TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Patched 1 Expression Correlates with Biochemical Relapse in High-Risk Prostate Cancer Patients

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There is an unmet clinical need for adequate biomarkers to aid risk stratification and management of prostate cancer (PCa) patients. Even within the high-risk PCa category, not all patients will invariably have a poor prognosis, and improved stratification of this heterogeneous group is needed. In this context, components of the hedgehog (Hh) pathway may have promise as biomarkers, because the available evidence suggests increased Hh pathway activity may confer a poorer outcome in advanced and castrate-resistant PCa. In this study, potential associations between Hh pathway protein expression and clinicopathological factors, including time to biochemical recurrence (BCR), were investigated using a tissue microarray constructed from benign and malignant prostate samples from 75 predominantly high-risk PCa patients who underwent radical prostatectomy. Hh signaling activity was found to differ between benign and malignant prostate tissue, with a greater amount of active Hh signaling present in malignant than benign prostate epithelium. High expression of Patched 1 in malignant prostate epithelium was found to be an independent predictor of BCR in high-risk PCa patients. Glioma-associated oncogene 1 may potentially represent a clinically useful biomarker of an aggressive tumor phenotype. Evaluation of Hh signaling activity in PCa patients may be useful for risk stratification, and epithelial Patched 1 expression, in particular, may be a prognostic marker for BCR in high-risk PCa patients. (*Am J Pathol* 2018; 188: 795–804; <https://doi.org/10.1016/j.ajpath.2017.11.019>)

Prostate cancer (PCa) is the most common solid organ malignancy diagnosed in men in developed countries, and it is the second leading cause of male cancer-related death.¹ Patients with PCa who will potentially receive radical treatment [radical prostatectomy (RP) or radical radiotherapy] are currently stratified as having low-, intermediate-, or high-risk disease based on the risk of post-treatment relapse, and these risk categories use clinicopathological factors, including prostate-specific antigen level, clinical T stage, and biopsy Gleason sum score (GS).^{2,3} However, even within the different risk categories, a wide range of clinical outcomes of treatment exist. At the present time, clinicians do not routinely evaluate the underlying molecular features of PCa that might promote increased tumor aggressiveness, or use this information in decision making, and this is partly attributable to a lack of robust clinically

applicable biomarkers of aggressive disease.^{4,5} Robust biological markers of potentially aggressive disease are therefore necessary to improve the current prognostic groupings and better identify patients most likely to benefit

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Figure 1 Schematic illustration of the main hedgehog (Hh) pathway components. The Hh pathway is activated when Sonic Hh ligand binds to the Patched 1 (PTCH1) membrane receptor, thereby relieving the inhibitory effect of PTCH1 on Smoothened (SMO). Activated SMO is released into the cytoplasm and accumulates in the primary cilium, which, in turn, facilitates the dissociation of the glioma-associated oncogene (GLI) transcription factors from suppressor of fused (SUFU). The GLI transcription activators translocate to the nucleus, where they promote the transcription of Hh target genes, including GLI1, PTCH1, snail family transcriptional repressor 1 (SNAIL), snail family transcriptional repressor 3 (SNAI3), and cyclin D1.

from radical treatment based on their high risk of otherwise developing systemic or lethal disease.

Previous studies indicate that elevated hedgehog (Hh) signaling pathway activity occurs in advanced and androgen-resistant PCa.^{6–12} We hypothesize that increased Hh signaling pathway activity may represent a promising biomarker of increased disease aggressiveness in high-risk PCa patients receiving RP with curative intent. For this patient group in particular, further risk stratification is warranted because these patients have the highest risk of biochemical failure, metastatic progression, and eventual PCa-related death, but large variations in outcome are still notable within this group.^{5,13}

Aberrant Hh signaling in PCa is thought to be ligand dependent; however, it remains unclear whether this is mediated in a paracrine and/or autocrine manner.⁸ The Hh signaling cascade (Figure 1) is activated when Sonic Hh (SHH) ligand binds to the Patched 1 (PTCH1) membrane receptor. PTCH1 is a negative regulator of Hh signaling and inhibits activation of the downstream target Smoothened (SMO). Binding of SHH to PTCH1 overcomes this inhibition, releasing SMO into the cytoplasm. This activates the glioma-associated oncogene (GLI) transcription factors by facilitating their dissociation from suppressor of fused (SUFU).^{14,15} The GLI transcription factor family consists of three members (GLI1, GLI2, and GLI3). GLI1 is the primary transcriptional activator, whereas

GLI2 and GLI3 can have activator or repressor function, depending on cellular context.^{16–22}

To investigate the potential clinical utility of evaluating the expression level of key Hh signaling components in a cohort of predominantly high-risk localized PCa, Hh protein expression in malignant and surrounding benign prostate tissue was compared in a tissue microarray. To investigate the mode of Hh signaling in PCa and surrounding benign tissue, potential correlations between the expression levels of different Hh proteins were assessed. The clinical relevance of Hh signaling in localized high-risk PCa was evaluated through potential associations between Hh protein expression and clinicopathological factors, and the prognostic value of Hh signaling was evaluated.

Materials and Methods

Tissue Microarray Construction

Tissue microarrays were constructed from 450 samples of whole-mount formalin-fixed, paraffin-embedded specimens from 75 RP patients from KU Leuven,²³ with four cores taken from the index tumor, and two cores taken from benign tissue, to account for potential intratumor heterogeneity. Full clinicopathological and outcome data

Table 1 Patient and Tumor Characteristics (*n* = 75)

Characteristics	Values
Median age at surgery, years (range)	66 (49–74)
Median PSA level, ng/mL (range)	10.4 (1.5–70.9)
Clinical T stage	
≤1c	1 (1.3)
2a-2c	19 (25.3)
≥3a	55 (73.3)
Biopsy Gleason score	
≤6 (3 + 3)	3 (4.0)
7 (3 + 4)	23 (30.7)
7 (4 + 3)	16 (21.3)
≥8 (4 + 4)	33 (44.0)
EAU risk category	
Low	0 (0)
Intermediate	5 (6.7)
High	70 (93.3)
Pathologic T stage	
≤1c	0 (0)
2a-2c	33 (44.0)
≥3a	42 (56.0)
RP Gleason score	
≤6 (3 + 3)	0 (0)
7 (3 + 4)	18 (24)
7 (4 + 3)	25 (33.3)
≥8 (4 + 4)	32 (42.7)
Median tumor volume, cc (range)	4.43 (0.32–23.96)
Median tumor volume, % (range)	9.69 (0.38–37)
pN status	
Negative	38 (50.7)
Positive	37 (49.3)
Surgical margin status	
Negative	59 (78.7)
Positive	16 (21.3)
Seminal vesicle invasion	
Absent	59 (78.7)
Present	16 (21.3)
Capsular invasion	
Absent	33 (44)
Present	42 (56)
Extraprostatic extension	
Absent	37 (49.3)
Present	38 (50.7)
Vascular invasion	
Absent	64 (85.3)
Present	11 (14.7)
Perineural invasion	
Absent	4 (5.3)
Present	71 (94.7)
Biochemical recurrence	16 (21.3)
Median follow-up, years (range)	5.2 (0.01–7.3)

Data are expressed as *n* (%) unless otherwise indicated.

EAU, European Association of Urology; pN, pathologic nodal status; PSA, prostate-specific antigen; RP, radical prostatectomy.

[including biochemical recurrence (BCR)] were available (Table 1).

The tissue microarray was built by the Oxford Centre for Histopathology Research, Oxford University Hospitals

National Health Service Foundation Trust (Oxford, UK) under the approval of the Oxford Radcliffe Biobank Ethics Committee (reference number 09/H0606/5+5). The study itself had full approval from the local ethics committee of KU Leuven (Leuven, Belgium) (reference number S55726).

Immunohistochemistry

Slides were deparaffinized and hydrated, heat-induced antigen retrieval with citrate buffer was performed, and endogenous peroxidases were blocked with 0.3% hydrogen peroxide before a serum-free protein block (X0909; Dako/Agilent Technologies, Santa Clara, CA). Slides were incubated overnight at 4°C with antibodies (1:50, unless stated otherwise) against SHH (ab53281; Abcam, Cambridge, UK), PTCH1 (1:300, sc-6147; Santa Cruz Biotechnology, Dallas, TX), SMO (1:100, ab72130; Abcam), SUFU (1:100, sc-28847; Santa Cruz Biotechnology), GLI1 (sc-20687; Santa Cruz Biotechnology), GLI2 (1:1000, 600-401-845; Rockland, Limerick, PA), GLI3 (AF3690; R&D Systems, Minneapolis, MN), snail family transcriptional repressor 1 (SNAIL; AF3639; R&D Systems), snail family transcriptional repressor 3 (SNAI3; NBPI-90661; Novus Biologicals, Littleton, CO), cyclin D1 (M364229; Dako/Agilent Technologies), or CD31 (IR610 Clone JC70A; Dako/Agilent Technologies). Secondary antibodies (ImmPRESS REAGENT Anti-Rabbit Ig peroxidase MP-7401, Anti-Rat Ig peroxidase MP-7404, Anti-Mouse Ig peroxidase MP-7402, or Anti-Goat Ig peroxidase MP-7405; Vector Laboratories, Burlingame, CA) were added, followed by 3,3'-diaminobenzidine substrate (ImmPACT DAB, SK-4105; Vector Laboratories) and counterstained with hematoxylin. Positive control specimens from patients who underwent a transurethral resection of the prostate were used to validate the specificity of the primary antibodies, which was histopathologically assessed on the basis of the expected subcellular localization [based on previous validations of these antibodies, as reported elsewhere,^{24–27} and/or as observed in The Human Protein Atlas, available from <http://www.proteinatlas.org> (GLI1, GLI2, SNAI3; last accessed October 13, 2017)] and lack of nonspecific background staining. Blood vessel staining was used as an internal positive control for the validation of the anti-Snail antibody. Negative controls were performed without addition of any primary antibody to exclude nonspecific staining of secondary antibodies.

Immunohistochemistry Scoring

Immunohistochemistry staining of SHH, PTCH1, SMO, SUFU, GLI1, GLI2, GLI3, SNAIL, SNAI3, and cyclin D1 was independently scored by researchers (S.I., A.G., and/or E.L.) blinded to clinicopathological and outcome data. Potentially discordant scores were resolved by joint review of the corresponding cores. The percentage of cell immunoreactivity (0% to 100%) and the staining intensity (score 0 to 3, with 0 indicating negative; 1, weak; 2, moderate; and

Table 2 Differential Expression of Hh Pathway Proteins between Malignant and Benign Prostate Tissue

Paired <i>t</i> -test	Epithelial expression		Stromal expression	
	Mean difference (HS tumor – HS benign)	<i>P</i> value	Mean difference (HS tumor – HS benign)	<i>P</i> value
SHH	–0.099	0.302	–0.340	<0.001*
PTCH1	–0.690	<0.001*	–0.321	<0.001*
SMO	0.280	0.005*	–0.120	0.188
SUFU	–0.056	0.58	–0.258	0.004*
GLI1	0.197	0.041*	–0.300	<0.001*
GLI2	–0.221	0.029*	–0.338	<0.001*
GLI3	0.015	0.827	–0.210	<0.001*
Cyclin D1	0.915	<0.001*	NA	NA
SNAIL	0.206	0.027*	NA	NA
SNAI3	0.565	<0.001*	–0.192	0.009*

**P* < 0.05.
GLI, glioma-associated oncogene; Hh, Hedgehog; HS, histoscore; NA, not applicable (no stromal staining detected); PTCH, Patched; SHH, Sonic Hh; SMO, Smoothened; SNAI3, snail family transcriptional repressor 3; SNAIL, snail family transcriptional repressor 1; SUFU, suppressor of fused.

3, strong) were recorded for each protein for both epithelial and stromal cells. Histoscores (HSs) (0 to 3) were calculated as the product of the percentage immunoreactivity and the staining intensity. In the malignant cores, only tumor glands were scored. If multiple staining intensities were observed in a single core, then the average HS of the whole core was recorded. For some statistical analysis, binary HSs were used (low HS was defined as <1.5, and high HS was defined as ≥1.5, with 1.5 representing the mean HS). The subcellular localization of each protein (membranous, cytoplasmic, and/or nuclear expression) was also recorded. Microvessel density was determined using CD31 staining and counting of blood vessels per tissue core.

Statistical Analysis

The Pearson correlation coefficient was used to evaluate potential correlations between expression levels of different Hh proteins. Any dependency between binary Hh expression level and clinical subgroup was determined using a Fisher exact test or Pearson χ^2 test. Intratumoral variation was assessed as the SD of the protein expression level within different tumor cores from each patient, and potential relationships with clinicopathological factors were investigated using a two-sample independent *t*-test. A paired *t*-test was used to investigate potential differences between protein levels in the malignant and benign cores.
Time to BCR after RP is defined as the time interval between surgery and an increase in prostate-specific antigen level to >0.2 ng/mL (where this occurred within the cohort). Relationships between BCR and protein expression were investigated using Kaplan-Meier analysis. The influence of different risk factors on time to BCR was estimated by a log-rank test based on the binary HS of the studied proteins

and clinicopathological PCa factors, including pretreatment prostate-specific antigen level, GS, clinical and pathologic T stage, lymph node involvement, and surgical margin status. A multivariate Cox proportional hazard regression model was used to determine the relative risk of BCR for these factors. Differences were considered statistically significant at *P* < 0.05. SPSS Statistics version 23/24 (IBM, Armonk, NY) was used for statistical analyses. The original data sets used during this study are available as [Supplemental Tables S1 and S2](#).

Results

Patient Cohort

Patient and tumor characteristics are shown in [Table 1](#). The patient cohort predominantly comprised high-risk PCa patients (93.3%) based on the latest European Association of Urology risk classification system [ie, prostate-specific antigen level > 20 ng/mL or biopsy GS > 7 or clinical T (cT) stage ≥cT2c]. A total of 73.3% of patients had ≥cT3a disease, and 44% of patients had biopsy GS ≥ 8 disease. Pathologic analysis of RP specimens revealed that 56% of patients had ≥pT3a disease and 42% had a pathologic GS score ≥8. Almost half of the patients had positive lymph nodes. One in five patients developed BCR during a median follow-up of 5.2 (range, 0 to 7.3) years.

Differential Expression of Hh Proteins in Benign and Malignant Prostate Tissue

Protein expression of the primary Hh signaling components was compared between benign and malignant prostate tissue ([Table 2](#)). Representative examples of low and high protein expression of studied proteins are shown in [Figure 2](#). Significantly higher expression levels of SMO (*P* = 0.005) and GLI1 (*P* = 0.041) were observed in malignant epithelium compared with benign prostate epithelium. Epithelial PTCH1 (*P* < 0.001) and GLI2 (*P* = 0.029) expression was significantly lower in malignant prostate epithelium than benign prostate epithelium.
These results suggest that Hh signaling activity is higher in malignant epithelium than benign epithelium. In accordance with this possibility, cyclin D1 (*P* < 0.001), SNAIL (*P* = 0.027), and SNAI3 (*P* < 0.001), which are known target genes of Hh signaling and involved in cell cycle progression and epithelial-to-mesenchymal transition, respectively, were more highly expressed in tumor cells than benign epithelium ([Table 2](#)). Lower expression of multiple Hh components (SHH, PTCH1, SUFU, GLI1, GLI2, and GLI3; *P* < 0.005 for each) was observed in the stroma of PCa tissue compared with stroma in surrounding benign tissue. In addition, protein expression of SNAI3 (*P* = 0.009) was lower in tumor-associated stroma than benign tissue stroma. Microvessel density was higher in

Figure 2 Representative images of low and high hedgehog pathway protein expression in malignant prostate tissue cores. Tissue cores were stained for Sonic hedgehog (SHH), Patched 1 (PTCH1), Smoothened (SMO), suppressor of fused (SUFU), glioma-associated oncogene (GLI) 1, GLI2, GLI3, cyclin D1, snail family transcriptional repressor 1 (SNAIL), and snail family transcriptional repressor 3 (SNAI3). Original magnification, $\times 100$.

malignant prostate tissue compared with benign prostate tissue ($P < 0.001$). Taken together, these results suggest that Hh signaling activity is higher in malignant prostate epithelium than in surrounding tumor-associated stroma and that autocrine Hh signaling may play a role in PCa.

Correlations in Expression between Different Hh Proteins

Potential relationships among differential expression of Hh signaling proteins were evaluated in benign and malignant prostate tissue using a Pearson correlation. Significant correlations were observed between Hh proteins (Table 3), with strong correlations being defined as a correlation coefficient ≥ 0.5 . In malignant epithelium, SHH was coexpressed with SMO ($r = 0.52$) and GLI1 ($r = 0.54$), these being important activators of Hh signaling. In addition, SMO expression was coexpressed with SNAI3 ($r = 0.58$) in malignant epithelium. In malignant stroma, SMO was strongly expressed with GLI3 ($r = 0.65$), SNAI3

($r = 0.53$), and SHH ($r = 0.61$). Furthermore, stromal SMO expression was associated with epithelial SNAIL expression ($r = 0.59$).

In benign prostate epithelium, SMO expression was correlated with GLI3 ($r = 0.53$), SNAI3 ($r = 0.57$), and GLI2 ($r = 0.70$) expression. Stromal and epithelial SNAI3 expression was also correlated ($r = 0.67$). In benign stroma, SMO expression was correlated with GLI3 ($r = 0.59$) and SHH ($r = 0.60$) expression, and a correlation was seen between GLI3 and SHH expression ($r = 0.57$).

GLI1 Expression in the Tumor Epithelium Correlates with Vascular Invasion and Higher Pathologic Gleason Score

Potential correlations between expression of Hh proteins and known clinicopathological factors were investigated. Increased total GLI1 expression in malignant prostate epithelium was observed to correlate with an increased number of blood vessels ($P = 0.046$). A distinction was

Table 3 Correlations between Hh Proteins in the Epithelial and Stromal Cells in the Tumor and Benign Prostate Tissue

Variable	Epithelial expression									Stromal expression								
	SNAIL	GLI1	SMO	SNAI3	SHH	GLI3	GLI2	SUFU	Cyclin D1	PTCH1	GLI1	SMO	SNAI3	SHH	GLI3	GLI2	SUFU	PTCH1
Tumor																		
Epithelial																		
SNAIL	1.00 [‡]	0.45 [†]	0.41 [†]	0.33*	0.28*	0.25*	0.35*	0.39*		0.26*		0.59 [†]	0.38*	0.36*	0.40 [†]	0.25*		
GLI1	0.45 [†]	1.00 [‡]	0.41 [†]	0.40 [†]	0.54 [†]	0.47 [†]	0.30*	0.40 [†]			0.27*	0.45 [†]	0.35*					
SMO	0.41 [†]	0.41 [†]	1.00 [‡]	0.58 [†]	0.52 [†]	0.38 [†]	0.27*		0.31*			0.38*	0.28*					
SNAI3	0.33*	0.40 [†]	0.58 [†]	1.00 [‡]	0.44 [†]	0.40 [†]	0.39*					0.29*	0.42 [†]					
SHH	0.28*	0.54 [†]	0.52 [†]	0.44 [†]	1.00 [‡]			0.34*				0.29*	0.27*					
GLI3	0.25*	0.47 [†]	0.38*	0.40 [†]		1.00 [‡]	0.50 [†]					0.39*	0.43 [†]		0.26*			
GLI2	0.35*	0.30*	0.27*	0.39*		0.50 [†]	1.00 [‡]	0.25*				0.39*	0.40 [†]			0.30*		0.27*
SUFU	0.39*	0.40 [†]			0.34*		0.25*	1.00 [‡]			0.32*	0.49 [†]	0.35*		0.40 [†]		0.53 [†]	
Cyclin D1			0.31*						1.00 [‡]									
PTCH1	0.26*									1.00 [‡]								0.26*
Stromal																		
GLI1		0.27*						0.32*			1.00 [‡]	0.41 [†]		0.29*	0.44 [†]	0.34*	0.44 [†]	
SMO	0.59 [†]	0.45 [†]	0.38*	0.29*	0.29*	0.39 [†]	0.39*	0.49 [†]			0.41 [†]	1.00 [‡]	0.53 [†]	0.61 [†]	0.65 [†]	0.42 [†]	0.32*	0.26*
SNAI3	0.38*	0.35*	0.28*	0.42 [†]	0.27*	0.43 [†]	0.40 [†]	0.35*				0.53 [†]	1.00 [‡]		0.35*			
SHH	0.36*										0.29*	0.61 [†]		1.00 [‡]	0.49 [†]	0.52 [†]	0.26*	
GLI3	0.40 [†]					0.26*		0.40 [†]			0.44 [†]	0.65 [†]	0.35*	0.49 [†]	1.00 [‡]	0.43 [†]	0.30*	
GLI2	0.25*						0.30*				0.34*	0.42 [†]		0.52 [†]	0.43 [†]	1.00 [‡]		
SUFU								0.53 [†]			0.44 [†]	0.32*		0.26*	0.30*		1.00 [‡]	
PTCH1							0.27*			0.26*		0.26*						1.00 [‡]
Benign																		
Epithelial																		
SNAIL	1.00 [‡]	0.26*			0.33*				0.37*									0.31*
GLI1	0.26*	1.00 [‡]	0.45 [†]			0.34*	0.30*		0.28*		0.30*							
SMO		0.45 [†]	1.00 [‡]	0.57 [†]		0.53 [†]	0.70 [‡]		0.30*			0.37*	0.34*					
SNAI3			0.57 [†]	1.00 [‡]		0.48 [†]	0.57 [†]		0.27*			0.45 [†]	0.67 [‡]	0.24*				0.40 [†]
SHH	0.33*				1.00 [‡]	0.36*	0.24*	0.28*										
GLI3		0.34*	0.53 [†]	0.48 [†]	0.36*	1.00 [‡]	0.67 [‡]		0.39*			0.39*	0.44 [†]	0.25*		0.35*		0.37*
GLI2		0.30*	0.70 [†]	0.57 [†]	0.24*	0.67 [‡]	1.00 [‡]		0.33*			0.30*	0.46 [†]		0.27*			0.34*
SUFU					0.28*			1.00 [‡]										
Cyclin D1	0.37*	0.28*	0.30*	0.27*		0.39*	0.33*		1.00 [‡]			0.40 [†]		0.29*				0.33*
PTCH1										1.00 [‡]			0.29*					0.42 [†]
Stromal																		
GLI1		0.30*									1.00 [‡]	0.42 [†]		0.25*	0.38*	0.34*	0.24*	
SMO			0.37*	0.45 [†]		0.39*	0.30*		0.40 [†]		0.42 [†]	1.00 [‡]	0.46 [†]	0.60 [†]	0.59 [†]	0.33*		0.45 [†]
SNAI3			0.34*	0.67 [‡]		0.44 [†]	0.46 [†]			0.29*		0.46 [†]	1.00 [‡]			0.44 [†]		0.46 [†]
SHH				0.24*		0.25*			0.29*		0.25*	0.60 [†]		1.00 [‡]	0.57 [†]			0.36*
GLI3											0.38*	0.59 [†]		0.57 [†]	1.00 [‡]	0.35*		0.36*
GLI2						0.35*	0.27*				0.34*	0.33*	0.44 [†]		0.35*	1.00 [‡]		0.35*
SUFU											0.24*						1.00 [‡]	
PTCH1	0.31*			0.40 [†]		0.37*	0.34*		0.33*	0.42 [†]		0.45 [†]	0.46 [†]	0.36*	0.36*	0.35*		1.00 [‡]

Pearson correlation coefficients of statistically significant ($P < 0.05$) correlations are given. Empty cells indicate that there was no significant result.

*Weak correlation.

[†]Moderate correlation.

[‡]Strong correlation.

GLI, glioma-associated oncogene; Hh, Hedgehog; PTCH, Patched; SHH, Sonic Hh; SMO, Smoothened; SNAI3, snail family transcriptional repressor 3; SNAIL, snail family transcriptional repressor 1; SUFU, suppressor of fused.

then made between nuclear GLI protein expression (irrespective of cytoplasmic expression) and sole cytoplasmic expression, to investigate active and inactive Hh signaling. In malignant prostate cells with positive nuclear GLI1 expression, higher GLI1 expression was observed to correlate with a higher pathologic GS ($GS > 7$) ($P = 0.041$) (Table 4 and Supplemental Figure S1).

Intratumor Heterogeneity

The expression of each Hh pathway protein in each of four tumor cores from each individual patient was evaluated to investigate potential intratumor heterogeneity²⁸ and to assess whether any observed heterogeneity may relate to a particular patient subgroup (Supplemental Table S3). SDs of the Hh

Table 4 Correlation between GLI1 Expression and Clinicopathological Factors

	Nuclear GLI1			Cytoplasmic GLI1			Total GLI1		
	<i>n</i>		<i>P</i> value	<i>n</i>		<i>P</i> value	<i>n</i>		<i>P</i> value
Fisher exact test	Low	High		Low	High		Low	High	
PSA level			0.500			0.186			0.322
PSA ≤10 ng/mL	20	6		2	4		22	10	
PSA >10 ng/mL	20	12		3	3		23	15	
Clinical T stage			0.342			0.417			0.098
cT ≤2	13	3		1	0		14	3	
cT >2	27	15		4	7		31	22	
Biopsy Gleason score			0.565			1.000			0.454
GS ≤7	26	10		2	3		28	13	
GS >7	14	8		3	4		17	12	
Pathologic T stage			0.571			1.000			0.313
pT ≤2	20	7		1	1		21	8	
pT >2	20	11		4	6		24	17	
RP Gleason score			0.041*			1.000			0.080
GS ≤7	28	7		2	4		30	11	
GS >7	12	11		3	3		15	14	
pN stage			0.682			0.472			0.261
Negative	22	9		1	1		23	22	
Positive	18	9		4	6		10	15	
Surgical margin status			0.689			0.372			0.241
Negative	34	14		3	4		37	18	
Positive	6	4		2	3		8	7	
Seminal vesicle invasion			0.483			1.000			0.370
Negative	36	14		3	4		37	18	
Positive	6	4		2	3		8	7	
Capsular invasion			1.000			0.417			0.614
Negative	19	9		1	0		20	9	
Positive	21	9		4	7		25	16	
Extraprostatic extension			0.781			1.000			0.457
Negative	22	9		1	1		23	10	
Positive	18	9		4	6		22	15	
Vascular invasion			0.238			0.205			0.046*
Negative	36	14		5	4		41	18	
Positive	4	4		0	3		4	7	
Perineural invasion			0.528						1.000
Negative	1	1		5	7		1	1	
Positive	39	17		5	7		44	24	

**P* < 0.05.

GLI, glioma-associated oncogene; GS, Gleason sum score; pN, pathologic nodal status; PSA, prostate-specific antigen; RP, radical prostatectomy.

pathway proteins were compared with different patient subgroups to investigate whether heterogeneous expression patterns may be associated with clinicopathological features. Less intratumor variation of epithelial SUFU expression was observed in patients with a high versus low biopsy GS (*P* = 0.026). Patients with positive lymph node metastases demonstrated more homogeneous PTCH1 expression (*P* = 0.013) within the primary tumor epithelium. Stromal SHH expression was less variable in patients with a high biopsy GS (*P* = 0.009), high pathologic GS (*P* = 0.016), or positive lymph node metastases (*P* = 0.027). Epithelial expression of SNAIL (*P* = 0.036) and stromal expression of SMO (*P* = 0.029) and GLI3 (*P* = 0.038) were more homogeneous in PCa cases with capsular invasion (stage ≥pT3)

than in cases of ≤pT2 stage disease. Taken together, these data suggest that patients with a more aggressive PCa phenotype have a more consistent (ie, homogeneous) staining pattern of several important Hh pathway proteins.

Higher Epithelial PTCH1 Expression Correlates with Unfavorable Patient Outcome

Univariate analysis revealed that higher epithelial expression of PTCH1 in malignant prostate cells correlated with a shorter time to BCR (Figure 3 and Supplemental Table S4). This observation was also seen in a multivariate analysis (Table 5), indicating that epithelial PTCH1 expression in malignant tissue may be a prognostic marker for BCR.

Figure 3 Correlations between epithelial Patched 1 (PTCH1) and patient outcome. Kaplan-Meier analysis of time to biochemical relapse (BCR) for prostate cancer patients with high (green) or low (blue) epithelial PTCH1 expression in the tumor (log-rank test: $\chi^2 = 7.728$, $P = 0.005$). Low and high PTCH1 expression levels are defined as such based on binary histoscores of <1.5 and ≥ 1.5 , respectively.

Tumor volume was also observed to be an independent predictor for BCR.

Discussion

Increasing evidence suggests a role for active Hh signaling in the development and progression of PCa to an advanced phenotype. Although Hh signaling plays an essential role in the embryonic development of the prostate, its role in the adult prostate is usually limited. In PCa, uncontrolled reactivation of Hh signaling appears to occur, which is more pronounced in the advanced stages of this malignancy. A potential relationship between Hh signaling and androgen-independent PCa has been described by several research groups. Moreover, preclinical data demonstrate that inhibition of Hh signaling may reduce prostate cancer invasiveness and metastatic potential.⁸

In this study, the expression profile of the principal Hh signaling pathway components was determined in a cohort of predominantly high-risk PCa patients treated by RP. The management of high-risk PCa is one of the most important challenges in contemporary clinical practice because of the large variation in treatment response observed in this group. Although many high-risk PCa patients have a good prognosis after RP performed with curative intent, unfortunately a significant number of patients still succumb to recurrent and progressive disease, with potentially lethal consequences.²⁹ There is therefore an unmet clinical need for further stratification tools, such as robust biological markers of potentially aggressive disease, within this specific high-risk cohort, such that these biomarkers might be used in routine clinical

practice to improve patient outcomes.^{5,13} We hypothesized that increased Hh signaling pathway activity may represent a promising biomarker of increased disease aggressiveness in high-risk PCa patients receiving RP with curative intent.

The expression levels of Hh signaling proteins differ between benign and malignant prostate tissue. Higher expression of the Hh pathway activators SMO and GLI1, and lower expression of the negative regulator PTCH1, was observed in this high-risk PCa cohort. GLI2 expression was also reduced in malignant versus benign prostate epithelium. Depending on the cellular context, GLI2 is processed to a truncated (GLI2-78) repressor form,³⁰ but little is known about the specific mechanisms that trigger this processing. It is known that when the Hh pathway is inactive, with no ligand bound to PTCH1, the GLI transcription factors are phosphorylated by protein kinase A, casein kinase 1, and glycogen synthase kinase 3 β , which results in targeting of GLI proteins to the proteasome for degradation.³⁰ Full-length GLI2 (GLI2-185) and truncated GLI2 (GLI2-78) could not be discriminated; therefore, clear conclusions cannot be made regarding the exact function of GLI2 in this setting. Nevertheless, the observations that both GLI1 and SMO appear to be up-regulated in the tumor, whereas PTCH1 is down-regulated, suggest that the repressor form of GLI2 is also down-regulated in PCa, indicating that Hh signaling activity is higher in PCa than in benign prostate epithelium. In accordance with this possibility, epithelial expression of cyclin D1, SNAIL, and SNAI3 was higher in malignant than in benign epithelium, consistent with greater Hh pathway signaling and potentially resulting in enhanced proliferation and epithelial-to-mesenchymal transition. In contrast, down-regulation of Hh signaling activity was observed in tumor-associated stroma compared with benign stroma. This finding is consistent with that of a previous report by Tzelepi et al.¹² Higher epithelial and lower stromal Hh expression may indicate autocrine Hh signaling in PCa tissue. Previous reports have also demonstrated an important role for autocrine Hh signaling in PCa.^{10,11,31,32} In contrast, Fan et al³³ found, in a xenograft PCa model, that paracrine Hh signaling stimulated the tumor microenvironment to induce tumor growth by the production of growth factors. A strong correlation was observed between stromal SMO and SNAI3 expression, suggesting that paracrine Hh signaling may also be biologically relevant in PCa. Taken together, the available evidence suggests that Hh signaling in PCa is likely to be a combination of both autocrine and paracrine signaling.

Table 5 Multivariate Cox Proportional Hazard Regression Analysis of Time to BCR

Variable	Hazard ratio	95% CI	P value
Tumor volume, cc	1.216	1.079–1.371	0.001*
Epithelial PTCH1	4.977	1.585–15.626	0.006*

* $P < 0.05$.
BCR, biochemical relapse; PTCH, Patched.

Nuclear GLI1 expression was positively correlated with pathologic GS, indicating that this may be a marker of an aggressive tumor phenotype. This accords with a study by Kim et al,³⁴ in which a correlation was observed between GLI1 and GS. Although Kim et al³⁴ observed that expression of other Hh proteins (SHH, PTCH1, and SMO) also correlated with GS, this finding was not observed in our study. One reason for this might be that in this study most of the patients had high-risk PCa, whereas the study by Kim et al³⁴ included patients with low-, intermediate-, and high-risk PCa. Kim et al³⁴ also observed that epithelial SHH in the tumor was an independent prognostic marker for BCR, whereas in this study epithelial PTCH1 expression was associated with an unfavorable patient outcome.

A significant association was observed between epithelial PTCH1 expression and BCR in this predominantly high-risk PCa cohort. Although this finding may appear to contradict the known function of PTCH1 as a negative regulator of Hh signaling, PTCH1 activity is dependent on its precise subcellular localization. Membranous PTCH1 localization is associated with an inhibitory function, whereas nuclear and cytoplasmic PTCH1 localization indicates active Hh signaling. PTCH1 expression was found to be mostly present in the cytoplasm of malignant epithelium, and this particular localization is indicative of active Hh signaling.

There are inherent limitations in our study attributable to its retrospective nature and the use of immunohistochemistry. Furthermore, for each patient, all tumor cores ($n = 4$) were taken from the same macroblock, and for some patients, not all cores could be evaluated, which might compromise a detailed analysis of intratumor heterogeneity. Also, because the number of patients ($n = 75$) in this study is modest, the data should be validated in a larger patient cohort.

In conclusion, active Hh signaling appears to be greater in malignant prostate epithelium than in benign prostate epithelium. In addition, the available evidence suggests that both autocrine and paracrine Hh signaling may play roles in PCa progression, and GLI1 expression might represent a clinically useful biomarker of tumor aggressiveness in high-risk PCa treated with RP. PTCH1 may also be a good candidate for a clinically useful biomarker of outcome in high-risk PCa patients treated with RP; however, this requires validation in larger studies.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.11.019>.

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