Increased Expression of SIM2-s Protein Is a Novel Marker of Aggressive Prostate Cancer

Ole Johan Halvorsen,[1] Kari Rostad,[4] Anne Margrete Øyan,[2] Hanne Puntervoll,[5] Trond Hellem Bø,[6] Laila Stordrange,[6] Sue Olsen,[2] Svein Andreas Haukaas,[3,7] Leroy Hood,[9] Inge Jonassen,[6,8] Karl-Henning Kalland,[2,4] and Lars Andreas Akslen[1,5]

Abstract

Purpose: The human *SIM2* gene is located within the Down's syndrome critical region of chromosome 21 and encodes transcription factors involved in brain development and neuronal differentiation. *SIM2* has been assigned a possible role in the pathogenesis of solid tumors, and the *SIM2-short* isoform (*SIM2-s*) was recently proposed as a molecular target for cancer therapy. We previously reported *SIM2* among the highly up-regulated genes in 29 prostate cancers, and the purpose of our present study was to examine the expression status of *SIM2* at the transcriptional and protein level as related to outcome in prostate cancer.

Experimental Design: By quantitative PCR, mRNA *in situ* hybridization, and immunohistochemistry, we evaluated the expression and significance of *SIM2* isoforms in 39 patients with clinically localized prostate cancer and validated the expression of SIM2-s protein in an independent cohort of 103 radical prostatectomies from patients with long and complete follow-up.

Results: The *SIM2* isoforms (*SIM2-s* and *SIM2-l*) were significantly coexpressed and increased in prostate cancer. Tumor cell expression of SIM2-s protein was associated with adverse clinicopathologic factors like increased preoperative serum prostate-specific antigen, high histologic grade, invasive tumor growth with extra-prostatic extension, and increased tumor cell proliferation by Ki-67 expression. SIM2-s protein expression was significantly associated with reduced cancer-specific survival in multivariate analyses.

Conclusions: These novel findings indicate for the first time that SIM2 expression might be important for clinical progress of human cancer and support the recent proposal of *SIM2-s* as a candidate for targeted therapy in prostate cancer.

The human *SIM2* gene was identified within a region of chromosome 21 (21q22.2) referred to as the Down's syndrome critical region (1) associated with trisomy 21 (2). Proteins encoded by the *SIM2* gene belong to a family of transcriptional repressors (3, 4) and may control brain development and neuronal differentiation (5, 6). In animal studies, *SIM2*$^{-/-}$ knockout mice died following craniofacial abnormalities (7) and respiratory failure (8). Two differentially spliced isoforms of the *SIM2* transcript, *SIM2-long* (*SIM2-l*) and *SIM2-short* (*SIM2-s*), have been reported (3), but details on their differential function in humans are not available. In mice, the ratio of *SIM2-l* to *SIM2-s* differs between normal tissues (9). Although SIM-s is missing one of two repressive domains present in SIM2-l, SIM2-s activates gene expression from a central midline element through ARNT, whereas a repressive effect on gene expression induced by hypoxia and dioxin response element was observed (9). In addition to a role in brain and neuronal development, *SIM2* has recently been involved in the pathogenesis of solid tumors (10, 11), and overexpression of *SIM2-s* was associated with tumors of the colon, pancreas, and prostate (11, 12). Applying gene expression profiling, *SIM2* ranked second among highly up-regulated genes in prostate cancer, as recently reported by our group (13). Contrary to a previous report (11), we now report a basal expression of *SIM2* transcripts in benign prostate tissue, as well as coexpression and increase of both *SIM2* isoforms in prostate cancer. Current evidence points to the *SIM2-s* isoform as a therapeutic target (11), and on this basis, we examined SIM2-s at the protein level by immunohistochemistry. A highly significant relationship between SIM2-s protein expression and adverse outcome was established in an independent series of

Authors' Affiliations: Sections for [1]Pathology and [2]Microbiology and Immunology, The Gade Institute and [3]Department of Surgery, Haukeland University Hospital; Sections for [4]Microbiology and Immunology and [5]Pathology, The Gade Institute; Departments of [6]Informatics and [7]Surgical Sciences; and [8]Computational Biology Unit, Bergen Center for Computational Science, University of Bergen, Bergen, Norway; and [9]Institute for Systems Biology, Seattle, Washington

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Requests for reprints: Lars A. Akslen, Department of Pathology, The Gade Institute, Haukeland University Hospital, N-5021 Bergen, Norway. Phone: 47-55-97-31-82/47-55-97-31-73 (secr.); Fax: 47-55-97-31-58; E-mail: Lars.Akslen@gades.uib.no.

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prostate cancer patients with long follow-up, and this is the first report describing SIM2 expression as a marker of disease progress in human cancer. Our findings are of potential practical importance because the *SIM2-s* isoform was recently proposed as a molecular target for antisense cancer therapy (11).

Materials and Methods

Patient series and tissues. During the period of 1997 to 2003, fresh prostate tissues were collected from consecutive patients treated by radical prostatectomy for clinically localized and biopsy verified prostate cancer at the Department of Surgery, Section of Urology, Haukeland University Hospital, Bergen, Norway. Fresh tissue samples were immediately frozen in liquid nitrogen and stored for later use at −80°C. The percentage of tumor tissue was determined in the area from where fresh tissue was also collected. Samples were finally included in this series after histopathologic confirmation of benign or tumor tissue, evaluation of tumor content (>50%; mean, 76% in our cases), and availability of high-quality RNA. For cDNA microarray studies, 52 tissue samples from 33 patients (median age, 60 years; range, 47-69) were used, including 29 histologically verified primary tumors and 19 paired benign and malignant samples (13). The mean Gleason score for these 29 carcinomas was 6.2 (median, 6; range, 5-8); the median preoperative serum prostate-specific antigen (PSA) was 5.9 ng/mL; and 22 patients had clinical stage T_{1c}, and 6 patients had T_2 (missing information on one patient). After histopathologic examination, 21 cases were classified as stage pT_2, 8 cases were pT_3, and 15 cases had positive surgical margins. For validation purposes (quantitative PCR), this series of 29 carcinomas was expanded to a total of 37 malignant tumors and 39 benign samples, including 27 tumor/benign pairs. Finally, validating SIM2-s protein expression, a consecutive series of 104 men (median age, 62.0 years) treated by radical prostatectomy for clinically localized prostate cancer during 1988 to 1994 with long and complete follow-up was included (14). One case that did not contain tumor tissue on the tissue microarrays when stained for SIM2-s was omitted. The following variables were recorded: patient age, clinical stage (tumor-node-metastasis category), largest tumor diameter, WHO histologic grade, capsular penetration, seminal vesicle invasion, involvement of surgical margins, pathologic stage (pathologic tumor-node-metastasis), presence of lymph node metastasis, and serum PSA before and after surgical treatment. Time from surgery until biochemical failure, defined as serum PSA elevation ≥0.5 ng/mL in two consecutive blood samples, was recorded, as was time to clinical locoregional recurrence, skeletal metastases, and cancer-specific survival. The last date of follow-up was December 31, 2001. Skeletal metastases were present in 15 patients, and nine patients died as a result of prostate cancer. No patients were lost to follow-up. None of the patients examined by cDNA microarray or quantitative PCR was part of this series (n = 103), which was a strictly independent cohort. The study was approved by The Data Inspectorate of Norway and The Regional Committee for Medical Research.

RNA purification, enzymatic modification, and fluorochrome labeling. Individual biopsies were ground to powder under liquid N_2. Total RNA was extracted according to standard protocols (ref. 15; Invitrogen Trizol LS protocol and Qiagen RNeasy mini-kit protocol; Invitrogen, Carson City, CA). T7 RNA polymerase promoter-containing double-stranded cDNA and T7 RNA polymerase-amplified RNA (cRNA) were generated as previously described (16) and according to the Ambion T7 Megakit protocol. Aminoallyl-U (aminoallyl-UTP from Ambion, Austin, TX) incorporation into cRNA followed by cross-coupling Cy5 and Cy3 by means of reactive Cy-NHS compounds (Amersham, Piscataway, NJ) was used for fluorochrome labeling of nucleic acids. Cy5 and Cy3 incorporations were measured by absorption readings at 649 and 550 nmol/L, respectively, using a PowerWave Spectrophotometer. Simultaneous recording of absorptions at 260 nmol/L allowed calculation of specific labeling.

DNA microarray analysis. The Research Genetics human 40K cDNA microarray printed at the Institute for Systems Biology in Seattle has been described previously (13, 17). The Agilent human 1A oligonucleotide microarray (21K) was used for validation purposes according to the Agilent protocols except for a more stringent wash (0.1× SSC at 25°C for 10 min). The oligonucleotide microarrays were scanned, and features were automatically extracted, recorded, and analyzed using the Agilent Microarray Scanner Bundle. Normalization, flooring, or filtration of data was done as described (13, 17).

Real-time quantitative PCR. Synthesis of hexamer-primed cDNA was done according to the MMLV reverse transcriptase kit instructions (Ambion). cDNA corresponding to 5 ng total RNA was used in each PCR reaction. Real-time quantitative PCR was done in a 96-well format in the ABIPrism 7900HT thermocycler according to User Bulletin #2 and SDS2.2 program manuals (Applied Biosystems, Foster City, CA). The SIM2 isoforms have the first nine exons and the first part of exon 10 in common, but the long isoform is spliced to an 11th exon on chromosome 21q22 (Supplementary Fig. S1). The primers used were *SIM2-s*, *5′-GGTGGGTGGCAGATGGA-3′* (sense) and *5′-GCAGAAAGAGGGCAAGTTTGC-3′* (antisense); SIM2-l, *5′-CAGCCTGGTGCCAAGCTA-3′* (sense) and *5′-GTGTCCTCGCCGAACCT- 3′* (antisense); and *β-actin*, *5′-CCCAGCACAATGAAGATCAAGATCA-3′* (sense) and *5′-GCGAGGCCAGGATGGA-3′* (antisense). Supplementary Table S1 shows the Genbank accession numbers and Taqman custom-made assays for the specific detection of the short and long isoforms of *SIM2*, and the β-actin Taqman assay was used as endogenous control. The Applied Biosystems assay Hs_00231927_m1 (Applied Biosystems) common to both *SIM2* isoforms (across exons 3 and 4 boundary of NM_009586) was also used.

Real-time PCR in the low-density array format. Taqman low-density arrays are customizable, 384-well microfluidic cards for real-time PCR (Applied Biosystems). Each low-density array card was configured for 96 different genes in duplicates, including Hs_00231925 m1. Hs_00231925 m1 targets the boundary of exons 10 and 11 of Genbank accession nos. NM_005069 and U80456 and is thus specific for the *SIM2-l* isoform. Hexamer-primed single-stranded cDNA corresponding to 5 ng of prostate total RNA was diluted in Taqman Universal buffer (Applied Biosystems) and added to each loading well. Using the above configuration, each sample occupied four wells or one half of each card. The samples were distributed to the microwells by centrifugation for 1 min at 343 × g. The cards were sealed and placed in an ABIPRISM7900HT thermocycler for 40 cycles of 95°C to 60°C. The SDS2.2 software was used for qualitative analysis.

RNA synthesis and* in situ *hybridization. *SIM2* (accession no. NM005069) antisense (nucleotides 1395-541) and sense (nucleotides 541-1395) and *β-actin* (accession no. NM001101) antisense (nucleotides 1137-356) DIG-cRNA probes were made by a PCR-based approach. Initially, RNA was isolated from a prostate tumor sample with known elevated *SIM2* mRNA expression, and single-stranded cDNA was transcribed using gene-specific primers (*SIM2*, *5′-TTCGAATGAAATGTGTCT-3′*; *β-actin*, *5′-ATCGTCACCAACTGGGAC-3′*) and Superscript III Reverse Transcriptase as described by the manufacturer (Invitrogen). Next, cDNA was PCR amplified using the following primers: *SIM2*, *5′-TTCGAATGAAATGTGTCT-3′* and *5′-AGTGTCCGTAATGGTAGG-3′*; β-actin, *5′-ATCGTCACCAACTGGGAC-3′* and *5′-ATCTGCTGGAAGGTGGAC-3′*. Ampli-Taq Gold PCR Master Mix (Applied Biosystems) was used with 0.15 μmol/L of each primer. Samples were subjected to an initial denaturation at 95°C for 15 min and 50 cycles (95°C for 30 s, 50°C for 1 min, 72°C for 2 min), with a final extension at 72°C for 7 min. Additionally, one primer in the primer pair was designed with T7 promoter sequence (*5′-TAATACGACTCACTATAGG-3′*) in the 5′-end. The promoter sequence was hence incorporated into the PCR product. All PCR fragments were verified by sequencing. DIG-cRNA synthesis was done using 1 μg DNA and 1.9 mmol/L ATP/CTP/GTP, 1.3 mmol/L UTP, and 0.7 mmol/L DIG-UTP according to the MEGA script High Yield Transcription kit T7 manual instructions

(Ambion). DIG-cRNA was fragmented to 60- to 200-long nucleotides by Fragmentation Reagents from Ambion.

Slides of paraffin-embedded tumor tissue were deparaffinized in xylene twice for 5 min, rehydrated in 100% ethanol twice for 5 min, and incubated in DEPC water for 5 min. The slides were then boiled in 10 mmol/L citrate buffer (pH 6) for 30 min, treated with 0.2 mol/L HCl for 15 min, and 0.25% acetic anhydride in TEA-HCl for another 15 min. Fragmented DIG-cRNA probes were diluted to 100 ng/μL in hybridization solution (Sigma, St. Louis, MO) and incubated at 42°C overnight. Post-hybridization wash was done in 2× SSC at 52°C for 10 min, twice. Slides were RNase treated using 10 μg/mL RNase A at 37°C for 10 min. Re-fixation of the slides was done in 1:1 formamide/2× SSC at 42°C for 10 min followed by brief washing in 1× SSC and 0.5× SSC, respectively. The slides were blocked with 2% horse serum for 30 min at room temperature, before incubation with anti-DIG-AP (Roche, Basel, Switzerland) 1:250 for 1 h at room temperature. Staining was done by Liquid Permanent Red Chromogen (DakoCytomation, Copenhagen, Denmark) for 5 to 20 min. Hematoxylin was used as counter stain.

Tissue microarray and immunohistochemistry. After formalin fixation, radical prostatectomy specimens were totally embedded and studied by whole mount step sections. For immunohistochemistry, the area of highest tumor grade was selected for tissue microarray construction, using three parallel cores (0.6-mm diameter) from each case (14, 18). Tissue microarray slides were subjected to microwave epitope retrieval for 20 min in Tris-EDTA buffer at pH 9 and incubated for 60 min with an anti-SIM2-s antibody, SC-8715 (C15; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature, diluted 1:800 using DAKO Autostainer and the EnVision chain-polymer method (DakoCytomation). The anti-SIM2-s antibody is raised against a peptide mapping at the COOH-terminal end that differs from SIM2-l. Negative controls were treated with an excess of blocking peptide SC-8715 P (Santa Cruz Biotechnology). A staining index (range, 0-9) was obtained as a product of nuclear or cytoplasmic staining intensity (range, 0-3) and proportion of immunopositive tumor cells (≤10%, 1; 10-50%, 2; >50%, 3). Cutoff points were defined by median, tertiles, or quartiles. Tumor cell proliferation by Ki-67 expression was estimated in "hotspot" areas on regular slides as earlier presented (19), and the results were included in the present study for comparison. Data regarding expression of p16 and p27 were included from previous studies (14, 20, 21).

Statistical analysis. Associations between different variables were assessed by Pearson's χ^2 test or the Mann-Whitney or Kruskal-Wallis tests, when appropriate (SPSS 11.5, SPSS, Inc., Chicago, IL). Correlations between continuous variables not categorized by quartiles or tertiles were assessed by Spearman rank correlation. Sensitivity and specificity were calculated by ROC analysis. Survival analysis was done by the product-limit method (Kaplan-Meier) and log-rank test. Cox' regression models were applied for multivariate survival analysis (likelihood ratio test), including only significant variables ($P < 0.05$) from univariate analyses.

Results

Differential gene expression of malignant and benign prostate samples and validation by oligonucleotide arrays. We previously reported that the *SIM2* gene was up-regulated in 29 malignant prostate tumors using cDNA microarrays (13). In the present study, *SIM2* expression was associated with high Gleason score ≥7 (Fisher's exact test comparing two groups by the upper tertile, $P = 0.01$, $n = 29$; Supplementary Table S2). There were no consistent associations between *SIM2* expression and other clinicopathologic data. The increased expression of *SIM2* was now confirmed in a subset of cases ($n = 7$) and correlated significantly between cDNA and oligonucleotide microarrays (Spearman rho = 0.79, $P = 0.036$).

Real-time quantitative PCR of SIM2 transcripts. Both *SIM2-s* and *SIM2-l* isoforms were detected in benign ($n = 39$) and malignant ($n = 37$) prostate tissues at the mRNA level. *SIM2-total*, *SIM2-s*, and *SIM2-l* isoforms were all significantly elevated in malignant versus benign samples by 3.8-, 3.9-, and 6.7-fold, respectively ($P < 0.001$ for all, Mann-Whitney test; Fig. 1). The median (range) expression in benign versus tumor tissues was 2.2 (0.1-13.2) versus 8.2 (1.1-55.0), 1.3 (0.1-10.9) versus 5.2 (0.4-65.7), and 3.6 (0.1-80) versus 24.0 (1.36-1085.2) for *SIM2-total*, *SIM2-s*, and *SIM2-l*, respectively. The expression level of *SIM2-s* and *SIM2-l* isoforms correlated significantly in both benign and malignant prostate tissues, as did both isoforms with *SIM2-total* across all (i.e., tumor and benign) samples ($P < 0.001$ for all; Supplementary Table S3). *SIM2* expression by cDNA microarray correlated to *SIM2-s*, *SIM2-l*, and *SIM2-total* mRNA by real-time quantitative PCR across all samples, reaching significance for the subset of benign tissues only (Supplementary Table S4).

There were no consistent associations between the expression of *SIM2* isoforms and clinicopathologic features of prostate cancer in the subset of 37 cases, except a tendency for high expression of *SIM2-s* to be associated with increased preoperative serum PSA ($P = 0.054$, Mann-Whitney test).

Increased expression of the *SIM2-l* transcript has not been previously reported in prostate cancer, and the expression of *SIM2-l* mRNA by real-time quantitative PCR was therefore confirmed by low-density array in a subset of samples ($n = 10$). The *SIM2-l* expression ratios were significantly correlated between the two formats (Spearman rho = 0.95, $P < 0.001$).

Optimal cutoff values (based on highest sum of sensitivity and specificity) for classifying samples as malignant were determined by ROC analysis and found to be 2.0 for *SIM2-s*, 8.0 for *SIM2-l*, and 3.2 for *SIM2-total*. The sensitivity and specificity for classifying samples as malignant were 0.95 and 0.62 for *SIM2-s*, 0.86 and 0.72 for *SIM2-l*, and 0.89 and 0.69 for *SIM2-total*, respectively (Supplementary Fig. S2).

In situ *hybridization.* Cases expressing *SIM2-s* at high levels by quantitative PCR were selected for *SIM2* mRNA *in situ* hybridization. Positive expression was confirmed in malignant

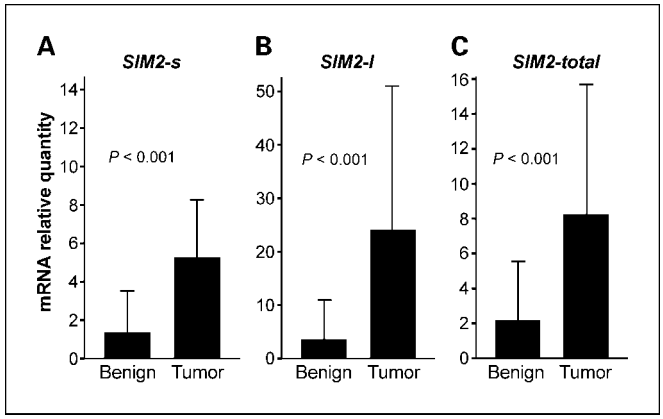

Fig. 1. Relative mRNA quantity of *SIM2* isoforms by real-time quantitative PCR versus *β-actin* used as endogenous control. Comparisons between 37 tumor and 39 benign prostate samples for *SIM2-s* and *SIM2-l* isoforms and common primer *SIM2-total*. Columns, median; bars, 75 percentiles. *A, SIM2-s. B, SIM2-l. C, SIM2-total. SIM2-s* and *SIM2-l* isoforms and common primer *SIM2-total* were significantly elevated in malignant versus benign prostate tissues ($P < 0.001$, Mann-Whitney test).

prostate glands and prostatic intraepithelial neoplasia compared with benign glands (Fig. 2). In benign glands, luminal cells were negative, whereas basal cells often showed some *SIM2* expression (Fig. 2).

SIM2-s *protein expression by immunohistochemistry.* The SIM2-s nuclear staining index ranged from 0 to 9 (median staining index = 2.0) in prostate cancer, with variable cytoplasmic SIM2-s expression. A moderate/strong (defined as positive) nuclear staining of SIM2-s protein (staining index = 3-9) was noted in 44 of the 103 paraffin-embedded prostate carcinomas, as opposed to a weak or negative (defined as negative) staining (staining index = 0-2) in the remaining cases (Fig. 2). Positive nuclear SIM2-s expression was significantly associated with adverse prognostic variables like preoperative serum PSA >11.15 (median; $P = 0.004$), high histologic grade (WHO; $P = 0.043$), extra-prostatic extension ($P = 0.044$), and increased tumor cell proliferation by Ki-67 expression ($P = 0.013$; Table 1). In addition, increased expression of SIM2-s was associated with reduced expression of p27 protein ($P = 0.038$, Mann-Whitney test).

SIM2-s *expression and survival.* In a univariate survival analysis of 103 prostate cancer patients, positive SIM2-s expression (cut off by median staining index) was significantly associated with reduced prostate cancer–specific survival (Fig. 3). The estimated 10-year (13-year) survival was 98.1% (98.1%) versus 79.5% (72.8%) in patients with negative versus positive SIM2-s expression, respectively ($P = 0.008$, log-rank test). Additionally, a trend for positive SIM2-s expression to be associated with time to skeletal metastasis ($P = 0.084$) was found. Among preoperative serum PSA, histologic grade (WHO), pathologic stage, and SIM2-s expression, only histologic grade ($P = 0.018$) and SIM2-s expression ($P = 0.008$) were significantly associated with survival in univariate analysis. Although only one single event was recorded among low SIM2-s expressors, a multivariate survival model, including WHO histologic grade and SIM2-s expression, revealed that only SIM2-s remained in the model as a significant independent predictor of reduced cancer-specific survival ($P = 0.028$; hazard ratio, 6.9; 95% confidence interval, 0.8-57.7), whereas histologic grade was of borderline significance only ($P = 0.059$; hazard ratio, 3.7; 95% confidence interval, 0.9-15.1).

In our independent series of 37 cases of prospectively collected prostate cancers analyzed by real-time quantitative PCR, five biochemical failures were recorded (median follow-up, 2.3 years), and a trend for cases with high levels of *SIM2-s* mRNA to be associated with early recurrences was noted ($P = 0.084$, log-rank test), as was a similar tendency for high *SIM2-l* ($P = 0.18$, log-rank test).

Discussion

Although the *SIM2* gene, which is located within the Down's syndrome critical region, is known to be important for brain development and neuronal differentiation, studies have indicated that it might also be involved in the pathogenesis of solid tumors (10–12). We recently reported *SIM2* as the second most consistently up-regulated gene in human prostate cancer (13). In our extended series, the short and long isoforms of *SIM2* were significantly coexpressed and elevated in prostate tumors compared with benign tissue. The *SIM2-l* isoform has not been previously identified in prostate cancer.

We here show for the first time that SIM2-s protein expression might be important for the clinical progress of human cancer, as shown for prostate tumors by multivariate survival analysis. Our findings may be of practical importance because *SIM2-s* was recently suggested as a candidate for targeted therapy. Thus, inhibition of tumor growth by antisense blocking of the *SIM2-s* isoform was shown in colon cancer xenografts in mice (11). Whether this effect is due to an influence on cell cycle regulation is not known, but high SIM2-s expression was significantly associated with increased tumor cell proliferation as indicated by our data. Alternatively, a stimulatory effect of *SIM2-s*

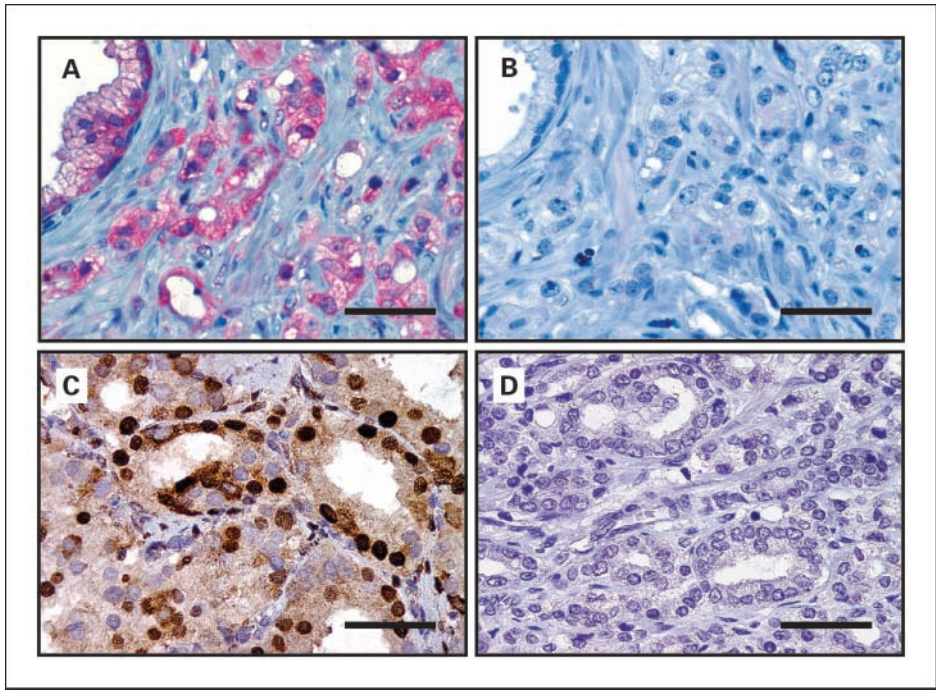

Fig. 2. *SIM2* mRNA *in situ* hybridization. *A, SIM2* antisense, staining in carcinomatous prostate glands, and negative staining of luminal cells in a benign gland (*top left*). Note some positive staining of basal cells in benign gland. *B, SIM2* sense, negative staining. SIM2-s protein expression by immunohistochemistry in 103 prostate carcinomas. *C,* positive nuclear staining in 44 of 103 carcinomas. *D,* negative nuclear staining in 59 of 103 carcinomas. Bar, 50 μm.

Table 1. SIM2-s protein expression by immunohistochemistry and clinicopathologic variables in prostate cancer

		SIM-s		
	No.	Negative*	Positive*	P†
Clinical stage				
T_{1b}	9	5	4	NS
T_{1c}	3	3	0	
T_{2a}	57	33	24	
T_{2b}	25	12	13	
T_{2c}	9	6	3	
Tumor dimension‡ (mm)				
≤28	50	32	18	NS
>28	52	26	26	
Histologic grade (WHO)				
Well	10	8	2	0.04
Moderate	65	40	25	
Poor	28	11	17	
Extra-prostatic extension				
Absent	32	23	9	0.04
Present	71	36	35	
Seminal vesicle invasion				
Absent	69	41	28	NS
Present	34	18	16	
Surgical margins				
Negative	49	30	19	NS
Positive	54	29	25	
Lymph nodes				
Negative	96	56	40	NS
Positive	7	3	4	
Serum PSA§ (ng/mL)				
≤11.15	49	35	14	<0.01
>11.15	49	21	28	
Ki-67 index‖ (%)				
≤4.8	24	19	5	0.01
>4.8	79	40	39	

*Cut point: median staining index.
†Pearson's χ^2.
‡Cut point: median tumor diameter; tumor diameter available in 102 cases.
§Cut point: median preoperative serum PSA; preoperative serum PSA available in 98 cases.
‖Tumor cell proliferation by Ki-67 expression; cut point: lower quartile.

antisense on tumor cell apoptotic regulation has been suggested (22). When looking at transcription factor binding sites by modules of the Genomatix program,[10] an AHRR site (a potential binding site for PER, ARNT, SIM1, and SIM2) was predicted in the promoter regions of *p27* and *p16* and could represent a putative link between *SIM2* and proliferation (data not shown). Notably, an association between increased SIM2-s protein and reduced p27 expression was found in our study, indicating a possible repressive effect of *SIM2*. However, the significance of *SIM2* in cell cycle regulation presently remains unknown.

The specific role of *SIM2* in prostate cancer compared with other *SIM2*-overexpressing cancers like colon and pancreatic tumors has not been extensively explored. Differences in the expression of *SIM2-s* and *SIM2-l* isoforms have been reported for various normal tissues (9), and the two *SIM2* isoforms may thus differ in a possible oncogenic or tumor-suppressive activity in a tissue-specific manner. In addition, SIM2-s and SIM2-l have different effects on central midline response elements and gene expression under control of hypoxia and dioxin (9), although the functions of the SIM2 proteins remain unknown in humans. Given the significance of androgen responsiveness of prostate cancer cells, a preliminary transcription factor binding site analysis did not reveal androgen-responsive elements within the *SIM2* promoter (data not shown). Conversely, no *SIM2* binding site was found within the androgen receptor promoter.

In our study, increased expression of SIM2-s protein was found to be associated with adverse prognostic factors, such as increased preoperative serum PSA, high histologic grade, increased tumor cell proliferation, and invasive growth with extra-prostatic extension, indicating an association with a subset of aggressive tumors. Supporting this, high SIM2-s expression was associated with significantly reduced cancer-specific survival as indicated by multivariate analysis. All prostate cancer–related deaths, with one exception, belonged to the SIM2-s–positive group.

Both isoforms of *SIM2* were expressed at low levels in benign prostate tissue. In a previous report, the *SIM2-s* and *SIM2-l* isoforms were not seen in normal colon, pancreas, or prostate, and *SIM2-l* was not detected in tumor tissues (11). Later, both isoforms have been detected in a pancreatic cancer cell line but not in normal tissues (12). We presently found that *SIM2-s* was paralleled by the expression of *SIM2-l* transcript in both benign and malignant prostate samples. Especially, the ratio between expression levels in malignant tumors compared with benign tissues was even higher for *SIM2-l* than for the *SIM2-s* isoform. The contrast between our findings and earlier reports may be explained by differences in methodologies such as the primer sequences applied, increased sensitivity of the assay, and differences in RNA quality. Our findings indicate that *SIM2-l* expression might also be significant for prostate cancer

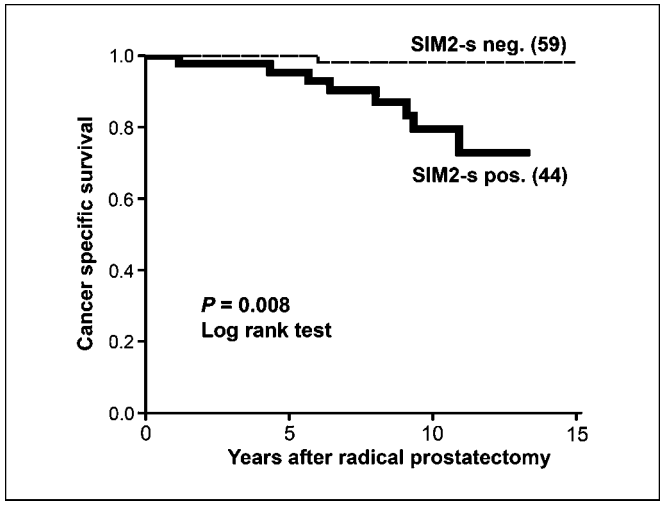

Fig. 3. SIM2-s protein expression by immunohistochemistry. Kaplan-Meier survival analysis of time to death from prostate cancer in 103 patients treated by radical prostatectomy.

[10] http://www.genomatix.de

development and should be further validated for antisense strategies.

In conclusion, the two different isoforms *SIM2-s* and *SIM2-l* were both found in benign prostate tissue, and they were significantly coexpressed and elevated in prostate cancers. SIM2-s protein expression was found to be associated with adverse prognostic variables such as increased preoperative serum PSA and high histologic grade, extra-prostatic tumor extension, increased tumor cell proliferation by Ki-67 expression, and reduced patient survival as shown by multivariate analysis. These novel findings support the proposal of *SIM2* as a candidate for targeted therapy of prostate cancer.

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