

A predictive role for noncancerous prostate cells: low connexin-26 expression in radical prostatectomy tissues predicts metastasis

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BACKGROUND: It is important to identify markers that predict whether prostate cancer will metastasise. The adjacent noncancerous cells (influenced by the tumour cells) may also express potential markers. The objective of this study was to determine the influence of cancer cells on noncancerous cells and to assess the value of the cell-communication protein connexin-26 (Cx26) as a marker to predict the development of metastasis.

METHODS: The effect of conditioned medium (CM) from PrCa cells on *in vitro* noncancerous cell proliferation, migration and invasion and Cx26 expression was determined. Connexin-26 expression was investigated in prostatectomy tissues from 51 PrCa patients by immunohistochemistry and compared with various clinicopathological parameters.

RESULTS: Proliferation, migration and invasion of noncancerous cells were influenced by CM from the PrCa cell lines. Importantly, a clear relation was found between low Cx26 expression in the noncancerous tissue in prostatectomy sections and the risk of development of metastasis ($P < 0.0002$). Kaplan–Meier analysis showed a relation between low Cx26 expression in noncancerous tissues and time to biochemical recurrence ($P = 0.0002$).

CONCLUSION: Measuring Cx26 expression in the adjacent noncancerous tissues (rather than cancer tissues) of prostatectomy sections could help to identify high-risk patients who may benefit from adjuvant therapy to decrease the risk of metastasis.

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Prostate cancer is the most common cancer diagnosed in males and the second leading cause of cancer-related death (Jemal *et al*, 2010). Many cases present with a slow growing, clinically unapparent form of prostate cancer. Others present with a fast growing, aggressive tumour that quickly metastasises. The cause of this variability is largely unknown. When prostate cancer has metastasised, no curative therapy is available. Hence, it is very important to find predictive factors that can identify the more aggressive forms of prostate cancer. Mostly, prostate cancer biomarkers are studied in prostate cancer tissues. However, the morphological normal (noncancerous) prostate cells may also be influenced by the tumour, as tumour and tumour-infiltrated cells (e.g., endothelial cells, fibroblasts and immune cells) secrete many factors that may change the behaviour of these noncancerous cells (Hanahan and Weinberg, 2000; Cunha *et al*, 2002; 2003). Thereby, the expression of genes and proteins may be changed in these noncancerous tissues of the prostate (Jia *et al*, 2011; Trevino *et al*, 2011). This aspect has not been studied thoroughly and may provide new perspectives in the biomarker research.

For tumour growth, cell-to-cell communications are very important. Gap junction connexions (Cxs) are structures that are involved in cell–cell communications (Cronier *et al*, 2009, Naus and Laird, 2010). The gap junction is a specialised cell membrane channel that interconnects the cytoplasm of adjacent cells. Gap junction intercellular communication is involved in the growth and differentiation of cells and it has a role in maintaining homeostasis of tissues (Krutovskikh *et al*, 2002). Gap junctions are described to be involved in cancer progression when down-regulated in cancer cells (Krutovskikh *et al*, 1994; Kamibayashi *et al*, 1995; Tsai *et al*, 1996; Jinn *et al*, 1998; Czyż *et al*, 2012). Connexions are gap junction proteins that allow the transport of factors between cells, supporting synchronised post-translational modifications such as phosphorylation cascades (intracellular signalling). Gap junctions that are composed of Cxs have an essential role in intercellular communication, adhesion, cell proliferation and cell differentiation. Dysregulation of these Cxs has been related to tumour progression (Kamibayashi *et al*, 1995; Mehta *et al*, 1999; Habermann *et al*, 2002; Li *et al*, 2008). It is possible that the intercellular exchange of molecules involved in the growth and death of cells may occur through these gap junctions (Trosko and Chang, 2001).

Connexin-26 (Cx26) is a protein that is involved in gap junctions (Cronier *et al*, 2009). Connexin-26 has been previously described to have a role in the progression of various cancers, including

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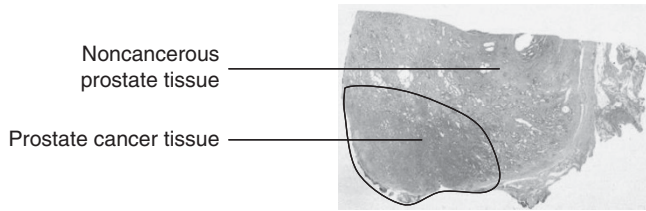


Figure 1 Haematoxylin- and eosin-stained tissue section derived from a radical prostatectomy specimen is shown, and both the tumour part and the noncancerous cells present in the rest of the specimen are indicated.

melanoma and colorectal cancer (Ezumi *et al*, 2008; Haass *et al*, 2010). However, a relation between Cx26 expression of the adjacent noncancerous tissues and progression has not been described. In this study, we determined the effect of prostate cancer cells on noncancerous prostate cell proliferation, migration and invasion, and the expression of Cx26. In addition, we assessed the expression levels of Cx26 in prostate tumour and in adjacent noncancerous tissues present in radical prostatectomy specimens (Figure 1). Connexin-26 expression in this material was characterised in relation to metastasis of the prostate cancer. We found a relation between the expression of Cx26 in the adjacent noncancerous tissues and the development of metastasis, which was detected months to years after radical prostatectomy.

MATERIALS AND METHODS

Cell lines and preparation of conditioned medium

The noncancerous prostate epithelial cells (PrECs) and prostate stromal cells (prSCs) were obtained from Lonza (Basel, Switzerland) and were cultured in EGM and SCGM bullet kit media (Lonza), respectively. LNCaP and PC3 cells (both derived from metastasis) were obtained from the American Type of Culture Collection (Rockville, MD, USA) and were cultured as monolayers in RPMI supplemented with 10% FCS. Cells were maintained in a humidified 5% CO₂ atmosphere at 37°C. The conditioned medium (CM) from the cancer cells was prepared as described previously (Bijnsdorp *et al*, 2011). In brief, cancer cells were seeded ($2\text{--}2.5 \times 10^6$ cells per T75 culture flask). After 24 h, the medium was replaced by exactly 10 ml serum-free RPMI. After 2 days, the medium was collected and filtered through a 0.22- μm filter. Subsequently, the medium was concentrated $20 \times$ using Amicon Ultra 15 Centrifugal filters (Millipore, Billerica, MA, USA). For each experiment, the concentrated medium was diluted in cell line-specific medium. The CM is referred as CM-L (LNCaP) and CM-P (PC3). As controls, concentrated medium from RPMI was used (CM-R).

Proliferation assay

Prostate epithelial cell and prSC proliferation was determined by the sulforhodamine B (SRB) assay, as described previously (Bijnsdorp *et al*, 2010). In brief, 7000 cells per well were seeded in 96-well plates (Greiner Bio One, Frickenhausen, Germany). After 24 h, cells were exposed to the CM of the cancer cells. Every day the medium was refreshed with CM. After 72 h of stimulation, cells were precipitated with trichloroacetic acid for 1 h at 4°C, stained with SRB, solubilised with Tris and the optical density was measured at 540 nm. Unstimulated controls were set to 100% cell proliferation.

Transwell assay

The transwell migration and invasion assay were carried out using transwell chambers with a fluorescence blocking 8- μm pore filter insert (no. 35-1152; HTS Fluoroblock Insert, Falcon, Becton

Dickinson Labware, Bedford, MA, USA; Bijnsdorp *et al*, 2011). For the invasion assay, the insert was coated overnight at room temperature (RT) with 100 μl matrigel (50 ng ml^{-1} in PBS; Sigma Aldrich Chemicals, Zwijndrecht, The Netherlands). For the migration assay, the insert was left uncoated. Cells (150 000 per insert) were seeded in serum-free medium. In the lower compartment, the CM was diluted in appropriate cell growth medium. Cells were allowed to migrate/invade for 8 h. About 5 μM calcein-AM was added 30 min before analysis to the lower compartment and fluorescently labelled cells were counted using a fluorescence microscope.

Fluorescent staining

Fluorescent staining was performed as described previously (Bijnsdorp *et al*, 2010). In brief, cells were grown to 90–100% confluency, a confluency at which the cells were able to form detectable intercellular channels, and were subsequently exposed to the CM for 6 h, after which cells were fixed, permeabilised with 0.2% Triton X-100 in PBS, blocked with 3% BSA (RT) and stained for Cx26 (1:200; Invitrogen, Camarillo, CA, USA) overnight at 4°C. The secondary antibody (α -mouse-FITC; 1:200; Dako, Glostrup, Denmark) was added together with Hoechst 33342 (Sigma Aldrich Chemicals) (1:2000) for 1 h at RT. Fluorescence microscopy analysis was carried out using an inverted Leica (Leica Cambridge Ltd, Cambridge, UK) DMIRB/E fluorescence microscope.

Western blotting

Western blotting was performed as described previously (Bijnsdorp *et al*, 2010). In brief, cells were exposed to the CM for 6 h and subsequently lysed in lysis buffer (Cell Signalling Technology Inc., Danvers, MA, USA). From each condition, 30 μg of protein was separated on a 10% SDS-PAGE and electroblotted onto polyvinylidenedifluoride (PVDF) membranes (Millipore, Billerica, MA, USA Immobilon-FL PVDF). Subsequently, the membranes were blocked for 1 h at RT in blocking buffer and incubated overnight at 4°C with the primary antibody (Anti-Cx26, (clone CX-1E8, 1:1000; Invitrogen) and subsequently incubated with the secondary antibody (1:10 000 goat- α -mouse-IRDye or goat- α -rabbit-IRDye (Westburg, Leusden, The Netherlands)) for 1 h at RT. The bands were scanned using the Odyssey Infrared Imager (Westburg).

Patients and tissues

Fifty-one patients with clinically localised adenocarcinoma of the prostate were selected for this study. Selection criteria were follow-up time and tumour present in the tissue blocks. Radical prostatectomies were performed between 1994 and 2011 at the Department of Urology, VU University Medical Center in Amsterdam, The Netherlands. Of these patients, the preoperative PSA values, clinical and pathological classification and Gleason scores were available. Postoperatively, the patients were scheduled for regular follow-up visit at the institutional outpatient clinic (5–178 months). Biochemical progression was defined as rising postoperative PSA levels by $>0.2 \text{ ng ml}^{-1}$, confirmed in a consecutive visit. Clinical progression was defined as detection of metastasis, which was detected 1–44 months after surgery.

Immunohistochemistry

Immunohistochemistry was performed on 3- μm thick paraffin-embedded sections of radical prostatectomy specimens. Slides were deparaffinised by steps of xylene and subsequently rehydrated by decreasing concentrations of ethanol. Endogenous peroxidase expression was blocked by incubation with 0.3% H₂O₂ in methanol for 30 min. Slides were heated for antigen retrieval in

1 mM Tris/EDTA buffer (pH 9.0). Slides were blocked with 2% normal goat serum (Dako) for 10 min at RT and exposed for 1 h (RT) to the primary antibody against anti-Cx26 (1:100; Invitrogen). After incubation, sections were washed and incubated with post-antibody blocking solution (Immunologic, Duiven, The Netherlands) for 15 min (RT). Subsequently, slides were washed and incubated with Brightvision-plus (Immunologic) for 60 min at RT. Peroxide was revealed by AEC solution (Invitrogen). Slides were counterstained with haematoxylin and were covered in Kaisers glycerol (Merck KGaA, Darmstadt, Germany).

Evaluation of IHC

Blinded for the clinical parameters, including age, PSA values, Gleason, progression and metastasis development, immunohistochemically stained slides were reviewed independently by an experienced pathologist and an experienced researcher. Connexin-26 staining of the whole-tissue section was scored for percentage of glands positive and staining intensity (0–3) for both cancer and noncancerous tissues (Figure 1). These values were multiplied, providing scores from 0 to 300. Statistical analysis was performed by χ^2 -test for the significance between the patient groups.

RESULTS

In vitro behaviour of noncancerous prostate cells

To evaluate the effect of prostate cancer cells on noncancerous prostate cells, CM from LNCaP and PC3 cells was collected and concentrated. This medium was subsequently used to stimulate the noncancerous prEC and prSCs. The effect of the CM-L and CM-P on proliferation, migration and invasion of prECs and prSCs was determined by the SRB assay (proliferation) and the transwell assay (migration/invasion). Interestingly, prECs and prSCs responded differently to the CM. Prostate stromal cell proliferation was increased by CM-P, whereas prEC cell proliferation decreased by both CM-L and CM-P (Figure 2A). The migration and invasion of prEC cells were highly increased, while not significantly affected in prSCs (Figures 2B and C).

As cell communication is important in the behaviour of cells, we determined the expression level of the communication protein, Cx26 by western blotting. The expression of Cx26 was decreased in both prECs and prSCs after incubation with CM-L and CM-P (Figure 3A). In addition, we evaluated Cx26 expression by immunofluorescent staining. The prECs and prSCs were grown to about 90% confluency and subsequently exposed to CM-L or CM-P. We observed channels to a high extent in untreated controls, while still detectable but to a very low extent after exposure to both the CM-L and CM-P. Taken together, the decreased Cx26 expression and decreased formation of

intercellular channels indicate a disturbed intercellular communication (Figure 3B).

Connexin-26 expression in patient tissues and clinical outcome

To study whether the expression of Cx26 was decreased in the adjacent noncancerous region of prostate cancer, we evaluated its expression in prostate cancer tissues of 51 patients. In Table 1, the patients characteristics are summarised. The mean and median age at time of radical prostatectomy were 64 years. Gleason scores ranged from 5 to 10. During the follow-up, 35% developed metastasis and 47% showed biochemical recurrence.

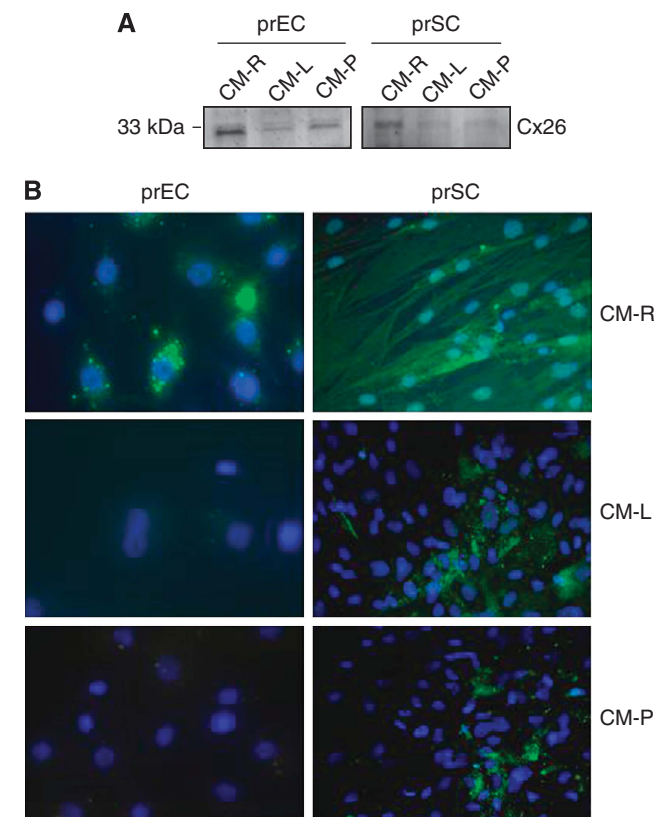


Figure 3 (A) Western blot of Cx26 expression in prECs and prSCs after 6-h stimulation with either CM-L or CM-P. (B) Immunofluorescent staining of Cx26 in prECs and prSCs after 6-h stimulation with CM-L or CM-P.

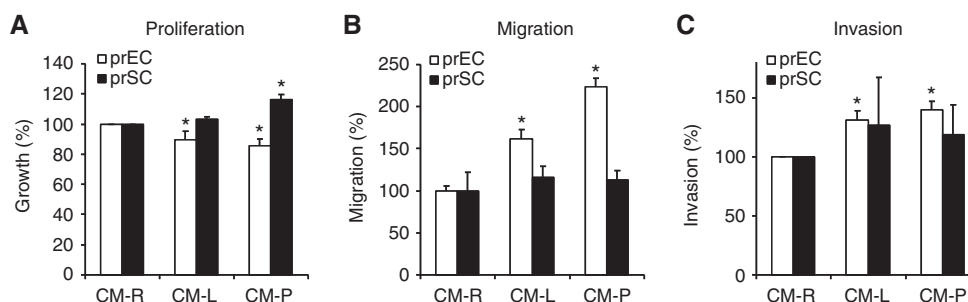


Figure 2 Cell behaviour of prECs and prSCs after stimulation with the conditioned media of LNCaP cells (CM-L) or PC3 cells (CM-P). (A) proliferation after 72-h stimulation with the CM. (B) Migration after 8-h stimulation with the CM. (C) Invasion after 8-h stimulation with the CM. The values of the three graphs represent means of at least three to five independent experiments \pm s.e.m., relative to CM-R responses. *Significant differences between the CM-stimulated cells and untreated controls (CM-R) are indicated in the graph ($P < 0.05$).

Table 1 Characteristics of study participants (n = 51)

Age (median)	51–83 (64)
Gleason score, n (%)	
5–6	20 (39)
7	18 (36)
8–10	13 (26)
Clinical stage, n (%)	
cT1	17 (33)
cT2	28 (57)
cT3	5 (10)
Metastasis, n (%)	
Yes	18 (35)
No	33 (65)
Biochemical recurrence, n (%)	
Yes	24 (47)
No	27 (53)
Cx26 expression tumour tissue, n (%)	
Score < 100 (low)	40 (87)
Score > 100 (high)	11 (22)
Cx26 expression noncancerous tissue, n (%)	
Score < 100 (low)	26 (51)
Score > 100 (high)	25 (49)

Abbreviation: Cx26 = connexin-26.

Connexin-26 expression was observed immunohistochemically in 94% of the patients. Connexin-26 was almost exclusively expressed in the cytoplasm of the luminal epithelial cells (Figure 4). The benign prostatic hyperplastic regions showed a comparable staining distribution compared with 'healthy' tissues, but the atrophic regions were negative for Cx26 expression. A significant association ($P < 0.0002$) was found between a low Cx26 score in noncancerous tissues and the subsequent formation of distant metastasis (Figure 5A). These metastases were detected between 1 month to 4 years after radical prostatectomy. No relation was found between Cx26 expression in the tumour tissue and occurrence of metastasis (Figure 5B). The expression levels of Cx26 in the tumour tissues were low, with a median score of 30, which is considerably lower than the average score in the noncancerous prostate tissue (120; $P = 0.0007$; data not shown).

The Kaplan–Meier estimates by high or low Cx26 expression in noncancerous tissues are shown in Figure 5C. For discrimination between a high or low expression, we chose a cutoff value of 100, which was based on the differences in expression between patients who developed metastasis and patients without metastasis (Figure 5A). A value of > 100 was therefore considered as a high Cx26 expression. With this cutoff, the time to biochemical recurrence was significantly shorter for patients with a low Cx26 expression ($P = 0.0002$), which is in relation to the increased number of metastatic patients in this group (with a hazard ratio of 3.449 in a multivariate analysis). Univariate Cox proportional hazards analysis showed that Cx26 expression in the nontumour-containing tissue, Gleason score and PSA levels significantly correlated independently with the time to progression (Table 2). Multivariate Cox proportional hazards analysis revealed that time to progression was associated with tumour stage, Gleason score and Cx26 expression in the noncancerous prostate tissues (Table 2), but not with the PSA levels.

DISCUSSION

This study shows that a decreased Cx26 expression in noncancerous prostate tissue was related to an increased risk to develop

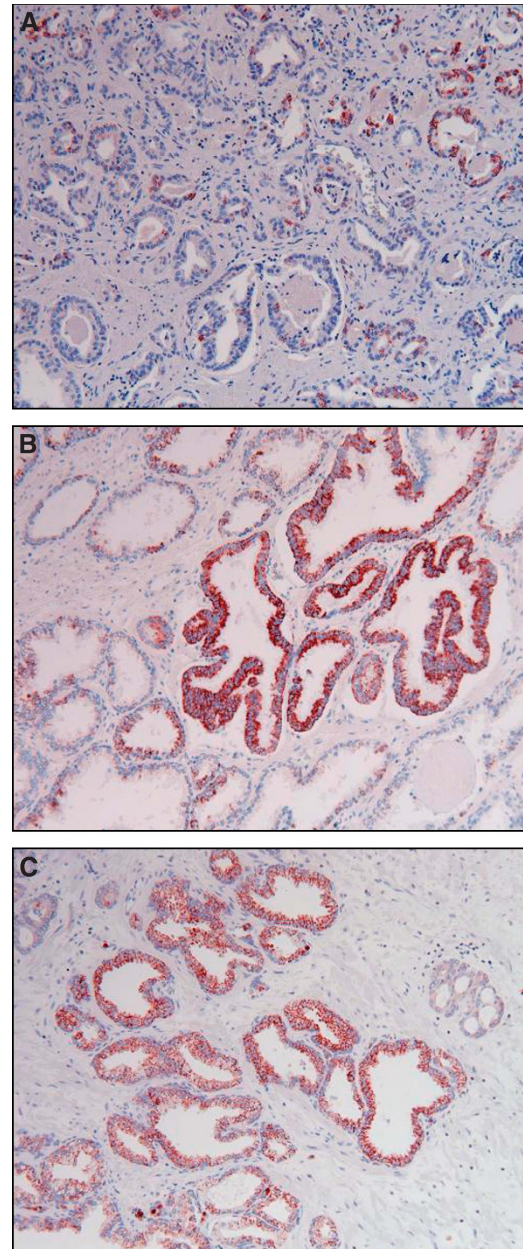


Figure 4 Expression pattern of Cx26 in prostate tissues. (A) Representative example of Cx26 expression in tumour tissue. (B) Connexin-26 expression in the noncancerous prostate tissue (within the same slide as A) of the radical prostatectomy specimen. (C) Connexin-26 expression in the noncancerous prostate tissue of another patient.

metastasis. It is especially notable that the expression of Cx26 was decreased, months to years before the diagnosis of the metastasis. The approach to study biomarkers in the noncancerous parts of the prostate will provide novel opportunities in the investigation of prostate cancer biology and metastasis. The expression of Cx26 may be used to identify metastatic prostate cancer patients after radical prostatectomy in a relatively early stage. When Cx26 is expressed at low levels, these patients may benefit from adjuvant (postoperative) therapy to decrease the risk of development of distant metastasis.

Mostly, biomarkers are studied in the tumour or tumour-associated stromal cells. At this moment the knowledge about the complexity of the role of the tumour microenvironment in

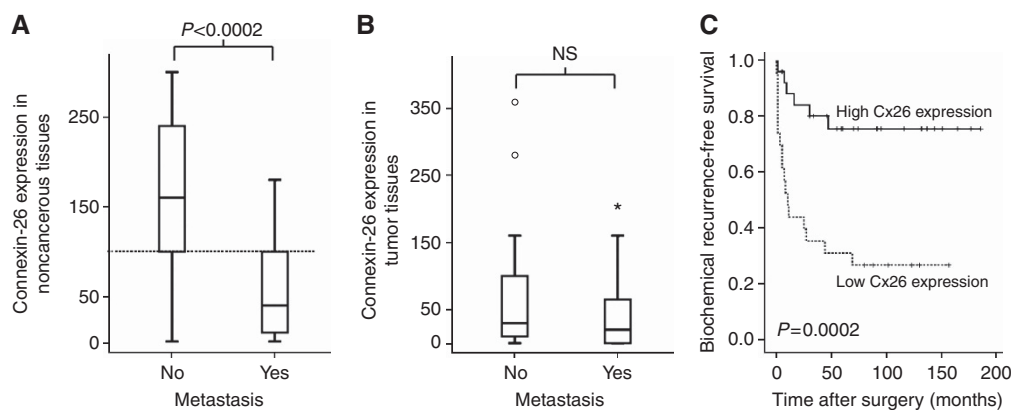


Figure 5 Connexin-26 expression in relation to postoperative development of metastasis. **(A)** Box plot showing the relation between Cx26 expression in the noncancerous tissues and the development of metastasis. The line within the box plot corresponds to the median value, the box length to the interquartile range, and the lines emanating from the box (whiskers) extend to the smallest and largest observations. The dotted line indicates the cutoff value of 100, which represents a high/low expression of Cx26. **(B)** Box plot showing the relation between Cx26 expression in the tumour tissues and the development of metastasis. **(C)** Kaplan–Meier biochemical recurrence-free survival curves according to the expression of Cx26 in the noncancerous prostate tissue. High expression was defined when Cx26 score was ≥ 100 , and a low expression was defined when the Cx26 score was < 100 . The cutoff value of 100 was chosen based on the differences between Cx26 expression in the noncancerous tissues of patients with and without metastasis. Statistical difference was analysed by the log-rank test ($P = 0.0002$). Outliers are indicated by * and °.

Table 2 Uni- and multivariate analyses of prognostic factors for time to biochemical recurrence

Variables	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Tumour stage	2.829 (1.370–5.841)	0.005	4.853 (1.740–13.533)	0.003
Gleason	1.818 (1.377–2.401)	0.00003	5.368 (1.742–16.546)	0.003
PSA	1.019 (1.001–1.039)	0.05	1.016 (0.991–1.041)	0.2
Biomarker				
Cx26 expression in nontumorous prostate tissue	4.785 (1.875–12.214)	0.001	3.449 (1.221–9.748)	0.02

Abbreviations: CIs = confidence intervals; Cx26 = connexin-26; HR = hazard ratio; PSA = prostate-specific antigen.

(prostate) cancer progression is increasing rapidly (Cunha *et al*, 2002; 2003; Alberti, 2006; Hanahan and Weinberg, 2011). Cancer cells and tumour-infiltrated cells are known to secrete many factors, such as growth factors and hormones, that have a role in cell signalling. These factors may potentially diffuse to the noncancerous parts of the prostate and subsequently affect these normal cells in their behaviour. Connexins are regulated by growth factors and hormones, which has been described in various cell systems (Maldonado *et al*, 1988; Madhukar *et al*, 1989; Stagg and Fletcher, 1990; Yu *et al*, 1994; Carruba *et al*, 2002). The disturbed Cxs expression in prostate cancer may be related to the hormones and growth factors that are secreted by the cancer and its microenvironment. The relation behind the decreased Cx26 expression in the noncancerous regions and the increased risk of metastasis might be caused by a decreased adhesion regulation, mediated by an interaction with the focal adhesion kinase (Tate *et al*, 2006). The disturbed intercellular communication between the tumour and noncancerous tissue may finally result in more aggressive cancer cells with a higher metastatic potential. The molecular mechanisms underlying this hypothesis need to be investigated in future studies.

The number of positive surgical margins in the patient group who eventually developed metastasis was higher (79%) than in the nonmetastatic group (32%). For patients with positive surgical margins, Cx26 staining may therefore be a predictive stratification

marker for prostate cancer metastasis. In addition, the morphological healthy prostate cells in prostatectomy tissues are often used as healthy controls (Tsai *et al*, 1996; Habermann *et al*, 2002). Our data suggest that these morphologically healthy prostate cells may not be proper controls. In addition, some studies already indicated that the adjacent benign (normal) prostate cells can have a predictive role for the presence of cancer (Isharwal *et al*, 2010; Jia *et al*, 2011). Therefore, caution is recommended for using these noncancerous cells as healthy controls.

Connexin-26 was previously described to be highly expressed in the luminal cells of the normal prostate epithelium (El-Alfy *et al*, 2000). This is in agreement with the observations in this study. The loss of gap junctions is considered to be a critical step to prostate cancer progression (Mehta *et al*, 1999). Connexin-26 expression has been studied in several other tumour types, such as colorectal, gastric and oesophageal squamous cell carcinoma (Inose *et al*, 2009; Liu *et al*, 2010; Nomura *et al*, 2010). A loss of Cx expression in the cancer cells has been described for various of these tumour types (Mehta *et al*, 1999; Nomura *et al*, 2010; Benko *et al*, 2011). This is in agreement with our findings in this study. Although the roles of Cxs in cancer progression seem to vary, in general Cxs appear to have a tumour-suppressive function.

We show a novel correlation between a low Cx26 expression in noncancerous prostate tissue and the development of metastasis within 4 years after radical prostatectomy. This expression can therefore be considered as a marker, which can potentially be used to identify high-risk patients with localised disease. When patients have a low Cx26 expression in noncancerous prostate tissue, adjuvant postoperative therapy can be given to decrease the chance of developing tumour metastasis. Additional studies examining the association between Cx26 expression and the development of metastasis are recommended.

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Conflict of interest

The authors declare no conflict of interest.

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