Prognostic value of connexin43 expression in patients with clinically localized prostate cancer

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Connexins (Cxs) are a family of transmembrane proteins that build cell-to-cell channels in gap junctions. Gap junctions composed of Cxs have an essential role in intercellular communication, adhesion and cell differentiation. Several studies investigated the role of connexin43 (Cx43) in different carcinomas; however, none investigated its prognostic role in prostate cancer. Cx43 expression and relationship with established prognostic features were assessed in a cohort of 102 patients treated with radical prostatectomy for clinically localized prostate adenocarcinoma. Cx43 expression in prostate cancer was significantly associated with established features indicative of worse prognosis, such as follow-up time (P < 0.001) and preoperative PSA (P < 0.007). Patients with lower Cx43 expressions in tumours have shorter follow-up time, which indicated shorter disease-free survival and higher preoperative PSA values. Furthermore, tumours with positive surgical margins (P < 0.001) showed significantly lower Cx43 expression compared with tumours without this feature. In univariate (P < 0.001) and multivariate (P = 0.014) analyses, decreased Cx43 expression was found to be a significant predictor of biochemical recurrence free-survival. Study results show the association of decreased Cx43 expression with prostate cancer progression. Moreover, Cx43 could serve as an additional prognostic marker and used together with traditional prognostic markers might help in further stratifying the risk of disease progression in patients with prostate cancer.

Prostate Cancer and Prostatic Diseases (2010) 6, 000–000. doi:10.1038/pcan.2010.51

Keywords: connexin43; prognosis

Introduction

Connexins (Cxs) are a family of transmembrane proteins forming cell-to-cell channels in gap junctions and provide a direct intercellular pathway for the passage of small signalling molecules (<1 kD) between the cytoplasmic interiors of adjoining cells.1-3 The channels are bicontinuous structures formed by the members of a family of about 20 related but distinct Cxs.4,5 Six Cx monomers assemble to form a hemi-gap junction channel or connexon, which, in turn, forms a complete gap junction channel by docking with connexon from an adjacent cell.6

The phosphorylation of connexin43 (Cx43) in its life cycle has been implicated in the regulation of a broad variety of Cx processes, such as the trafficking, assembly/disassembly, degradation, as well as the gating of gap junction channels. In addition, Cx43 phosphorylation is known to have a role in gap junctional plaque formation and activity.6

Gap junctions composed of Cxs have an essential role in intercellular communication, adhesion, cell proliferation and cell differentiation.7 Dysregulation of Cxs expression is thought to have a role in carcinogenesis.2,7 Cxs are differentially expressed in cell and tissue in developmental manner.8 The expression of some Cxs is altered and often reduced during tumour progression. Cx43 is also a tumour suppressor gene.2,10,11 Several studies also suggest that gap junctional intercellular communication and Cxs contribute to suppression of tumour growth by participating in the regulation of cell death.9,12

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Several studies investigated the role of Cx43 in different carcinomas,6,9,10 and some studies focused on Cx43 in prostate cancer2,4,7,13 but none on its prognostic role in prostate cancer.

Cx43 expression and relationship with established prognostic features were assessed in a cohort of 102
patients treated with radical prostatectomy for clinically localized prostate adenocarcinoma.

Patients and methods

Patients
The study included 102 patients, median age 65.0 (range 52–74, interquartile range 61.0–68.0) years, treated with radical retropubic prostatectomy and bilateral lymphadenectomy for clinically localized prostate adenocarcinoma at University Department of Urology, Sestre Milosrdnice University Hospital, Zagreb between 1 January 2002 and 31 December 2003. All patient identifiers were removed and replaced by unique study numbers, linked to the original identifiers by a single file kept under high security. A minimum of 60 months of follow-up for the patients without biochemical disease progression and the retrieval of archival tissue block was conducted under institutional review board approval.

After radical prostatectomy, patients were scheduled for clinical examination and serum PSA evaluation every 3 months for the first year, semiannually from the second year and annually thereafter. The study end point was the time of biochemical recurrence and was defined as two consecutive rising serum PSA values greater than 0.2 ng ml\(^{-1}\).

Overall follow-up ranged from 6 to 84 (median 63, interquartile range 49.5–70.0) months. A total of 30 of 102 (29.4%) patients had biochemical disease progression with a median time of progression of 18.5 (range 6–63, interquartile range 9.0–31.0) months.

Preoperative PSA ranged from 3.2 to 38.4 ng ml\(^{-1}\) (median 8.5, interquartile range 6.5–11.8), and PSA of biochemical disease progression ranged from 0.3 to 3.2 ng ml\(^{-1}\) (median 0.9, interquartile range 0.6–1.7).

The clinical stage was assigned by the operative surgeon according to the criteria of the International Union against Cancer.\(^{14}\) The Gleason distribution with Gleason patterns is shown in Table 1. Overall, 27 of 102 (26.5%) patients had extraprostatic extension of disease; 15 (55.6%) of them had extension through the capsule, 5 (18.5%) had seminal vesicle invasion and 7 (25.9%) had both extension through the capsule and seminal vesicle invasion. Only three (2.9%) patients had metastases of lymph nodes. None of the patients was treated with hormone or radiation therapy before radical prostatectomy, and none had secondary cancer.

Methods
Specimens were fixed in 10% buffered formalin, embedded in paraffin, cut at 5-µm thickness and routinely stained with haematoxylin and eosin. The diagnosis of adenocarcinoma was histologically confirmed in all cases. All slides submitted to immunohistochemistry analysis were so chosen that Gleason patterns in the slides most closely recapitulated postoperative Gleason score and also contained areas of non-neoplastic prostate tissue.

Deparaffinization and immunohistochemical staining were performed following the Microwave Streptavidin ImmunoPeroxidase (MSIP) protocol on a DAKO TechMateTM Horizon automated immunostainer (DAKO, Copenhagen, Denmark). We used primary monoclonal antibodies to Cx43 (sc-59949, dilution 1:500, Santa Cruz Biotechnology, USA). The used antibody was made against amino acids 362–381 of Cx43 of human origin and detected total Cx43. Normal colon tissue served as a positive control, and removal of the primary antibody was used as a negative control.

To evaluate the intensity of Cx43 expression in prostate cancer and non-neoplastic prostate tissue, the percentage of positively stained carcinoma and benign glands was examined in the whole mount of the chosen slide two times. First examination was under low (× 40) and second examination was under medium magnifications (× 200). In the two tumour cases with Gleason pattern 5, a percentage of positively stained carcinoma cells were counted as previously stated. Immunostained sections were examined by three independent pathologists (AD, BK and DT), who were blinded to the clinical course of the patients, and the final score was determined as a mean of the values counted by each pathologist.

Statistical analysis was performed using Mann–Whitney U-test, Kruskal–Wallis test, \(t^2\)-test, Kaplan–Meier test and Cox proportional hazards regression test. The levels of statistical significance were set at least at \(P<0.05\). Computations were performed using data analysis software system MedCalc for Windows, version 11.3.1. (MedCalc Software, Mariakerke, Belgium).

Results
The results of the Cx43 expression analysis in prostatic carcinoma and benign tissue are summarized in Table 2. Variable degree of Cx43 expression was present in all the

| Table 1 Preoperative and postoperative Gleason grade with Gleason scores |
|-----------------------------|-----------------------------|-----------------------------|
| **Gleason grade** | **Preoperative (%)** | **Postoperative (%)** |
| 6 (3+3) | 57 (55.8) | 34 (33.3) |
| 6 (4+2) | 1 (1) | 0 (0) |
| 7 (3+4) | 22 (21.6) | 41 (40.2) |
| 7 (4+3) | 22 (21.6) | 25 (24.5) |
| 8 (3+5) | 0 (0) | 1 (1) |
| 8 (5+3) | 0 (0) | 0 (0) |
| **Total** | **102 (100)** | **102 (100)** |

Abbreviation: Cx43, connexin43.
102 analyzed tumours and benign tissue samples. In benign tissue, over 50% of the analyzed samples expressed Cx43 in 85% or more glands, whereas less than 10% of the tumours showed similar Cx43 expression. On the contrary, 1 (1%) benign tissue specimen and 40 (39.2%) tumours showed expression of Cx43 in less than 60% of the glands.

Immunohistochemical reaction was intracytoplasmic in benign and neoplastic epithelial cells. In benign cells, reaction was clumped, paranuclear, apically oriented and has not reached luminal cell membrane (Figures 1a-e).

Prostate stromal cells did not express Cx43, and any staining in the stroma was considered background immunoreactivity.

The extent of Cx43 expression in prostatic carcinomas ranged from 5 to 100% with a median value of 60% (interquartile range 30.0–80.0). The extent of Cx43 expression in benign prostatic tissue ranged from 40 to 100% with a median value of 85% (interquartile range 80.0–90.0). In our analysis, the extent of Cx43 expression was significantly higher in benign than in malignant prostatic tissue (P<0.001) (Table 2).

We found no significant correlation between the Cx43 expression in the tumours and patient’s age (rho = 0.058, P = 0.563), preoperative (rho = -0.161, P = 0.105) and postoperative (rho = -0.176, P = 0.077) Gleason score, biochemical recurrence time (rho = -0.156, P = 0.411) and value of PSA relapse (rho = -0.329, P = 0.076). In addition, Cx43 expression was not associated with seminal vesicle invasion or extracapsular extension in T3 stage tumours (P = 0.200) or with lymph node metastasis (N1 stage tumours) (P = 0.999). Patients with seminal vesicle invasion had no significantly different Cx43 expression compared with patients with extracapsular extension of the tumour.

In contrast, Cx43 expression showed a statistically significant negative correlation with follow-up time (rho = -0.432, P < 0.001), which indicated shorter disease-free survival and earlier appearance of biochemical recurrence (study end point) in these patients. Preoperative PSA (rho = -0.266, P < 0.007) also showed negative correlation with Cx43 expression, and patients with low Cx43 expression had higher preoperative PSA.

Furthermore, tumours in T3 stage (P = 0.021) and tumours with positive surgical margins (P < 0.001) showed significantly lower Cx43 expression compared with T2 tumours and tumours without positive surgical margins.

To determine whether the Cx43 expression in tumours correlates with patient biochemical recurrence-free survival, we performed receiver–operator characteristic analysis. On the basis of the receiver–operator characteristic analysis results, cases having Cx43 expression involving at least 30% of the tumour glands were considered to show extensive Cx43 expression (sensitivity 63.33%, confidence interval: 43.91–80.10% and specificity 87.50%, confidence interval: 77.60–94.11) (Figure 2). A total of 11 tumours (10.8%) showed 30% Cx43 expression (the cutoff value), and an additional 19 tumours (18.6%) showed values close to the cutoff (20% in 12 and 40% in 7 cases, respectively) (Table 2).

On Kaplan–Meier analysis, biochemical recurrence-free survival was significantly longer in patients with extensive Cx43 expression over the 30% compared with patients without extensive Cx43 expression (P < 0.001) (Figure 3).
In multivariate, Cox proportional hazard regression analysis, preoperative Gleason score \((P = 0.025)\), Cx43 expression \(\leq 30\) \((P = 0.014)\), higher T stage \((P < 0.001)\) and preoperative PSA \((P = 0.003)\) were significant predictors of biochemical recurrence-free survival, whereas age \((P = 0.676)\), postoperative Gleason score \((P = 0.465)\), N stage \((P = 0.707)\) and positive surgical margins \((P = 0.060)\) showed no influence on biochemical recurrence-free survival (Table 3, Figure 4).

### Discussion

Decreased Cx43 expression has been reported in prostate carcinoma cells compared with adjacent benign prostate cells.\(^7\),\(^13\)

Habermann et al.\(^7\) showed that 93% of the BPH specimens express Cx43, whereas expression of Cx43 in carcinoma diminished and only 10% of poorly differentiated prostate cancers (Gleason score 8–10) showed Cx43 expression. These results indicated that Cx43 expression was connected with Gleason score and expression decreased with increased tumour grade. Similar results of diminished Cx43 expression in more advanced, anaplastic prostatic cancer cells were previously published by Tsai et al.\(^13\).

In our study, Cx43 expression was present in all malignant and benign analyzed tissue but the extent of Cx43 expression was significantly higher in benign compared with malignant prostatic tissue, which is similar to the published results.\(^7\),\(^13\) We have found no significant correlation between the Cx43 expression in the tumours and preoperative or postoperative Gleason score but postoperative Gleason score showed tendency to reach significant values \((P = 0.077)\). Study group of Habermann et al.\(^7\) and Tsai et al.\(^13\) included 40 and only 3 carcinomas, respectively. Our study group was significantly larger, but additional investigations are necessary to resolve this issue and confirm or reject connection between Cx43 expression and Gleason score.

The majority of neoplastic cells has fewer gap junctions, and gap junctional intercellular communication has been impaired compared with non-neoplastic cells.\(^7\) These results could point to conclusion that decreased Cx43 expression resulted in decreased gap junctional intercellular communication that may lead to aberrant and uncontrolled epithelial growth.\(^7\)

Umhauser et al.\(^15\) showed that the expression of Cx43 was nearly absent in ovarian adenocarcinomas compared with its greater expression in normal ovarian tissue. Brehm et al.\(^6\) study showed that progression from CIS to seminoma is associated with an intratubular reduction or even loss of Cx43 gene expression and Cx43 protein synthesis mainly in Sertoli cells, indicating that regulation of their Cx43 expression takes place at transcriptional level.

Studies of Mehta et al.\(^7\) showed that Cxs were localized at cell-to-cell contact areas in epithelial cells of well-differentiated prostate tumours, and they began to accumulate intracellularly as the tumours progressed to more invasive and undifferentiated stages, with an eventual loss of expression in advanced stages. The loss of gap junctions is a critical step to prostate cancer progression.\(^2\) These findings are consistent with their in vitro studies that indicated that epithelial cells from prostate cancer exhibit gross alterations in Cxs expression, with a severe loss in advanced and metastatic stages.\(^2\)

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**Table 3** Cox proportional hazard regression analysis for various clinicopathologic tumour features and extent of Cx43 expression

<table>
<thead>
<tr>
<th>Covariate</th>
<th>b</th>
<th>s.e.</th>
<th>P-value</th>
<th>OR</th>
<th>95% CI of OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.020</td>
<td>0.049</td>
<td>0.676</td>
<td>0.98</td>
<td>0.893–1.077</td>
</tr>
<tr>
<td>Cx43 &lt;30</td>
<td>1.197</td>
<td>0.491</td>
<td>0.014</td>
<td>3.31</td>
<td>1.271–8.626</td>
</tr>
<tr>
<td>Preoperative Gleason score</td>
<td>1.134</td>
<td>0.505</td>
<td>0.025</td>
<td>3.10</td>
<td>1.160–8.326</td>
</tr>
<tr>
<td>Postoperative Gleason score</td>
<td>0.451</td>
<td>0.618</td>
<td>0.465</td>
<td>1.57</td>
<td>0.477–5.241</td>
</tr>
<tr>
<td>Positive surgical margins</td>
<td>0.940</td>
<td>0.501</td>
<td>0.060</td>
<td>2.56</td>
<td>0.963–6.809</td>
</tr>
<tr>
<td>Preoperative PSA</td>
<td>0.086</td>
<td>0.028</td>
<td>0.003</td>
<td>1.08</td>
<td>1.030–1.153</td>
</tr>
<tr>
<td>T stage</td>
<td>2.209</td>
<td>0.496</td>
<td>&lt;0.001</td>
<td>9.09</td>
<td>3.460–23.917</td>
</tr>
<tr>
<td>N stage</td>
<td>0.302</td>
<td>0.803</td>
<td>0.707</td>
<td>1.35</td>
<td>0.282–6.480</td>
</tr>
</tbody>
</table>

Abbreviations: 95% CI of OR, 95% confidence interval for odds ratio; b, logistic regression coefficient; Cx43, connexin43; OR, odds ratio; P, significance level; s.e., standard error of logistic regression coefficient.

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**Figure 3** Biochemical recurrence-free survival was significantly longer in patients with extensive connexin43 (Cx43) expression over the 30% compared with patients without extensive Cx43 expression \((P < 0.001; \text{Kaplan–Meier analysis})\).

**Figure 4** Cox proportional hazard regression analysis showed that the extent of connexin43 expression could serve as an independent prognostic factor for assessment of disease-free period in patients with prostatic carcinoma \((\text{odds ratio} = 3.31, 95\% \text{confidence interval:} \ 1.27–8.63, P = 0.014)\).
Xu et al.\textsuperscript{10} reported similar results in lung cancer tissues with reduced expression of Cx43, and E-cadherin was associated with poor differentiation, advanced TNM stages and lymph node metastasis in lung cancer.

We found that diminished Cx43 expression in tumours was associated with advanced tumour stage (T3 stage tumours) but was not different in tumour with seminal vesicle invasion compared with tumour with extracapsular extension. In addition, diminished Cx43 expression was correlated with higher preoperative PSA values and with positive surgical margins. In a case of lymph node metastasis (N1 stage tumours) that showed no connection with Cx43 expression, any possible conclusions are hampered by the fact that only three patients in our study group had nodal metastasis, which is an insufficient number for a reliable statistical analysis.

Several studies investigated the Cxs expression in different carcinomas but rarely pointed out their prognostic significance.\textsuperscript{16–19} Recently, there were more and more studies that investigate Cxs, especially Cx26, and its prognostic value in different carcinomas.\textsuperscript{16–18}

Nomura et al.\textsuperscript{16} evaluated 153 patients with colorectal carcinoma. Cx26 had a statistically significant relationship with disease recurrence and histological type.\textsuperscript{16} The patients with Cx26-negative tumours had a significantly worse survival than those with positive tumours.\textsuperscript{16} Cx26 was an independent prognostic factor according to their multivariate analysis.\textsuperscript{16}

Liu et al.\textsuperscript{17} assessed Cx26 expression in 205 gastric carcinoma cases, and their multivariate regression analysis revealed that positive Cx26 expression was an independent prognostic predictor of intestinal-type gastric carcinoma and indicated favourable prognosis.

Inose et al.\textsuperscript{18} aimed to clarify the clinicopathologic outcome and prognostic significance of Cx26 in human oesophageal squamous cell carcinoma on surgical specimens obtained from 123 patients with oesophageal squamous cell carcinoma. Their results showed that there was no positive staining for Cx26-specific expression in normal oesophageal squamous cells, and 5-year survival rates of oesophageal squamous cell carcinoma patients with Cx26-positive expression were significantly lower than those with Cx26-negative expression.\textsuperscript{18}

To our knowledge, there was only one study that assessed prognostic role of Cx43 expression in cancer. Conklin et al.\textsuperscript{18} analyzed expression of Cx26, Cx32 and Cx43 in 438 invasive breast cancer. Cx43 was downregulated at various stages of breast cancer progression and showed strong negative correlation with Ki67 and strong positive correlation with oestrogen and progesterone receptor status.\textsuperscript{19} However, Kaplan–Meier survival analysis showed that Cx proteins (Cx26, Cx32 and Cx43) do not appear to be reliable markers for breast cancer prognosis.\textsuperscript{19} Cox analysis was not performed in this study.

Present study is the first that assesses the prognostic significance of Cx43 expression in prostate carcinoma. In our study, loss of Cx43 expression in prostatic carcinoma was clearly associated with tumour features that indicated a more aggressive tumour phenotype (higher preoperative PSA, T3 tumour stage and positive surgical margins). In addition, the patients with tumours that expressed Cx43 in less than 30% of tumour tissue had shorter biochemical recurrence-free survival in both univariate and multivariate analyses.

Several studies investigated the role of Cx43 in prostate cancer therapy. Wang et al.\textsuperscript{3} showed that Cx43 increased the sensitivity of prostate cancer cells to tumour necrosis factor-\textalpha-induced apoptosis. Fukushima et al.\textsuperscript{2} showed that combination therapy of Cx43 and docetaxel was significantly more cytotoxic when cells were treated with docetaxel after 24h of Cx43 transfection, suggesting that Cx43 affected sensitivity to docetaxel.

Our results support the potential role for Cx43 in prostatic cancer progression and showed their prognostic significance. In this way, Cx43 could serve as an additional prognostic marker that might help in further stratifying risk of progression in patients with clinically localized prostate cancer. In addition, further studies of the biological effect of this protein may help develop targeted therapeutics that upregulate Cx43 and by Cxs enhancement inhibit cellular growth and suppress malignant progression.

Conflict of interest

The authors declare no conflict of interest.

References


