Induction of Centrosome Amplification and Chromosome Instability in Human Bladder Cancer Cells by p53 Mutation and Cyclin E Overexpression

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ABSTRACT

Centrosome amplification frequently occurs in human cancers and is a major cause of chromosome instability (CIN). In mouse cells, centrosome amplification can be readily induced by loss or mutational inactivation of p53. In human cells, however, silencing of endogenous p53 alone does not induce centrosome amplification or CIN, although high degrees of correlation between p53 mutation and CIN/centrosome amplification in human cancer can be detected, suggesting the presence of additional regulatory mechanism(s) in human cells that ensures the numeral integrity of centrosomes and genomic integrity. Cyclin E, a regulatory subunit for CDK2 that plays a key role in centrosome duplication, is overexpressed in human cancers. We found that cyclin E overexpression, together with loss of p53, efficiently induces centrosome amplification and CIN in human bladder cancer cells but not by either cyclin E overexpression or loss of p53 alone. We extended these findings to bladder cancer specimens and found that centrosome amplification is strongly correlated with concomitant occurrence of cyclin E overexpression and p53 inactivation but not with either cyclin E overexpression or p53 inactivation alone. Because cyclin E expression is strictly controlled in human cells compared with mouse cells, our findings suggest that this stringent regulation of cyclin E expression plays an additional role underlying numeral homeostasis of centrosomes in human cells and that deregulation of cyclin E expression, together with inactivation of p53, results in centrosome amplification.

INTRODUCTION

Chromosome instability (CIN) is widely accepted as a formidable feature of cancer because loss or gain of a single chromosome can introduce multiple mutations responsible for malignant phenotypes. Thus, CIN, generally resulting from mitotic defects, greatly accelerates tumor progression. A number of studies have shown that centrosome amplification is a contributing factor of CIN in tumor cells (1, 2). The centrosome, a major microtubule-organizing center of animal cells, is composed of a pair of centrioles and various surrounding proteins (3). During mitosis, centrosomes direct the formation of bipolar mitotic spindles, which is an essential event for accurate chromosome segregation (4). Because each daughter cell receives only one centrosome on cytokinesis, the centrosome must duplicate once before the following mitosis, which normally occurs at the time of S-phase entry (5). Thus, a cell contains either one unduplicated centrosome or two duplicated centrosomes at any given time, and the numeral homeostasis of centrosomes is strictly controlled. When this control is lost, centrosome amplification occurs, leading to aberrant mitotic spindle formation and increased frequency of chromosome segregation errors (3, 4).

Centrosome amplification can occur through several mechanisms, including deregulated centrosome duplication, uncontrolled splitting of a centriole pair, and cytokinesis failure (4, 6), and is induced by mutations of various cell cycle-related proteins and tumor suppressor proteins, most notably p53 and BRCA1 (7, 8). Centrosome amplification caused by loss or mutational inactivation of p53 has been studied extensively in cultured mouse cells. For example, cells derived from p53-deficient (p53−/−) mice (9) and cells transfected with dominant negative p53 mutants (10, 11) show high frequencies of centrosome amplification. Centrosome amplification also can be detected in the tissues of p53−/− mice (12) and human papillomavirus E6 (a potent destabilizer of p53) transgenic mice (13). In contrast, loss of p53 does not confer centrosome amplification in cultured human cells and appears to require additional mutation(s) for efficient induction of centrosome amplification and CIN. For example, short interference RNA (siRNA)-mediated silencing of p53 in normal human fibroblasts or expression of human papillomavirus E6 in normal human keratinocytes do not result in centrosome amplification at a significant level (14)x or CIN (15). However, examination of human cancer tissues and cultured cells has revealed a significant correlation between loss or mutational inactivation of p53 and occurrence of centrosome amplification, supporting the idea that p53 mutation alone is not sufficient to induce centrosome amplification in human cells but rather requires additional mutation(s) (16, 17).

The activity of CDK2/cyclin E, a well-known inducer of DNA synthesis (18), is required for the initiation of centrosome duplication (19). Some of the centrosomal target proteins of CDK2/cyclin E have been identified, including nucleophosmin, Mps1 kinase, and CP110 (20–22). The activity of CDK2/cyclin E primarily is controlled by temporal expression of cyclin E, which normally occurs at mid-late G1 phase of the cell cycle (18). In cultured normal human cells, cyclin E expression is strictly controlled, limited to a short period at late G1 phase (23). In contrast, in cultured mouse cells, cyclin E expression is controlled less stringently, and increased levels of cyclin E often can be detected during early-mid G1 phase.6 There is a remarkable difference in the cyclin E promoter sequences between human and mouse: the critical E2F binding site found in the mouse cyclin E promoter is absent in the human promoter, and the mouse promoter sequence does not confer negative regulation of cyclin E expression (24), which is present in the human promoter (25). We have shown that cyclin E overexpression (constitutive activation of CDK2/cyclin E in p53−/− mouse cells accelerates centrosome amplification (26). Moreover, cyclin E overexpression frequently is observed in various human cancers (27). These observations converge to a hypothesis that the additional mutation required for loss of p53 to induce centrosome amplification in human cells may be uncontrolled expression of cyclin E. Here, we tested this hypothesis in human bladder cancer cell lines and bladder cancer tissues because cyclin E overexpression (38–30) and p53 mutation (31–33) are common, and appropriate bladder
cancer cell lines with known p53 status are available (34). We found that centrosome amplification and CIN were readily observed in bladder cancer cell lines that show p53 mutation and cyclin E overexpression. Moreover, centrosome amplification was efficiently induced in the bladder cancer cell line and normal human fibroblasts that retain wild-type p53 and normal cyclin E expression by silencing of endogenous p53, together with ectopic expression of cyclin E. We extended these in vitro findings to 65 clinical bladder cancer specimens with different grades and stages. We found a statistically significant correlation between occurrence of centrosome amplification and double mutations of p53 inactivation and cyclin E overexpression in these clinical samples. Together, deregulated expression of cyclin E may be the additional mutation required for centrosome amplification in human cells that harbor p53 mutations, and the stringent control of cyclin E expression serves as an additional control for numeral integrity of centrosomes in human cells.

MATERIALS AND METHODS

Cell Lines and Transfection. Human bladder cancer cell lines (RT-4, HT-1197, and HT-1376) and HEL299 diploid normal human fibroblast-like cells were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in medium [DMEM supplemented with 10% fetal bovine serum with penicillin (100 units/ml) and streptomycin (100 µg/ml)] and grown in an atmosphere containing 10% CO₂. For subcloning RT-4 cells with 2N = 86, ~1 × 10⁶ cells were seeded on 100-mm culture dish, and single cell colonies were subcloned. These cells were examined for chromosome numbers by counting of the metaphase spreads, and one subclone with 2N = 86 was maintained for additional experimentation. Transfection was performed using a FuGENE 6 transfection system (Roche, Basel, Switzerland).

Bladder Cancer Tissue Samples. The tissue samples came from 65 patients, who underwent surgery for bladder cancer between December 1997 and October 2001 (total cystectomy, 8 cases; transurethral bladder biopsy, 57 cases). Fifty-seven patients were men and 8 were women, with a mean age at diagnosis of 68 years (range, 45–91 years). Normal bladder epithelium was obtained from patients with different grades and stages. We found a statistically significant correlation between occurrence of centrosome amplification and double mutations of p53 inactivation and cyclin E overexpression in these clinical samples. Together, deregulated expression of cyclin E may be the additional mutation required for centrosome amplification in human cells that harbor p53 mutations, and the stringent control of cyclin E expression serves as an additional control for numeral integrity of centrosomes in human cells.

RESULTS

Levels of Cyclin E in Human Bladder Cancer Cell Lines with Known p53 Status. To examine the role of cyclin E overexpression and p53 inactivation in centrosome amplification and CIN in human cells, we selected three established bladder cancer cell lines with known p53 statuses. RT-4, a low grade, papillary tumor cell line derived from a well-differentiated transitional cell carcinoma (35), retains wild-type p53 (34). HT-1197 and HT-1376 cell lines were derived from poorly differentiated transitional cell carcinomas involving the bladder muscularis (36) and have point mutations in p53 genes at codons 365 and 250, respectively (34). These cell lines were first characterized for p53 and cyclin E expression. Immunoblot analysis using anti-p53 antibody showed the presence of high levels of p53 in HT-1197 and HT-1376 cell lines compared with RT-4 cell line (Fig. 1A, left), which was expected from the stabilization (and inactivation) of p53 in HT-1197 and HT1376 cells because of point mutations and the unstable nature of wild-type p53 in RT-4 cells. Immunoblot analysis using anti-cyclin E antibody revealed a significant increase in the level of cyclin E in HT-1197 and HT-1376 cells compared with RT-4 cells (Fig. 1A, right). These results were further corroborated by immunohistochemical analysis. Cyclin E and p53 were barely detectable in RT-4 cells, whereas HT-1197 and HT1376 cells showed readily detectable levels of cyclin E and p53, both of which were predominantly localized in the nucleus (data not shown; see Fig. 1B).

To test whether cyclin E levels in these bladder cancer cell lines were proportionally translated to the activity of CDK2/cyclin E, the extracts prepared from exponentially growing cells were subjected to immunoprecipitation using anti-cyclin E antibody. The immunoprecipitates (cyclin E and CDK2/cyclin E) were tested for the kinase activity using histone H1 as a substrate. The immunoprecipitates from RT-4 cells showed a low level of histone H1 kinase activity, whereas those from HT-1197 and HT-1376 cell lines showed several-fold
Analysis of cyclin E and p53 expression, chromosome instability (CIN), and centrosome amplification in human bladder cancer cell lines. A, the lysates prepared from three human bladder cancer cell lines (RT-4, HT-1197, and HT-1376) were subjected to immunoblot analysis using anti-p53 monoclonal antibody (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-cyclin E polyclonal antibody (C-19; Santa Cruz Biotechnology).

B, a summary of RT-4, HT-1197, and HT-1376 cell lines for their tumor grades, p53 status and expression levels, cyclin E expression levels, and CDK2/cyclin E kinase activities. The p53 genotypes were reported previously (34). For immunohistochemical (IHC) analysis, cells were immunostained with either anti-p53 (Pab1801; Santa Cruz Biotechnology) or anti-cyclin E (C-19) antibody.

C, analysis of CIN in the human bladder cancer cell lines by direct counting of metaphase spreads. Metaphase spreads prepared from the bladder cancer cell lines under an optimal growth condition were subjected to immunoprecipitation using anti-cyclin E antibody (C-19), and the immunoprecipitates were tested for 

D, analysis of CIN using fluorescence in situ hybridization (FISH). RT-4, HT-1197, and HT-1376 cells were examined by FISH using fluorescent probes for chromosomes 7 and 11. RT-4 cells were found to be tetrasomy for chromosomes 7 and 11, whereas HT-1197 and HT-1376 cells show extensive polysomy for chromosomes 7 and 11. More than 100 cells were examined for each cell line. The numbers of chromosomes 7 and 11 in RT-4 cells are stable, whereas those in HT-1197 and HT-1376 cells were variable, indicating the CIN phenotype in HT-1197 and HT-1376 cells.

E, centrosome profiles of the human bladder cancer cell lines. RT-4, HT-1197, and HT-1376 cells under an optimal growth condition were subjected to immunostaining using anti-α-tubulin antibody. The antibody-antigen complexes were detected with FITC-conjugated goat antirabbit IgG antibody. The number of centrosomes per cell was scored under a fluorescence microscope. More than 300 cells were examined for each cell line, and the results are shown as number of centrosomes per cell; n = 1, 2, and ≥3.

Fig. 1. Analysis of cyclin E and p53 expression, chromosome instability (CIN), and centrosome amplification in human bladder cancer cell lines. A, the lysates prepared from three human bladder cancer cell lines (RT-4, HT-1197, and HT-1376) were subjected to immunoblot analysis using anti-p53 monoclonal antibody (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-cyclin E polyclonal antibody (C-19; Santa Cruz Biotechnology). B, summary of RT-4, HT-1197, and HT-1376 cell lines for their tumor grades, p53 status and expression levels, cyclin E expression levels, and CDK2/cyclin E kinase activities. The p53 genotypes were reported previously (34). For immunohistochemical (IHC) analysis, cells were immunostained with either anti-p53 (Pab1801; Santa Cruz Biotechnology) or anti-cyclin E (C-19) antibody. +++, high level expression; ++, medium level expression; +, low level expression. For analysis of CDK2/cyclin E kinase activity, the lysates prepared from the bladder cancer cell lines under an optimal growth condition were subjected to immunoprecipitation using anti-cyclin E antibody (C-19), and the immunoprecipitates were tested for in vitro histone H1 kinase activity as described previously (26). +++, high activity; ++, low activity. C, analysis of CIN in the human bladder cancer cell lines by direct counting of metaphase spreads. Metaphase spreads prepared from RT-4, HT-1197, and HT-1376 cells were stained with Giemsa, and the number of chromosomes was counted under a microscope. More than 100 metaphase spreads were examined for each cell line. Arrow indicates G1 peaks of two populations of RT-4 cells. D, analysis of CIN using fluorescence in situ hybridization (FISH). RT-4, HT-1197, and HT-1376 cells were examined by FISH using fluorescent probes for chromosomes 7 and 11. RT-4 cells were found to be tetrasomy for chromosomes 7 and 11, whereas HT-1197 and HT-1376 cells show extensive polysomy for chromosomes 7 and 11. More than 100 cells were examined for each cell line. The numbers of chromosomes 7 and 11 in RT-4 cells are stable, whereas those in HT-1197 and HT-1376 cells were variable, indicating the CIN phenotype in HT-1197 and HT-1376 cells.

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higher activities, demonstrating that the activity of CDK2/cyclin E is up-regulated in these two cell lines (data not shown; see Fig. 1B). The genetic and biological profiles of RT-4, HT-1197, and HT-1376 cell lines are summarized in Fig. 1B.

CIN and Centrosome Amplification in the Bladder Cancer Cell Lines. We next examined three bladder cancer cell lines for CIN by counting the metaphase chromosomes and by FISH analysis using the probes specific for chromosomes 7 and 11. The direct counting of the metaphase spreads revealed that the RT-4 line carried in our laboratory consisted of two distinct populations in respect to chromosome number [4N = 66 (10–15%) and 4N = 86 (85–90%), indicated by arrows in Fig. 1C]. The chromosomes of these two populations appeared to be stable because we seldom detected cells with the chromosome number that differs from these two populations. The preliminary characterization of RT-4 cells revealed that chromosomes 7 and 11 were tetrasomy in both populations (data not shown). The FISH analysis of RT-4 cells using chromosome 7 and 11 probes revealed that >96% of cells showed four spots for chromosomes 7 and 11, indicating the stability of chromosomes in RT-4 cells (Fig. 1D). In contrast, the HT-1197 line was karyotypically heterogeneous, and chromosome numbers ranged from 4N = 43–142 (Fig. 1C), and the number of spots for chromosomes 7 and 11 was unstable (Fig. 1D). Similarly, the HT-1376 line showed karyotypic heterogeneity with the chromosome number ranging from 52–204 and the unstable number of spots for chromosomes 7 and 11 (Fig. 1). C and D). From these results, we concluded that RT-4 cells are karyotypically stable, whereas HT-1197 and HT-1376 cells are karyotypically unstable.

We next examined centrosome profiles of the bladder cancer cell lines by immunostaining using antibody against γ-tubulin, one of the major centrosome components (Fig. 1E). More than 98% of RT-4 cells contained either one or two centrosomes, and mitotic RT-4 cells showed bipolar spindles (data not shown). In contrast, >15% of HT-1197 and >30% of HT-1376 cells contained amplified (≥3) centrosomes, and aberrant mitotic spindles with multiple spindle poles frequently were observed in these cells lines (data not shown). We further dissected centrosome amplification in the bladder cancer cell lines by laser scanning cytometry, which allows examination of the centrosome profile and the DNA content and the cell cycle phase of the same individual cell (see Supplemental Data). We found that centrosome amplification in many of the HT-1197 and HT-1376 cells was not accompanied with genome doubling, indicating that cytokinesis failure is not the cause of centrosome amplification in these cells.

Cyclin E Overexpression Together with Loss of p53 Induce Centrosome Amplification and CIN in Human Bladder Cancer Cells. To corroborate the induction of centrosome amplification and CIN by p53 inactivation and cyclin E overexpression, we genetically manipulated RT-4 cells, which show a normal centrosome profile and retain wild-type p53 and normal levels of cyclin E expression. As described previously (Fig. 1), the RT-4 cell line consists of two karyotypically distinct populations (2N = 66 and 86). To avoid complication for data analysis, we first subcloned RT-4 cells with 2N = 86 (see “Materials and Methods”). The subcloned RT-4 cells were expanded and stably transfected with plasmids encoding either cyclin E or siRNA for p53 together with a plasmid encoding neomycin-resistance gene (pKOneo) as a selection marker (20:1 molar ratio; Ref. 37). RT-4 cells also were cotransfected with the cyclin E and p53 siRNA plasmids, together with pKOneo (20:20:1 molar ratio). As a control, RT-4 cells were transfected with an siRNA vector (pSuper). After 4–6 weeks of G418 treatment, the surviving cells were pooled, and expression of p53 and cyclin E was examined by immunoblot analysis (Fig. 2A). Expression of p53 in the cells transfected with p53 siRNA (RT4/p53i) and with p53 siRNA and cyclin E (RT4/cycE+p53i) was successfully silenced. Cyclin E levels in cells transfected with cyclin E (RT4/cycE) and RT4/cycE+p53i cells were increased threefold to fourfold compared with the vector-transfected cells (RT4/vec) and RT4/p53i cells. There was a slight increase in cyclin E levels in RT4/p53i cells compared with the control RT4/vec cells, which probably reflected silencing of p53 because cyclin E expression is controlled by series of activation and inactivation of cell cycle-associated proteins, and p53 is likely involved in such events.

We first examined the centrosome profiles of the RT-4-derived cells (Fig. 2B). The frequency of centrosome amplification in RT4/vec cells was low as expected (<3%). There was a small increase in the frequency of centrosome amplification in RT4/p53i and RT4/cycE cells (7–9%). In contrast, RT4/cycE+p53i cells showed a pronounced amplification of centrosomes (>20%). Thus, loss of p53 and cyclin E overexpression efficiently induce centrosome amplification in human bladder epithelial cells.

We next examined whether centrosome amplification induced by cyclin E overexpression and loss of p53 in RT-4 cells was translated to CIN. To this end, the RT-4-derived cells were subjected to FISH analysis using the probes specific for chromosomes 3 and 8 (Fig. 2C). The subcloned RT-4 cells (2N = 86) used for generation of these cell lines are tetrasomy for chromosomes 3 and 8 (data not shown). The frequency of karyotypic alteration in RT4/vec cells was low (average 4.7%), whereas there were small increases in the overall frequencies of karyotypic alterations in RT4/cycE and RT4/p53i cells (8.3% and 14.8%, respectively). A similar finding previously was reported for ectopic expression of cyclin E in human breast epithelial cells (38). In contrast, RT4/cycE+p53i cells showed a more pronounced increase in the overall frequency of karyotypic alteration (32.4%). These observations demonstrate that CIN is efficiently induced by loss of p53 together with cyclin E overexpression in human cells compared with either cyclin E overexpression or loss of p53 alone. Statistical analysis of centrosome amplification and CIN in these RT-4-derived cells revealed a strong linear correlation (P < 0.0001 and r = 0.961; Fig. 2D). Thus, cyclin E overexpression and loss of p53 together, but neither cyclin E overexpression nor loss p53 alone, result in high frequencies of centrosome amplification and CIN in cultured human bladder cancer cells.

We next tested whether efficient induction of CIN and centrosome amplification by cyclin E overexpression and loss of p53 is specific to bladder epithelial cells. HEL299 diploid normal human fibroblasts were genetically manipulated as described for RT-4 cells. The immunoblot analysis of cyclin E and p53 in these genetically manipulated HEL299 cells (HEL299/vec (vector-control), HEL299/p53i (p53 siRNA-transfected cells), HEL299/cycE (cyclin E-transfected cells), and HEL299/cycE+p53i (transfected with p53 siRNA and cyclin E)) is shown in Fig. 3A. These cell lines then were examined for their centrosome profiles. More than 99% of HEL299/vec cells contained one or two centrosomes. Similar to RT-4 cells, a small increase in the frequency of centrosome amplification was detected for HEL299/p53i (3.8%) and HEL299/cycE cells (1.6%). However, HEL299/cycE+p53i showed a significant increase in the frequency of centrosome amplification (>15%).

We also tested these HEL299-derived cells for CIN by FISH analysis using the probes specific for chromosomes 3 and 8. The frequency of karyotypic alteration in HEL299/vec cells was low (average 1%) as expected, whereas there were small increases in the overall frequencies of karyotypic alterations in HEL299/cycE and HEL299/p53i cells (1.8% and 2.8%, respectively). In contrast, HEL299/cycE+p53i cells showed a marked increase in the overall frequency of karyotypic alteration (6.3%). Compared with RT-4, centrosome amplification appears not to be efficiently translated to CIN in HEL299 cells. However, this is not unexpected because gain...
or loss of chromosomes introduces mutations that affect positively and negatively on cell growth and survival. It is likely that normal cells may be more sensitive to karyotypic changes than cancer cells; therefore, some HEL cells that have suffered karyotypic alterations because of centrosome amplification may be disposed to cell death.

However, efficient centrosome amplification and readily recognizable frequency of CIN were induced by loss of p53 and cyclin E overexpression in normal fibroblasts, demonstrating that this is not specific to either an RT-4 cell line or bladder epithelial cells but rather is more general.
**Centrosome Amplification in Clinical Materials.** We extended our *in vitro* findings to bladder cancer specimens of various grades and stages from 65 patients. As a control, normal tissues adjacent to the tumor area were examined. The touch preparations of normal bladder epithelial and bladder cancer tissues were immunostained with anti-αH9253-tubulin antibody. The normal bladder mucosal cells contained either one (66.7%) or two centrosomes (33.3%), and none of the cells examined contained amplified centrosomes [Fig. 4A (a); representative immunostaining images are shown in Fig. 4A (b)]. In contrast, we detected centrosome amplification in 34 of 65 (52.3%) cancer specimens. Statistical analysis of centrosome amplification in relation to tumor grades evaluated according to the World Health Organization classification (G1, G2, and G3) revealed a striking association: centrosome amplification was detected in 1 of 13 (7.7%) G1 tumors, 11 of 23 (47.8%) G2 tumors, and 22 of 29 (75.9%) G3 tumors (*P* < 0.01; Table 1).

Because the frequencies of cells with centrosome amplification (n ≥ 3) vary greatly among tumor samples, we graded the tumors into three groups: abnormal amplification of centrosome (AAC) 0–2. On examination of >200 cells within a tumor area, if no cell displayed centrosome amplification, it was assigned as “AAC0.” Among the AAC-positive tumors, the tumors with <10% of cells with n ≥ 3 centrosomes were assigned as “AAC1,” and those with ≥10% of cells with n ≥ 3 centrosomes were assigned as “AAC2.” Using this system, 31 cases were found to be AAC0, 12 cases were AAC1, and 22 cases were AAC2 (Table 1). The representative immunostaining images of tumor specimens with different AAC grades are shown in Fig. 4B. When AAC grades and tumor grades were statistically compared, we found an interesting trend: a higher percent of G2 tumors are graded as ACC2 (AAC1, 2 cases; AAC2, 9 cases) than G3 tumors (AAC1, 10 cases; AAC2, 12 cases). However, this was not unexpected: it is known that the CIN...
phenotype frequently is suppressed in highly advanced tumors. During tumor progression, the tumor population often undergoes karyotypic convergence, which is depicted by karyotypic heterogeneity of less advanced tumors and karyotypic homogeneity of highly advanced tumors (39). During tumor progression, cells acquire CIN phenotypes (i.e., centrosome amplification), leading to karyotypic heterogeneity of the tumor population, which promote acquisition of additional malignant phenotypes. At a certain time point, some cells acquire the most desirable karyotypes for growth and gradually dominate the tumor population. For those cells, to maintain this specific karyotype becomes a priority, forcing the selection of cells that have acquired mutations that suppress the cause of CIN (i.e., centrosome amplification; Ref. 40). Thus, the decrease in the severity of centrosome amplification in highly advanced bladder cancer likely reflects the karyotypic convergence of tumor progression.

Association between Centrosome Amplification and Overexpression of Cyclin E and p53 in Bladder Cancer Tissues.

We examined the bladder cancer specimens for the levels of p53 and cyclin E. It has been shown that in transitional cell carcinomas of bladder cancer tissues. The touch preparations of 65 bladder cancer specimens and 13 adjacent normal bladder epithelium samples were subjected to immunostaining with anti-γ-tubulin antibody and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The number of centrosomes per cell was scored by fluorescence microscope. A, centrosomes in normal bladder epithelium. Thirteen normal bladder epithelium specimens that were adjacent to the tumor areas were examined for centrosomes. For each normal bladder epithelium, >200 cells were examined, and the result of one normal epithelium is shown in (a). Twelve other normal epithelia showed similar centrosome profiles. The representative immunostaining images of normal epithelium are shown in (b); original magnification, ×600. B, centrosomes in bladder cancer tissues. Touch preparations from 65 bladder cancer specimens were examined for centrosomes. For each tumor, >200 cells were examined. Various degrees of centrosome amplification were detected in these specimens. We graded each tumor in respect to the degree of centrosome amplification: abnormal amplification of centrosome (AAC) 0, no centrosome amplification; AAC1, <10% of cells with n ≥ 3 centrosomes; and AAC2, ≥10% of cells with n ≥ 3 centrosomes. The representative immunostaining images of ACC0, ACC1, and ACC2 tumors are shown. Arrows indicate cells with n ≥ 3 centrosomes. Two investigators (K. K. and R. I.) independently evaluated the degrees of AAC. Magnified images of some of those cells are shown on the right; original magnification, ×600. Among 65 specimens, 31 cases were found as ACC0, 12 cases were ACC1, and 22 cases were ACC2 (see Table 3).

Table 1 Pathologic information in relation to centrosome amplification, cyclin E levels, and p53 levels

<table>
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<th>Tumor grade</th>
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<td>0.01b</td>
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<td>&lt;0.01b</td>
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a AAC, abnormal amplification of centrosome.

b P value was determined using Mantel-Haenszel χ² test.

c Associations of tumor grades (G1 vs. G2/G3) with cyclin E and p53 overexpression were analyzed by Fisher’s exact test.
bladder, immunohistochemical detection of p53 overexpression alone is a good indicator for occurrence of p53 mutation (41, 42). The immunohistochemical analysis detected p53 overexpression in 18 of 65 cases and cyclin E overexpression in 24 cases (Table 1). Of 27 cases that were positive for either p53 or cyclin E overexpression, 15 cases showed overexpression of p53 and cyclin E.

When tumor grades and occurrence of either cyclin E or p53 overexpression were compared, we found a statistically significant association between tumor grades and cyclin E (P < 0.01) and p53 overexpression (P < 0.01), especially G1 tumors versus G2/G3 tumors (Table 1), consistent with the previous findings that cyclin E overexpression was significantly more frequent in pT1 (lamina propria invasive) tumors (TNM pathologic staging system) than in pTa (papillary) bladder tumors (43) and was strongly associated with aggressive tumor growth in bladder cancer (29). Moreover, p53 mutation (p53 overexpression) frequently is observed in advanced bladder cancers (31–33).

We next tested the association of centrosome amplification versus either p53 overexpression or cyclin E overexpression as a single (independent) event. We found that cyclin E and p53 overexpression strongly associated with occurrence of centrosome amplification and AAC grades (P < 0.001 for all the associations; Table 2). However, as described previously, 15 cases showed concomitant overexpression of p53 and cyclin E. Thus, we statistically analyzed the tumors for the association of occurrence of centrosome amplification versus p53 overexpression and cyclin E overexpression as conditional events. To this end, each tumor was assigned to one of the four groups in respect to p53 and cyclin E expression: (a) tumors with neither p53 nor cyclin E overexpression; (b) tumors with cyclin E overexpression but no p53 overexpression; (c) tumors with p53 overexpression but no cyclin E overexpression; and (d) tumors with p53 and cyclin E overexpression.

We then statistically compared these tumors with occurrence of centrosome amplification and AAC grades (Table 3). Among the tumors with neither p53 nor cyclin E overexpression (38 cases), 27 cases (71%) were found as AAC0, 8 cases (21%) as AAC1, and 2 cases (8%) as AAC2. Thus, if the tumor retains wild-type p53 and normal cyclin E expression, it is likely that this tumor shows a normal centrosome profile (P < 0.001). Surprisingly, although the sample number is too small to firmly conclude, there was no association between AAC grades and cyclin E overexpression in the presence of wild-type p53 (P = 1.000). Similarly, no association was observed between AAC grades and p53 overexpression in the presence of normal levels of cyclin E (P = 0.564). Thus, the strong association between centrosome amplification and either cyclin E overexpression or p53 overexpression, which was revealed when analyzed as an independent event (Table 2), was rejected when cyclin E and p53 overexpression was analyzed conditionally. In contrast, among the tumors with p53 and cyclin E overexpression, all of the tumors were found to suffer centrosome amplification, in which 1 case was graded as AAC1 and 14 cases were graded as AAC2 (P < 0.001). Thus, in transitional cell carcinoma of bladder, occurrence of centrosome amplification and AAC grades do not correlate with either p53 overexpression or cyclin E overexpression alone but strongly correlate with double mutations of p53 and cyclin E overexpression.

### DISCUSSION

Centrosome amplification, which is believed to be a major cause of CIN, frequently occurs in human cancers and is known to be associated with loss or mutational inactivation of p53. However, unlike mouse cells, loss of p53 function alone is not sufficient to disturb the numeral integrity of centrosomes in human cells. Thus, human cells are likely equipped with additional regulatory mechanism(s) for numeral homeostasis of centrosomes. The critical role of CIN in carcinogenesis stresses the importance of identifying the additional regulation(s) in human cells to maintain the karyotypic integrity and numeral integrity of centrosomes. Here, we examined bladder cancer cell lines and tissues to test whether deregulated expression of cyclin E might be the additional mutation required for efficient induction of CIN and centrosome amplification in human cells that harbor p53 mutations. The RT-4 cell line, a low histologic grade and well-differentiated tumor, retains wild-type p53 and normal levels of cyclin E and shows virtually no centrosome amplification. In contrast, high frequency of centrosome amplification was detected in two cell lines derived from highly invasive poorly differentiated tumors, which harbor point mutations in p53 genes and show high levels of cyclin E. Moreover, when RT-4 cells were genetically manipulated either to knock down endogenous p53 or overexpress cyclin E, there was a small increase in the frequencies of karyotypic alteration and centrosome amplification. However, introduction of p53 knockdown and cyclin E overexpression into RT-4 cells resulted in a significant increase in the frequencies of karyotypic alteration and centrosome amplification. These results indicate that in human cells the highly controlled cyclin E expression serves as an additional regulation for maintaining numeral integrity and stabilization of chromosomes. This conclusion agrees with the studies reported previously, in which human papillomavirus E6 and E7 proteins, which target p53 and Rb proteins, respectively, synergistically induce centrosome amplification in normal human keratinocytes (14). It has been shown that loss of Rb results in uncontrolled activation of the E2F family of tran-

### Table 2: Association between centrosome amplification and cyclin E levels and between centrosome amplification and p53 levels

<table>
<thead>
<tr>
<th>Cyclin E overexpression</th>
<th>p53 overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
</tr>
<tr>
<td>AAC0</td>
<td>28</td>
</tr>
<tr>
<td>AAC+</td>
<td>13</td>
</tr>
<tr>
<td>AAC0</td>
<td>28</td>
</tr>
<tr>
<td>AAC1</td>
<td>8</td>
</tr>
<tr>
<td>AAC2</td>
<td>5</td>
</tr>
</tbody>
</table>

* Associations of AAC grades with either cyclin E or p53 overexpression as a single event were analyzed by Mantel-Haenszel χ² test.

### Table 3: Association between centrosome amplification and concurrent overexpression of cyclin E and p53

<table>
<thead>
<tr>
<th>Neither cyclin E overexpression nor p53 overexpression (%)</th>
<th>Cyclin E overexpression, but no p53 overexpression (%)</th>
<th>p53 overexpression but no cyclin E overexpression (%)</th>
<th>Both cyclin E overexpression and p53 overexpression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAC0+</td>
<td>11 (29)</td>
<td>6 (67)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>AAC0−</td>
<td>27 (71)</td>
<td>3 (33)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>AAC0</td>
<td>27 (71)</td>
<td>3 (33)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>AAC1</td>
<td>8 (21)</td>
<td>3 (33)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>AAC2</td>
<td>3 (8)</td>
<td>P &lt; 0.001</td>
<td>3 (33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Associations between AAC grades and cyclin E and/or p53 overexpression as a conditional event were analyzed by χ² goodness of fit test.

AAC, abnormal amplification of centrosome.
Continuously operated, preventing premature activation of CDK2/cyclin E (25, 44). We extended our in vitro findings to bladder cancer specimens. Overexpression of p53 (a good indication of mutational inactivation of p53 in bladder cancer) and cyclin E, when analyzed as an independent event in respect to centrosome amplification, showed a significant association with centrosome amplification. However, when cyclin E and p53 overexpression was analyzed conditionally to each other, the association between centrosome amplification and either p53 or cyclin E overexpression was rejected. However, the conditional analysis revealed a significant association between centrosome amplification and concomitant occurrence of p53 and cyclin E overexpression. These data agree with the results obtained from cultured cells, in which cyclin E overexpression together with p53 inactivation efficiently induce centrosome amplification in human cells. This finding provides one possible explanation for the contradicting findings made by examination of clinical samples in respect to correlation between p53 mutation and CIN (as well as centrosome amplification); although many studies have found the correlation between these two events, equally many studies failed to detect a significant correlation.

If cyclin E overexpression is a frequent event in the tumor types or the tumor population under investigation, significant correlation between p53 mutation and CIN/centrosome amplification will be likely detected. In contrast, if cyclin E overexpression is a rare event, correlation between p53 mutation and CIN/centrosome amplification will likely be insignificant. An important issue to be considered for this scenario is the effect of overexpression of cyclin A, another cyclin that complexes with CDK2, on induction of centrosome amplification. CDK2/cyclin A also has been implicated in centrosome duplication (45), and frequent overexpression of cyclin A has been reported in several types of cancers (46). Because of the toxicity associated with cyclin A overexpression in cultured cells, it is difficult to directly address whether cyclin A overexpression and loss of p53 together induce centrosome amplification in human cells. However, correlative investigation of the clinical specimens in respect to cyclin A levels, p53 status, and occurrence of centrosome amplification will certainly provide critical information on this issue.

The major question remaining to be answered is the mechanism underlying efficient induction of centrosome amplification by cyclin E overexpression in human cells harboring p53 mutations. It has been shown that when Chinese hamster ovary cells are exposed to DNA synthesis inhibitor [i.e., aphidicolin (Aph) or hydroxyurea (HU)], cells continue to duplicate centrosomes without DNA synthesis, resulting in centrosome amplification (47). It was later found in mouse cells that this phenomenon is observed in the absence of functional p53 (48). Chinese hamster ovary cells are known to harbor p53 mutations (49). Thus, in normal cells, Aph or HU treatment blocks DNA synthesis and centrosome duplication (48). Moreover, in normal cells, p53 was up-regulated in response to Aph or HU treatment, which in turn up-regulates p21Waf1/Cip1 (p21; Ref. 48), a major transactivation target of p53 and a potent inhibitor of CDK (CKI), including CDK2/cyclin E (49). The p21 then suppresses CDK2 kinase activity, resulting in failure of centrosomes to undergo reduplication (48). However, the control of CDK2 activity is multifold, including temporal expression and programmed degradation of cyclins, site-specific phosphorylation and dephosphorylation of CDK2, and CKI-mediated inhibition (50). Thus, the p53-p21 pathway comprises one of several mechanisms operating in parallel in human cells to suppress CDK2/cyclin E during Aph or HU treatment. In mouse cells, cyclin E expression is less stringently regulated, and an increase in cyclin E level often can be observed in early-mid G1 phase. It has been suggested that in mouse cells basal levels of the p53-p21 pathway are continuously operated, preventing premature activation of CDK2/cyclin E; therefore, loss of p53 alone can lead to promiscuous activation of CDK2/cyclin E to the level sufficient for centrosome reduplication when DNA synthesis inhibitor is administered, resulting in centrosome amplification (26, 48). In contrast, in human cells treated with DNA synthesis inhibitor, loss of p53 function alone may not be sufficient for promiscuous activation of CDK2/cyclin E. One explanation is that loss of p53 cannot override the stringent control of cyclin E expression in human cells. Only when cyclin E is exogenously introduced, centrosomes can undergo multiple duplication. In support of this scenario, silencing of endogenous p53 by siRNA in RT-4 cells alone allowed only minimal induction of centrosome amplification when treated with HU, whereas introduction of silencing of p53 and cyclin E overexpression allowed efficient induction of centrosome amplification on exposure to HU.6 It should be noted that ectopic expression of cyclin E alone in RT-4 cells did not confer efficient centrosome reduplication in the presence of HU, suggesting that the p53-p21 pathway activated in response to HU treatment is sufficient for suppressing CDK2 binding to endogenous and exogenously introduced cyclin E and that loss of p53 is prerequisite for centrosome reduplication in the presence of DNA synthesis inhibitors in human cells. Thus, we propose the following model. Cycling cells in any given environment (either in vitro or in vivo) are subjected to internal and external stresses that temporarily halt DNA synthesis. Under such condition, normal human cells elicit the p53-dependent and p53-independent responses, resulting in suppression of CDK2 activity and thus inhibiting centrosome duplication. However, in human cells lacking functional p53, when DNA synthesis is blocked, p53-independent pathways still suppress CDK2 kinase activity (i.e., unavailability of cyclin E), hence not allowing centrosome reduplication. Only in human cells that harbor p53 mutations and uncontrolled expression of cyclin E, centrosomes continue to duplicate, albeit DNA synthesis is blocked, leading to centrosome amplification.

ACKNOWLEDGMENTS

We thank Drs. Brunmelkamp and Agami for providing a p53 siRNA plasmid, and Dr. S. Reed for discussion on cyclin E expression in human cells. We also thank K. George for his technical assistance.

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Induction of Centrosome Amplification and Chromosome Instability in Human Bladder Cancer Cells by p53 Mutation and Cyclin E Overexpression

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