High-Throughput Tissue Microarray Analysis of Cyclin E Gene Amplification and Overexpression in Urinary Bladder Cancer

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Studies by comparative genomic hybridization revealed that the 19q13 chromosomal region is frequently amplified in bladder cancer. The cyclin E gene (CCNE), coding for a regulatory subunit of cyclin-dependent kinase 2, has been mapped to 19q13. To investigate the role of cyclin E alterations in bladder cancer, a tissue microarray of 2,317 specimens from 1,842 bladder cancer patients was constructed and analyzed for CCNE amplification by fluorescence *in situ* hybridization and for cyclin-E protein overexpression by immunohistochemistry. Fluorescence *in situ* hybridization analysis showed amplification in only 30 of the 1,561 evaluable tumors (1.9%). Amplification was significantly associated with stage and grade ($P < 0.0005$ each). Immunohistochemically detectable cyclin E expression was strong in 233 (12.4%), weak in 354 (18.9%), and negative in 1,286

of the 1,873 interpretable tumors. The majority (62.1%) of CCNE-amplified tumors were strongly immunohistochemistry-positive ($P < 0.0001$). The frequency of protein expression increased from stage pTa (22.2%) to pT1 (45.5%; $P < 0.0001$) but then decreased for stage pT2-4 (29.4%; $P < 0.0001$ for pT1 versus pT2-4). Low cyclin E expression was associated with poor overall survival in all patients ($P < 0.0001$), but had no prognostic impact independent of stage. It is concluded that cyclin E overexpression is characteristic to a subset of bladder carcinomas, especially at the stage of early invasion. This analysis of the prognostic impact of CCNE gene amplification and protein expression in >1,500 arrayed bladder cancers was accomplished in a period of 2 weeks, illustrating how the tissue microarray technology remarkably facilitates the evaluation of the clinical relevance of molecular alterations in cancer. (*Am J Pathol* 2000, 157:787-794)

Gene amplification plays an important role in the development and progression of many solid tumors. More than 20 different genomic loci have been found highly amplified in urinary bladder cancer.¹⁻⁶ Some of these amplifications contain known oncogenes such as HER-2 at 17q21, CCND1 at 11q13, CMYC at 8q24, EGFR at 7p13, as well as MDM2 and CDK4 at 12q13-15.⁷⁻¹¹ The target genes of amplifications are primarily unknown in many other chromosomal regions that are sites of recurrent DNA amplifications, such as 1q21-31, 2q13, 3p22-24, 6p22, 8p11, 8q21, 9p21, 10p13-14, 13q13, 13q31-33, 18p11, 20q, 21p11, 22q11-13, Xp11-13, and Xq21-22.2. Most of the current knowledge about amplification sites in bladder cancer has been derived from studies using comparative genomic hybridization (CGH).¹² Certain GC-rich regions of the genome, such as 1p and 16p, as well as chromosomes 19 and 22, are prone to hybridization variability that may result in CGH artifacts. Therefore, these regions are often excluded from CGH analysis, and very little is known about amplifications at these sites in bladder cancer.

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Figure 1. CGH results in tumors with 19q amplification. CGH profile examples of four tumors showing distinct peaks in the 19q13 region suggesting amplification. The mean green:red fluorescence ratio profile (**thick line**) and its SD (**thin lines**) are shown for each chromosome from pter to qter. The chromosome identification and the number of observations are shown on the bottom of each profile. The baseline ratio is printed in black (1.0), the **vertical lines** besides indicate the threshold ratio values of 0.8 (red) and 1.2 (green).

Altogether 278 bladder carcinomas have been analyzed by CGH in our laboratory.^{3,4,13-15} A critical review of several CGH profiles revealed high-level amplifications around the 19q13 region in bladder cancer. These amplifications centered around the 19q13 chromosomal region suggesting amplification in the area where the cyclin E gene (CCNE) resides. CCNE, a regulatory subunit of cyclin-dependent kinase, is an important regulator of entry into S phase in the mammalian cell cycle¹⁶⁻¹⁸ and has previously been reported to undergo amplification in a small fraction of breast and ovarian cancers.^{19,20} It is therefore possible that CCNE would have an oncogenic role if the protein is overexpressed. We therefore decided to apply our recently developed tissue microarray technology²¹ to examine the role of cyclin E amplification and expression in bladder cancer. Tissue microarrays containing 2,317 specimens were constructed, and applied to determine the cyclin E involvement at the DNA and protein level, as well as to assess the association of cyclin E with tumor phenotype and clinical outcome.

Materials and Methods

Comparative Genomic Hybridization

The CGH results of 278 bladder cancers have been published previously.^{3,4,13-15} A review of these CGH profiles revealed 11 tumors (4%) with distinct peaks around 19q13. Examples are shown in Figure 1.

Material and Tissue Microarray Construction

A total of 2,317 formalin-fixed, paraffin-embedded tissue samples of urinary bladder carcinomas were available from the archives of the Institute of Pathology at the University of Basel, the Cantonal Hospitals at St. Gallen, Baden, Winterthur, and Münsterlingen, and the Triemli Hospital in Zurich, Switzerland. Approval for the study was granted by the University of Basel Ethics Committee. The tumors were from 1,842 patients and included 1,071 primary tumors, 207 recurrent tumors, and 1,039 biopsies with no associated information on previous history.

Among the patients with multiple tumors there were 234 with two, and 92 with three or more different tumor specimens. All slides of all tumors were reviewed by one pathologist (GS). Tumor stage and grade were defined according to International Union Against Cancer and World Health Organization classifications.^{22,23} Stage pT1 was defined by the presence of both unequivocal tumor invasion of the suburothelial stroma and tumor-free fragments of the muscular bladder wall. One hundred and one carcinomas with stroma invasion but absence of muscular bladder wall in the biopsy were classified as at least pT1 (pT1-). There were 2,108 transitional cell (TCC), 73 squamous cell, 22 adeno, 24 sarcomatoid, 31 small cell, and two adenosquamous carcinomas of the bladder. The series contained 277 pTaG1, 567 pTaG2, 107 pTaG3, 194 pT1G2, 299 pT1G3, 142 pT2-4G2, and 423 pT2-4G3 TCC. A papillary tumor growth was assumed if at least one unequivocal papilla with similar atypia as in the invasive tumor area was present. There were 1,658 papillary and 640 solid tumors. No data had been recorded for histological tumor type ($n = 57$), stage ($n = 13$), and tumor growth pattern ($n = 19$) in small subsets of tumors.

For tissue microarray construction, a hematoxylin and eosin (H&E)-stained section was made from each block to define representative tumor regions. Tissue cylinders with a diameter of 0.6 mm were then punched from tumor areas of each donor tissue block and brought into a recipient paraffin block using a custom-made precision instrument as described.²¹ Samples for all tumors were distributed in five regular-sized paraffin blocks each containing 400 to 600 tumors. Five- μ m sections of the resulting tissue microarray blocks were transferred to glass slides using a paraffin-sectioning aid system (adhesive coated slides, adhesive tape, UV-lamp; Instrumedics Inc., Hackensack, NJ). An overview of an H&E-stained microarray section is shown in Figure 2A.

Clinical Follow-Up Information

Of the 1,842 patients, 1,375 were males and 463 females. The gender was unknown in four patients. The average age of these patients was 68 years (range, 20 to 100 years). Clinical histories of 1,317 patients were retrospectively evaluated by reviewing the patients' charts and contacting the attending physicians. Patients whose cause of death remained unknown were excluded from analyses of tumor-specific survival. The median follow-up period was 42 months (range, 1 to 236 months). Among 243 patients with pT2-4 carcinomas and follow-up data, 127 had undergone total or partial cystectomy, 48 had radiation therapy, and 63 had received chemotherapy.

For patients with pTa and pT1 tumors, time to recurrence and time to progression (to stage pT2 or higher) were selected as study endpoints. The clinical follow-up information was considered complete enough to include pTa/pT1 cancer patients in the analysis of time to recurrence if regular follow-up cystoscopies had been performed at least at 3, 9, and 15 months, then annually until

Figure 2. Tissue microarrays for cyclin E analysis. **A:** Overview of a bladder cancer array section (H&E). **B:** CCNE amplification; cells show a massive increase in the number of their red CCNE as compared to the green centromere 17 signals. **C:** Nonamplified tumor with two copies of CCNE and centromere 17 per cell. **D–G:** Cyclin E immunohistochemistry: in normal urothelium expression was limited to the umbrella cell layer (**D**). Cyclin E immunostained tumors with no (**E**), weak (**F**), and strong cyclin E positivity (**G**).

the endpoint of this study (recurrence, last control). To include a patient for analyses of time to progression longer intervals between controls were accepted if the last follow-up control ruled out progression. According to these criteria 528 patients were evaluable for time to

recurrence and 559 for time to progression. The medium follow-up period was 53 months (range, 1 to 188 months). Intravesical treatment had been performed in 337 of these patients (mitomycin in 154, BCG in 159, epirubicin in 65, adriablastin in 10). Recurrences were defined as

cystoscopically visible tumors. Tumor progression was defined as the presence of muscle invasion (stage pT2 or higher) in a subsequent biopsy.

Fluorescence in Situ Hybridization (FISH)

The tissue microarray sections were treated according to the Paraffin Pretreatment Reagent Kit protocol (Vysis, Downers Grove, IL) before hybridization. FISH was performed with a Spectrum Orange-labeled CCNE probe and a Spectrum Green-labeled chromosome 17 centromeric probe (CEP17) as a reference (Vysis). Hybridization and posthybridization washes were according to the LSI procedure (Vysis). Slides were then counterstained with 125 ng/ml 4',6-diamino-2-phenylindole in an antifade solution. FISH signals were scored with a Zeiss fluorescence microscope equipped with double-band pass filters for simultaneous visualization of Spectrum Green and Spectrum Orange signals. Amplification was defined as presence (in $\geq 5\%$ of tumor cells) of either >10 gene signals or tight clusters of at least five gene signals or more than three times as many CCNE than centromere 17 signals.

Immunohistochemistry

Standard indirect immunoperoxidase procedures were used for immunohistochemistry (ABC-Elite; Vector Laboratories, Burlingame, CA). Four different monoclonal antibodies for cyclin E, ie, C-19 (Santa Cruz Biotechnology, Santa Cruz, CA), Ab-2 (Neomarkers, Fremont, CA), NCL (Novocastra, Newcastle, UK), and Mob181 (DBS), were tested for optimal staining on sections from formalin-fixed, paraffin-embedded human placenta. Staining conditions were optimized to obtain a strong staining in a high fraction of the cytotrophoblast nuclei while stroma cells remained negative. Optimal staining could best be achieved for the antibody from DBS (Mob181, mouse, 1:120) after pressure cooker pretreatment for 5 minutes for antigen retrieval. Diaminobenzidine was used as a chromogen. The primary antibody was omitted for negative controls. Cyclin E staining was defined as presence of negative (total absence of staining), weakly positive (only a faint staining, or moderate to strong staining in ≤ 20 of tumor cell nuclei), and strongly positive (moderate or strong nuclear positivity in $>20\%$ of cells).

Statistics

All tissue samples on the array were used for comparisons of amplification and overexpression of cyclin E. Only the first biopsy was used for further statistical analyses in patients having more than one tumor in the array. Contingency table analysis and chi-square tests were used to study the relationship between histological tumor type, grade, stage, and cyclin E expression/amplification. Survival curves were plotted according to Kaplan-Meier.²⁴ A log rank test was applied to examine the relationship between grade, stage, or cyclin E alterations and tumor recurrence, progression, or survival. Patients with pTa/

pT1 tumors were censored at the time of their last clinical control showing no evidence of disease or at the date when cystectomy was performed. Patients with pT2-4 carcinomas were censored at the time of their last clinical control or at the time of death if they died from causes not related to their tumor.

Results

CCNE Amplification

FISH analysis was successful in 1,561 of 2,317 arrayed tumors. FISH-related problems (weak hybridization, background, tissue damage) were responsible for approximately one third of the noninformative cases, whereas the rest of the reasons for analysis failure were because of the array technology, such as missing samples or too few tumor cells in a tissue spot. CCNE amplification was detected in 30 of 1,561 interpretable tumors. Examples of CCNE-amplified and CCNE-nonamplified tumors are shown in Figure 2, B and C. The association of CCNE amplification with the histological tumor type, stage, and grade is given in Table 1. The comparison of different histological subtypes was limited to stage pT2-4 carcinomas. This was done because nontransitional cell carcinomas are almost always detected at this stage and an inclusion of pTa/pT1 tumors (almost always TCC) could have introduced a stage bias into the comparison of different histological tumor types. Besides TCC, CCNE amplification was only found in small-cell carcinomas. Within the group of TCC, by far the most common bladder cancer subtype, CCNE amplification was significantly associated with high tumor grade and advanced stage.

Cyclin E Protein Expression

In two normal urothelium samples from patients without a bladder cancer history, cyclin E positivity was only detected in the umbrella cell layer (Figure 2D). Among 1,873 interpretable tumors, 1,286 (68.7%) were considered cyclin E-negative, 354 (18.9%) were weakly positive, and 233 (12.4%) strongly positive. Examples of cyclin E-positive and cyclin E-negative tumors are shown in Figure 2, E-G. There was a significant association between cyclin E expression (as defined by either weak or strong immunostaining) and amplification ($P < 0.0001$). Expression was found in 24 of 29 amplified (82.8%) but in only 450 of 1,358 nonamplified (33.1%) tumors for which both FISH and immunohistochemistry data were available. There were no significant differences in the expression pattern between tumors of different histological subtypes (Table 1). Within transitional cell carcinomas, cyclin E expression was significantly more frequent in pT1 than in pTa tumors ($P < 0.0001$) but then decreased from pT1 to pT2-4 ($P < 0.0001$; Table 1). At the same time, there was a significant increase in the positivity rate from grade 1 to grade 3 tumors ($P < 0.0001$; Table 1).

Table 1. Cyclin E Amplification/Overexpression and Tumor Phenotype

	Cyclin E FISH			Cyclin E immunohistochemistry			
	<i>n</i>	Amplified cases (%) [*]	<i>P</i> value [†]	<i>n</i>	Strongly positive (%) [*]	Weakly positive (%) [§]	<i>P</i> value [†]
Stage [‡]							
pTa	522	1.2	0.0022	626	9.9	12.3	<0.0001
pT1	281	2.5		338	19.8	25.7	
PT2–4	283	3.5		344	7.3	22.1	
Grade [‡]							
G1	149	0.7	0.0002	187	2.1	7.5	<0.0001
G2	514	0.8		614	9.9	16	
G3	478	4		567	18.3	25.2	
Stage/grade [‡]							
pTaG1	149	0.7	0.0005	187	2.1	7.5	<0.0001
pTaG2	318	0		373	9.1	14.8	
pTaG3	55	9.1		66	36.4	12.1	
pT1G2	117	0.9		139	13.7	17.3	
pT1G3	164	3.7		199	24.1	31.7	
pT2–4G2	65	4.6		86	5.8	19.8	
pT2–4G3	218	3.2		258	7.8	22.9	
Histologic type [§]							
TCC	356	3.1	0.96	439	7.8	21.2	0.61
Squamous	44	0		53	3.8	20.8	
Adeno	6	0		8	12.5	25	
Small cell	20	5		22	9.1	9.1	
Sarcomatoid	17	0		15	0	40	

*For definition see text.

†Contingency table analysis.

‡Only first biopsies of patients with TCC included.

§Only muscle invasive carcinomas (pT2–4) included.

Cyclin E Involvement and Prognosis

The prognostic significance of CCNE amplification could not be formally assessed because only 21 patients with CCNE amplification had clinical follow-up information. Eleven of these tumors were stage pTa/pT1. One of them progressed but none of the patients died from disease. Strong cyclin E expression was associated with a longer tumor-specific survival when all patients were included in the analysis ($P = 0.0011$; Figure 3A). However, cyclin E provided no prognostic discrimination among pTa tumors, neither in terms of recurrence ($P = 0.96$; Figure 3B) nor in progression ($P = 0.96$). The same was true for pT1 tumors, $P = 0.93$ for recurrence and $P = 0.40$ for tumor progression (Figure 3C) as well as for pT2–T4 tumors and the tumor-specific survival ($P = 0.25$; Figure 3D).

Discussion

This study on CCNE amplification and overexpression in a large series of bladder tumors illustrates, how the tissue microarray technology substantially facilitates evaluation of new molecular markers not only for their involvement at various stages of cancer development and progression,^{21,25} but also for their clinical and prognostic significance. The tissue microarray constructed for this project contained samples from 2,317 different bladder carcinomas, covering all stages of cancer progression in this organ, and making it possible for us to study a substantially higher number of tumor specimens by FISH and immunohistochemistry than in any previous report on bladder cancer. Currently, this bladder cancer microar-

ray, constructed on five regular-sized paraffin blocks, represents the largest organ-specific tissue microarray resource that we have developed. Despite the very large number of tumors included on the microarray, the immunohistochemical interpretation could be done in ~4 hours and the FISH scoring in ~6 days.

In addition to speed, the other significant advantage of the tissue microarrays is that it makes it possible to retrieve dozens of punched samples from each donor block without significantly damaging it. This enables generation of multiple replicate tissue microarray blocks, each having samples from the same tumor specimens at identical coordinates. Depending on the thickness of the original tissue, between 100 and 400 sections can be cut from each array block. Therefore, thousands of replicate tissue microarray slides can be generated, each being stained with different probes or antibodies, as well as being evaluated for the morphological representativity. This approach enables extensive analyses of even small primary tumors, thereby preserving often unique and precious tumor specimens. The limitation of the tissue microarray is that it may not detect molecular alterations that are only present in a small fraction of the cancer cells, especially as foci of aberrant cells. However, our analysis of the prognostic impact of ER, PR, and HER-2 expression in breast cancer indicates that for homogeneously staining biomarkers, even a single 0.6-mm diameter biopsy specimen, as used in the tissue microarrays, is very representative of the entire tumor and is appropriate for the analysis of the prognostic significance of such a marker (Torhorst, unpublished data). The tissue microarray technology should be viewed as a method for

Figure 3. Cyclin E immunostaining and prognosis. The curves show the associations of cyclin E expression with tumor-specific survival in all stages (**A**), recurrences in pTa tumors (**B**), progression in pT1 carcinomas (**C**), and tumor-specific survival in pT2–4 TCC (**D**).

the analysis of molecular alterations at the population level, not as a means of extensively analyzing any single cancer specimen. Tissue microarray results can provide suggestions as to which molecular biomarkers should be further evaluated using conventional whole section analyses.

Our results suggest that CCNE could be one, but probably not the only target gene for the 19q13 amplification seen in bladder cancer by CGH. The frequency of gene amplification by FISH was only 2%, which is lower than the frequency of CGH positivity (4%) seen at 19q13. There was only a small overlap between the tumors an-

alyzed previously by CGH, and those analyzed here with the tissue microarray FISH. However, out of the eight 19q13 amplified tumors (by CGH) present on the array, only two had CCNE amplification by FISH (data not shown). It is therefore possible that other genes besides CCNE contribute to the malignant potential of 19q13 amplified tumors. Previously, CCNE amplification has been reported in 5% of breast cancer cell lines and in 21% of ovarian carcinomas, detected by Southern blot analysis.^{19,20} Other candidate oncogenes in the 19q13 area include AKT2 (19q13.1-13.2), FOSB (19q13.3), BCL3 (19q13), and SUPT5H (19q13.1-13.2).^{26–29} AKT2

has been previously found to be amplified and overexpressed in two of eight ovarian carcinoma cell lines and two of 15 primary ovarian tumors.²⁶

Despite of the possibility of involvement of other flanking genes at 19q13, there was a strong association between CCNE amplification and overexpression ($P < 0.0001$). There were only a few amplified tumors with no expression, but there were many tumors that showed weak or strong protein expression in the absence of DNA amplification. This suggests that other mechanisms than amplification are responsible for cyclin E expression in most cases. The only other study previously performed on cyclin E expression in bladder cancer³⁰ was done on 48 tumors representing different stages and grades using a polyclonal antibody. We also tested this antibody (Santa Cruz Biotechnology), but abandoned it, as it produced cytoplasmic staining and showed positivity in most normal bladder epithelium samples. Del Pizzo and co-workers³⁰ suggested that CCNE would mostly be down-regulated in advanced tumor stages. The cell cycle activating role of CCNE, as well as our results on the association of CCNE amplification and its association with overexpression are inconsistent with this hypothesis. However, both we and Del Pizzo and co-workers did find the same correlation between cyclin E expression and favorable patient prognosis. Our stratified analysis of tumors of different stages revealed that this prognostic relevance was mostly driven by expression differences between different tumor stages. Cyclin E expression was not associated with the clinically most relevant endpoints in pTa (recurrence), pT1 (progression), and pT2-4 tumors (tumor-specific survival).

In summary, CCNE is sometimes included in a bladder cancer amplicon at 19q13. The cell-cycle activating role of CCNE and its expression in amplified tumors suggest that CCNE is one of possibly many amplification target genes at 19q13. Overexpression of cyclin E by mechanisms other than amplification seems common and is characteristic to a subset of bladder carcinomas, especially at the stage of early invasion. The tissue microarray constructed in this study will be highly instrumental to rapidly examine these and other genes and proteins in a large series of bladder carcinomas. The tissue microarray strategy will remarkably speed up the evaluation of the clinical relevance of molecular changes in cancer.

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