Modern Pathology (2014) 27, 87–95

CCND1/CyclinD1 status in metastasizing bladder cancer: a prognosticator and predictor of chemotherapeutic response

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The *CCND1* gene encodes the protein CyclinD1, which is an important promoter of the cell cycle and a prognostic and predictive factor in different cancers. *CCND1* is amplified to a substantial proportion in various tumors, and this may contribute to CyclinD1 overexpression. In bladder cancer, information about the clinical relevance of *CCND1*/CyclinD1 alterations is limited. In the present study, amplification status of *CCND1* and expression of CyclinD1 were evaluated by fluorescence *in situ* hybridization and immunohistochemistry on tissue microarrays from 152 lymph node-positive urothelial bladder cancers (one sample each from the center and invasion front of the primary tumors, two samples per corresponding lymph node metastasis) treated by cystectomy and lymphadenectomy. *CCND1* amplification status and the percentage of immunostained cancer cells were correlated with histopathological tumor characteristics, cancer-specific survival and response to adjuvant chemotherapy. *CCND1* amplification in primary tumors was homogeneous in 15% and heterogeneous in 6% (metastases: 22 and 2%). Median nuclear CyclinD1 expression in amplified samples was similar in all tumor compartments (60–70% immunostained tumor nuclei) and significantly higher than in non-amplified samples (5–20% immunostained tumor nuclei; $P<0.05$). *CCND1* status and CyclinD1 expression were not associated with primary tumor stage or lymph node tumor burden. *CCND1* amplification in primary tumors ($P=0.001$) and metastases ($P=0.02$) and high nuclear CyclinD1 in metastases ($P=0.01$) predicted early cancer-related death independently. Subgroup analyses showed that chemotherapy was particularly beneficial in patients with high nuclear CyclinD1 expression in the metastases, whereas expression in primary tumors and *CCND1* status did not predict chemotherapeutic response. In conclusion, *CCND1* amplification status and CyclinD1 expression are independent risk factors in metastasizing bladder cancer. High nuclear CyclinD1 expression in lymph node metastases predicts favorable response to chemotherapy. This information may help to personalize prognostication and administration of adjuvant chemotherapy.
Modern Pathology (2014) **27,** 87–95; doi:10.1038/modpathol.2013.125; published online 26 July 2013

Keywords: bladder cancer; *CCND1*; CyclinD1 chemotherapy response prediction; survival

Approximately 25% of clinically staged N0M0 and surgically treated bladder cancer patients have lymph node metastases upon histological evaluation.[1,2] These patients are at high risk for disease recurrence and about two-thirds will die from cancer.[2,3] Better prediction of their clinical course and response to adjuvant therapies is necessary to improve patient management.

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Received 29 January 2013; revised 17 May 2013; accepted 17 May 2013; published online 26 July 2013

Aberrant expression of the protein CyclinD1 and alterations in its coding gene *CCND1* are frequent in human cancers[4] and may harbor prognostic[5–9] and predictive[10–12] information. CyclinD1 is primarily known as a key regulator of the cell cycle. It forms complexes with cyclin-dependent kinase 4 or 6 in the cytoplasm, these complexes then enter the nucleus[4] and inactivate the cell-cycle suppressive retinoblastoma protein, thereby promoting progression from G1 to the S-phase. CyclinD1 expression is physiologically controlled by different signal transduction pathways.[13] In cancer, CyclinD1 is frequently overexpressed. Different mechanisms may drive this process, including upregulation of transduction pathways and *CCND1*

amplification.[14–16] However, amplification does not necessarily leads to protein overexpression, suggesting complex expression mechanisms.[17–19]

In bladder cancer, information on frequency of *CCND1*/CyclinD1 alterations and their clinical relevance are limited. The prognostic impact has primarily been evaluated in non-muscle invasive bladder cancer for CyclinD1 and with regard to recurrence- and progression-free survival. However, data were inconsistent showing CyclinD1 expression as a marker of adverse[20–22] or favorable[23–27] prognosis or without prognostic impact.[28] Only two studies investigated the prognostic relevance of *CCND1* amplification in bladder cancer: while CCND1 amplification was an adverse risk factor in non-muscle invasive bladder cancers,[29] it failed to predict survival in muscle invasive tumors.[30] Finally, CyclinD1 expression has been shown to predict response to chemotherapies in cancers of the head and neck;[10,11] however, this potential has never been tested in bladder cancer. We evaluated these open questions on *CCND1*/CyclinD1 status in a homogeneous cohort of lymph node-positive bladder cancer patients treated by cystectomy and extended lymphadenectomy as well as adjuvant chemotherapy in a subset of patients.

Materials and methods

Patient Selection

Bladder cancer patients ($n = 152$) preoperatively staged cN0cM0 (physical examination, chest X-ray, abdominal and pelvic computerized tomography and bone scan) but lymph node metastases upon pathological examination were enrolled for the study. A standardized extended pelvic lymphadenectomy with cystectomy was performed between January 1985 and April 2008 at the Department of Urology, University of Bern, Bern, Switzerland. No patient received neoadjuvant therapy.

Follow-Up and Adjuvant Chemotherapy

Patients were followed prospectively according to a standard protocol as follows: In general, clinical evaluation, blood examination and ultrasound were performed postoperatively at 3 and 6 months, then at 6-month intervals until 5 years after cystectomy and yearly thereafter. Additionally, an abdominal and pelvic computerized tomography was performed at 6 and 12 months postoperatively when becoming available. In case of aggressive disease (eg, high number of positive lymph nodes, presence of extranodal extension (ENE)) or recurrence, the recommendation for adjuvant chemotherapy was made after a multidisciplinary discussion. Chemotherapy was given in 41% (63/152). A platin-based regimen was administered in 59% (37/63) of the patients; the others ($n = 26$) received Vincristine, Methotrexat, Leucovorin, Navelbine or Vinflunine in various combinations for different health reasons.

Surgical Technique and Pathology

Bilateral pelvic lymphadenectomy was performed in all the patients according to a standard protocol.[31] All lymphatic tissues were meticulously removed from the common iliac bifurcation along the external iliac vessels to the inguinal ligament, from the obturator fossa and from the ventral aspect of the internal iliac vein as well as the internal iliac artery and branches. The cystectomy protocol has previously been described in detail.[32]

The opened bladder and lymphadenectomy specimens were fixed overnight in neutral-buffered formalin and processed at the Institute of Pathology, University of Bern, Bern, Switzerland. Samples taken from the bladder included the deepest macroscopic invasion of the tumor. Microscopically, tumor stage and grade were determined. The lymphadenectomy specimens from each anatomical region were examined by inspection and palpation. All macroscopically detected lymph nodes were embedded completely. Whenever no identification of nodes was possible, the entire tissue was embedded for histological examination. All tumors were staged according to the seventh International Union Against Cancer classification of 2009.[33]

Construction of Tissue Microarray

Tissue microarray[34] was constructed with four samples (diameter: 0.6 mm) per patient, two from primary tumors (center and invasion front) and two from a corresponding lymph node metastases (samples A and B).

Fluorescence *In Situ* Hybridization (FISH) and Immunohistochemistry (IHC)

Two consecutive sections of 3-μm thickness were taken for FISH and IHC assays.

For FISH analysis of *CCND1* status, the Vysis *CCND1* /CEP 11 FISH Probe Kit was used (Vysis, Downers Grove, IL). It is a mixture of LSI *CCND1* gene (11q13) labelled in SpectrumOrange and CEP11 SpectrumGreen, directed against the centromeric satellite repeat of chromosome 11. Detailed instructions for hybridization procedures are provided by the manufacturer. Briefly, tissue array sections (3-μm thick) were dewaxed and rehydrated in graded ethanol. After pre-treatment, tissues were denatured for 5 min at 73 °C in a 70% formamide/2X-SSC solution. Samples were then dehydrated and subsequently treated with proteinase K. The hybridization mixture contained 1 μl LSI probe diluted in the hybridization mix. After overnight hybridization at 37 °C, slides were washed and

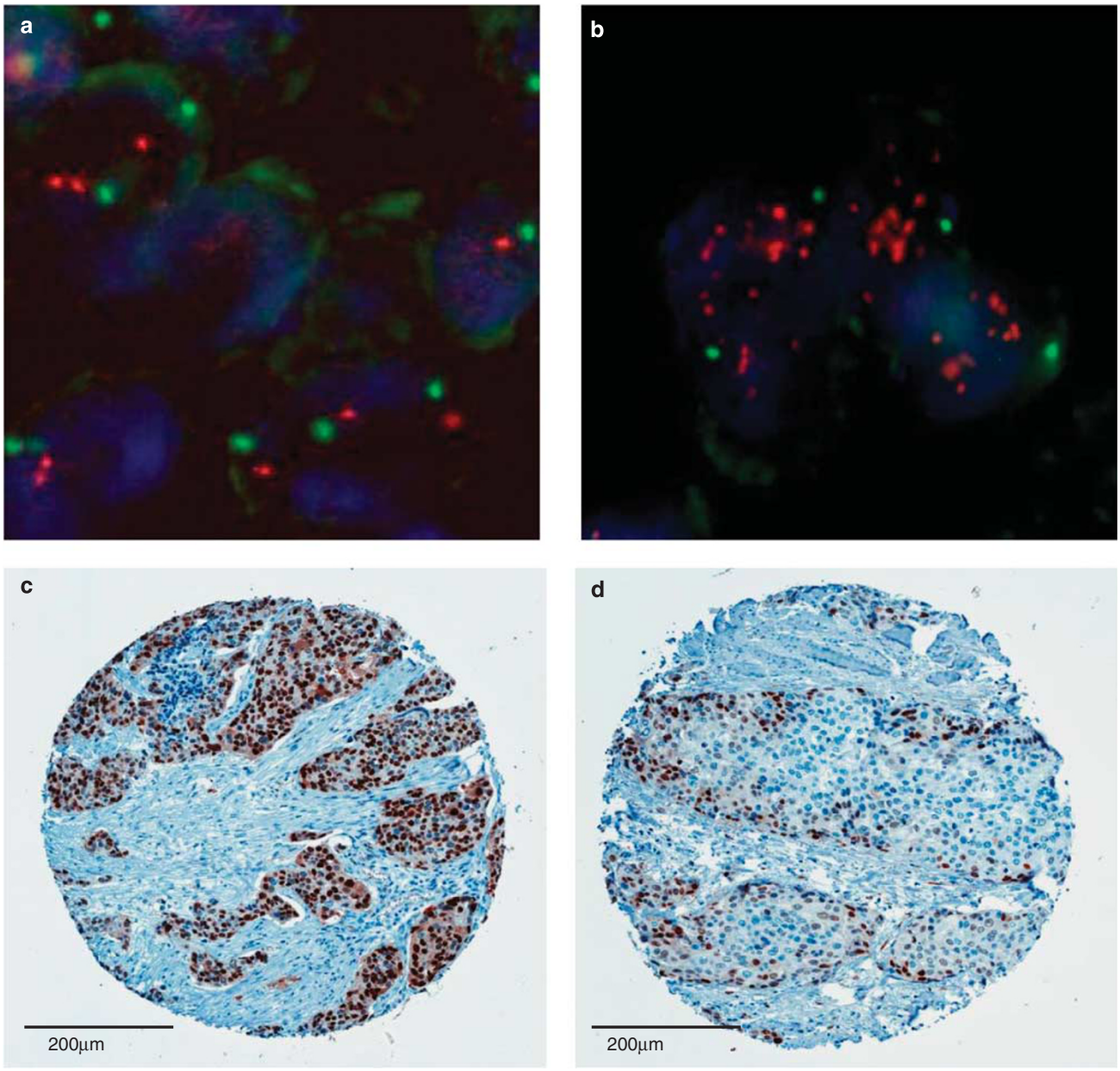

Figure 1 Bladder cancer samples without *CCND1* amplification (**a**) show a gene/centromer 11 (red signals/green signals) ratio <2 and those with amplification (**b**) a ratio ≥ 2. Immunostains of tumors with high (**c**; $>80\%$ positive cancer nuclei) and low (**d**) nuclear CyclinD1 expression.

counterstained with DAPI II (125 ng/ml, Vysis) in an antifading solution. A minimum of 25 non-overlapping nuclei were evaluated on each spot, and the individual spot was considered normal or amplified according to the *CCND1*/CEP11 fluorescence ratios <2 or ≥ 2[18] (Figures 1a and b).

For IHC staining, before incubation slides were dewaxed, rehydrated and boiled in 1 mM EDTA–10 mM Tris, pH 9.0 in a microwave oven. CyclinD1 expression was assessed using a monoclonal rabbit antibody (clone SP4; Thermo Fisher Scientific, Kalamazoo, MI, USA). Optimal staining was achieved at 1:25 antibody dilution. Bound primary antibodies were visualized using the Envision Plus system (Dako, Glostrup, Denmark). CyclinD1 expression was observed in the nuclei and cytoplasm[27] and evaluated separately for percentage of positive tumor cells (Figures 1c and d).

A summary of the number of samples that were available for analysis is shown in Figure 2.

Statistical Analyses

For each primary tumor and each corresponding lymph node metastasis, (a) a *CCND1* status based on the presence (homogeneous or heterogeneous) or absence of amplification and (b) a CyclinD1

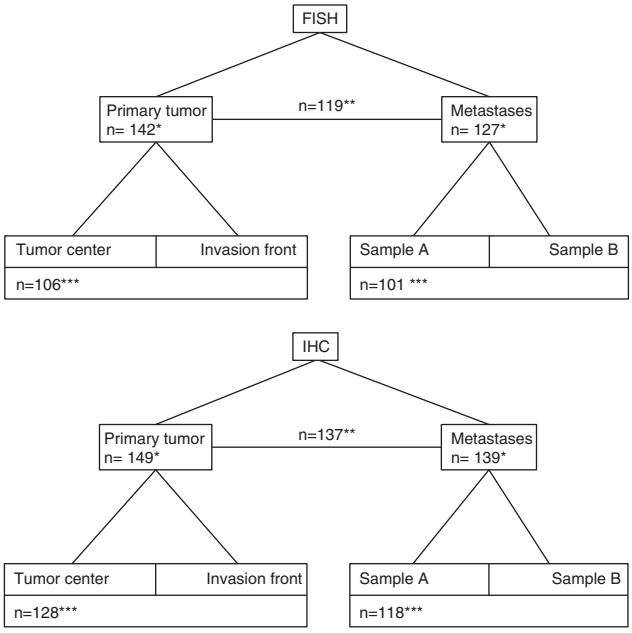

Figure 2 Flow chart summarizing the number of samples that are included in a given analysis.

expression level (nuclear, cytoplasmic) based on the sample with the highest percentage of immunostained tumor cells was assigned. These parameters were used to compare primary tumors with metastases and for correlations with histopathological tumor features (primary tumor stage; ENE and number of lymph node metastases; two-sided Wilcoxon's rank-sum test for non-categorical data and Fisher's test for categorical data) and cancer-specific survival (CSS). Concordance in FISH results between (a) the two samples of a primary tumor, (b) the two samples of a lymph node metastasis and (c) a primary tumor and its lymph node metastasis was evaluated with Cohen's kappa test; corresponding investigations for CyclinD1 expression used Pearson's correlation. Kaplan–Meier plots were used to estimate CSS from surgery to the date of cancer-related death (data available for 148 patients). This end point was chosen, because qualification for adjuvant chemotherapy might already be a feature with impact on overall survival that would bias such analyses. Patients still living and those who died without evidence of tumor recurrence were censored at the date of last follow-up and death, respectively. Differences in CSS between the subgroups defined according to *CCND1* status (non-amplified *vs* amplified) and CyclinD1 expression level were assessed using the log-rank test. Survival according to the expression level was analyzed in quartiles. Metastases but not primary tumors showed similar outcome in the first three quartiles, which were therefore clustered (low expression) and compared with the fourth quartile (high expression; >80% positive cancer nuclei) in both the tumor compartments. To identify independent prognostic factors, Cox proportional hazards models were applied. *P*-values <0.05 were considered significant. All statistical analyses were performed using the R software package, Version 2.9.1., http://www.r-project.org.

Table 1 Clinico-pathological data of 152 lymph node-positive patients with urothelial cancer of the bladder

Patient data (n = *152)*	
Age at surgery (years), median (range)	67 (35–89)
Female/male (*n*)	29/123
Follow-up (years), median	7.2
5-year cancer-specific survival	37%
Cystectomy data	
Tumor stage (*n*)	
pT1/2	4/17
pT3/4	93/38
Lymphadenectomy data	
Evaluated nodes per patient (*n*), median (range)	27 (10–56)
Positive nodes per patient (*n*), median (range)	3 (1–46)
Lymph node stage (*n*)	
pN1	44
pN2/3	108
Extranodal extension (*n*)	
No	71
Yes	81

Results

Clinico-pathological data of the cohort are given in Table 1.

CCND1 Amplification and CyclinD1 Expression in Primary Tumors and Lymph Node Metastases and Their Association with Tumor Characteristics

CCND1 amplification status in the tumor center and at the invasion front was concordant in 94% of the 106 tested primary tumors with two informative samples (15% ($n=16$) amplified, 79% ($n=84$) non-amplified) and discordant in 6% ($n=6$); concordance between samples A and B in the metastases ($n=101$) was 97% (22% ($n=22$) amplified, 75% ($n=76$) non-amplified), discordance was 3% ($n=3$). *CCND1* amplification status of both the primary tumor and a corresponding metastasis could be tested in 119 patients and was highly concordant (Cohen's Kappa: 0.9). Only one of the 22 amplified primary tumors had no evidence for amplification in the metastasis, and four of the 25 patients with amplified metastases showed no amplification in their primary tumors.

Nuclear and cytoplasmic Cyclin D1 expressions were significantly higher in metastases (median immunostained cancer cells: 50 and 15%) compared with primary tumors (30 and 0%, $P<0.05$). Nuclear

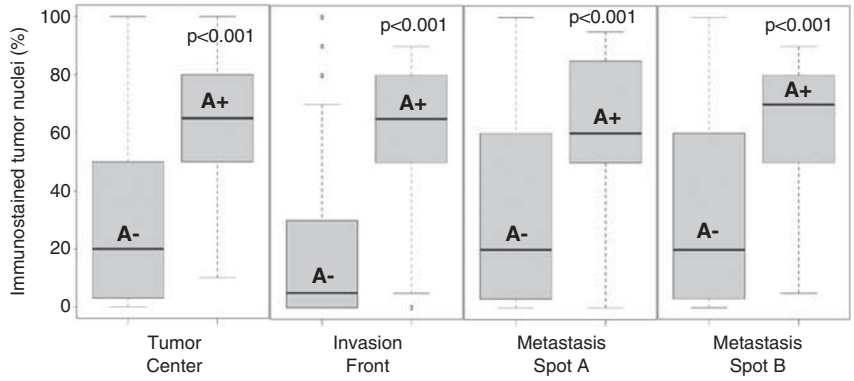

Figure 3 Box plots showing nuclear CyclinD1 expression in all the four tumor areas segregated for *CCND1* non-amplified (A−)- and amplified (A+) tumor samples. In all the areas, median nuclear CyclinD1 expression in the amplified samples is similar and significantly higher than in the non-amplified samples, while the range of CyclinD1 expression is virtually independent of the amplification status.

expression in primary tumors and their metastases was well correlated (correlation coefficient (CC): 0.65), as was the expression in both samples of the primary tumors (CC: 0.72) and the metastases (CC: 0.78), respectively. Cytoplasmic expression in primary tumors and metastases was only weakly correlated (CC: 0.32), but the correlation between the two samples from the primary tumors (CC: 0.7) and from the metastases (CC:0.7) was good.

Median nuclear CyclinD1 expression level of the *CCND1* amplified samples was similar in each region (median of immunostained tumor nuclei 60–70%; Figure 3) and significantly ($P<0.05$) higher than in non-amplified samples (5–20% immunostained tumor nuclei). However, the range of CyclinD1 expression in the amplified and in non-amplified samples was the same (0–100% immunostained tumor nuclei).

CCND1/CyclinD1 status in the primary tumors and in the metastases was not significantly related to the stage of the primary tumor or lymph nodes or to ENE of metastases. However, there was a trend ($P>0.1$) for higher amplification rate (22%) and median nuclear expression (38%) in locally advanced pT3/4 compared with organ-confined pT1/2 (5 and 5%) primary tumors.

Univariate and Multivariate Survival Analysis

For survival analyses, FISH data were available from 142 primary tumors and from 127 lymph node metastases with at least one informative tissue sample per tumor component (for IHC in 149 primary tumors and 139 metastases). CSS was significantly reduced when primary tumors ($P<0.001$, Figure 4a) or lymph node metastases ($P=0.02$, Figure 4b) were amplified and when nuclear CyclinD1 expression in metastases was high (expression $\geq$4th quartile, $P=0.001$, Figure 4d). These three prognosticators were independent from other known risk factors ($P=0.01$, $P=0.001$, $P=0.02$, Table 2). High nuclear CyclinD1 expression in the primary tumors ($P=0.8$, Figure 4c) and cytoplasmic CyclinD1 in any tumor compartment were no prognosticators.

CCND1/CyclinD1 Status and Adjuvant Chemotherapy

Patients with high nuclear expression of CyclinD1 in their metastasizing tumor component had significantly different CSS depending on chemotherapy status: Virtually all patients without adjuvant chemotherapy died from cancer during the first 2.5 years after surgery (Figure 5a) while CSS significantly ameliorated when any chemotherapy (5-year CSS: 22%, Figure 5b) and particularly platin-based chemotherapy (5-year CSS: 37%, Figure 5c) was applied. No similar effects after adjuvant chemotherapy were observed in the subgroup of patients with low CyclinD1 expression in the metastases and also not in any other subgroup defined according to the amplification status or protein expression.

Discussion

The *CCND1* gene and expression of its protein product CyclinD1 are frequently altered in human cancers and may predict tumor progression.[35] However, in bladder cancer data on this well-established oncogene are still limited. Therefore, we investigated *CCND1* amplification and CyclinD1 expression in a homogeneous cohort of lymph node-positive bladder cancer patients with regard to tumor heterogeneity, correlation of amplification and protein expression as well as association with histopathological tumor characteristics, survival and response to chemotherapy.

Genomic instability is frequent in human cancers, which as a consequence harbor genetically diverse populations of tumor cells.[36] This intra-tumor heterogeneity can influence disease progression and therapeutic response via cell subclones able to

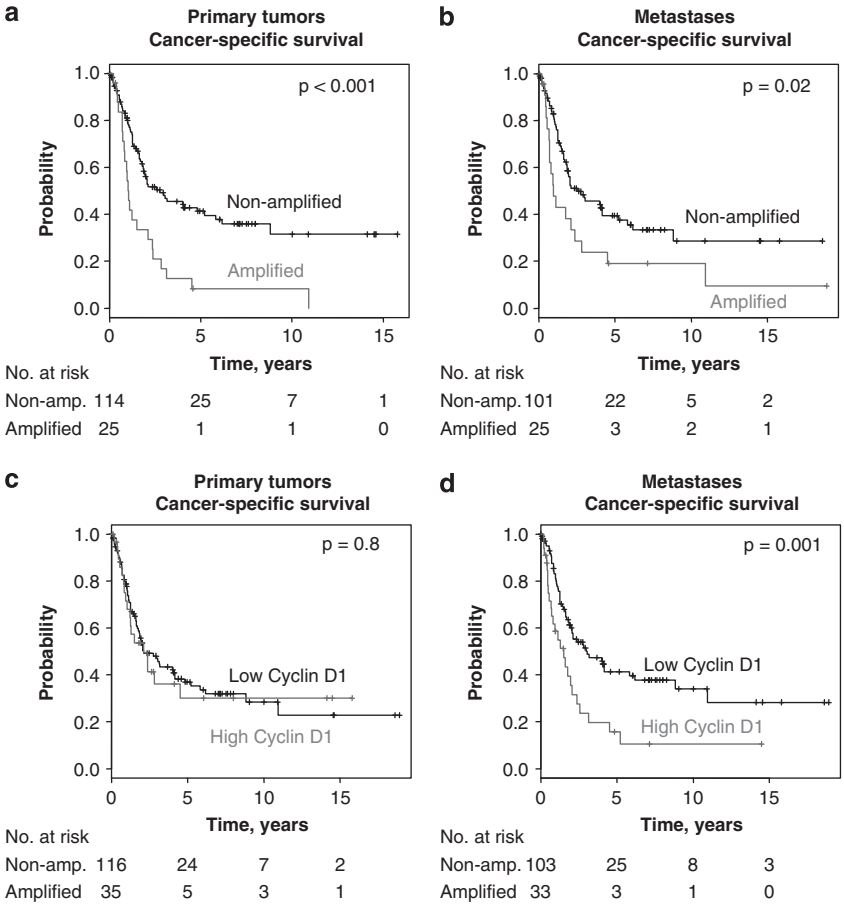

Figure 4 Cancer-specific survival stratified according to the *CCND1* amplification status (**a**, **b**) and nuclear CyclinD1 expression (<4th *vs* 4th quartile; **c**, **d**) in primary tumors (**a**, **c**) and lymph node metastases (**b**, **d**).

Table 2 Multivariate analysis for cancer-specific survival: *CCND1* amplification and high nuclear CyclinD1 expression in lymph node metastases predict early cancer-related death independently from other established risk factors

	Localization	*Variable*	*Reference level*	*HR*	*95% CI*		P
Fluorescence *in situ* hybridization	Primary tumor	Amplified	Non-amplified	2.35	1.44	3.83	**0.001**
		Extranodal extension of metastases	Without	2.07	1.27	3.37	**0.003**
		pN2/3	pN1	1.41	0.78	2.57	0.26
		pT3/4	pT1/2	3.41	1.40	8.55	**0.008**
	Metastasis	Amplified	Non-amplified	1.75	1.02	3.0	**0.04**
		Extranodal extension of metastases	Without	1.53	0.91	2.6	0.1
		pN2/3	pN1	1.62	0.8	3.3	0.18
		pT3/4	pT1/2	2.48	0.98	6.27	**0.05**
Immunohistochemistry (nuclear)	Primary tumor	Expression ≥4th quartile	≤3rd quartile	1.37	0.8	2.32	0.25
		Extranodal extension of metastases	Without	1.94	1.2	3.16	**0.007**
		pN2/3	pN1	1.5	0.82	2.74	0.19
		pT3/4	pT1/2	3.03	1.3	7.11	**0.01**
	Metastasis	Expression ≥4th quartile	≤3rd quartile	1.92	1.19	3.1	**0.008**
		Extranodal extension of metastases	Without	1.54	0.95	2.5	0.08
		pN2/3	pN1	1.43	0.73	2.77	0.3
		pT3/4	pT1/2	3.12	1.23	7.89	**0.02**

Abbreviations: CI, confidence interval; HR, hazard ratio.
Significant *P* values are indicated in bold.

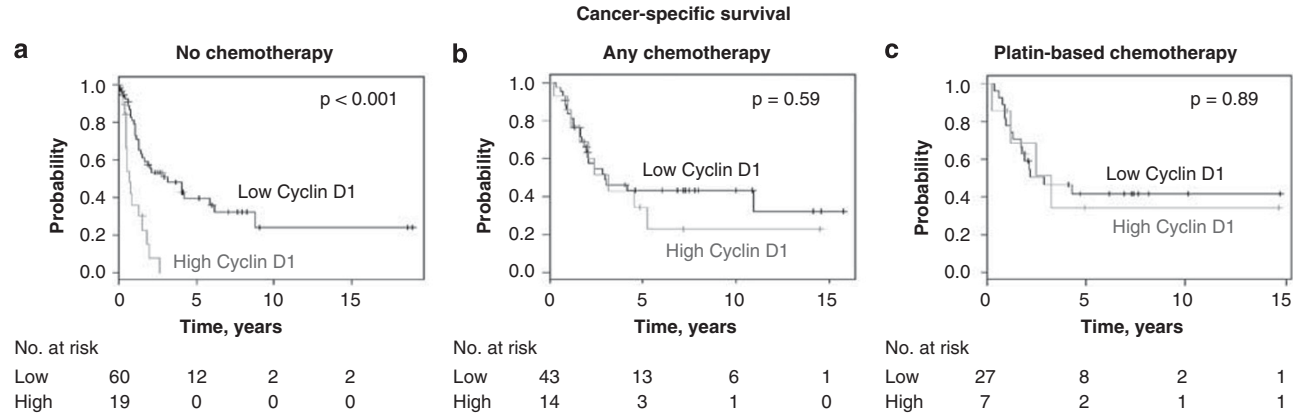

Figure 5 Cancer-specific survival stratified according to the CyclinD1 expression in lymph node metastases. All patients without chemotherapy and with high expression die within 2.5 years after surgery (**a**). This prognostic impact of high CyclinD1 expression in the metastases is lost after any chemotherapy (**b**) and particularly after platin-based therapy (**c**).

seed metastases and to survive therapy.[36,37] Knowledge about intra-tumor heterogeneity is important to assess the probability of a sampling error when evaluating prognostic and predictive biomarkers, and understanding of this heterogeneity is incomplete without the analysis of the metastasizing component, which can differ substantially from their primary tumor.[38] In bladder cancer, *CCND1* status was identical between tumor center and invasion front in 94% of the tested primary tumors (79% non-amplified, 15% amplified) and different in 6%. In the metastases, concordance between samples A and B was 97% (76% non-amplified; 22% homogeneously and 2% heterogeneously amplified). Finally, *CCND1* amplification status between primary tumor and corresponding metastasis was also highly concordant; only one patient with an amplified primary tumor had a non-amplified metastasis and four patients showed an inverse genotype with no evidence of amplification in the primary tumors but amplified metastases. These data were generated from small cancer samples of 0.6 mm in diameter and defined clinically relevant subgroups. This suggests high intra-tumor homogeneity of *CCND1* status in a given tumor and a minor sampling error in its determination.

The relation of *CCND1* amplification status and CyclinD1 expression in bladder cancer has not been investigated yet. In all the tumor compartments (tumor center, invasion front, metastases), median CyclinD1 expression level in amplified samples was significantly higher compared with non-amplified samples. However, an amplified tumor sample could express the lowest CyclinD1 levels and vice versa non-amplified tumor samples could show the highest. The same phenomenon has also been observed in breast cancer[18] and indicates that overexpression of CyclinD1 in cancer is a complex mechanism in amplified cases also. This suggests that increased *CCND1* gene copy numbers are multipliers of intracellular signals for protein expression but, on their own, not sufficient for CyclinD1 overexpression.

Amplification of *CCND1* is an adverse prognostic factor in cancers of the breast,[39] colon[39] and head and neck.[40] In our bladder cancer cohort also, *CCND1* amplification in either the primary tumor or the metastasis predicted poor outcome. Patients with this genetic defect doubled their probability of dying from bladder cancer compared with patients without this alteration, and this prognostic information was independent from the established risk factors. The high concordance of the *CCND1* status between a primary tumor and its metastasis explains their aligned prognostic potential. However, the pathophysiological background for the aggressivity of amplified tumors is unclear and not necessarily mediated by CyclinD1. *CCND1* amplification might also reflect a high degree of genetic instability,[41] that by itself is associated with a particularly aggressive phenotype or, alternatively, other genes in the amplicon may contribute to malignancy.[29] Contrarily to amplification status, CyclinD1 expression level in primary tumors was not strongly linked to the one of their metastases, and CyclinD1 overexpression was an adverse independent risk factor only in metastases. These differences in biomarker status and in its prognostic potential between both tumor components show that the primary tumor is not necessarily a surrogate for the metastasizing component. In general, the latter drives the disease and needs to be explored adequately to determine the relevance of prognostic and predictive molecules. Finally, the ability to stratify survival may differ between *CCND1* amplification status and CyclinD1 expression level as previously reported for other cancers.[18,42] Consequently, *CCND1* and CyclinD1 are also no surrogates for each other, and investigations on genetic and protein level are necessary to determine the respective prognostic potential.

Bladder cancer, in general, has a limited chemosensitivity and better prediction of chemotherapeutic

response in the individual tumor is highly warranted to personalize this therapy and avoid overtreatment.[43] Interestingly, CyclinD1 has previously been reported to predict favorable response to chemotherapy in head and neck cancers.[10,11] Most importantly, in our bladder cancer cohort also, CyclinD1 is a predictor of response to chemotherapy: while virtually all patients with high CyclinD1 expression in metastases and without chemotherapy die from cancer within 2.5 years after surgery, CSS significantly increases when adjuvant chemotherapy is given (5 year CSS: 22%) and particularly after platin-based chemotherapy (5 year CSS: 37%). No similar effect of chemotherapy was observed in the subgroup with low CyclinD1 expression. The successful chemotherapy treatment of the metastasizing tumor component with high CyclinD1 expression might help to personalize this application, and targeting CyclinD1[35] might supplement therapeutic options in such patients with reduced health condition not qualifying for conventional chemotherapy.

In conclusion, *CCND1* amplification and CyclinD1 overexpression are independent adverse risk factors in metastasizing bladder cancer. Additionally, high CyclinD1 expression in the metastasizing component predicts favorable response to adjuvant chemotherapy. This information may help to individualize prognostication and administration of adjuvant therapies, thus improving patient management.

Acknowledgements

Received funding by Novartis Stiftung für medizinisch-biologische Forschung.

Disclosure/conflict of interest

The authors declare no conflict of interest.

Disclaimer

We confirm that the design and conduct of the work was performed by all of us. Manuscript has been written, read and approved by all of us. This work, or parts of it, have not been and will not be submitted elsewhere for publication.

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