Amplification/Overexpression of a Mitotic Kinase Gene in Human Bladder Cancer

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Background: **The mitotic kinase-encoding gene STK15/BTAK/AuroraA is associated with aneuploidy and transformation when overexpressed in mammalian cells. STK15 overexpression activates an unknown oncogenic pathway that involves centrosome amplification and results in missegregation of chromosomes. Because clinical prognosis and tumor aneuploidy are tightly linked in human bladder cancer, we examined whether increased STK15 copy number and protein levels are linked to aneuploidy in bladder cancers.** *Methods:* **STK15 protein was visualized by immunohistochemistry in 205 formalin-fixed, paraffin-embedded human bladder tumors. STK15 gene copy number was evaluated in 61 tumors by Southern blot hybridization and in 21 of these 61 tumors by fluorescence *in situ* hybridization (FISH). Copy numbers of chromosomes 3, 17, 20, and 21 were evaluated by FISH with chromosome-specific probes. STK15 expression levels were related to histologic grade, stage, and DNA ploidy of the tumors and to the patients' follow-up data. The chi-square test for association was used to analyze the relationship between STK15 expression and pathologic features. All statistical tests were two-sided.** *Results:* **Tumors with low levels of STK15 amplification (3–4 copies) showed minimal deviation in their chromosome copy number and diploid or near-diploid total nuclear DNA content. Tumors with higher levels of STK15 amplification (>4 copies) had a major increase of chromosome copy number and of their total nuclear DNA content, i.e., exhibited pronounced aneuploidy. Elevated expression of STK15 was strongly associated with parameters of clinical aggressiveness including high histologic grade (*P*<.001), invasion (*P*<.001), increased rate of metastasis (*P*<.001), and decreased metastasis-free (*P*<.001) and overall (*P*<.001) survival of patients with bladder cancer.** *Conclusion:* **STK15 gene amplification and associated increased expression of the mitotic kinase it encodes are associated with aneuploidy and aggressive clinical behavior in human bladder cancer. [J Natl Cancer Inst 2002;94:1320–9]**

Numeric chromosomal aberrations or aneuploidy are the most prevalent somatic alterations identified in solid human tumors *(1,2).* Aneuploidy has been proposed to drive tumor progression by enhancing genomic instability, resulting in alterations of cellular phenotypes *(3).* This hypothesis has gained credibility in view of recent reports showing that, during the process of transformation, human and rodent cell lines display elevated rates of chromosome instability and that aneuploidy precedes immortalization *(4–6).* The degree of chromosomal instability and tumor behavior are also strongly related. Tumors with minimal deviation in chromosome copy number, i.e., near-diploid, are clinically less aggressive than those with major deviation in chromosome copy number—that is, with increases in total nuclear DNA content resulting from extra copies of multiple chromosomes *(7–12).* Therefore, measurements of total nuclear DNA content by image analysis or flow cytometry are frequently used to assess the clinical aggressiveness of many solid human tumors *(13,14).*

During normal cell proliferation, centrosomes ensure equal segregation of chromosomes to the postmitotic daughter cells by organizing the bipolar mitotic spindle. Cancer cells, by contrast, frequently have multipolar mitotic spindles and various centrosomal anomalies, such as supernumerary centrosomes, centrosomes of abnormal size and shape, aberrantly phosphorylated centrosomal proteins, and prematurely split centrosomes *(15–21).* It is conceivable that such abnormalities would disrupt normal chromosomal segregation, producing aneuploid cells.

The molecular mechanisms of centrosome function, including the pathways through which they regulate chromosomal segregation, remain to be elucidated. However, a recently cloned and characterized mitotic kinase-encoding gene, STK15/BTAK/AuroraA *(22,23)*, has been implicated in the regulation of centrosome duplication and has been reported to be frequently amplified/overexpressed in human tumors, suggesting that the STK15 protein may represent a critical regulatory component of chromosomal segregation that can cause aneuploidy and transformation *(23,24).*

STK15 is a member of the serine/threonine kinase family that includes the prototypic yeast IPL1 and *Drosophila* aurora kinases, as well as other mammalian and nonmammalian aurora kinases involved in regulating chromosomal segregation *(25,26).* In yeast, the temperature-sensitive ipl1 gene mutants missegregate chromosomes, resulting in polyploidy *(27).* Loss of function of aurora kinases in *Drosophila* inhibits centrosome separation, leading to formation of abnormal mitotic spindles *(26).* STK15 has high homology with other aurora kinases *(28).*

In the present study, we attempted to relate the levels of STK15 gene amplification and/or overexpression with the degree of aneuploidy and the biologic behavior of human bladder tumors *in vivo.* Bladder tumors are among the most common human cancers, with approximately 55 000 new cases detected each year in the United States. Bladder cancers, which represent

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a group of tumors with diverse morphologic and clinical behavior, exhibit one of the strongest relationships seen in any cancer between clinical aggressiveness and degree of aneuploidy *(29–31)*. Bladder cancers arise from at least two distinct, albeit sometimes overlapping, pathways that lead to the development of papillary and solid or nonpapillary tumors *(31,32)*. Most superficially growing, low-grade papillary lesions are diploid or near-diploid. Although they often recur, they are unlikely to invade the bladder wall and metastasize. By contrast, virtually all nonpapillary tumors are highly aneuploid and have a strong propensity to invade the stroma and metastasize. Superficial bladder tumors that are aneuploid are also likely to progress to invasive clinically aggressive carcinomas, which may metastasize. In this study, we used human bladder tumors as a model system to determine whether higher than normal STK15 gene copy number and/or protein expression levels are linked to aneuploid, clinically aggressive variants of bladder cancer.

Materials and Methods

Tumor Samples

These studies were conducted on a retrospective cohort of 205 patients with urinary bladder carcinoma who were treated and/or followed at The University of Texas M. D. Anderson Cancer Center. All patients had primary urothelial carcinomas of the bladder, and none of the patients whose tumor samples were used in this study had received prior chemotherapy or radiotherapy. The patients ranged in age from 37 years to 85 years (mean 71 years). One hundred fifty-four patients were male and 51 were female. Fresh tumor tissues were obtained by transurethral resection or from cystectomy specimens and were stored at –70 °C. Tumor samples and follow-up data were obtained according to The University of Texas M. D. Anderson Cancer Center Institutional Review Board-approved laboratory protocol.

For processing, the frozen tissue samples were sectioned, stained with hematoxylin–eosin, and evaluated microscopically. Portions of tissue containing almost pure tumor cells were microdissected from the frozen block and used for preparation of touch prints *(31)*. One portion of the tumor tissue was fixed in formalin, embedded in paraffin, and sectioned (4 μm thick) for use in microscopic assessment of the tumor and immunohistochemical studies of STK15 expression; another was used for DNA extraction. The tumors were classified according to the three-tier World Health Organization histologic grading system, growth pattern (papillary versus nonpapillary), and DNA ploidy *(33)*. The depth of invasion was recorded according to the TNM (Tumor–Node–Metastasis) staging system *(34)*. Stage T_1 (lamina propria invasion) has been divided into T_{1a} (no muscularis mucosae invasion) and T_{1b} (muscularis mucosae invasion), which have a clinically significant higher risk of progression *(35)*. Consequently, the tumors were dichotomized into superficial (T_a–T_{1a}) and invasive (T_{1b} and higher) groups. Of 205 bladder tumors, 104 were classified low-grade (grade 1–2) and 101 were high-grade (grade 3).

Paraffin-embedded tumor tissue from all 205 patients was used to analyze the relationship between STK15 expression and pathologic parameters of the tumors and clinical follow-up data. For 61 of the tumor samples, extracted genomic DNA was available for Southern blot hybridization analysis of STK15 gene copy number. For 21 of these 61 tumor samples, touch prints from the fresh tumor samples were used for quantitative fluorescence *in situ* hybridization (FISH) of the STK15 gene and selected chromosomes (3, 17, 20, and 21) copy number analysis.

For DNA ploidy measurements, touch prints of tumor tissue and slides of a diploid standard (human peripheral blood lymphocytes from healthy volunteers) were fixed in 10% neutral buffered formalin and stained with the Feulgen reaction *(31)*. Total nuclear DNA content in individual tumor cells was measured by visual inspection with the SAMBA 4000 (Ampersand Medical, Chicago, IL) computer-assisted image analysis system. Frequency histograms of DNA content were generated on the basis of measurements from 200 to 300 morphologically identifiable intact tumor nuclei. The DNA index was calculated as the ratio of the mean nuclear DNA content of the tumor cells to the mean nuclear DNA content of peripheral blood lymphocytes. Tumors with DNA indices ranging from 0.9 to 1.2 were classified as diploid/near-diploid; tumors in which more than 20% of cells formed a distinct peak on the histogram with a DNA index of greater than 1.2 were classified as aneuploid. The aneuploid bladder tumors with DNA index greater than 1.2 have more aggressive clinical behavior than diploid/near-diploid tumors and have a higher risk for progression and development of distant metastases *(31)*.

Immunohistochemical Analysis of STK15 Expression

A rabbit polyclonal anti-STK15 antibody developed in our laboratory and raised against a carboxy terminal peptide was used for immunohistochemical localization of STK15 protein expression in the tumor tissues as described *(23,36)*. Slides of formalin-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated, and then digested with 0.05% trypsin. After blocking in 15% (vol/vol) normal goat serum, the slides were first incubated at room temperature with anti-STK15 antibody (diluted 1 : 40 in phosphate-buffered saline [PBS]) and then with biotinylated goat anti-rabbit immunoglobulin G (IgG) antibody (Vector Laboratories, Burlingame, CA) (diluted 1 : 200 in PBS). The bound antibodies were detected by the avidin–biotin–peroxidase complex assay (Vector Laboratories) and were visualized with the chromogen amino-ethyl carbazole (Sigma Chemical Co., St. Louis, MO). Semiquantitative evaluation of the staining intensity for immunohistochemical localization of STK15 was performed by three investigators (B. Czerniak, S. Sen, F. Vakar-Lopez) independently in a blinded manner. The intensity of immunohistochemical staining was scored on a three-point scale as follows: –, no detectable expression; +, weak to moderate expression; ++, strong expression. The tumors were classified as positive for STK15 overexpression when more than 20% of the cells showed strong cytoplasmic expression, as has been conventionally done in other studies *(35)*. Paraffin-embedded tumor sections of a human breast carcinoma cell line (MCF7) with known eightfold amplification of the STK15 gene and overexpression of the STK15 protein identified in our previous study were used as positive controls *(36)*. The baseline expression level of STK15 protein in normal urothelium was determined by using sections of paraffin-embedded normal ureters obtained from nephrectomy specimens.

STK15 Copy Number Analysis by Southern Blot Hybridization

Southern blot hybridizations using the STK15 cDNA probe developed in our laboratory *(36)* were performed on *Bam*HI-digested total genomic DNA (10 μg per sample) from 61 tu-

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mors, according to a previously described standard protocol *(23,36).* The STK15 cDNA probe identifies three DNA bands, of 13.5 kb, 9.4 kb, and 7.9 kb. We measured the signal intensity of the 13.5-kb band, representing the STK15-specific sequence, and the signal intensity of the 7.9-kb band, representing an unamplified STK15–pseudogene sequence, by PhosphorImager analysis using a Storm 860 system (Molecular Dynamics, Sunnyvale, CA). To assess the STK15 gene copy number, the ratio of the signal intensities of these two bands for each tumor sample was compared with the ratio obtained from normal lymphocyte DNA that was run on the same gel. *Bam*HI-digested MCF7 DNA was used as a positive control.

STK15 Gene Amplification Detected by FISH

FISH analyses were performed on 21 touch prints of 61 frozen tumor tissues. The STK15 probe contained in a 3B23 bacterial artificial chromosome clone identified in our laboratory was co-hybridized with D20S894, a chromosome 20p arm-specific probe (provided by Dr. Joe Gray, University of California, San Francisco) as previously described *(23).* Briefly, the STK15 probe was labeled with digoxigenin, and the D20S894 probe was labeled with biotin. After hybridization to the tumor tissues, the bound probes were detected with fluorescein isothiocyanate-conjugated mouse anti-digoxigenin antibody (Sigma Chemical Co.) and Cy3-labeled streptavidin (Amersham Pharmacia, Piscataway, NJ), respectively.

Chromosome 3, 17, and 21 copy numbers in interphase tumor nuclei were analyzed in the same 21 tumor samples by FISH with chromosome-specific centromeric probes (Vysis, Abbott Park, IL). Quantitative FISH analysis was performed with fluorescence microscopy by counting hybridization signals in 100–200 intact tumor nuclei. Normal human urothelial cells from nephrectomy specimens resected for renal carcinoma were used both to test the performance of FISH probes in normal human diploid tissue and to serve as a negative control. All images were collected on a Leica DMRXA fluorescence microscope equipped with a high-resolution, high-sensitivity Hamamatsu CCD camera (model No. C4742-95-12; Hamamatsu Photonics K.K., Sunayama-CHO, Hamamatsu-city, Japan) and were digitally processed by using Openlab imaging software (Improvision, Boston, MA). Complete quantitative data on STK15 and chromosome 3, 17, 20, and 21 copy numbers were obtained for 16 of the 21 tumors. Only partial quantitative FISH data could be obtained from the remaining five tumors because of technical problems.

Cell Culture and Western Blot Analysis

Nu201 normal human urothelial cells were derived in our laboratory from primary cultures of normal ureters removed during nephrectomy for renal carcinoma *(37).* The UC14 cell line was derived in our laboratory from a highly aneuploid grade 3 nonpapillary urothelial carcinoma of the bladder *(38,39).* Cells were cultured in serum-free keratinocyte medium (Life Technologies Inc., Carlsbad, CA) containing 0.04 *M* calcium.

The specificity of the anti-STK15 antibody was verified by western blot analysis of total protein lysates prepared from Nu201 and UC14 cells grown *in vitro.* Subconfluent cell monolayers were lysed in extraction buffer as described *(23).* Lysates containing approximately 100 μg of total cell protein were loaded onto 125% sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS–PAGE)–10% Tris–glycine gels (NOVEX; Invitrogen, Carlsbad, CA), subjected to electrophoresis, and transferred by electroblotting (BioRad, Hercules, CA) to nitrocellulose membranes. The membranes were first blocked in 5% bovine calf serum (BCS) in 10 m*M* Tris, 150 m*M* NaCl, 0.05% Tween 20 (TBST) and then incubated either with the affinity-purified rabbit polyclonal anti-STK15 antibody (1 : 400 dilution in 0.5% BCS in TBST) or with the antibody preincubated with the cognate peptide against which it was raised (0.1 mg of antibody to 1 mg of peptide). The STK15 protein band was detected with a peroxidase-conjugated anti-rabbit secondary antibody (Amersham) and visualized with the use of the enhanced chemiluminescence (ECL)-Plus (Amersham) detection system. Finally, western blots were stripped and reincubated with a primary mouse monoclonal anti-β-actin antibody (Amersham) (1 : 300 in 5% BCS in TBST) and a secondary peroxidase-conjugated anti-mouse antibody (Amersham) to normalize the amount of protein loaded in each lane.

Statistical Analysis

The relationship between expression and amplification levels of STK15 and various clinicopathologic parameters was analyzed using chi-square and Student's *t* tests. Continuous measures, such as STK15 expression, were analyzed both with and without logarithmic transformation, with similar results for each. Sample sizes were large enough to permit parametric tests. Where appropriate, the associated nonparametric tests were also performed and yielded similar results to the parametric tests. The expression levels of STK15 in relation to time between diagnosis and recurrence, to time between diagnosis and metastasis, and to overall survival were tested by Kaplan–Meier analysis by the Gehan–Wilcoxon and Peto log-rank tests *(40).* Correlation between STK15 and chromosome copy number estimated by FISH was analyzed by the Spearman's rank correlation coefficient *(41).* Statistical analyses were performed by using Statistica software (StatSoft, Inc., Tulsa, OK) and Statistical Package for Social Scientists Software (SPSS Inc., Chicago, IL). In all statistical analyses, two-sided P values $\leq .05$ were considered statistically significant.

RESULTS

This study was designed to investigate whether increased STK15/BTAK/AuroraA copy number and protein levels are linked to chromosomal instability and aneuploidy in human bladder cancer. STK15 copy number was initially analyzed by Southern blot hybridization of genomic DNA from tumor tissues representing various pathologic subsets, grades, and stages of the disease (representative results are shown in Fig. 1, *a*). To determine whether STK15 was amplified, we measured the ratio of the hybridization signal intensities of the 13.5-kb and 7.9-kb bands (Fig. 1, *a*) for each tumor and normalized this ratio to the ratio of the hybridization signal intensities of the same bands obtained from normal peripheral blood lymphocytes. The STK15 gene was amplified two- to sevenfold in 35% of high-grade (grade 3) invasive urothelial carcinomas with pronounced aneuploidy (data not shown). Examples of STK15 amplification identified by Southern blot hybridization are shown in Fig. 1, *a* (lanes 15–19). No STK15 amplification was identified in low-grade (grades 1–2) superficial papillary tumors that were diploid/near-diploid (i.e., that showed only a minor increase in their total DNA content, with a DNA index of 1–1.2).

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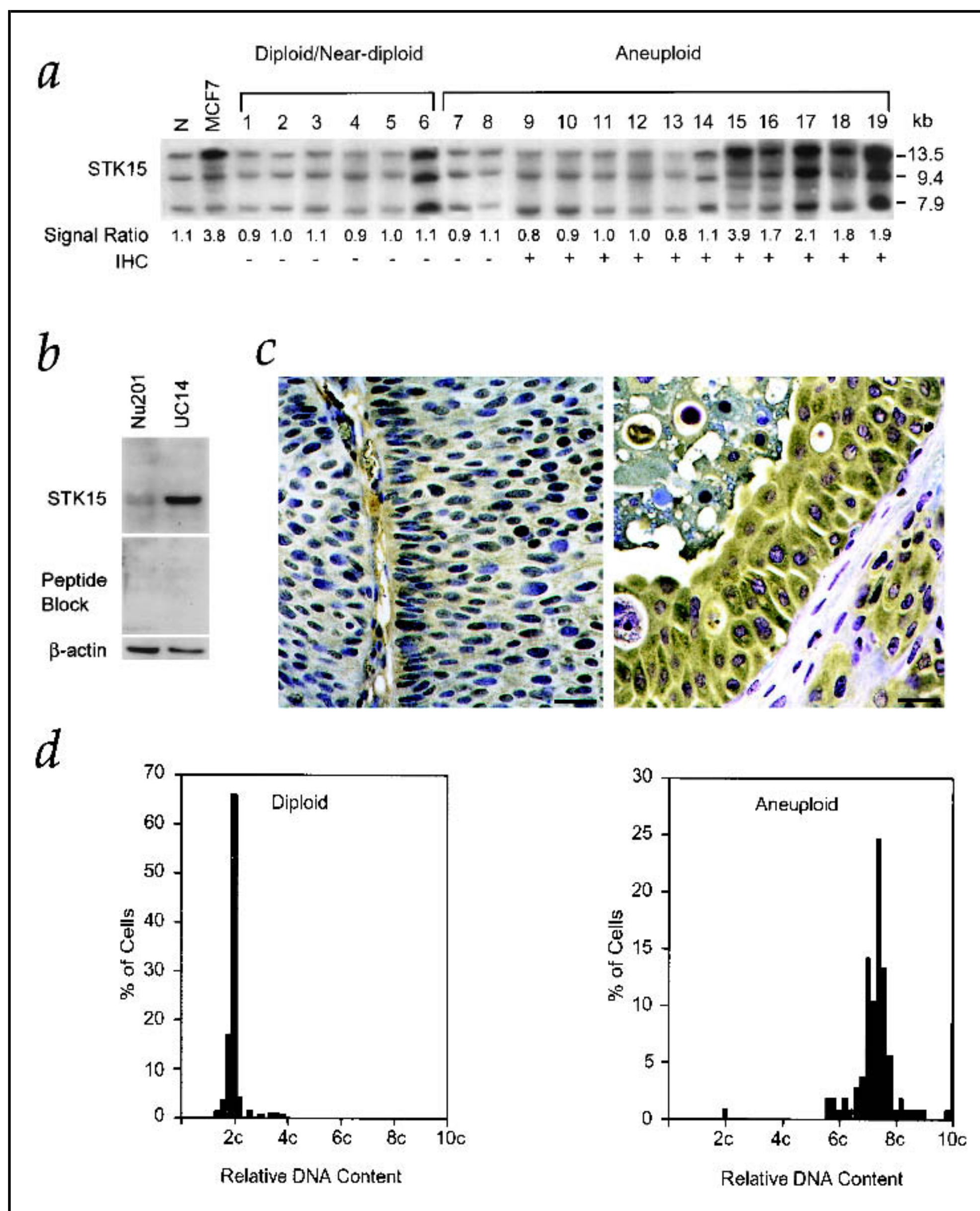

Fig. 1. Analysis of STK15 copy number, protein expression, and DNA ploidy in human bladder cancers. ***a***) Genomic DNA was isolated from 61 human bladder cancers, digested with the restriction enzyme *Bam*HI, and analyzed for STK15 gene amplification by Southern blot hybridization. Representative results from 19 tumors are shown. Differences in signal intensity (signal ratio) between the top band of approximately 13.5 kb, which represents STK15-specific sequences, and the bottom band of approximately 7.9 kb, which represents unamplified sequences of a pseudogene, were normalized to the signal ratio from *Bam*HI-digested normal human peripheral blood lymphocyte DNA (**lane N**) and used to estimate gene copy number. The signal ratios are given below each lane. *Bam*HI-digested DNA from the MCF7 human breast tumor cell line, which has a high level of STK15 amplification *(36),* was used as a positive control. Tumor case numbers are shown above each lane. The size of each DNA band (in kb) is shown to the right of the figure. Immunohistochemical detection of overexpressed STK15 protein is indicated by a **+** below each lane. After staining the samples with the Feulgen reaction *(13)*, the DNA content was determined by generating frequency histograms with the SAMBA 4000 computer-assisted image analysis system. DNA indices were calculated by comparing the DNA content of the tumor sample with the DNA content of a diploid standard (human peripheral blood lymphocytes from healthy volunteers). Tumors with DNA indices ranging from 0.9 to 1.2 were classified as diploid/near-diploid; tumors in which more than 20% of cells formed a distinct peak on the histogram with a DNA index of greater than 1.2 were classified as aneuploid. ***b***) Western blot analysis of STK15 expression in Nu201 normal human urothelial cells and in the aneuploid UC14 bladder cancer cell line using a rabbit polyclonal anti-STK15 antibody *(36).* The specificity of the antibody is seen from the detection of a single protein band in the **top panel** and the absence of any band in the **middle panel** in which the antibody was blocked by the peptide antigen against which the antibody was made. The presence of similar intensity β-actin bands in **both lanes** in the **lower panel** indicates that comparable amounts of proteins were loaded from the two cell types. ***c***) Representative immunohistochemical staining of STK15 expression in human bladder cancers with the rabbit polyclonal anti-STK15 antibody. The antibody–antigen complexes were detected by using the avidin–biotin–peroxidase method and amino-ethyl carbazole as the chromogen. The slides were counterstained with hematoxylin. **Left panel,** immunohistochemical staining of tumor No. 3, a diploid/near-diploid low-grade papillary urothelial carcinoma showing minimal expression of STK15. **Right panel,** immunohistochemical staining of tumor No. 15, an aneuploid high-grade invasive nonpapillary urothelial carcinoma showing strong overexpression of STK15. **Solid bar** indicates 50 μm. ***d***) DNA histograms generated by measurements of total nuclear DNA content of Feulgen-stained human bladder cancer cells of diploid/near-diploid tumor No. 3 (**left panel**) and aneuploid tumor No. 15 (**right panel**).

To determine STK15 protein expression levels in tumor tissues, we used a specific anti-STK15 antibody (Fig. 1, *b*) to perform immunohistochemistry (Fig. 1, *c*). The semiquantitative expression levels were subsequently related to the total nuclear DNA contents (ploidy) (Fig. 1, *d*). The STK15 protein was overexpressed, defined as strong positive staining in more than 20% of tumor cells in all of the 10 bladder tumors identified by Southern blotting as having increased STK15 gene copy number (data not shown) and in an additional 46% of the high-grade aneuploid carcinomas with no evidence of gene amplification (data not shown). In low-grade (grades 1–2) superficial papillary tumors that were diploid/near-diploid (Fig. 1, *d,* left panel), minimal levels of STK15 expression were typically detected (Fig. 1, *c,* left panel). In general, there was no difference in the intensity of staining among normal urothelium, bladder wall stromal tissue, and low-grade superficial papillary tumors (data not shown).

These data suggested that STK15 protein overexpression was associated with high-grade aneuploid tumors and could occur in the absence of gene amplification. However, it is possible that STK15 gene amplification occurred in only a proportion of the tumor cells and was not detected by Southern blot analysis. It is also possible that an increase in STK15 gene copy number was the result of multiple copies of chromosome 20, on which the

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STK15 gene resides, and not *bona fide* amplification of the STK15 gene alone. To distinguish between these possibilities, dual-color FISH analysis on bladder tumor tissue touch prints was performed.

For this analysis, tumor cells were co-hybridized with an STK15-containing genomic bacterial artificial chromosome probe and a probe for the p arm of chromosome 20. To assess the relationship between levels of STK15 amplification and overall aneuploidy, sequential touch prints from the same tumors were hybridized with centromeric probes for chromosomes 3, 17, and 21. These studies allowed us to identify the exact levels of STK15 gene amplification and to detect small populations of aneuploid cells and/or gene amplification in otherwise predominantly diploid tumor tissues. The STK15 gene amplification estimates by Southern blotting and FISH, the immunohistochemical gene expression levels, and the degree of aneuploidy measured as the DNA index of total nuclear content in the 16 bladder tumors for which we could obtain these data are listed in Table 1. The patterns of signal distribution in individual nuclei (representative images are shown in Fig. 2, *a–d*) exemplify the various STK15 gene copy number and chromosome 20 ploidy alterations. The presence of two discrete signals for each probe reflected normal chromosome 20 disomy and a diploid STK15 gene copy number (Fig. 2, *a*). More than two STK15 signals in the presence of two signals for the chromosome 20p-arm probe indicated that the gene was amplified in tumor cells (Fig. 2, *b* and *c*). The same number of signals, but more than two for both probes, indicated polysomy for chromosome 20 (Fig. 2, *d*).

Using the patterns of signal distribution, the 16 tumors were classified according to their levels of STK15 gene amplification and chromosomal ploidy status (Fig. 2, *e* and *f*). We found that an increase of STK15 gene copy number was ubiquitous in bladder tumors: at least a minimal level (three to four copies) of gene amplification involving a fraction of the tumor cell population could be identified in every bladder cancer tested. There were, however, striking differences in the levels of STK15 gene amplification between diploid/near-diploid and highly aneuploid tumors. In diploid/near-diploid tumors, approximately 40%–50% of the tumor cells contained three to four copies of the STK15 gene, and only about 2%–20% of the tumor cells contained more than four copies of the STK15 gene (Fig. 2, *e*). By contrast, highly aneuploid tumors were characterized by the presence of at least 20% of the tumor cells containing more than four copies of the STK15 gene (Fig. 2, *e*). Such relationships were observed in all (9 of 10) but one of the tested cases in which strong overexpression of STK15 was associated with pronounced aneuploidy but not with a major increase in gene copy number (Fig. 2, *e* and *f,* case 16). This case supports our previous observations implicating another mechanism involved in increased STK15 gene expression in the absence of STK15 gene amplification *(23).*

For some tumors, the prominent tumor cell population contained an increased STK15 copy number, primarily due to polysomy of chromosome 20 (Fig. 2, *d*). Overall, high levels of STK15 amplification were associated with a major increase in total nuclear DNA content and chromosomal copy number (Fig. 2, *g* and *h*). By contrast, tumors with low levels of STK15 amplification showed only a minimal increase in chromosomal copy number and total nuclear DNA content (Fig. 2, *g* and *h*). Overall, these data suggest that the degree of aneuploidy is proportional to the level of STK15 gene amplification (Spearman's correlation coefficient, $r = .82$) (Fig. 2, *i*).

Table 1. Comparison of STK15 gene copy estimates by fluorescence *in situ* hybridization (FISH) and Southern blotting, immunohistochemical expression levels of STK15 protein, and DNA ploidy in 16 bladder tumors classified according to their histologic grade, pathogenetic subset, and stage*

	STK15 copy No.						
	% of cells by FISH						
Case No.	3–4 copies	>4 copies	By Southern blotting†	STK15 protein expression‡	DNA ploidy§ (DNA index)	Histologic grade and pathogenetic subset‖	Stage¶
1	42	2	–	–	1.2	2(P)	T_{1a}
2	32	2	–	–	1.2	2(P)	T_a
3	36	4	–	–	1.1	2(P)	T_a
4	38	7	–	–	1.1	2(P)	T_2
5	50	13	–	–	1.2	2(P)	T_{1a}
6	47	18	–	–	1.3	2(P)	T_{1a}
7	47	22	–	–	2.2	3(NP)	T_3
8	54	24	–	+	2.8	3(NP)	T_3
9	55	32	–	+	2.9	3(NP)	T_4
10	50	55	–	+	2.3	3(NP)	T_3
11	36	60	+ (2.5)	+	2.1	3(NP)	T_3
12	25	67	+ (2.5)	+	4.6	3(NP)	T_3
13	22	70	+ (3)	+	3.1	3(P)	T_3
14	10	83	+ (3)	+	3.7	3(NP)	T_3
15	14	86	+ (5)	+	3.2	3(P)	T_2
16	63	2	–	+	1.7	3(NP)	T_3

*P = papillary; NP = nonpapillary.

†+ indicates increased gene copy number documented by Southern blotting. Numbers in parentheses designate the increased gene copy number based on densitometric analyses of STK15 gene-specific fragment in reference to an unamplified internal control genomic DNA fragment detected on the same Southern blots. – indicates that no increased gene copy number could be documented by Southern blotting.

‡+ indicates immunohistochemical evidence of STK15 overexpression in at least 20% of tumor cells. – indicates that no overexpressed STK15 protein level could be documented in at least 20% of tumor cells.

§DNA index = the ratio of total nuclear DNA content of tumor cells to that of a diploid standard. The tumors with a DNA index of 0.9–1.2 were classified as diploid/near-diploid. The tumors with a DNA index of greater than 1.2 were classified as aneuploid.

‖Histologic grading is according to World Health Organization (WHO) grading system for bladder tumors *(33).*

¶Tumors were staged according to the Tumor–Node–Metastasis (TNM) classification of malignant tumors with $T_{1a–b}$ substaging *(34,35).*

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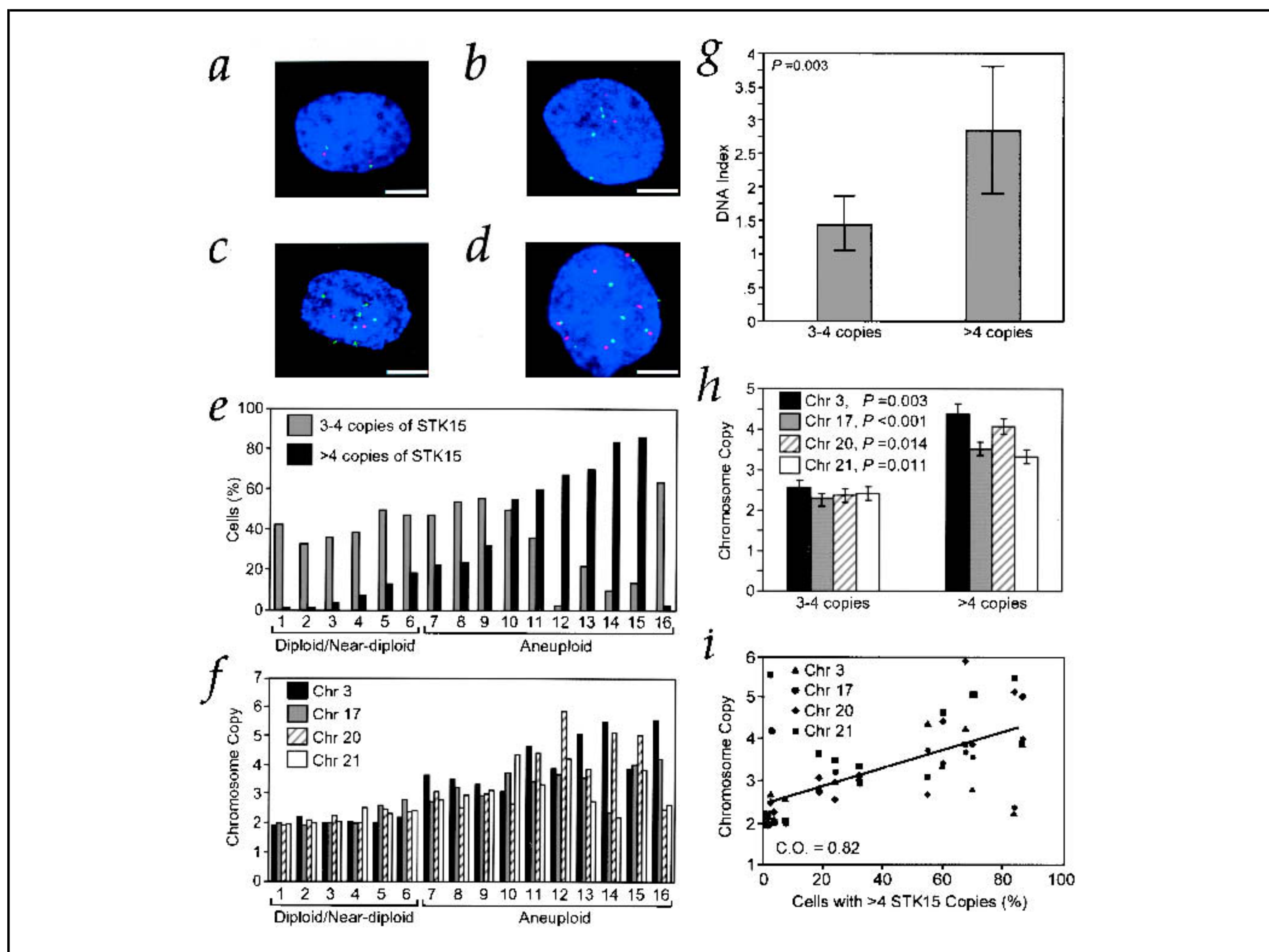

Fig. 2. Quantitation of the copy number of the STK15 gene and chromosomes 3, 17, 20, and 21 in 16 human bladder cancers by fluorescence *in situ* hybridization (FISH). ***a–d***) Examples of dual-color FISH, demonstrating localization of the STK15 probe (**green** signals) and a chromosome 20p arm-specific probe (**red** signals). ***a***) Diploid copy number of STK15 in a normal urothelial cell. ***b***) Low level of STK15 gene amplification in tumor No. 5, a diploid/near-diploid urothelial carcinoma cell containing four copies of the STK15 gene and two copies of the p arm-specific probe. ***c***) High level of STK15 gene amplification in tumor No. 10, a urothelial carcinoma cell containing seven copies of STK15 and only two copies of the p arm-specific probe. ***d***) Polysomy of chromosome 20 in tumor No. 14, a urothelial carcinoma cell containing multiple copies of STK15 and of the p arm-specific probe. ***e***) Quantitative FISH analysis of STK15 copy number in 16 human bladder cancers. FISH signals were counted in 100–200 intact tumor nuclei. The percentage of cells showing low (3–4 copies) and high (>4 copies) amplification levels of STK15 in the individual cases are shown. In diploid/near-diploid tumors, approximately 40%–50% of the cells have a low level of STK15 gene amplification and less than 20% have a high level. In the majority of aneuploid tumors, more than 20% of the cells have high levels of STK15 amplification. ***f***) FISH analysis of chromosomes 3, 17, 20, and 21 copy number using centromere-specific probes of the same cases as shown in ***b–e.*** Tumors with low levels of STK15 amplification show no or minimal increases in chromosome copy numbers. Tumors with high levels of STK15 gene amplification show increases in chromosome copy numbers in all but tumor No. 16. In tumor No. 16, pronounced aneuploidy was not associated with a major increase in STK15 copy number, although this tumor showed strong overexpression of STK15 protein by immunohistochemical staining. ***g***) The analysis of degree of aneuploidy (DNA index; the ratio of the total nuclear DNA content of tumor cells to that of a diploid standard) in relation to STK15 gene amplification levels (3–4 copies versus >4 copies). Values greater than 1 indicate an increase in total nuclear content and reflect the degree of aneuploidy. **Bars** represent mean DNA index ± 95% confidence interval (CI). Statistical significance of the difference between the two groups was determined by a two-sided Student's *t* test. ***h***) Degree of chromosomal instability (increased chromosome copy number) in relation to STK15 gene amplification levels (3–4 copies versus >4 copies). **Bars** represent mean chromosome copy number ± standard deviation. Statistical significance of the difference between the two groups for each chromosome was determined by two-sided Student's *t* tests. ***i***) Scatter plot showing the relationship among the proportion of cells with high STK15 gene amplification levels (>4 copies) and average chromosome copy number in 16 tumors studied by FISH. The regression line shows a predicted average chromosome copy number (Spearman's correlation coefficient = 0.82). **Solid bars** within FISH images shown in ***a–d*** indicate 5 μm. Note: Although the tumor numbers within the figure are consistent with each other, they do not correspond to the tumor numbers used in Fig. 1.

We next tested whether the correlation between STK15 gene amplification levels and aneuploidy could be used as a marker to identify clinically aggressive bladder cancers and whether immunohistochemical levels of STK15 expression were related to various clinicopathologic parameters and long-term follow-up data in a cohort of 205 patients with bladder cancer. Strong staining indicative of STK15 overexpression could be identified in approximately 50% of all bladder tumors and was strongly associated with high histologic grade, a nonpapillary growth pattern, invasion, and aneuploidy (Table 2), all indicators of highly aggressive human bladder cancer. STK15 overexpression was detected in 76.9% of invasive, high-grade aneuploid bladder

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Table 2. Relationship between STK15 overexpression and pathologic features of 205 human bladder cancers

Pathologic features	Tumors with STK15 overexpression, %*	*P* value†
Growth pattern		
Papillary	20.4	<.001
Nonpapillary	76.9	
Histologic grade‡		
Low grade (grade 1–2)	3.9	<.001
High grade (grade 3)	74.0	
Invasion		
Superficial (T_a–T_{1a})	7.5	<.001
Invasive (T_{1b}–T_4)	73.6	
DNA ploidy§		
Diploid/near-diploid	0	<.001
Aneuploid	76.6	

*STK15 overexpression was determined by immunohistochemical analysis. STK15 expression in at least 20% of tumor cells was considered overexpressed or positive.

†Chi-square tests of association.

‡Histologic grading is according to World Health Organization (WHO) grading system for bladder tumors *(33)*. Tumors were staged according to the Tumor–Node–Metastasis (TNM) classification of malignant tumors with $T_{1a–b}$ substaging *(34,35)*.

§DNA ploidy was determined by measuring the total nuclear DNA content of cells with the Feulgen reaction. A DNA index was generated by the ratio of total nuclear DNA content of tumor cells to that of a diploid standard. The tumors with a DNA index of 0.9–1.2 were classified as diploid/near-diploid. The tumors with a DNA index of greater than 1.2 were classified as aneuploid.

cancers but in only 7.5% of superficial bladder tumors. Overall, superficial noninvasive tumors that overexpressed STK15 protein were highly aneuploid and had a high likelihood of progression to invasive clinically aggressive disease. Tumors that overexpressed the STK15 protein had an increased propensity for distant metastasis and decreased patient survival (Table 3). Kaplan–Meier analyses revealed that patients whose bladder cancers overexpressed STK15 protein had dramatically decreased metastasis-free and overall survival compared with patients whose tumors did not overexpress STK15 protein (Fig. 3 and Table 4).

Discussion

Genetic instability has been suggested to be responsible for malignant transformation and tumor progression *(42–44)*. Recent studies have shown that several forms of genetic instability associated with distinct molecular mechanisms can be found in human tumors *(41–48)*. The most well characterized form of instability involves the inactivation of DNA mismatch repair genes, which is reflected in the expansion or shortening of microsatellite sequences; however, this form of instability can be documented in only a minor proportion of tumors, which typi-

Table 3. Relationship between STK15 overexpression and clinical behavior of 205 human bladder cancers

Clinical behavior	Proportion of patients, %	*P* value
Recurrence		
STK15 −	74	.057*
STK15 +	71	
Distant metastases		
STK15 −	13	<.001*
STK15 +	31	
5-y survival		
STK15 −	68	<.001*
STK15 +	19	
Overall survival		
STK15 −	73	<.001†
STK15 +	27	

*Chi-square tests of association.

†Kaplan–Meier two-sided log-rank test.

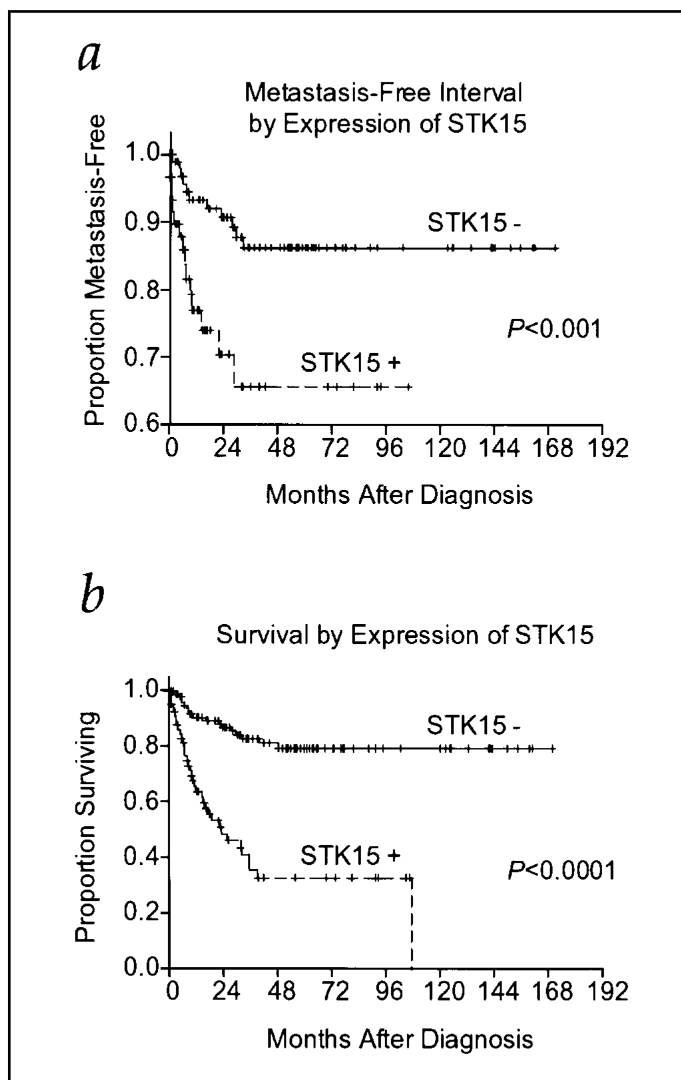

Fig. 3. Kaplan–Meier plots showing metastasis-free (***a***) and overall survival (***b***) of 205 patients with bladder cancer, grouped on the basis of tumor STK15 overexpression. STK15 expression was determined on the basis of immunohistochemical analysis in which tumors were classified as positive when more than 20% of the tumor cells showed strong cytoplasmic STK15 staining. *P* values are derived from two-sided log-rank tests. ***a***) For patients with no STK15 overexpression, the mean metastasis-free survival was 145.1 months (95% confidence interval [CI] = 132.8 months to 157.5 months); 1-year survival was 88.3% (95% CI = 81.6% to 94.9%); 3-year survival was 83.9% (95% CI = 75.7% to 92.0%). For patients with STK15 overexpression, the mean metastasis-free survival was 79.1 months (95% CI = 63.9 months to 94.3 months); 1-year survival was 74.2% (95% CI = 60.0% to 88.4%); 3-year survival was 69.1% (95% CI = 50.9% to 87.4%). ***b***) For patients with no STK15 overexpression, the mean overall survival was 133.5 months (95% CI = 119.8 months to 147.4 months); 1-year survival was 84.3% (95% CI = 77.0% to 91.5%); 3-year survival was 77.5% (95% CI = 68.7% to 86.4%). For patients with STK15 overexpression, the mean overall survival was 48.7 months (95% CI = 34.8 months to 62.5 months); 1-year survival was 51.9% (95% CI = 37.3% to 66.5%); 3-year survival was 35.8% (95% CI = 18.8% to 52.8%).

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Table 4. Summary of Kaplan–Meier analyses of STK15 expression levels, histologic grade, invasion, and DNA ploidy as predictors of recurrence, metastasis, and survival in patients with bladder cancer (n = 205)

	Recurrence-free survival	Metastasis-free survival	Overall survival
STK15 expression levels (no overexpression vs. overexpression)*	.578†	.001	<.001†
Histologic grade (low grade [grade 1–2] vs. high grade [grade 3])‡	.230	.007	<.001
Invasion (superficial [T_a–T_{1a}] vs. invasive [T_{1b}–T_4])	.784	.002	<.001
Aneuploidy (diploid/near-diploid vs. aneuploid)§	.018	.036	<.001

*STK15 overexpression was determined by immunohistochemical analysis. STK15 overexpression in at least 20% of tumor cells was considered positive.

†*P* value, two-sided log-rank test.

‡Histologic grading is according to World Health Organization (WHO) grading system for bladder tumors *(33).* Tumors were staged according to the Tumor–Node–Metastasis (TNM) classification of malignant tumors with T_{1a-b} substaging *(34,35).*

§DNA ploidy was determined by determining the total nuclear DNA content of cells with the Feulgen reaction. A DNA index was generated by determining the ratio of total nuclear DNA content of tumor cells to that of a diploid standard. A DNA index of 0.9–1.2 was classified as diploid/near-diploid. A DNA index of greater than 1.2 was classified as aneuploid.

cally retain a diploid or near-diploid karyotype *(45,46).* Inactivation of mitotic spindle checkpoint genes, such as BUB and MAD2, have been implicated in aneuploidy, but mutant BUB genes and decreased expression of MAD2 protein could be documented in only a small fraction of aneuploid solid tumors *(47,48).* Our finding of STK15 amplification in virtually all bladder tumors tested by FISH and the strong association of the gene amplification and overexpression levels with the degree of aneuploidy suggests that STK15 may play an important role in bladder carcinogenesis by contributing to the development of aneuploid cell populations with aggressive phenotypes.

Although a high level of STK15 expression appeared to be common in bladder tumors, approximately 30% of highly aneuploid aggressive bladder tumors showed no evidence of STK15 overexpression. This suggests that, in a fraction of bladder tumors, other genes are associated with the development of aneuploidy. In fact, two recently identified mammalian members of the aurora kinase family, AuroraB (also referred to as Aurora1/Aik2) and AuroraC (also referred to as Aik3), have increased expression levels in several human cancers *(49,50).* Because of the complexity of genomic alterations seen in the vast majority of solid tumors, it is unlikely that STK15 is the sole contributor to the development of aneuploidy. Rather, overexpressed STK15 most likely acts in concert with the products of other genes involved in the process of chromosomal segregation.

It is important to note that the STK15 gene is localized to chromosome 20q13 and was identified by searching for overexpressed sequences from the long arm of chromosome 20, which is frequently amplified in a wide range of human cancers *(51).* Increased copies of 20q have also been associated with aggressive variants of several epithelial and mesenchymal malignancies including bladder cancer *(52,53).* The 20q region is typically amplified in high-grade invasive urothelial carcinomas of the bladder, virtually all of which exhibit pronounced aneuploidy *(54–56).* Moreover, *in vitro* studies have shown that virally transformed human urothelial cells contain an increased copy number of chromosome 20, suggesting that the amplification of genetic material on this chromosome may play a role in early phases of urothelial neoplasia *(57).* All these features support the hypothesis that STK15 represents a major target gene within the 20q amplicon. The role of this gene in human carcinogenesis must be viewed, however, in concert with those of other genes mapped to the same region, which may be amplified and overexpressed together with or independently of STK15. Several such genes were recently identified and include AIB1, CAS, TFAP2C, ZNF217, NABC1, and CYP24 *(58–62).* The involvement of this segment of the genome in human carcinogenesis was further confirmed by the recent mapping of human chromosome 20 with microarray comparative genomic hybridization technology, which revealed several discontinuous gains and losses along the 20q arm that varied in individual tumors *(63).*

In summary, on the basis of our results, we hypothesize that high expression of STK15 disrupts the signaling cascade that regulates equal segregation of chromosomes, which leads to pronounced aneuploidy and an aggressive phenotype. Thus, expression profiling of this kinase may have the potential to be of diagnostic and prognostic significance. Identification of additional genes involved in the STK15 regulatory pathway should further improve our understanding of mechanisms causing aneuploidy in human cancers. In fact, we have recently proposed that protein phosphatase type 1 acts in a feedback regulatory pathway with STK15 kinase to control chromosome segregation during mitosis *(64).* Abrogation of this regulation was shown to cause anomalous mitotic segregation of chromosomes. The genes involved in the STK15 pathway and their products may also be attractive novel targets for the development of detection markers and therapeutic strategies. Such approaches might be particularly useful in early premalignant phases of human neoplasia, providing tools for monitoring and, ultimately, even preventing chromosomal instability.

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Notes

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