Fluorescence *in Situ* Hybridization Evaluation of c-*erbB-2* Gene Amplification and Chromosomal Anomalies in Bladder Cancer[1]

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ABSTRACT

Oncogene amplification and chromosomal anomalies are found in many solid tumors and are often associated with aggressiveness of cancer. We evaluated the frequency and the role of c-*erbB-2* gene amplification, relative increase in c-*erbB-2* gene copy number, and gain of chromosome 17 in bladder cancer. A total of 29 bladder cancer specimens were examined using fluorescence *in situ* hybridization (FISH). Dual labeling hybridization with a directly labeled centromere probe for chromosome 17 together with a probe for the c-*erbB-2* locus was performed. c-*erbB-2* gene amplification was found in 3.4% (1 of 29) of specimens. Relative increase in c-*erbB-2* gene copy number was found in 41.4% (12 of 29) of specimens and was significantly associated with tumor grade ($P = 0.044$ by Fisher's exact test). Gain of chromosome 17 was identified in 65.5% (19 of 29) of specimens and was significantly associated with tumor grade ($P = 0.002$ by Fisher's exact test) and tumor stage ($P = 0.003$ by Fisher's exact test). Our results suggest that c-*erbB-2* gene amplification, relative increase in c-*erbB-2* gene copy number, and gain of chromosome 17 may play important roles in the development and progression of bladder cancers. Moreover, the use of c-*erbB-2* amplification, relative increase in c-*erbB-2* gene copy number, and gain of chromosome 17 using FISH, together with tumor grade and stage, may provide a more useful clinical indicator in bladder cancer.

INTRODUCTION

Bladder cancer is the fifth most common type of cancer in the United States, with an annual incidence of ~18 cases per 100,000/year. In Japan, the incidence is lower, 7–8 cases per 100,000/year, although gradually increasing. The major difficulty in treating bladder cancer is the limited tools available to accurately predict the behavior of tumors. Although some factors have been demonstrated to correlate with patient outcome, histological grade and clinical stage still remain the most important prognostic variables in determining the clinical management of bladder cancer. Very few cytogenetic or genetic markers have been identified for predicting the progression or recurrence of bladder cancer.

Oncogene amplification is found in many solid tumors and is often associated with aggressiveness and poor outcome of cancer (1, 2). The proto-oncogene c-*erbB-2* is one of the most frequently amplified regions in tumors of various origins, such as breast and ovarian cancers (3). c-*erbB-2* gene amplification and overexpression have been suggested as potentially useful prognostic markers in several malignancies, including those of breast and ovary. Both amplification and overexpression are associated with poor prognosis (3).

The c-*erbB-2* gene, also known as *HER-2/neu*, is located on chromosome 17q21 and has a product that is a tumor antigen, p185. The c-*erbB-2* gene encodes a transmembrane phosphoglycoprotein that is serologically related to the epidermal growth factor receptor. c-erbB-2 protein is a cell surface receptor for tyrosine kinase and has the ability to stimulate cell growth.

Although c-*erbB-2* gene amplification has also been reported in bladder cancer using FISH[4] (4), the correlation between c-*erbB-2* gene amplification, development and progression, and its clinical significance of bladder cancers is unknown. Moreover, molecular studies in bladder cancer have shown a discrepancy between gene amplification and overexpression of c-*erbB-2* (4–7), and the clinical significance of c-*erbB-2* gene amplification remains controversial (7–9).

Structural and numerical chromosomal anomalies such as translocation, inversion, deletion, and gain of chromosomes are usually associated with tumor aggressiveness and progression. No specific chromosomal change, such as Ph chromosome in chronic myelocytic leukemia, has been established in bladder cancer.

Several reports have identified chromosomal numerical aberrations in bladder cancers (10). In a previous comparative genomic hybridization study, gain of chromosome 1p22, 1q31, 3p22–24, 3q24–26, 6p, 8q, 8q21, 10p13–14, 10q, 12q13–15,

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[4] The abbreviations used are: FISH, fluorescence *in situ* hybridization; CEP, chromosome enumeration probe.

Table 1 Normal value study in five normal bladder samples

	Copy number[a]	
	≤2	≥3
c-erbB-2[b]	93.8 ± 8.1	3.4 ± 4.7
Chromosome 17	83.2 ± 8.6	16.8 ± 8.6

[a] Mean and SD of percentage of copy numbers for five samples of normal bladder.

[b] CEP17 ratio, 0.92 ± 0.04.

13q21–34, 17q, 17q22–23, 18p11, or 22q11–13 or loss of chromosome 3p, 8p, 9, 10q, 11p, 11q, 12q, 17p, or Y in bladder cancer was reported (11–13).

Using FISH, numerical changes of chromosome 1, 7, 9, 11, 15, 17, and Y have been shown in previous studies (10, 14–19). Loss of chromosome 9 was associated with low-grade bladder cancers and did not correlate with tumor grade or stage. Gain of chromosome 7 was correlated with increasing tumor grade and stage. Loss of chromosome Y has been shown to have prognostic value in bladder cancer. There are few reports about the correlation between numerical anomalies of chromosome 17 and tumor grade, stage, and clinical outcome.

It is important to clarify the correlation between c-*erbB-2* gene amplification or numerical anomalies of chromosome 17 and the development and progression of bladder cancers. Compared with conventional karyotypic analysis, FISH does not require cell culture and preparation of metaphase nuclei (20–23). Culture of bladder cancer tissue is not convenient; thus, FISH is a useful method for interphase cytogenetic analysis of bladder cancer. Recently, FISH analysis of interphase cells with a centromere-specific or a region-specific probe has been used for the detection of gene amplification and numerical chromosome alterations in solid tumors, such as breast and prostate cancers.

In this study, we evaluated the frequency and the role of c-*erbB-2* gene amplification and gain of chromosome 17 in 29 bladder cancer specimens, using dual labeling FISH.

MATERIALS AND METHODS

Patients. A total of 29 randomly selected cases of bladder cancer at Yokohama City University were analyzed. Specimens from a total of 29 cases of newly diagnosed bladder cancer were obtained by transurethral resection. To determine the criteria for FISH anomalies, five samples of normal bladder tissue obtained by transurethral resection were also analyzed. After the sample was flash-frozen in liquid nitrogen, it was immediately stored at −80°C until used for FISH. Frozen tumor tissue from 29 bladder cancers was examined histologically, using formalin-fixed, paraffin-embedded sections stained with H&E. Tumor grade was classified according to the pathological grade, and tumor stage was classified according to the tumor-node-metastasis system.

FISH. For isolation of cells, 0.075 M KCl was added to tissue for 10 min, and the tissue was minced with a scalpel to form a suspension of single cells. The cell suspension was fixed in methanol:acetic acid (3:1). The suspension of isolated fixed nuclei was dropped on a slide and fixed by 70°C steam. Target slides were denatured in 2× SSC/70% formamide, pH 7, at 75°C for 5 min and dehydrated in graded ethanol.

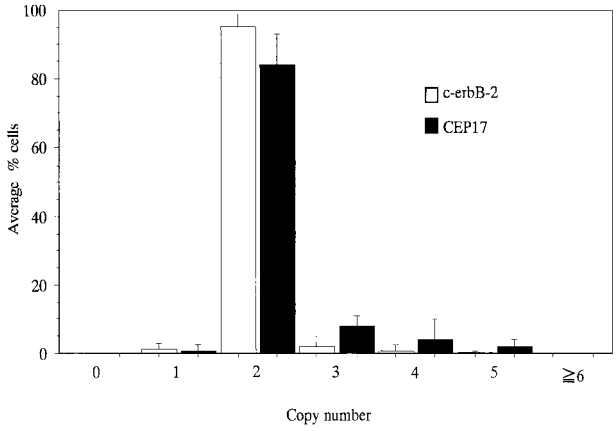

Fig. 1 Dual-color FISH with Spectrum Green-labeled probe specific for chromosome 17 centromere (*green signal*) and Spectrum Orange-labeled probe specific for c-*erbB-2* gene locus (*red signal*) counterstained with 4′,6-diamino-2-phenylindole dihydrochloride. *Bars,* SD.

Dual labeling hybridization with 10 μl of hybridization mix and a directly labeled centromere probe specific for chromosome 17 (Spectrum Green-labeled CEP 17) together with a Spectrum Orange-labeled probe for the c-*erbB-2* locus (17q21; Vysis) was performed. Probes were denatured at 75°C for 5 min and applied to the target slides. Hybridization was performed overnight at 37°C. Posthybridization washes were performed with 50% formamide/2× SSC for 10 min three times, 2× SSC for 5 min, and 2× SSC/NP40 for 5 min at 45°C. Counterstaining was performed with 4′,6-diamino-2-phenylindole dihydrochloride. The number of FISH signals was counted with a NIKON microscope equipped with a triple band pass filter. A minimum of 300 nuclei were evaluated. FISH signals were counted according to the criteria described previously (24) and were recorded as 0, 1, 2, 3, 4, 5, or more signals for each probe.

Normal Value Study. To determine FISH anomalies, five normal bladder samples were investigated using FISH. Detailed data of each probe are listed in Table 1 and Fig. 1.

Criteria for Gene Amplification and Chromosomal Aberrations. The criteria for FISH anomalies were based on FISH analysis of five samples of normal bladder tissue, and the criteria were described previously (24) with slight modifications. Cutoff values were determined based on the upper limit (mean + 3 SD) of the technical variation found in five normal bladder tissue samples, according to FISH studies: (*a*) c-*erbB-2* gene amplification (more c-erbB-2 signals than CEP 17 signals in >17% of cells and c-*erbB-2*:CEP 17 ratio ≥ 4.0); (*b*) relative increase in c-*erbB-2* gene copy number (including c-*erbB-2* gene amplification; more c-*erbB-2* signals than CEP 17 signals in >17% of cells and c-*erbB-2*:CEP 17 ratio ≥ 1.04); (*c*) gain of chromosome 17 (≥42% nuclei with three or more signals for CEP 17).

Statistics. Fisher's exact test was used to analyze the correlation between copy number aberrations and tumor grade and stage.

Table 2 FISH results in 29 bladder cancers

No.	Grade	Stage	Relative increase in c-erbB-2	c-erbB-2 amplification	Gain of 17	c-erbB-2: CEP17 ratio
1	2	pT_a	−	−	−	0.98
2	2	pT_a	−	−	−	1.01
3	2	pT_a	−	−	−	1.01
4	2	pT_{1b}	−	−	−	0.99
5	2	pT_a	−	−	−	1.08
6	2	pT_a	−	−	−	0.96
7	2	pT_a	−	−	−	1.16
8	2	pT_{1a}	−	−	−	0.85
9	3	pT_{3a}	+	−	−	1.09
10	2	pT_{1a}	+	−	−	1.25
11	2	pT_a	−	−	+	0.71
12	3	pT_2	+	−	+	1.12
13	2	pT_a	−	−	+	0.77
14	3	pT_a	+	−	+	2.67
15	3	pT_2	+	+	+	10.5
16	3	pT_{3a}	+	−	+	1.04
17	3	pT_4	−	−	+	0.96
18	3	pT_2	−	−	+	0.96
19	3	pT_{1a}	+	−	+	1.11
20	3	pT_a	+	−	+	1.27
21	3	pT_2	+	−	+	1.31
22	3	pT_2	+	−	+	1.11
23	3	pT_2	+	−	+	1.33
24	3	pT_a	−	−	+	0.98
25	3	pT_a	+	−	+	0.98
26	3	pT_a	−	−	+	0.98
27	3	pT_2	−	−	+	0.98
28	3	pT_2	−	−	+	0.98
29	2	pT_a	−	−	+	0.98

RESULTS

c-*erbB-2* gene amplification, relative increase in c-*erbB-2* gene copy number, and gain of chromosome 17 are summarized in Table 2. Fig. 2 shows typical FISH results for c-*erbB-2* gene and chromosome 17. c-*erbB-2* gene amplification was found in 3.4% (1 of 29) of all tumors. This case was grade 3 transitional cell carcinoma with muscle invasion. Relative increase in c-*erbB-2* gene copy number was found in 41.4% (12 of 29) of all tumors. It was identified in 64.7% (11 of 17) of high grade (grade 3) tumors and 63.6% (7 of 11) of high-stage ($\geq pT_2$) tumors. It was identified in 8.3% (1 of 12) of intermediate grade (grade 2) tumors and 33.3% (6 of 18) of low-stage ($\leq pT_1$) tumors. There was a significant correlation between relative increase in c-*erbB-2* gene copy number and tumor grade (Table 3; $P = 0.0436$) in bladder cancers, but no correlation between relative increase in c-*erbB-2* gene copy number and tumor stage (Table 4).

Gain of chromosome 17 was found in 65.5% (19 of 29) of all cancer tissue samples. It was identified in 94.1% (16 of 17) of high-grade (grade 3) tumors and 90.9% (10 of 11) of high-stage ($\geq pT_2$) tumors. It was identified in 25.0% (3 of 12) of intermediate grade (grade 2) tumors and 50.0% (9 of 18) of low-stage ($\leq pT_1$) tumors. Gain of chromosome 17 was significantly associated with tumor grade (Table 3; $P = 0.0020$) and stage (Table 4; $P = 0.0032$) in bladder cancers.

DISCUSSION

Oncogene amplification is one mechanism that leads to stepwise progression of solid tumors. Moreover, oncogene amplification may be associated with aggressive growth and may be a useful indicator of progression and prognosis in various human cancers (1). A few studies using FISH showed c-*erbB-2* gene amplification in bladder cancers, and the clinical significance of c-*erbB-2* gene amplification is controversial.

Sauter *et al.* (4) reported that c-*erbB-2* gene amplification (defined as more than twice as many c-*erbB-2* signals as centromere 17 signals per tumor using FISH) was found in 10 of 141 bladder cancers, and overexpression was present without amplification in 51 tumors. All tumors with c-*erbB-2* gene amplification showed c-*erbB-2* overexpression. Oncogene overexpression is usually attributable to gene amplification, point mutation, translocation, or transcriptional up-regulation. These data show that c-*erbB-2* amplification may not be a frequent cause of c-*erbB-2* overexpression in bladder cancer.

Sauter *et al.* (4) also reported that c-*erbB-2* gene amplification was more frequent in pT_{2-4} tumors than in pT_{a-1} tumors by FISH study, but there was no correlation with tumor grade. Zhau *et al.* (6) found c-*erbB-2* gene amplification in 2 of 24 high-grade bladder cancers, and Mellon *et al.* (9) reported it in 1 of 24 bladder cancers using Southern blotting. Miyamoto *et al.* reported that c-erbB-2 gene amplification was found in 18 of 57 bladder cancers using PCR, and c-*erbB-2* amplification was an independent prognostic marker (25). Underwood *et al.* (7) reported that of 89 patients with recurrent bladder cancer, 43 had progressive disease, and of these, 14 exhibited c-*erbB-2* gene amplification by differential semiquantitative PCR, indicating a strong association between c-*erbB-2* gene amplification and tumor progression.

In our study, c-*erbB-2* gene amplification was only identified in 3.4% (1 of 29) of all tumors. But relative increase in c-*erbB-2* copy number was found in 41.4% (12 of 29) of all tumors and was significantly associated with tumor grade ($P = 0.0032$, grade 2 *versus* grade 3) in bladder cancers. There was no association between relative increase in c-*erbB-2* gene copy number and tumor stage. These results suggest that relative increase of c-*erbB-2* gene copy number is associated with aggressive bladder cancer and may play an important role in tumor progression.

Using FISH, numerical change of chromosome 1, 7, 9, 11, 15, 17, and Y has been shown in previous studies (10). Loss of chromosome 9 was associated with low-grade bladder cancers. Gain of chromosome 7 was correlated with increasing tumor grade and pathological stage. Loss of chromosome Y has been shown to have prognostic value in bladder cancer. Although numerical anomalies of chromosome 17 have been reported, there are few reports about the correlation between numerical anomalies of chromosome 17 and tumor grade, stage, and clinical outcome in bladder cancers. Hovey *et al.* (11) reported that gain of chromosome 17q11–21.3 (the locus of *erbB-2*) was one of the most common numerical aberrations (23.9%) in bladder cancers. Sauter *et al.* (4) reported that gain of chromosome 17 showed a correlation with tumor grade and stage.

In our normal value study, almost 17% of the normal cells evaluated for chromosome 17 centromere probe had

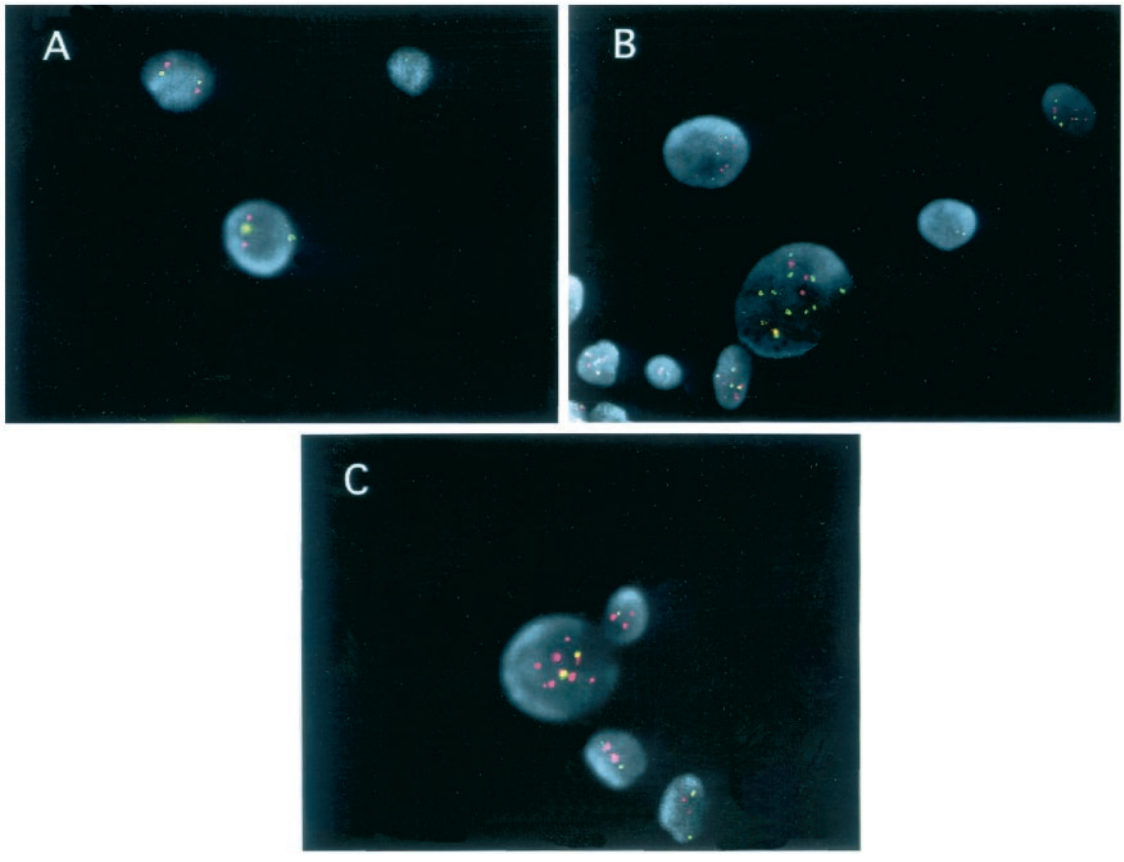

Fig. 2 *A*, nucleus of bladder cancer with two signals for each of *green* and *red*, showing no gain of chromosome 17, relative increase in c-*erbB-2* gene copy number, or c-*erbB-2* gene amplification. *B*, nucleus of bladder cancer with 4–8 signals for *green* and 4–11 signals for *red*, indicating relative increase in c-*erbB-2* gene copy number or gain of chromosome 17. *C*, nucleus of bladder cancer with 2 *green signals* and 4–8 *red signals*. c-*erbB-2* gene amplification was demonstrated.

Table 3 Relation between FISH results and grade in bladder cancers

	No. of cases		
	Grade 2	Grade 3	P^a
Relative increase in c-erbB-2 (n = 12)	1	11	0.0032
No relative increase in c-erbB-2 (n = 17)	11	6	
Gain of chromosome 17 (n = 19)	3	16	0.0020
No gain of chromosome 17 (n = 10)	9	1	

[a] Fisher's exact test, grade 2 *versus* 3.

Table 4 Relation between FISH results and stage in bladder cancers

	No. of cases		
	$\leq pT_1$	$\geq pT_2$	P^a
Relative increase in c-erbB-2 (n = 12)	5	7	0.1186
No relative increase in c-erbB-2 (n = 17)	13	4	
Gain of chromosome 17 (n = 19)	9	10	0.0436
No gain of chromosome 17 (n = 10)	9	1	

[a] Fisher's exact test, $\leq pT_1$ *versus* $\geq pT_2$.

three or more signals, and its rate was high compared with just >3% for the c-*erbB-2* probe. Cross hybridization to other centromere region may be possible. But we confirmed 99% of normal human lymphocytes evaluated for chromosome 17 centromere probe revealed two signals (data not shown). The other possibility was contamination with a small amount of malignant cells, although we confirmed the specimens as histologically normal.

In our study, we investigated numerical aberrations of chromosome 17 in 29 bladder cancers. Gain of chromosome 17 was identified in 65.5% (19 of 29) of all tumors and was significantly associated with tumor grade (P = 0.0020, grade 2 *versus* grade 3) and stage (P = 0.0436, $\leq pT_1$ *versus* $\geq pT_2$) in bladder cancers. These results are consistent with the results reported by Sauter *et al.* (4) and suggest that gain of chromosome 17 is associated with aggressive bladder cancer and may play an important role in tumor progression.

In this study, gene amplification of c-*erbB-2*, relative increase in c-*erbB-2* gene copy number, and gain of chromosome 17 were investigated in 29 bladder cancer patients. As a result: (*a*) a relative increase in c-*erbB-2* gene copy number was significantly associated with tumor grade in bladder cancers; and (*b*) a gain of chromosome 17 was significantly associated with tumor grade and stage in bladder cancers. Our data suggest

that these genetic and chromosomal changes may play important roles in the development and progression of bladder cancers.

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