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ORIGINAL ARTICLE

The epithelial–mesenchymal transition regulators Twist, Slug, and Snail are associated with aggressive tumour features and poor outcome in prostate cancer patients

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Abstract

The prognostic importance of transcription factors promoting epithelial–mesenchymal transition (EMT) and angiogenesis has not been well explored in prostate cancer patients with long follow-up, nor the interplay between these factors. The objective of this study was to assess the individual protein expression and co-expression of Twist, Slug (Snai2), Snail (Snai1), and hypoxia-inducible factor-1 alpha (Hif-1α) in prostate cancer in relation to EMT, angiogenesis, hypoxia, tumour features, disease recurrence, and patient survival. Immunohistochemical staining was performed on tissue microarray sections from 338 radical prostatectomies with long follow-up. In addition, 41 cases of prostatic hyperplasia, 33 non-skeletal metastases, 13 skeletal metastases, and 33 castration-resistant prostate carcinomas were included. Our findings were validated in external gene expression data sets. Twist was overexpressed in primary prostate cancer and markedly reduced in distant metastases ($p < 0.0005$). Strong expression of Twist and Slug was associated with Hif-1α in localised prostate cancer ($p \leq 0.001$), and strong Twist was associated with Hif-1α in castration-resistant carcinomas ($p = 0.044$). Twist, Slug, and increased Snail at the tumour stromal border were associated with vascular factors ($p \leq 0.045$). Each of the three EMT-regulating transcription factors were associated with aggressive tumour features and shorter time to recurrence and cancer-specific death. Notably, the co-expression of factors demonstrated an enhanced influence on outcome. In the subgroup of E-cadherin^{low} carcinomas, strong Slug was associated with shorter time to all end points and was an independent predictor of time to multiple end points, including cancer-specific death (hazard ratio 3.0, $p = 0.041$). To conclude, we demonstrate an important relation between EMT, hypoxia, and angiogenesis and a strong link between the investigated EMT regulators and aggressive tumour features and poor patient outcome in prostate cancer. Despite the retrospective nature of this long-term study, our findings could have a significant impact on the future treatment of prostate cancer, where tailored therapies might be directed simultaneously against epithelial–mesenchymal phenotypes, angiogenesis, and tumour hypoxia.

Keywords: prostate cancer; immunohistochemistry; epithelial–mesenchymal transition; Twist; Slug; Snail; Hif-1α; castration resistance; angiogenesis; co-expression

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No conflicts of interest were declared.

Introduction

Epithelial tumour cells can convert to a mesenchymal-like phenotype, thereby making invasion and metastasis possible [1,2]. This trans-differentiation programme, epithelial–mesenchymal transition (EMT), is seen not only in aggressive carcinomas but also during embryonic development, fibrosis, and wound healing [1–3]. Tumour EMT is induced by signalling pathways, including transforming growth factor β, Wnt, and Notch [1]. The stromal cells in the tumour microenvironment can secrete cytokines, chemokines, and growth factors

and thereby induce EMT [4], and hypoxia is known to induce EMT by upregulation of factors such as hypoxia-inducible factor-1 alpha (Hif-1α) [1,5].

EMT is driven and coordinated by master regulators, including Twist, Slug (Snai2), and Snail (Snai1), which are able to activate mesenchymal genes and repress epithelial genes [6]. These pathways are dependent on the context, as well as the tissue, and the EMT regulators have been shown to regulate each other in complicated, hierarchical, and interdependent manners [7]. Hence, in addition to examining the expression of individual EMT regulators in carcinomas, the impact of their co-expression is of great interest [5,8–10].

Twist, Slug, and Snail differ both structurally and functionally [7]. Although Slug and Snail share a similar structural organisation, it appears that they still play different roles in EMT [11] and are expressed differently [12]. Twist induces expression of mesenchymal markers such as N-cadherin [13–15], fibronectin, and vimentin [14,15] and promotes proliferation, facilitates intravasation [14], inhibits apoptosis, and reduces sensitivity to chemotherapy [14,16,17]. Slug and Snail act as repressors on the E-cadherin (*CDH1*) promoter [2,18]. They also affect proliferation and have pro-survival activity [18]. Increased Snail expression has been found especially at the invasive front in carcinomas [19,20].

In addition, a relationship between EMT and angiogenesis has been suggested. Twist is shown to have a pro-angiogenic effect through increased vascular endothelial growth factor (VEGF) in studies on pancreatic cancer [21] and breast cancer [14,22,23], and Slug and Snail have also been linked to angiogenesis via VEGF [24]. However, little is reported on this possible connection between EMT, hypoxia, and angiogenesis on clinical tumour specimens from prostate cancer patients.

Twist, Slug, and Snail are associated with aggressive features and disease progression in several cancers [8,25–30] but are studied to a lesser extent in prostate cancer [31–39], where studies presenting end points beyond biochemical recurrence are still unavailable. Here, we evaluated the expression of Twist, Slug, and Snail in different prostatic tissues. Hif-1α was re-evaluated, after being assessed in a previous study [40], in our current larger series with extended follow-up. We focused particularly on co-expression of the biomarkers, as well as on potential novel links between EMT, hypoxia, and angiogenesis. Furthermore, relations to clinicopathological features and patient survival were examined as factors that control epithelial–mesenchymal states are attractive targets for cancer therapy. Our study supports a central role for these EMT regulators, with findings that could affect the future management of prostate cancer.

Materials and methods

Patients and tissues

As previously described [41], series 1–5 include the following: series 1, radical prostatectomy specimens from patients (n = 338) with clinically localised prostate cancer, treated from 1986 to 2007 at Haukeland University Hospital, Bergen, Norway; series 2, prostate tissues (n = 41) of benign prostatic hyperplasia (BPH); series 3, non-skeletal metastases (27 lymph node metastases and 6 distant soft tissue metastases [testis, rectum, bronchial mucosa, orbita, skeletal muscle, and subcutaneous tissue]); series 4, skeletal metastases (n = 13); and series 5; prostate cancer tissues from castration-resistant prostate cancer (CRPC) patients (n = 33) treated by transurethral resection for palliation purposes from 1990 to 2005. Five lymph node metastases in series 3 match with patients in series 1 as pelvic lymphadenectomy was performed along with radical prostatectomy on these patients. The tumours are principally acinar adenocarcinomas as previously reported [41]. This study was approved by the Western Regional Committee for Medical and Health Research Ethics, REC West (REK 2015/2178).

Clinicopathological variables

For series 1, the following variables were retrieved from the clinical patient files: age at diagnosis, date of primary diagnosis, date of prostatectomy, preoperative and postoperative serum prostate-specific antigen (s-PSA), and clinical TNM stage [42]. From the pathology reports, Gleason grading, extra-prostatic extension, seminal vesicle invasion, involvement of surgical margins, pelvic lymph node status at prostatectomy, and largest tumour dimension were recorded (see supplementary material, Table S1). s-PSA was introduced in the early 1990s in Norway. Consequently, the patients in the first part of series 1 more often had palpable tumours, clinical stage T2, with locally advanced pathological stages compared with prostate-specific antigen (PSA)-detected tumours, which were largely clinical stage T1C, in the second part. Because of the long study period, series 1 was re-examined and Gleason graded according to the recommendations from the International Society of Urological Pathology

(ISUP) Consensus Conference in 2005 and 2014 [43,44]. For the CRPC patients (series 5), age at diagnosis (median 77.3 years) was recorded.

Follow-up

September 2016 was the last date of follow-up for series 1. The median follow-up time was 147.5 months for all patients and 151.5 months for surviving patients. Time from surgery until the following end points was recorded: biochemical recurrence (169/338 patients), clinical recurrence (101/338 patients), locoregional recurrence (77/338 patients), skeletal metastases (41/338 patients), and death (112/338 patients), including cancer-specific death (38/338 patients) (see supplementary material, Table S1). Two patients were lost to follow-up. An elevated s-PSA level in two consecutive blood samples was defined as biochemical recurrence. As a result of more sensitive s-PSA measurements, the elevated s-PSA level was set at ≥0.5 ng/ml if the blood sample was taken before 31 December 1994 and ≥0.2 ng/ml after 1 January 1995. A tumour in the prostatic fossa was defined as locoregional recurrence, as was a >50% reduction of s-PSA or a s-PSA level <0.1 ng/ml after local radiation therapy. Magnetic resonance imaging, X-ray, or bone scan identified skeletal metastases.

For series 5, disease progression during androgen ablation therapy defined castration resistance. Additional treatment with anti-androgens (bicalutamide) was given to 8 of 33 patients prior to palliative transurethral resection. Of 33 patients, 25 developed metastases of the bone, lung, liver, or testis. The median time from castration resistance to death was 28.5 months. All patients had died by the last follow-up (2018).

Tissue microarrays

Tissue microarray (TMA) blocks (series 1–3 and 5) were made by collecting three tissue cores (diameter 0.6–1.0 mm) from the area of the highest tumour grade per case and moving them to a new paraffin block. Regular sections were used for the skeletal metastases (series 4).

Immunohistochemistry

5-μm sections from formalin-fixed, paraffin-embedded (FFPE) tissue were stained by immunohistochemistry for Twist (rabbit polyclonal antibody [H-81]: sc-15393 [Santa Cruz Biotechnology, Santa Cruz, CA, USA]), Slug (monoclonal rabbit antibody [C19G7] [Cell Signaling Technology®, Danvers, MA, USA]), and Snail (polyclonal rabbit antibody [H-130]: sc-28199 [Santa Cruz Biotechnology]). Staining for Hif-1α (monoclonal mouse antibody clone H1α67 [sc-53546; Santa Cruz Biotechnology]) was performed according to previous protocols [40]. Further information regarding the immunohistochemical staining methods can be found in supplementary material, Table S2. Positive controls (multiorgan TMA sections with known expression of the relevant antigen) and negative controls (isotypic immunoglobulin or antibody diluent without the primary antibody) were included. In addition, smooth muscle cells in the intervening stroma served as the positive control for Slug.

Evaluation of staining in prostate tissues

All slides were examined by one pathologist (AB). Parts of series 1 (n = 104) and series 2–5 were examined by two pathologists (AB and KG). The evaluation was performed with pathologists blinded to patient information or earlier registration. To record the staining, we used a staining index (SI; values 0–9) by multiplying the staining intensity (values 0–3) by the proportion of positive tumour cells (0% = 0, 1–10% = 1, 11–50% = 2, >50% = 3) across the three tissue cores from each case.

Twist, Slug, Snail, and Hif-1α variably stained nuclei and cytoplasm (Figure 1). Nuclear expression is presented in this study for Twist and Hif-1α. For Slug, a super SI was calculated as the sum of the nuclear and cytoplasmic staining indices. A prominent finding for Snail was increased expression at the tumour–stromal border (Snail-SB). Snail-SB was defined subjectively by accentuated nuclear or cytoplasmic staining either in the margins of tumour cell islands or as increased staining of infiltrating single tumour cells in contrast to weaker staining in central parts of tumour islands, i.e. in tumour cells without apparent stromal contact (Figure 1H,I). Snail-SB was superior to nuclear Snail in evaluation of results, and only Snail-SB is presented in this study.

Survival patterns and frequency distribution of quartile and median values of the biomarkers were examined, and robust cut-off values were preferred; Twist (SI ≥4 versus others) and Slug (super-SI ≥8 versus others) were dichotomised by the median. Snail-SB was categorised as present or absent, and Hif-1α by the upper quartile (SI ≥6 versus others).

Intra-observer variability was tested by one pathologist (AB) on 25 randomly selected cases (series 1) with very good intra-observer agreement for Twist, Slug, Snail-SB, and Hif-1α (kappa values ≥0.82). Series 2–5 and a subset of series 1 (n = 104) were scored by two pathologists (AB and KG). Inter-observer agreement was moderate to very good for Twist (kappa values

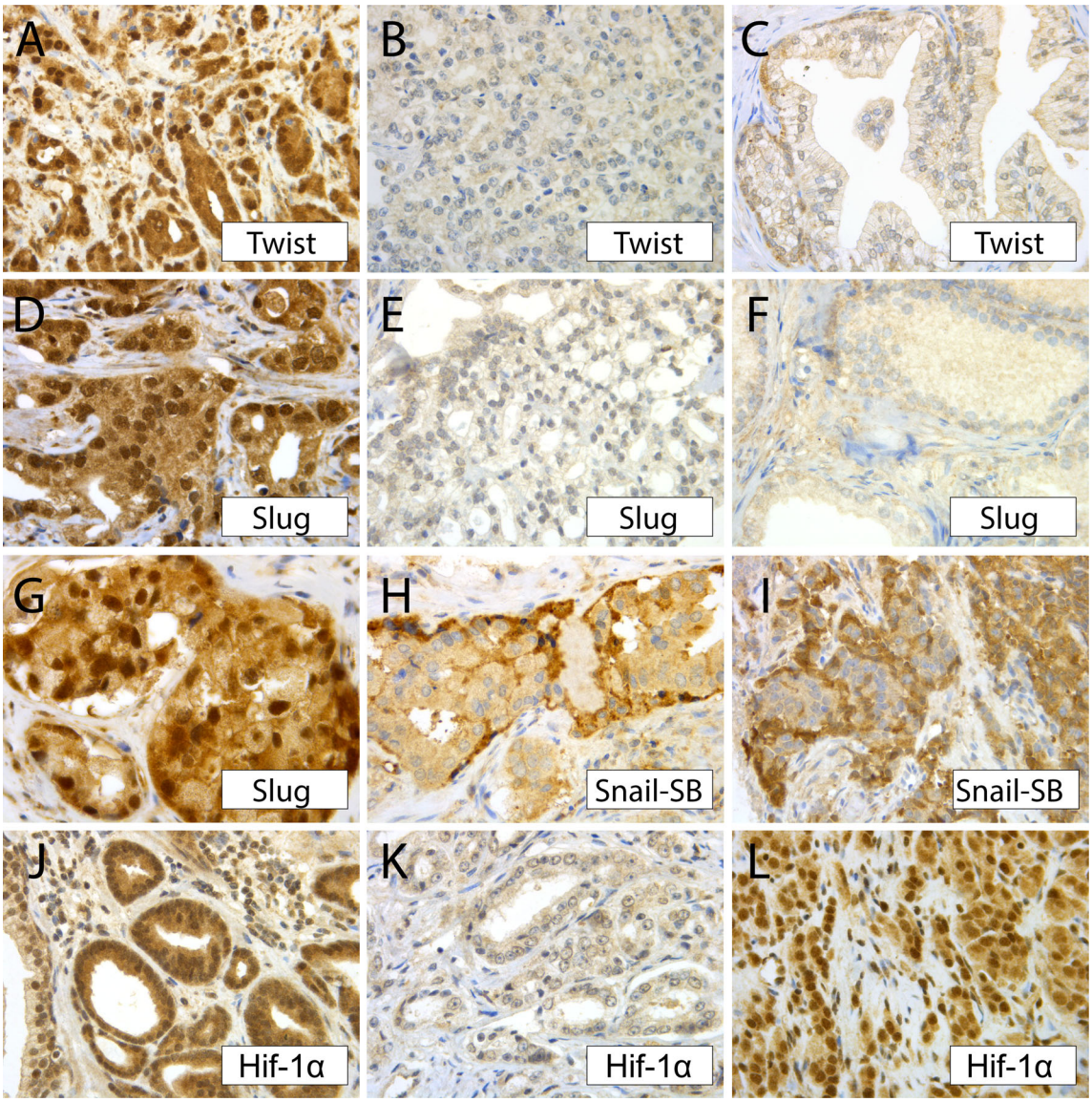

Figure 1. Immunohistochemical staining of Twist, Slug, Snail, and Hif-1α: strong nuclear expression of Twist in localised prostatic carcinoma (A) and weak Twist expression in localised prostatic carcinoma (B) and in BPH (C). Strong nuclear and cytoplasmic expression of Slug in localised carcinoma (D), weak Slug in localised carcinoma (E), weak Slug in BPH (F), and strong Slug in skeletal metastasis (G). Increased staining of Snail at the tumour–stromal border in localised prostatic carcinoma (H) and in CRPC (I). Strong nuclear Hif-1α in localised prostatic carcinoma (J), weak Hif-1α in localised prostatic carcinoma (K), and strong Hif-1α in castration-resistant carcinoma (L). Original magnification ×400.

0.45–0.94) and good to perfect for Slug, Snail-SB, and Hif-1α (kappa values 0.71–1.0).

Evaluation of TILs

The number of tumour-infiltrating lymphocytes (TILs) was assessed on the haematoxylin and eosin-stained TMA slides from series 1 and graded subjectively with four grades (0–3) (see supplementary material, Note S1). Associations were analysed using the categorisation of low (0–1) versus high (2–3) TILs. For survival analyses, subgroups with similar survival curves were merged and categorised into intermediate TILs versus absent or very high TILs.

Biomarkers from previous studies

Biomarkers from previous studies, including FOXC2 [41], E-cadherin, N-cadherin [41,45], β-catenin [45], microvessel density (FVIII) [46,47], VEGF-A [40],

vascular proliferation (Nestin/Ki-67) [40], glomeruloid microvascular proliferation (GMP) [48], Ki-67 [49], and p27 [50] were included in the analyses.

Biomarker gene expression in external cohorts

Three publicly available prostate cancer gene expression data sets were analysed for validation: GSE16560 ($n = 281$, FFPE tissue from transurethral resection of prostate) [51], GSE 10645 ($n = 596$, FFPE tissue from radical prostatectomies) [52], and The Cancer Genome Atlas Prostate Adenocarcinoma (TCGA-PRAD) database ($n = 497$, tissue from radical prostatectomies). GSE 16560 was downloaded from Gene Expression Omnibus (GEO), www.ncbi.nlm.nih.gov/geo, and GSE 10645 from GEO (mRNA data) and Oncomine, www.oncomine.org (clinical data). The TCGA-PRAD database was downloaded from cBioPortal for cancer genomics, www.cbioportal.org. If available, databases containing information on cancer-specific survival after radical prostatectomy were preferred. The patients were dichotomised by high and low expression of *TWIST1*, *HIF-1α*, and *SNAI1* using median as the cut-off for the GSE databases and z-score > 2.0 for the TCGA-PRAD database. Cancer-specific death was used as the end point for GSE 16560 and GSE 10645, and disease-free survival was used for the TCGA-PRAD database.

Statistics

Statistical analyses were performed using the SPSS statistical package (IBM Corp., Armonk, NY, USA), versions 24.0–26.0. The clinicopathological variables were dichotomised by clinically meaningful cut-off points or by merging groups with comparable outcomes on survival analyses. Pearson's chi-square or Fisher's exact test was performed for associations between categorical variables, and the Mann–Whitney U or Kruskal–Wallis test was used for continuous variables. The McNemar test and Wilcoxon signed rank test were used to compare related samples. Cohen's kappa statistics was used to evaluate inter- and intra-observer agreement. The product-limit method (log-rank test) was used for univariate survival analyses, and Kaplan–Meier plots were computed. The Cox proportional hazards method and the likelihood ratio test were performed for multivariate analysis, including variables with $p < 0.10$ in univariate analysis. Log–log plots were used to examine model assumptions of proportionality, and possible interactions were tested.

Results

Individual expression of EMT regulators associate with adverse clinicopathological features and predict survival

Twist expression was recorded as strong in 161 of 334 (48%), Slug in 157 of 332 (47%), Snail-SB in 115 of 333 (35%), and Hif-1α in 80 of 334 (24%) of the prostatectomy cases.

Strong expression of Twist, Slug, and Snail-SB was associated with high Gleason score (≥GG3 [4 + 3] versus ≤GG2 [3 + 4]) (Figure 2A), but only Snail-SB was associated with Gleason GG5 ($p < 0.0005$) (Figure 2B). Among 15 cases with heterogenous Gleason patterns, Twist or Slug did not differ between different Gleason patterns, whereas all cases with increased Snail-SB (9/15) were found in the higher and not in the lower Gleason patterns ($p = 0.004$). Furthermore, strong Slug and Snail-SB were associated with nearly all other unfavourable clinicopathological variables, and Hif-1α was associated with none (Table 1). Strong Slug was associated with strong FOXC2, strong β-catenin, and high Ki-67, and Snail-SB was associated with strong FOXC2, low E-cadherin, EN-switch, weak β-catenin, weak p27, and increased s-PSA (Mann–Whitney, $p = 0.001$). In addition, the biomarkers were associated with each other (see supplementary material, Table S3): strong Twist with strong Slug expression ($p < 0.0005$), strong Twist and Slug with strong Hif-1α ($p = 0.001$ and $p < 0.0005$), and strong Slug with Snail-SB ($p = 0.014$).

In univariate survival analyses, strong Twist, Slug, and Snail-SB were associated with shorter time to biochemical, clinical, and locoregional recurrence and cancer-specific death (Figure 3 and supplementary material, Table S4). Strong Hif-1α was associated with clinical recurrence (Figure 3 and supplementary material, Table S4). Evaluation of these biomarkers within the subgroup of Gleason score 7 carcinomas broadly gave a trend for similar, although less significant, results compared with using the complete series (data not shown).

In multivariate models, Twist, Slug, Snail-SB, and Hif-1α were introduced individually together with the three standard prognostic variables: Gleason score (≥GG3 [4 + 3] versus ≤GG2 [3 + 4]), pathological stage (≥pT3 versus pT2), and preoperative s-PSA (>13.3 versus ≤13.3, upper quartile). Survival data for

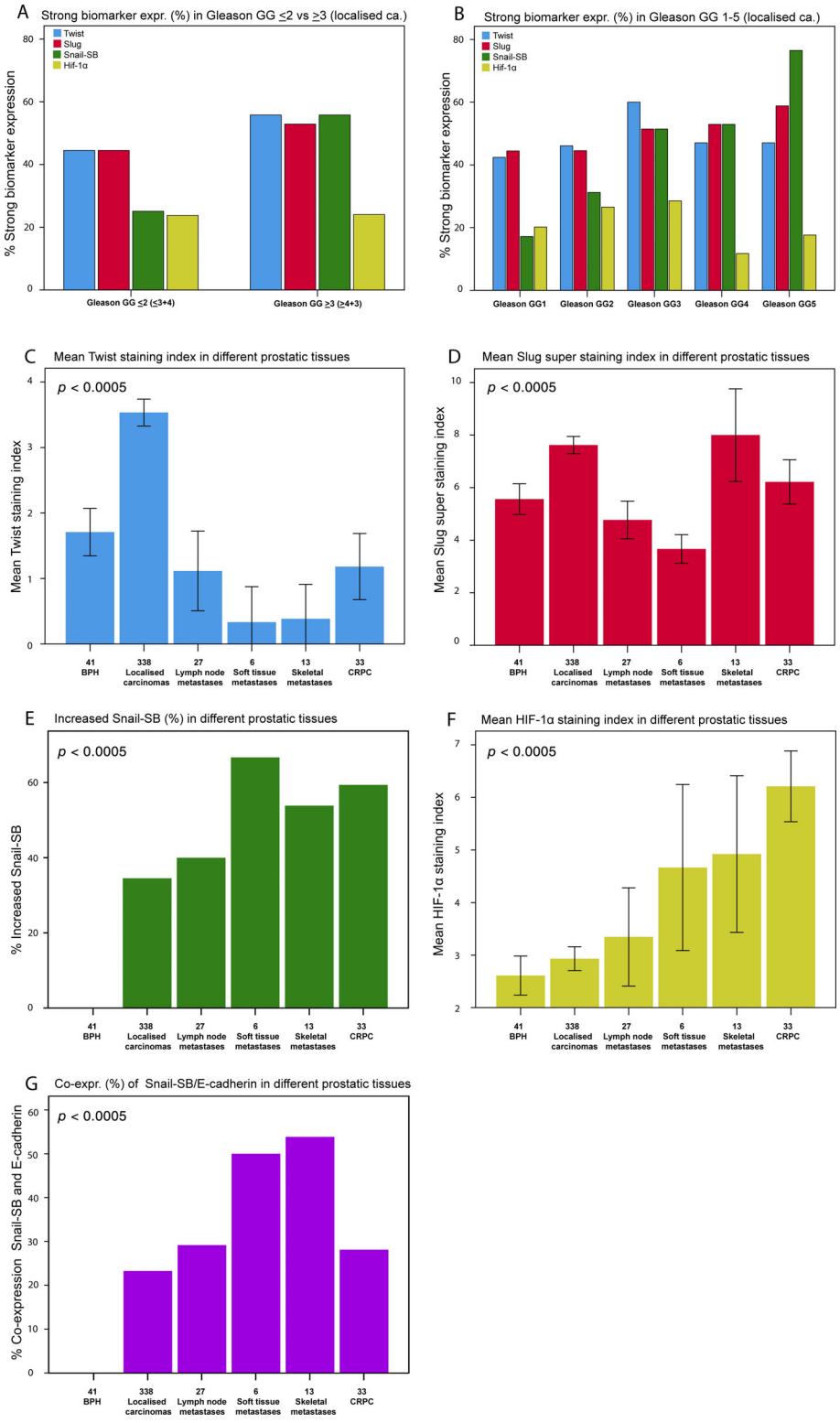

Figure 2. Strong expression of Twist, Slug, Snail-SB, and Hif-1α (%) in Gleason grade group ≥GG3 (4 + 3) versus ≤GG2 (3 + 4) (A) and in Gleason Grade Groups 1–5 (B) in localised prostatic carcinomas. Mean Twist SI (C), mean Slug super SI (D), Snail at tumour–stromal border (%) (E), mean Hif-1α SI (F), and co-expression (%) of Snail-SB and E-cadherin (G) in different prostatic tissues (95% CI).

Table 1. Associations between clinicopathological variables and expression of Twist, Slug, Snail-SB, and Hif-1α in addition to number of co-expressing factors (Twist, Slug, Snail-SB, and Hif-1α; 3–4 strong versus 0–2 strong) in patients with clinically localised prostatic adenocarcinoma (338 radical prostatectomies).

	Twist*			Slug†			Snail-SB†			Hif-1α*			Twist–Slug–Snail–SB-Hif-1α co-expression§		
Variables	Low, *n* (%)	High, *n* (%)	*P* value¶	Low, *n* (%)	High, *n* (%)	*P* value¶	Not increased, *n* (%)	Increased, *n* (%)	*P* value¶	Low, *n* (%)	High, *n* (%)	*P* value¶	0–2 Strong biomarkers, *n* (%)	3–4 Strong biomarkers, *n* (%)	*P* value¶
Gleason score**			0.036			0.032††			<0.0005			0.915			0.002
≤3 + 4	127 (56)	101 (44)		149 (66)	78 (34)		170 (75)	57 (25)		173 (76)	55 (24)		191 (84)	36 (16)	
≥4 + 3	46 (43)	60 (57)		56 (53)	49 (47)		48 (45)	58 (55)		81 (76)	25 (24)		72 (69)	32 (31)	
Extra-prostatic extension			0.060			<0.0005			<0.0005			0.813			0.006
Absent	112 (56)	88 (44)		121 (61)	78 (39)		149 (75)	50 (25)		153 (77)	47 (23)		168 (84)	31 (16)	
Present	61 (46)	73 (54)		54 (41)	79 (59)		69 (52)	65 (48)		101 (75)	33 (25)		95 (72)	37 (28)	
Seminal vesicle invasion			0.885			0.012			0.001			0.711			0.011
Absent	145 (52)	134 (48)		155 (56)	123 (44)		193 (69)	86 (31)		214 (76)	66 (24)		227 (82)	50 (18)	
Present	28 (51)	27 (49)		20 (37)	34 (63)		25 (46)	29 (54)		40 (74)	14 (26)		36 (67)	18 (33)	
Pathological stage‡‡			0.150			<0.0005			<0.0005			0.612			0.002
pT2	108 (55)	88 (45)		119 (61)	76 (39)		145 (74)	50 (26)		151 (77)	45 (23)		166 (85)	29 (15)	
≥pT3	65 (47)	73 (53)		56 (41)	81 (59)		73 (53)	65 (47)		103 (75)	35 (25)		97 (71)	39 (29)	
Lymph node infiltration§§			0.269			0.029			0.130			0.403			0.035
Absent¶¶	171 (52)	156 (48)		174 (54)	150 (46)		215 (66)	110 (34)		249 (76)	77 (24)		260 (80)	64 (20)	
Present	2 (29)	5 (71)		1 (13)	7 (87)		3 (38)	5 (62)		5 (63)	3 (37)		3 (43)	4 (57)	
Tumour dimension (mm)***			0.254			0.204			0.001			0.503			0.007
<25	86 (55)	70 (45)		88 (56)	68 (44)		117 (75)	39 (25)		122 (78)	35 (22)		133 (86)	22 (14)	
≥25	87 (49)	91 (51)		87 (49)	89 (51)		101 (57)	76 (43)		132 (75)	45 (25)		130 (74)	46 (26)	
Surgical margins			0.115			0.060			0.148			0.109			0.583
Negative	104 (56)	83 (44)		106 (57)	79 (43)		128 (69)	58 (31)		136 (73)	51 (27)		149 (80)	36 (20)	
Positive	69 (47)	78 (53)		69 (47)	78 (53)		90 (61)	57 (39)		118 (80)	29 (20)		114 (78)	32 (22)	
FOXC2			0.096			0.003			0.013			0.532			0.302
Low	73 (58)	53 (42)		79 (63)	46 (37)		92 (74)	33 (26)		98 (78)	28 (22)		103 (82)	22 (18)	
High	100 (49)	106 (51)		96 (47)	110 (53)		124 (60)	82 (40)		154 (75)	52 (25)		160 (78)	46 (22)	
E-cadherin			0.409			0.151			0.001			0.072			0.982
High	130 (51)	127 (49)		129 (51)	126 (49)		179 (70)	77 (30)		190 (74)	67 (26)		203 (80)	52 (20)	
Low	42 (56)	33 (44)		45 (60)	30 (40)		37 (49)	38 (51)		63 (84)	12 (16)		59 (80)	15 (20)	
N-cadherin			0.901			0.416			0.781			0.265			0.815
Low	115 (52)	108 (48)		120 (54)	101 (46)		146 (66)	76 (34)		174 (78)	49 (22)		176 (80)	44 (20)	
High	57 (52)	52 (48)		54 (50)	55 (50)		70 (64)	39 (36)		79 (73)	30 (27)		86 (79)	23 (21)	
EN-switch†††			0.687			0.883			0.004			0.425			0.299
Others	156 (52)	143 (48)		157 (53)	140 (47)		202 (68)	96 (32)		226 (76)	73 (24)		238 (80)	58 (20)	
Switch	16 (49)	17 (51)		17 (52)	16 (48)		14 (42)	19 (58)		27 (82)	6 (18)		24 (73)	9 (27)	

(Continues)

Table 1. Continued

Variables	Twist* Low, *n* (%)	Twist* High, *n* (%)	Twist* *P* value¶	Slug† Low, *n* (%)	Slug† High, *n* (%)	Slug† *P* value¶	Snail-SB‡ Not increased, *n* (%)	Snail-SB‡ Increased, *n* (%)	Snail-SB‡ *P* value¶	Hif-1α* Low, *n* (%)	Hif-1α* High, *n* (%)	Hif-1α* *P* value¶	Twist–Slug–Snail–SB-Hif-1α co-expression§ 0–2 Strong biomarkers, *n* (%)	Twist–Slug–Snail–SB-Hif-1α co-expression§ 3–4 Strong biomarkers, *n* (%)	Twist–Slug–Snail–SB-Hif-1α co-expression§ *P* value¶
β-Catenin			0.132			0.002			0.030			0.003			0.043
High	20 (35)	37 (65)		6 (11)	49 (89)		30 (54)	26 (46)		34 (60)	23 (40)		30 (54)	25 (46)	
Low	22 (50)	22 (50)		16 (36)	28 (64)		14 (32)	30 (68)		38 (86)	6 (14)		32 (74)	11 (26)	
GMP			0.203			0.183			0.016			0.021‡‡‡			0.021
Absent	34 (39)	53 (61)		21 (25)	64 (75)		42 (49)	44 (51)		59 (68)	28 (32)		57 (68)	27 (32)	
Present	8 (57)	6 (43)		1 (7)	13 (93)		2 (14)	12 (86)		5 (36)	9 (64)		5 (36)	9 (64)	
MVD_{max}§§§			0.373			0.036			0.530			0.470			0.494
Low	23 (46)	27 (54)		15 (31)	33 (69)		20 (41)	29 (59)		34 (68)	16 (32)		32 (67)	16 (33)	
High	19 (37)	32 (63)		7 (14)	44 (86)		24 (47)	27 (53)		38 (75)	13 (25)		30 (60)	20 (40)	
VEGF-A			0.894			0.391			0.036			0.271			0.715
Low	33 (41)	47 (59)		19 (24)	59 (76)		39 (49)	40 (51)		55 (69)	25 (31)		48 (62)	29 (38)	
High	9 (43)	12 (57)		3 (14)	18 (86)		5 (24)	16 (76)		17 (81)	4 (19)		14 (67)	7 (33)	
Ki67			0.143			0.036			0.961			0.084			0.494
Low	24 (49)	25 (51)		15 (31)	33 (69)		21 (44)	27 (56)		31 (63)	18 (37)		32 (67)	16 (33)	
High	18 (35)	34 (65)		7 (14)	44 (86)		23 (44)	29 (56)		41 (79)	11 (21)		30 (60)	20 (40)	
Nestin/Ki67¶¶¶			0.045			0.267			0.009			0.864			0.141
Low	31 (49)	32 (51)		16 (26)	46 (74)		34 (54)	29 (46)		46 (72)	18 (28)		42 (69)	19 (31)	
High	11 (29)	27 (71)		6 (16)	31 (84)		10 (27)	27 (73)		26 (70)	11 (30)		20 (54)	17 (46)	
p27			0.008			0.390			0.006			0.047			0.135
High	14 (29)	35 (71)		9 (19)	39 (81)		28 (57)	21 (43)		31 (62)	19 (38)		26 (55)	21 (45)	
Low	28 (55)	23 (45)		13 (26)	37 (74)		15 (30)	35 (70)		40 (80)	10 (20)		35 (70)	15 (30)	

MVD, microvessel density.
*Nuclear expression, cut-off by median (Twist) or upper quartile (Hif-1α).
†Cytoplasmic and nuclear expression, cut-off by median.
‡Nuclear or cytoplasmic expression of Snail at tumour–stromal border.
§Expression of nuclear Twist, nuclear and cytoplasmic Slug, and increased Snail at tumour–stromal border and nuclear Hif-1α.
¶Pearson chi-square or Fisher's exact test.
**Gleason score in radical prostatectomy specimens.
††Cut-off by upper tertile.
‡‡Pathological stage, UICC TNM Classification of Malignant Tumours, Eighth Edition, 2017 [42].
§§Pelvic lymph node infiltration at radical prostatectomy.
¶¶Includes cases without lymphadenectomy.
***Largest tumour dimension in prostatectomy specimens, divided by median.
†††Subgroup with combined weak membranous E-cadherin and positive membranous N-cadherin expression.
‡‡‡Cut-off by lower tertile.
§§§MVD by FVIII, the maximum count within any single field.
¶¶¶Proliferating MVD by dual Nestin/Ki67 staining.

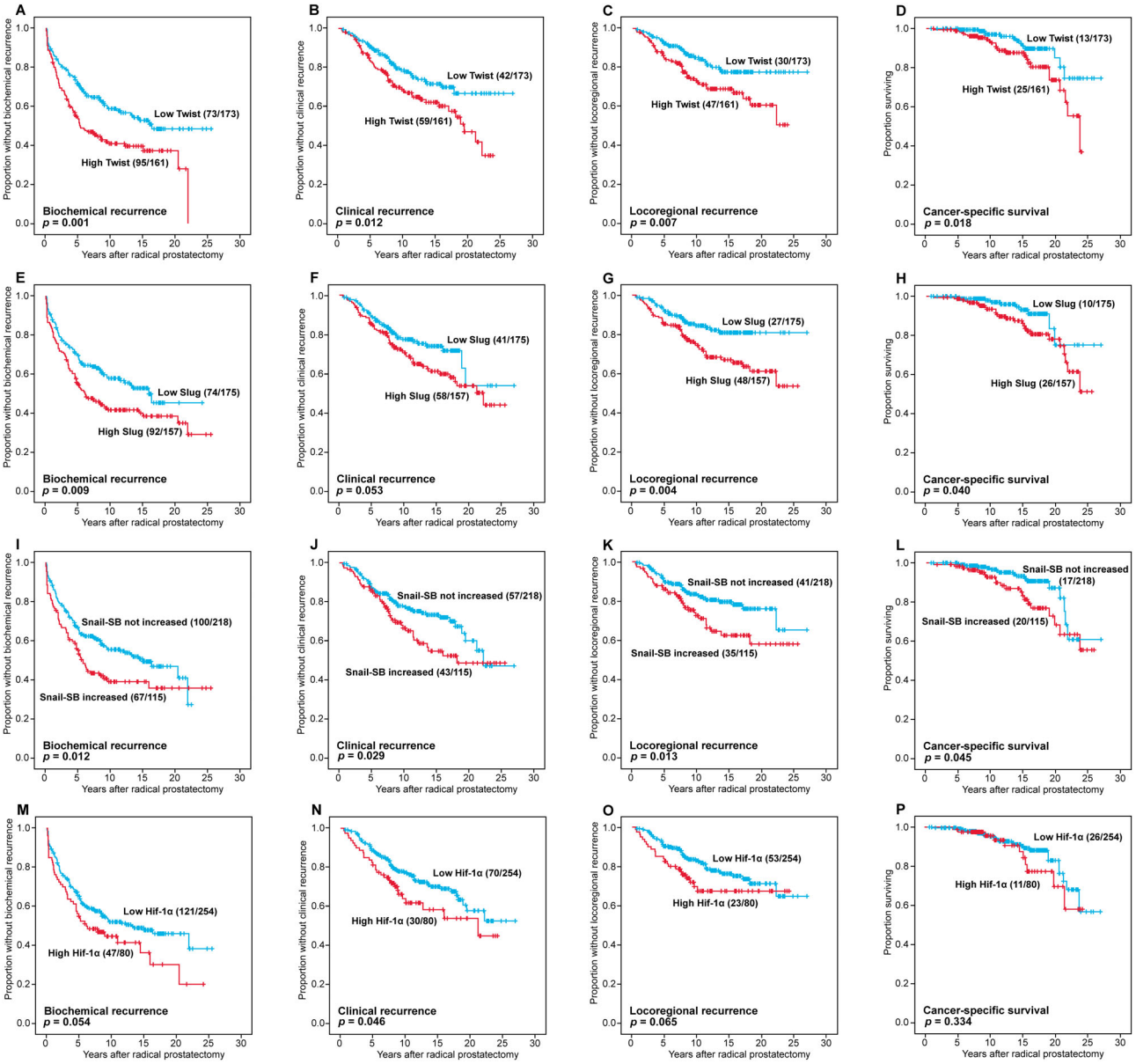

Figure 3. Univariate survival analyses (Kaplan–Meier) according to expression of Twist (A–D), Slug (E-H), Snail-SB (I-L), and Hif-1α (M–P) in patients with clinically localised prostatic adenocarcinoma (338 radical prostatectomies). End points: biochemical recurrence, clinical recurrence, locoregional recurrence, and cancer-specific death.

univariate clinicopathological variables are previously described [41]. Twist and Slug tended to be independent predictors of biochemical (hazard ratio [HR] 1.6, $p = 0.005$; HR 1.3, $p = 0.087$) and locoregional recurrence (HR 1.6, $p = 0.060$; HR 1.6, $p = 0.041$). Hif-1α tended to be an independent predictor of biochemical, clinical, and locoregional recurrence (HR 1.4–1.7, $p \leq 0.054$). Gleason score and pathological stage remained independent predictors in the models (Table 2). Snail-SB was not an independent predictor of any end point.

The link between EMT regulators, adverse features, and outcome is strongly sustained in carcinomas with E-cadherin^{low} phenotype

E-cadherin staining was weak in 76 of 335 (23%) of the cases in series 1, indicating ongoing EMT [41]. In

Table 2. Multivariate survival analysis (Cox proportional hazards method) according to expression of Twist, Slug, and Hif-1α and according to number of co-expressing factors (Twist, Slug, Snail-SB, and Hif-1α; 3–4 strong versus 0–2 strong) in patients with clinically localised prostatic adenocarcinoma (338 radical prostatectomies). End points: biochemical recurrence, clinical recurrence, and locoregional recurrence.

Twist					Slug					Hif-1α					Twist–Slug–Snail-SB-Hif-1α co-expression				
Variables	No.	HR	95% CI	*P* value*	Variables	No.	HR	95% CI	*P* value*	Variables	No.	HR	95% CI	*P* value*	Variables	No.	HR	95% CI	*P* value*
Biochemical recurrence					**Biochemical recurrence**					**Biochemical recurrence**					**Biochemical recurrence**				
Gleason score†					Gleason score†					Gleason score†					Gleason score†				
≤3 + 4	222	1.0			≤3 + 4	221	1.0			≤3 + 4	222	1.0			≤3 + 4	221	1.0		
≥4 + 3	106	2.5	1.8–3.4	<0.0005	≥4 + 3	105	2.6	1.8–3.6	<0.0005	≥4 + 3	106	2.7	1.9–3.7	<0.0005	≥4 + 3	104	2.5	1.8–3.5	<0.0005
Path. stage‡					Path. stage‡					Path. stage‡					Path. stage‡				
pT2	196	1.0			pT2	195	1.0			pT2	196	1.0			pT2	195	1.0		
≥pT3	132	2.0	1.4–2.8	<0.0005	≥pT3	131	1.9	1.3–2.7	<0.0005	≥pT3	132	2.0	1.4–2.8	<0.0005	≥pT3	130	1.9	1.3–2.6	<0.0005
Preoperative s-PSA§					Preoperative s-PSA§					Preoperative s-PSA§					Preoperative s-PSA§				
Low	246	1.0			Low	245	1.0			Low	247	1.0			Low	244	1.0		
High	82	1.9	1.3–2.6	<0.0005	High	81	1.8	1.3–2.5	0.001	High	81	1.8	1.3–2.4	0.001	High	81	1.8	1.3–2.5	0.001
Twist¶					Slug**					Hif-1α¶					Co-expression††				
Low	170	1.0			Low	170	1.0			Low	249	1.0			0–2 Strong	257	1.0		
High	158	1.6	1.1–2.2	0.005	High	156	1.3	1.0–1.8	0.087	High	79	1.4	1.0–2.0	0.054	3–4 strong	68	1.6	1.1–2.2	0.014
Clinical recurrence					**Clinical recurrence**					**Clinical recurrence**					**Clinical recurrence**				
Gleason score†					Gleason score†					Gleason score†					Gleason score†				
≤3 + 4	228	1.0			≤3 + 4	227	1.0			≤ 3 + 4	228	1.0			≤3 + 4	227	1.0		
≥4 + 3	106	2.7	1.8–4.1	<0.0005	≥4 + 3	105	2.6	1.7–4.0	<0.0005	≥4 + 3	106	2.8	1.9–4.3	<0.0005	≥4 + 3	104	2.6	1.7–4.0	<0.0005
Path. stage‡					Path. stage‡					Path. stage‡					Path. stage‡				
pT2	196	1.0			pT2	195	1.0			pT2	196	1.0			pT2	195	1.0		
≥pT3	138	2.1	1.4–3.3	0.001	≥pT3	137	2.0	1.3–3.2	0.001	≥pT3	138	2.1	1.4–3.3	0.001	≥pT3	136	2.0	1.3–3.1	0.002
Twist¶					Slug**					Hif-1α¶					Co-expression††				
Low	173	1.0			Low	175	1.0			Low	254	1.0			0–2 Strong	263	1.0		
High	161	1.4	0.9–2.0	0.127	High	157	1.2	0.8–1.8	0.403	High	80	1.7	1.1–2.6	0.021	3–4 Strong	68	1.9	1.2–2.9	0.005
Locoregional recurrence					**Locoregional recurrence**					**Locoregional recurrence**					**Locoregional recurrence**				
Gleason score†					Gleason score†					Gleason score†					Gleason score†				
≤ 3 + 4	228	1.0			≤3 + 4	227	1.0			≤3 + 4	228	1.0			≤3 + 4	227	1.0		
≥ 4 + 3	106	2.3	1.5–3.8	<0.0005	≥4 + 3	105	2.3	1.4–3.7	0.001	≥4 + 3	106	2.5	1.6–4.0	<0.0005	≥4 + 3	104	2.2	1.4–3.6	0.001
Path. stage‡					Path. stage‡					Path. stage‡					Path. stage‡				
pT2	196	1.0			pT2	195	1.0			pT2	196	1.0			pT2	195	1.0		
≥pT3	138	2.1	1.3–3.5	0.002	≥pT3	137	1.9	1.2–3.2	0.010	≥pT3	138	2.1	1.3–3.5	0.002	≥pT3	136	1.9	1.2–3.2	0.008
Twist¶					Slug**					Hif-1α¶					Co-expression††				
Low	173	1.0			Low	175	1.0			Low	254	1.0			0–2 Strong	263	1.0		
High	161	1.6	1.0–2.5	0.060	High	157	1.6	1.0–2.7	0.041	High	80	1.7	1.1–2.8	0.038	3–4 Strong	68	2.5	1.6–4.0	<0.0005

*Likelihood ratio test.
†Gleason score in radical prostatectomy specimens.
‡Pathological stage, UICC TNM Classification of Malignant Tumours, Eighth Edition, 2017 [42].
§s-PSA, cut-off by upper quartile.
¶Nuclear expression, cut-off by median (Twist) or upper quartile (Hif-1α).
**Cytoplasmic and nuclear expression, cut-off by median.
††Expression of nuclear Twist, nuclear and cytoplasmic Slug, increased Snail at tumour–stromal border, and nuclear Hif-1α.

this subgroup, strong Twist was associated with high Gleason score ($p = 0.028$) and lymph node infiltration ($p = 0.034$), whereas strong Slug and Snail-SB were associated with high Gleason score, extra-prostatic extension, seminal vesicle invasion, high pathological stage, and positive surgical margins ($p \leq 0.048$) (see supplementary material, Table S5). In univariate survival analyses, Slug was associated with all end points, and Twist, Snail-SB, and Hif-1α were associated with most end points (Figure 4, and supplementary material, Figure S1 and Table S6). Multivariate models showed that Slug was an independent predictor of clinical and locoregional recurrence, skeletal metastases, and cancer-specific death (HR 2.3–3.3, $p \leq 0.04$) (Table 3), and Hif-1α was an independent predictor of clinical and locoregional recurrence (HR 3.9–4.9, $p \leq 0.008$) together with Gleason score.

The above results support a convincing relation between EMT regulators and aggressive tumour features and outcome in prostate cancer.

Co-expression of factors strengthens relations to adverse features and outcome

Co-expression of Twist–Slug and Twist–Slug–Snail-SB revealed an even more striking association with unfavourable clinicopathological features and poor outcome (see supplementary material, Note S2, Tables S7 and S8, and Figure S2). Furthermore, the patients were classified into two groups based on expression of Twist, Slug, Snail-SB, and Hif-1α: zero to two strong biomarkers (263/331, 79%) versus three to four strong biomarkers (68/331, 21%). Co-expression of three to four biomarkers was associated strongly with all unfavourable clinicopathological features (Table 1) and all end points (Figure 4 and supplementary material, Table S8). In multivariate survival analysis, co-expression of three to four biomarkers independently predicted biochemical, clinical, and locoregional recurrence (HR 1.6–2.5, $P = 0.014$ to <0.0005) consistently together with Gleason score and pathological stage (Table 2).

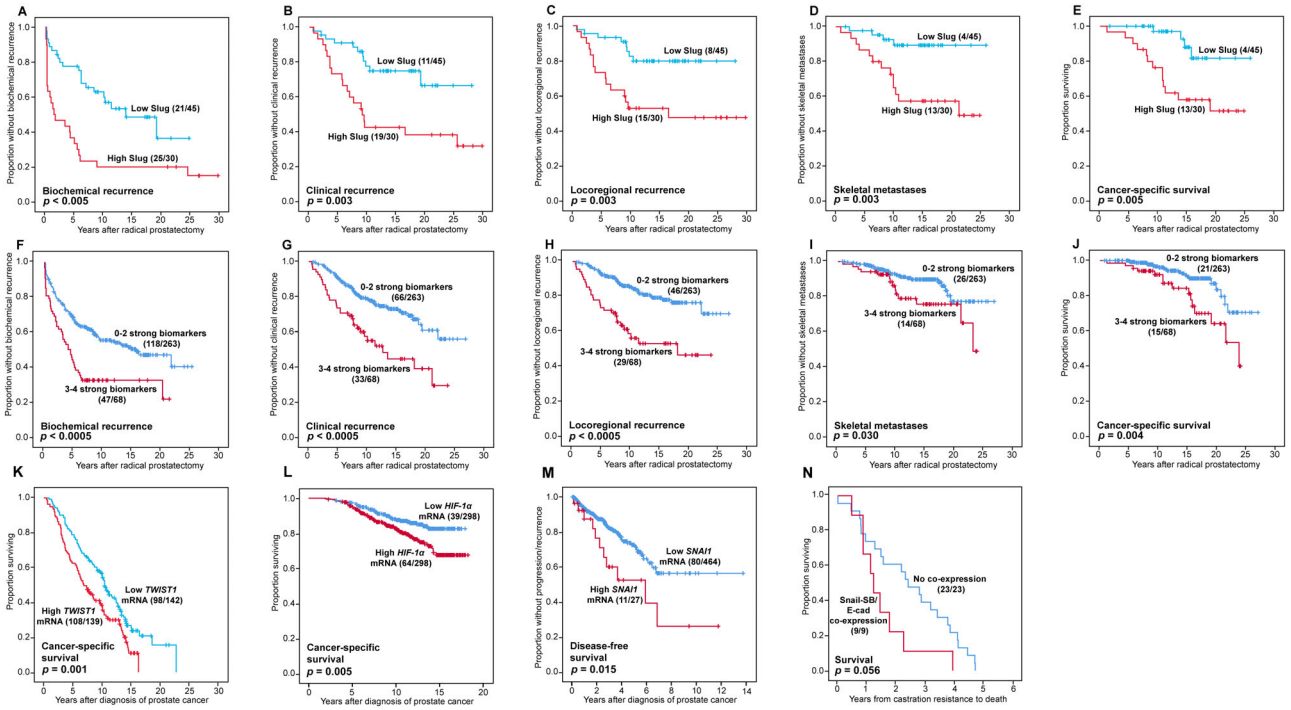

Figure 4. Univariate survival analyses (Kaplan–Meier) according to expression of Slug (A–E) in patients with E-cadherin^{low}, clinically localised prostatic adenocarcinoma (76 radical prostatectomies), according to number of co-expressing factors (Twist, Slug, Snail-SB, and Hif-1α; 3–4 strong versus 0–2 strong) (F–J) in patients with clinically localised prostatic adenocarcinoma (338 radical prostatectomies) (end points: biochemical recurrence, clinical recurrence, locoregional recurrence, skeletal metastases, and cancer-specific death) and according to mRNA expression of *TWIST1* in 281 prostate cancer patients with cancer-specific death as end point (K); mRNA expression of *HIF-1α* in 596 prostate cancer patients with cancer-specific death as end point (L), mRNA expression of *SNAI1 in* 497 prostate cancer patients with disease-free survival as end point (M), and co-expression of Snail-SB and E-cadherin in patients with castration-resistant prostatic carcinoma with time from castration resistance to death as end point (N).

Table 3. Multivariate survival analysis (Cox proportional hazards method) according to expression of Slug in patients with E-cadherin^{low}, clinically localised prostatic adenocarcinoma (76 radical prostatectomies). End points: clinical recurrence, locoregional recurrence, skeletal metastasis, and cancer-specific death.

Variables	No.	HR	95% CI	*P* value*
Clinical recurrence				
Gleason score†				
≤3 + 4	38	1.0		
≥4 + 3	37	2.2	1.0–5.0	0.055
Slug‡				
Low	45	1.0		
High	30	2.3	1.0–5.1	0.034
Locoregional recurrence				
Gleason score†				
≤3 + 4	38	1.0		
≥4 + 3	37	2.4	0.9–6.4	0.069
Slug‡				
Low	45	1.0		
High	30	2.5	1.0–6.2	0.042
Skeletal metastases				
Gleason score†				
≤3 + 4	38	1.0		
≥4 + 3	37	3.9	1.1–14.0	0.021
Slug‡				
Low	45	1.0		
High	30	3.3	1.0–10.4	0.031
Cancer-specific survival				
Gleason score†				
≤3 + 4	38	1.0		
≥4 + 3	37	5.9	1.3–26.4	0.005
Slug‡				
Low	45	1.0		
High	30	3.0	1.0–9.5	0.041

*Likelihood ratio test.
†Gleason score in radical prostatectomy specimens.
‡Cytoplasmic and nuclear expression, cut-off by median.

EMT regulators are associated with angiogenesis

As summarised in Table 1, both individual expression and co-expression of the biomarkers were associated with vascular factors. Strong Twist was associated with high vascular proliferation (Nestin/Ki-67); strong Slug with maximum microvessel density (FVIII); and Snail-SB with presence of GMP, strong VEGF-A, and increased Nestin/Ki-67. Hif-1α above the lower tertile and co-expression of three to four biomarkers were associated with the presence of GMP. Among E-cadherin^{low} carcinomas, strong Twist ($p = 0.018$) and Slug ($p = 0.073$, borderline) were associated with Nestin/Ki67, and strong Slug was associated with microvessel density ($p = 0.026$) (see supplementary material, Table S5). Taken together, the above data support a link between EMT and angiogenesis.

EMT regulators are associated with high numbers of TILs

Strong expression of Twist, Slug, and Hif-1α and co-expression of Twist, Slug, Snail-SB, and Hif-1α were associated with high TILs ($p \leq 0.044$) as seen in supplementary material, Table S9. Absence of TILs or very high TILs was associated with shorter time to most end points compared to the subgroup with an intermediate number of TILs (see Supplementary material, Figure S3).

Survival analyses by gene expression

In univariate survival analyses, high *TWIST1* (GSE 16560) and *HIF-1α* (GSE 10645) mRNA were associated with reduced cancer-specific survival, and high *SNAI1* (TCGA-PRAD database) mRNA was associated with reduced disease-free survival ($p = 0.001$, $p = 0.005$, and $p = 0.015$) (Figure 4K–M). In multivariate survival analyses, high *TWIST1* (GSE 16560) tended to be an independent predictor of cancer-specific death (HR 1.3, 95% confidence interval [CI] 1.0–1.7, $p = 0.053$) together with Gleason score (HR 3.6, 95% CI 2.7–4.7, $p < 0.0005$). *HIF-1α* (GSE 10645) was not an independent predictor of cancer-specific death (HR 1.4, 95% CI 0.9–2.3, $p = 0.167$) when including preoperative s-PSA, Gleason score, and pathological stage in the model. *SNAI1* (TCGA-PRAD database) was an independent predictor of disease recurrence with borderline significance (HR 1.8, 95% CI 1.0–3.5, $p = 0.088$) together with Gleason score and pathological stage. No survival differences were found for *SNAI2* (GSE 16560 and TCGA-PRAD database).

Biomarker expression in different prostatic tissues

Expression of the biomarkers was analysed in tissues from BPHs, localised carcinomas, lymph node metastases, distant soft tissue metastases, skeletal metastases, and castration-resistant carcinomas, as seen in Figure 2C–G. Twist expression was strong in the localised carcinomas but strikingly reduced in the distant metastases. Slug, on the other hand, was strongest in the skeletal metastases. Snail-SB was most frequently seen in the distant metastases and in the CRPCs.

Hif-1α expression has been evaluated previously [40]. After re-evaluation of the material, partition of series 3 into lymph node and distant soft tissue metastases, new patient follow-up, and expansion of series 1, Hif-1α expression was still strongest in CRPCs, skeletal metastases, and distant soft tissue metastases.

Snail-SB/E-cadherin co-expression was not found in BPHs. However, it was most frequent among the distant soft tissue metastases and skeletal metastases (Figure 2G).

Biomarker expression in matched samples

Five patients from series 1 had matching pelvic lymph node metastases in series 3. Strong Twist and Slug were observed in none and one of the five lymph node metastases, respectively, but were found in four of five and all five of the localised carcinomas ($p = 0.066$ and $p = 0.043$ [SI]). Significant differences were not found for Snail-SB or Hif-1α.

Biomarker expression in CRPC

In the CRPCs, Snail-SB was associated with positive N-cadherin ($p = 0.036$) and strong Slug (SI ≥ 8, upper quartile) ($p = 0.050$). Positive Twist (SI ≥ 1, median) was associated with strong Hif-1α (SI ≥ 6, median) ($p = 0.044$), and Snail-SB/E-cadherin co-expression was associated with positive N-cadherin ($p = 0.018$).

Strong Twist (SI ≥ 3, >upper quartile) was associated with shorter time from castration resistance to death ($p = 0.003$). Strong Hif-1α (SI ≥ 6, median, new follow-up) and Snail-SB/E-cadherin co-expression were borderline associated with reduced survival in these patients ($p = 0.084$ and $p = 0.056$) (Figure 4N and see supplementary material, Figure S4). In multivariate survival analysis, including Twist, Hif-1α, E-N-cadherin co-expression, Nestin/Ki67, and Snail-SB/E-cadherin, strong Twist and Snail-SB/E-cadherin were independent predictors of time from castration resistance to death (HR 9.2, 95% CI 2.2–38.7, $p = 0.009$; HR 2.5, 95% CI 1.1–5.9, $p = 0.045$). Strong Nestin/Ki67 was of borderline significance (HR 2.0, 95% CI 0.9–4.6, $p = 0.093$).

Discussion

In prostate cancer, transcription factors regulating EMT and angiogenesis have not been well studied, nor has their interplay. Here, we demonstrated a strong link between the EMT regulators Twist, Slug, and Snail and aggressive tumour features and disease progression with long follow-up in prostate cancer, with novel relations to hypoxia and angiogenesis, including a stronger impact by co-expression of the biomarkers.

Twist, one of the key EMT regulators central to carcinoma progression [6], was over-expressed in our localised prostatic carcinomas compared to BPH, and strong expression was associated with adverse factors and poor outcome. However, the EMT programme is plastic [3], and Twist activation may represent a transient phenomenon, with Twist downregulation favouring tumour initiation at the metastatic site [53]. In line with this, Twist was markedly reduced in the distant metastases in our material. Tumour cells with a hybrid epithelial–mesenchymal phenotype have been linked to stemness, aggressiveness, and drug resistance [54]. As these cancer cells have both adhesive and migratory abilities, they may move as clusters in the blood stream and more efficiently lead to metastases [55], and such plasticity is associated with poor patient outcome [56]. Corresponding with the above, a colon cancer cell line study demonstrated expression of both epithelial markers and Snail in circulating tumour cells [57]. Corroborating these studies, we found co-expression of Snail-SB and E-cadherin in approximately 50% of the distant metastases. Among our CRPC patients, this hybrid epithelial–mesenchymal phenotype was borderline associated with reduced survival and was an independent predictor of time to death. However, epithelial plasticity makes the targeting of EMT-inducing factors used as monotherapy complicated as reversal of EMT might lead to the establishment of macro-metastases in patients with disseminating disease or circulating tumour cells at the time of treatment [58]. Thus, a strategy for clinical application of EMT-based therapy may be to target the hybrid epithelial–mesenchymal phenotype [59].

The subgroup of carcinomas expressing low E-cadherin [41] in our material may represent tumours with ongoing EMT. As in localised carcinomas, Slug had an even more striking and strong relation to multiple adverse factors, disease progression, and cancer-specific death in this subgroup, probably reflecting the additional roles of the EMT inducer Slug, such as its effect on cell proliferation and pro-survival activity [18]. Likewise, in a study of colorectal carcinomas, tumours with both strong Slug and weak E-cadherin expression showed the worst prognosis [28].

Our finding of increased Snail expression at the tumour–stroma interface, possibly linked to the EN-switch, support that Snail, under certain conditions and tumour types, could be involved in the initial stages of invasion and be an early marker of EMT in contrast to Twist and Slug, which could be responsible for the maintenance of the migratory, malignant nature of the tumour cells [18]. While Slug and Snail repress E-cadherin expression directly by binding to the E-boxes of the *E-cadherin* promoter, Twist appears to depend on Slug activation to promote EMT by inducing Slug at the promoter level [60].

Expression of EMT biomarkers differed between our skeletal metastases and lymph node metastases and between the matched samples of localised carcinomas and lymph node metastases. The weaker expression of Slug and Snail in the lymph node metastases supports the theory that EMT might not be required for lymph node colonisation as collective sheet migration of cancer cells has been shown to have the ability to colonise lymphatics [53,61].

In prostate cancer cells, Hif-1α induces an EMT phenotype [62], and it has been demonstrated that Hif-1α directly binds to the hypoxia response element in the promoter region of Twist to increase transcription [5,63]. Hif-1α can also regulate the expression of Slug and Snail and lead to increased cancer stemness during tumour progression, thus mechanistically linking hypoxia to the EMT programme and increasing the metastatic potential [64]. In our study, strong Twist and Slug expression associated with strong Hif-1α in localised carcinomas and the relation between Twist and Hif-1α was sustained in CRPC, indicating an important association between hypoxia and EMT both before and after androgen deprivation therapy. It appears that our present study is the first to report this relationship in clinical tumour specimens from prostate cancer patients.

Associations were also found between strong Twist expression and strong Slug and between strong Slug and Snail-SB in our localised prostatic carcinomas. Co-expression of the EMT biomarkers was associated with aggressive tumour features and demonstrated an enhanced effect on outcome as suggested for other tumour types [8,9]. Furthermore, patients with co-expression of more than any two of Twist, Slug, Snail-SB, or Hif-1α demonstrated poorer outcome. Our data are in line with the experience that EMT regulators may act collectively and have an impact on each other [7], also in prostate cancer, and support a connection between EMT and hypoxia.

Hif-1α is a key regulator of angiogenesis [65,66]. Angiogenic factors, such as VEGF-A, have been shown to be upregulated in cancer cells during EMT and in cancer stem cells [67], and Twist, Slug, and Snail have been linked to angiogenesis via VEGF [21,22,24]. In addition to associations found between the EMT biomarkers and Hif-1α, strong expression of all three EMT-biomarkers was related to VEGF, microvessel density, microvascular proliferation, or GMP. Our data suggest a close relationship between EMT and angiogenesis in prostate cancer, possibly co-regulated and driven by Hif-1α.

By using a large retrospective cohort, we have achieved long and complete follow-up of the patients with several end points, including cancer-specific death. During 1986–2007, changes were made to the diagnosis and management of prostate cancer, especially by the introduction of s-PSA in the 1990s, and we are aware of the diversity of these patients. Furthermore, newer medications, such as abiraterone and enzalutamide, have been introduced after the patients in our series were treated. An effect of these newer medications cannot be assessed in our retrospective cohort of CRPCs. Validation of the results in independent, more up-to-date cohorts would be valuable.

Direct pharmacological inhibition of EMT transcription factors is difficult [68]. Alternative approaches to target EMT are described using metabolic inhibitors [69] or nanomaterials [70]. Examples include suramin for prostate cancer [71], inhibition of inducible nitric oxide synthase for triple negative breast cancer [72], and chitosan as a nanocarrier for SN38 and Snail-specific siRNA in prostate cancer cells [73]. These approaches have been shown to downregulate Twist [72], Slug [72,74], and Snail [72,73]. Furthermore, the crosstalk between tumour cells undergoing EMT and immune cells in the tumour microenvironment has garnered increasing interest and may provide possibilities for combining immunotherapy with EMT-targeted therapy or for using EMT score as a potential predictive marker for immunotherapy response [75,76]. In our study, we have investigated the relationship between EMT and tumour-associated chronic inflammation. In contrast to most other solid malignancies, a high number of TILs is often associated with poor patient outcome in prostate cancer [77]. Immune evasion in this situation can possibly be explained by the relative distribution of T-regulatory and effector cells [78]. Paralleling our findings that the absence of TILs is also linked to poor outcome, very low and very high numbers of $CD3^+$ T cells were associated with shorter time to biochemical recurrence compared to patients with intermediate numbers of $CD3^+$ T cells in prostate cancer [79]. Our findings of the significant associations between strong expression of Twist, Slug, and Hif-1α and high TILs may also be relevant for the future management of prostate cancer and invite further investigation. The potential for translational applicability of EMT may be improved by more knowledge about the plasticity of the EMT programme; its complex, dynamic, and interrelated regulation; and connection to cells in the tumour microenvironment. Treatment directed against epithelial–mesenchymal plasticity and related processes such as angiogenesis and hypoxia might be provided simultaneously and be tailored according to the expression of their respective transcriptional regulators and markers in different stages of the disease.

To summarise, our study provides new findings regarding the prognostic value of EMT in prostate

cancer using several end points, with increased prognostic influence by co-expression of EMT biomarkers. We identify a subgroup of patients with low expression of E-cadherin and strong expression of Slug with especially poor outcome; this may be a subgroup that may particularly benefit from future targeted therapy. We demonstrate an important, novel link between EMT and hypoxia, as well as angiogenesis in prostate cancer, and we find decreased Twist at the metastatic site, possibly indicating epithelial–mesenchymal plasticity. Taken together, our findings may contribute to prognostication and selection of prostate cancer patients for potential combined tailored treatment in the future.

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Author contributions statement

AB and KG performed the research. SAH and CB collected clinical data. MM downloaded gene expression data. LAA and OJH designed the study. All authors were involved in data analysis, interpretation of data, and writing the paper. All authors read and approved the final, submitted version of the paper.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Univariate survival analyses (Kaplan–Meier) according to expression of Twist, Snail-SB, and Hif-1α in patients with E-cadherin^{low}, clinically localised prostatic adenocarcinoma

Figure S2. Univariate survival analyses (Kaplan–Meier) according to co-expression of Twist–Slug and co-expression of Twist–Slug–Snail-SB in patients with clinically localised prostatic adenocarcinoma

Figure S3. Univariate survival analyses (Kaplan–Meier) according to the number of TILs in patients with clinically localised prostatic adenocarcinoma

Figure S4. Univariate survival analyses (Kaplan–Meier) according to expression of Twist and Hif-1α in patients with castration-resistant prostatic carcinoma

Table S1. Clinicopathological data and follow-up status in patients with clinically localised prostatic adenocarcinoma grouped by date of surgery

Table S2. Immunohistochemical staining methods

Table S3. Associations between expression of Twist, Slug, Snail-SB, and Hif-1α in patients with clinically localised prostatic adenocarcinoma

Table S4. Univariate survival analysis (Kaplan–Meier) according to expression of Twist, Slug, Snail-SB, and Hif-1α in patients with clinically localised prostatic adenocarcinoma

Table S5. Associations between clinicopathological variables and expression of Twist, Slug, Snail-SB, and Hif-1α in patients with E-cadherin^{low}, clinically localised prostatic adenocarcinoma

Table S6. Univariate survival analysis (Kaplan–Meier) according to expression of Twist, Slug, Snail-SB, and Hif-1α in patients with E-cadherin^{low}, clinically localised prostatic adenocarcinoma

Table S7. Associations between clinicopathological variables and co-expression of Twist–Slug and Twist–Slug–Snail-SB in patients with clinically localised prostatic adenocarcinoma

Table S8. Univariate survival analysis (Kaplan–Meier) according to co-expression of combinations of Twist, Slug, Snail-SB, and Hif-1α in patients with clinically localised prostatic adenocarcinoma

Table S9. Associations between TILs, clinicopathological variables, expression of EMT-related markers, and other variables in patients with clinically localised prostatic adenocarcinoma

Note S1. Evaluation of TILs

Note S2. Co-expression of biomarkers in radical prostatectomies