RNA interference-mediated COX-2 inhibition in metastatic PCa cells induced cell growth arrest and down-regulation of both androgen receptor and cyclin D1 [23]. Furthermore, there is evidence of p53-mediated repression of COX-2 protein and mRNA levels by wild-type p53 but not by mutant p53 [24]. Nevertheless, p53-induced activation of COX-2 has also been reported to occur via the Ras/Raf/ERK cascade [25], partly counteracting p53-mediated apoptosis via COX-2—mediated abrogation of p53 activity [26].

In an effort to examine the clinical usefulness of these important signaling proteins in the clinical course of patients with PCa, a number of relevant studies have been conducted. p53 nuclear accumulation detected by immunohistochemistry has been supported to be an independent prognostic marker in clinically localized PCa after RP [27–33]. In more recent studies, the addition of p53 immunohistochemical detection to the known panel of routinely used prognostic factors has offered a superior predictive ability of clinical outcome after RP [34, 35]. The combined examination of p53 gene mutations and immunohistochemical protein expression in a recent large scale study, demonstrated a

Table 2 Correlations between patterns of p53, COX-2 and cyclin D1 expression

	p53				COX-2				All
	−		+		−		+		
Cyclin D1	n	%	n	%	n	%	n	%	n
−	34	91.9	3	8.1	9	24.3	28	75.7	37
+	24	72.7	9	27.3	2	6.1	31	93.9	33
All	58		12		11		59		70
P value	0.055				0.05				

n number of patients

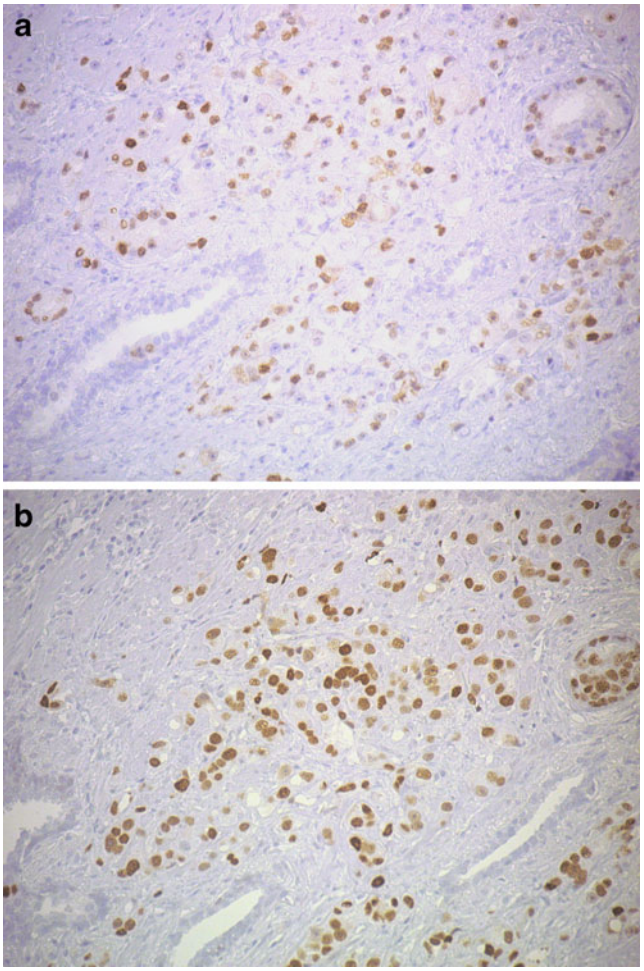

Fig. 2 Adenocarcinoma of the prostate. Gleason score 8 (3+5). **a** Positive immunostaining for Cyclin D1 X200; **b** The same case as figure 2a.Positive immunostaining for p53 X200

strong association between these two parameters as well as an independent prognostic role of p53 in the subgroup of low and intermediate grade carcinomas [36].

Despite this independent significance, p53 alone was able to correctly predict recurrence with only 61.1% accuracy thus implying a need to improve the clinical utility of this biomarker [29]. Moreover, not few controversies have emanated from the results of other studies supporting that the presence of increased p53 expression, was either univariately insignificant for risk of recurrence [2] or did not correlate with time to progression in the final multivariate model including known prognostic factors (pre-operative PSA, Gleason score, stage, surgical margins) [37, 38]. At least partially, the observed discordances between positive and negative studies may be attributed to the relatively small number of patients used as well as to differences in technical issues such as the selected reagents and protocols, also including subjective consideration of the degree of p53 immunostaining. With respect to the latter, a few authors tried to resolve this inconvenience by defining and applying clustered p53 staining, yet conflicting results were again unavoidable [39–41]. Even when other race populations were used (Japanese, Chinese), both positive [42] and negative studies [43, 44] have also been presented. In an effort to overcome frequent pitfalls of previous studies, all of which were based on a large number of cases in a clinically heterogenous population with a wide range of baseline descriptors, a nested, case–control study was recently designed. The authors' observations from paired analysis were that p53 upregulation had no prognostic value for biochemical recurrence after RP, even after considering stage, Gleason grade and pre-operative PSA level [45]. In our study, we have shown that p53 might be considered a potential prognostic marker, as patients with high protein expression had a shorter TTBF. Moreover, elevated p53 expression was also found to be an indepen-

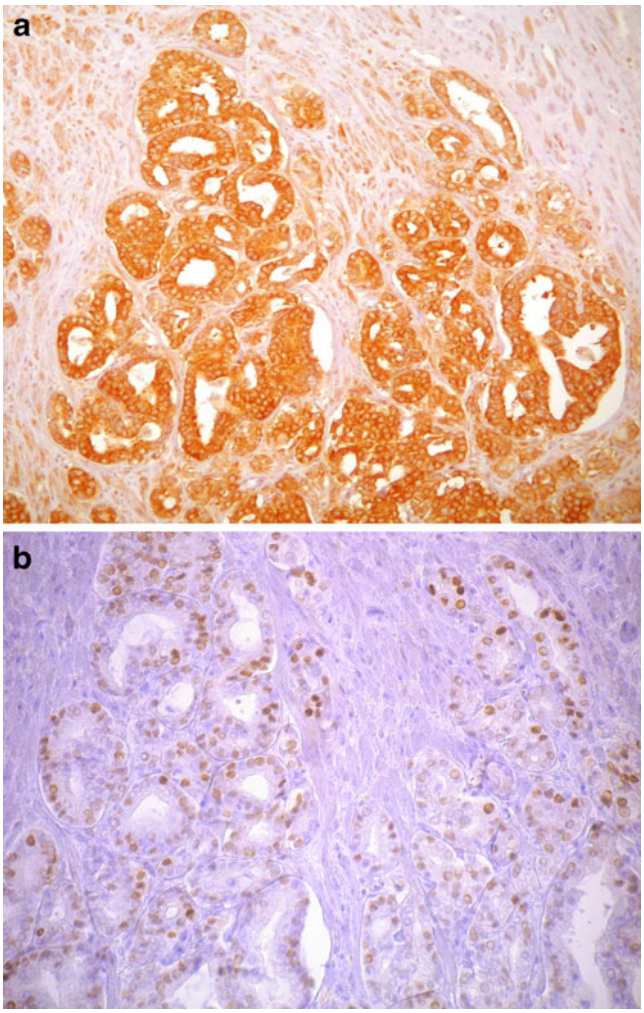

Fig. 3 Adenocarcinoma of the prostate. Gleason score 6 (3+3). **a** Positive immunostaining for COX-2 X200; **b** The same case as figure 3a.Positive immunostaining for Cyclin D1 X400

dent prognostic factor ($p = 0.023$) in a multivariate model including pathological stage and Gleason score.

The low cutoff value for p53 positivity (5%) used in our study was intended to be representative of the biological significance of p53 protein overexpression which is a result of p53 gene inactivation and a rare event in primary prostatic tumors, arising relatively late in neoplastic progression [2]. Thus, any detection of p53 protein immunoreactivity could be considered indirect evidence of p53 gene mutation. This explains why in many previous studies the expression of p53 was considered aberrant regardless of the percentage of cells stained [28–30, 32, 36, 37, 43, 44]. A low positive p53 immunostaining threshold (e.g. >1%, >10%) was used in other studies for the same reason [31, 46, 47]. On the other hand, it could be hypothesized that a higher cutoff value might be prognostically important for recurrence-free survival as a higher number of cells with p53 gene alteration would lead to a faster recurrence. However, when p53 expression was evaluated at higher cutoff values (e.g. >20%, >30%), little or no effect of high p53 expression was observed in certain subgroups, including older patients (>70 years), localized PCa and patients with better differentiated tumours (Gleason score <7) [33, 48, 49]. Even when the mean percentage of p53 was considered as representative staining, no statistically significant relationship was found between the prognosis and the mean value [42]. Further, p53 positive tumors typically display other features of high malignant potential such as a high histologic grade and a rapid proliferation rate which may blunt the putative independent prognostic value of p53. From another perspective, the presence of clusters of p53-positive nuclei may be of higher prognostic value in the group of moderately differentiated prostate cancers [39] as it delineates a group of patients with poor prognosis not identified by traditional scoring methods and supports the hypothesis that p53 dysfunction within PCa may exist in foci of tumor cells that are clonally expanded in metastases. [41]. Although all the above-mentioned studies are not directly comparable with the present, it may be suggested that in general, a patient with negative or low p53 staining is likely to have a good prognosis on prolonged follow-up.

The significance of COX-2 overexpression in PCa progression with regard to implications for a potential correlation of COX-2 overexpression with known prognostic variables has not been widely previously investigated. Early clinicopathological studies on immunohistochemistry localization and semiquantitative estimation of COX-2 expression had excluded any association of COX-2 with tumor grade [46] and had suggested that COX-2 is expressed by infiltrating lymphocytes and macrophages during inflammatory atrophy of the prostate [50]. However, later reports concluded that COX-2 has a significant association with tumor grade [51, 52], stage and PSA recurrence after RP, despite the fact that its value as an independent biological marker associated to disease relapse is limited [53–55]. The latter was doubted by a couple of other studies in which increased COX-2 staining independently predicted disease progression [56, 57]. In our study, we failed to confirm any statistically significant role of COX-2 expression as a predictor of BF in univariate analysis. However, COX-2 expression was directly correlated with p53, in line with the underlying biology described above.

Despite the influence of D-type cyclins on PCa proliferation, few studies have examined the expression of cyclin D1 in localized tumors or challenged its relevance to disease progression [58]. An almost universal finding was that although cyclin D1 positive immunoreactivity may be associated with a more aggressive phenotype, there was no significant correlation with standard clinicopathological prognostic factors of poor outcome, including time to PSA relapse [58–62]. Recently, a double marker combination of ErbB3-binding protein 1 (Ebp1) (+)/cyclin D1 (−) immunoreactivity was found to be an independent predictor of BF, but the study population was highly heterogenous, consisting of RP specimens as well as normal, non-cancerous adjacent and hormone refractory PCa tissues [63]. In our cohort, no statistically significant correlation was found between cyclin D1 expression and TTBF, although the former was directly associated with p53 expression. Again, this is highly expected from a preclinical point of view as the absence of functional p53, which is largely indicated by aberrant p53 protein expression, is synonymous with abrogation of p53-mediated cyclin D1 repression.

To conclude, although there is still a long road to walk until introducing novel biomarkers for early diagnosis of PCa biochemical progression, it seems that p53 is more likely to be added to the already existing panel of prognostic tools, whereas COX-2 and cyclin D1 need further studies to clarify whether they deserve a place in early prediction of PCa relapse after RP. We believe that the existence of significant interconnections between these three immunohistochemical markers both in vitro, as already known from gross amount of literature, and in vivo as we have demonstrated here, might necessitate their simultaneous examination in future studies.

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