Detection of non-papillary, non-invasive transitional cell G1 carcinoma as revealed by increased DNA instability and other cancer markers

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The method to reveal DNA-instability as demonstrated by immunohistochemical staining with anti-cytidine antibody after acid hydrolysis (DNA-instability test) was used as a marker of malignancy. The test was applied to paraffin-embedded sections taken from 15 urinary bladders, renal pelvic cavities, and ureters bearing multiple carcinoma in situ (CIS) and totally 31 papillary urothelial cancers. The serial sections of the same tissues were also subjected to immunohistochemical staining for PCNA, p53, DFF45, and VEGF. The DNA-instability test was positive in 100% cancer lesions irrespective of the grades, and apparently normal urothelium, and hyperplastic and dysplastic urothelial lesions also showed the areas with clones positively stained with DNA-instability testing, and the percent numbers of positive areas in them were 28.3%, 37.7%, and 61.5%, respectively. These clones, which were present in apparently normal urothelium and in hyperplastic and dysplastic urothelial lesions, showed higher percent values of PCNA-positive-cells, in comparison to the values estimated in the areas with negatively stained DNA-instability testing, and the former values were statistically not different from those in carcinoma lesions. Furthermore, the percent numbers of areas positive for p53, DFF45, and VEGF, with positive DNA-instability testing were also much higher than those with negative DNA-instability testing in apparently normal urothelium, and hyperplastic and dysplastic urothelial lesions, and the former values were again comparable to those in cancer lesions with no statistical differences. These clones were regarded as already being malignant and should be the direct precursors of progressed cancer lesions. They will make progression through two different pathways, one to papillary non-invasive G1 cancers by neovascularization induced by paracrine secretion of VEGF, and another to flat CIS G2 without secretion of VEGF; thus the clones should be regarded as non-papillary, non-invasive G1 TCC, or CIS G1. It should be always taken into account that the probability for apparently normal urothelium, and hyperplastic and dysplastic urothelial lesions to contain cancer clones, will be high already, especially in tumor-bearing bladders.

Key words: TCC, CIS G1, DNA-instability test, cancer marker, functional atypia, procancer

Urinary bladder cancer is now categorized into two genetic subtypes with marked difference in their degree of genetic instability corresponding to morphologically defined entities (Simon *et al.*, 2004). The genetically stable category includes low grade non-invasive papillary tumors (pTa G1 and G2), and the genetically unstable category contains high grade tumors including pTa G3 and carcinoma *in situ* (CIS) G2 and G3, and invasively growing carcinoma, stage pT1-4.

Non-invasive low- grade papillary bladder neoplasms, pTa G1 and G2, have only few genomic alterations and are viewed as *genetically stable*, according to Simon *et al.* (2004) who stated that losses of (often the entire) chromosome 9, and mutations of the fibroblast growth factor receptor 3 (FGFR3) are the most frequent genetic alterations in these early stages of bladder carcinogenesis.

In contrast, high grade non-invasive lesions (pTa G3 and CIS G2 and G3) are genetically unstable and very different from low-grade neoplasias (pTa G1 and 2) but resemble invasively growing tumors, having many genomic alterations (Simon *et al.*, 2004), such as different chromosomal aberrations, high level amplifications, and p53 mutations (Fujimoto *et al.*, 1992; Cordon-Cardo *et al.*, 1994; Pfister *et al.*, 1999; Hartmann *et al.*, 2000; 2002). Urothelial CIS or high grade-intra-urothelial neoplasia is defined as a non-papillary, flat lesion in which the surface epithelium contains cells that are cytologically malignant, NPT, TCC, G2 and G3. Furthemore, Simon *et al.* (2004) stated that it is unlikely that a relevant fraction of invasive cancer derives from non-invasive papillary low-grade tumor, based on marked differences in genomic alterations between them, and in consistence with the clinical observation that the vast majority of invasive bladder cancers was not preceded by pTa G1 and G2 tumors.

Furthermore, it is apparent that precursor lesions of either invasive or non-invasive urothelial tumors

may include hyperplasia, since significant chromo-somal aberrations can be found in these lesions, also in the absence of dysplasia (Hartmann et al., 1999; Simon et al., 2004). Urothelial hyperplasia may be seen in flat mucosa adjacent to low-grade papillary urothelial lesions, and dysplastic lesions are typically seen in bladders with urothelial neo-plasia and are uncommon in patients without it (Farrow et al., l976; Koss et al., 1979; Rübben et al., 1988; Epstein et al., 1998). Morphologically and genetically, dysplasia shares common features with CIS (Hofstäder et al., 1986), and also may be a precursor lesion of invasive carcinoma at least in some cases, although the natural history of dyspla-sia is poorly understood (Robertson et al., 1990).

If all the above reported data are taken into account, serious questions remain still open as to the direct precursor of CIS and the relationship between pTa and CIS; furthermore it is still unclear which position dysplasia occupies in the process of urothelial carcinogenesis.

In this regard, Fukuda et al., (l986, 1993) found that nuclear DNA of cancer cells is always, and without exception, much more unstable than that of normal and benign tumor cells of the same (either epithelial or mesenchymal) origin. Fukuda et al., (1986) also developed the method of differential fluorescent or immunohistochemical staining of cancer cells after denaturation of DNA by acid hydrolysis. Using this method (which they called the DNA-instability test), cancer clones can be identi-fied during early stages of carcinogenesis in so-called borderline or precancerous lesions such as colo-rectal adenoma (Nitta et al., l993), gastric adenoma (Otaki et al., 1994; Sun et al., 2003), otorhinolaryngeal borderline lesions (Tsuzuki et al., 1994), bone giant cell tumor (Azuchi et al., l998), uterine cervical dysplasia (Khaled et al., 2000), oral leukoplakia (Iwasa et al., 2001), and 20-methylcholanthrene -induced squamous cell carcinoma of mouse epidermis (Hirai et al., 2001).

In the present study, we used this DNA-instability test as a malignancy marker to identify cancer clones in various kinds of urothelial neoplasms as well as apparently normal urothelium, and in hyper-plastic and dysplastic urothelial lesions. Other can-cer markers were also immunolabeled in sections from the same lesions: namely, the proliferating cell nuclear antigen (PCNA: an auxiliary protein for DNA polymerase ‰, playing an important role in the initiation of cell proliferation: Bravo et al.,

1987; Wong et al., 1987; Jaskulski et al., 1988; Yang et al., 1993); p53 (a protein which inhibits the entrance of abnormal cells to S-phase through the GI-S checkpoint and pushes those to apoptosis even after slipping into S-phase: Finlay et al., 1988; Hollstein et al., 1991); the DNA fragmenta-tion factor 45 (DFF45: a protein which inhibits DNA fragmentation at the final step of apoptosis by forming a heterodimer with the DNase, DFF40: Liu et al., 1997; Enari et al., l998; Sabol et al., 1998; Sasaki et al., 1999); and the vascular endothelial growth factor (VEGF: vascular endothelial growth factor, a protein which induces vascular endothelial cell proliferation: Leung et al., 1989).

Materials and Methods

Tissue specimens

Surgically resected 15 urinary bladders, renal pelvic cavities and ureters bearing multiple CIS and totally 31 papillary urothelial cancers were used. After fixation with buffered 20% formalin for 36 h at room temperature, organs containing TCC lesions were cut into serial blocks of tissue with mapping of them at intervals of 5 mm thickness covering the entire mucosal areas, and 4μm paraffin-embedded sections were prepared.

Immunohistochemical staining of paraffin-embedded sections with polyclonal anti-cytidine antibody after acid hydrolysis to demonstrate DNA-instability (DNA-instability test).

(a) Preblocking

The sections were deparaffinized by xylene and replaced by ethanol, followed by washing in water, and intrinsic peroxidase was blocked with 0.03% H_2O_2 solution dissolved in absolute methanol at 20°C for 15 min and rinsed with PBS (pH 7.4). The sections were mounted with 2% skim-milk (Yukijirushi, Sapporo, Japan) dissolved in PBS (pH 7.4) at 37°C for 20 min to block the background adsorption of antiserum. Then they were treated with the blocking solution (Histofine, SAB-PO, Nichirei, Tokyo) at 20°C for 5 min.

(b) HCl hydrolysis and immunohistochemical stain-ing of single-stranded DNA by the ABC method

Sections were then washed in water, followed by

acid hydrolysis with 2N HCl at 30°C for 20 min, and then washed in water again. They were reacted overnight with polyclonal antibody to cytidine (Biogenesis, ONF, UK, 1:1,500 dilution with PBS, pH7.4) at 4°C and then rinsed with PBS (pH7.4). Sections were reacted with biotinylated goat anti-rabbit IgG (Histofine) at 37°C for 40 min, followed by rinsing with PBS (pH7.4) and allowed to react with the avidin-biotin peroxidase complex (Histofine) at 37°C for 30 min, and then rinsed with PBS (pH7.4).

(c) Co-DAB reaction

In order to visualize the peroxidase color reaction, sections were incubated with DAB (3,3′-diaminobenzidine tetrahydrochloride) cobalt solution (DAB, Dojin, Kumamoto, Japan, 50mg dissolved in 100 mL of 0.05M Tris-HCl buffer, pH7.4. added with 2 mL of 1% $CoCl_2$ and 10 μL of 30% H_2O_2) at 20°C for 10 min and washed in water. Nuclear counterstaining was carried out with Kernechtrot (Kernechtrot, Chroma, Stuttgart, Germany, 100 mg and 5g aluminum sulfate dissolved in 100 mL distilled water).

Immunohistochemical staining of PCNA, p53, DFF45, and VEGF

Serial 4 μm -thick sections were prepared from the same specimens used for the DNA-instability test as described above. After washing with water, the intrinsic peroxidase activity was blocked as described above and washed with PBS (pH 7.4). For PCNA staining, the sections were treated by microwave (500w) at 95°C for 5 min; for p53 and DFF45 staining, the sections were irradiated in a poly-propylene slide holder with a cap filled with 10 mM Na-citrate buffer (pH 6.0), over a period of 5 min, using an autoclave (Hirayama, 1.5 kw, Japan); and for VEGF staining, the sections were treated with proteinase K (DAKO, S-3020) for 8min at room temperature, respectively. Then the specimens were reacted with the blocking solution [Histofine, PO (M)] at 20°C for 5 min. This was followed by reactions at 4°C overnight with the following primary antibodies: monoclonal anti-PCNA antibody [DAKO, M-0879, 1:100 dilution in PBS, (pH7.4)] and monoclonal anti-human p53 tumor suppressor protein antibody [DAKO, Clone DO-7, 1:100 dilution in PBS (pH 7.4)]. Then sections were washed with PBS (pH7.4), and further reacted with

biotinylated rabbit anti-mouse IgG [Histofine, PO (M)] at 37°C for 30 min, followed by rinsing with PBS (pH 7.4). They were allowed to react with the avidin-biotin-peroxidase complex at 37°C for 30 min and rinsed with PBS (pH 7.4). In order to visualize the peroxidase color reaction, the sections were incubated with 0.02% DAB [20 mg DAB dissolved in 100 ml of PBS (pH 7.4)] at room temperature for 10 min and washed in water. Nuclear counterstaining was performed with hematoxylin.

Immunohistochemical stainings for DFF45 and VEGF were performed in a similar fashion except for using polyclonal anti-DFF45 antibody [NCL-DFFp, Novocastra, Newcastle, UK, 1:200 dilution in PBS (pH 7.4)] and polyclonal anti-human VEGF antibody[Santa-cruz A-20; 1:200 dilution in PBS (pH7.4)] as the primary antibody, respectively. Biotinylated goat anti-rabbit IgG was used this time as the secondary antiserum with the blocking solution for rabbit [Histofine, PO(R)].

Evaluations of immunohistochemical staining for the DNA-instability test, PCNA, p53, DFF45 and VEGF

As for the DNA-instability test, foci with more than several positively stained cells were regarded as positive. And percent numbers of positively stained areas were determined for all specimens. The PCNA-index of transitional cell carcinoma including pTa and CIS lesion, as well as dysplastic and hyperplastic urothelial lesions and normal urothelium represented the percentage of positively stained cells in each lesion, and was determined in both DNA-instability test-positive and negative areas, respectively. In the same areas in serial sections, foci with more than several positively stained cells for p53, DFF45 and VEGF were regarded positive, and the percent positive area numbers were evaluated in DNA-instability test positive- and negative areas, respectively.

Statistical Analysis

PCNA-indices were presented as mean ± S.D. Data were analyzed using Student's t-test with a p value less than 0.05 considered significant. Staining for p53, DFF45, VEGF, and DNA-instability test were shown as positive percentage. Data were compared in different cases and between DNA-instability test-positive and negative urothelial lesions by

Table 1: All 31 cases of papillary carcinoma lesions classified into 3 groups depending upon their peak-heights with the ratio of constituting Gl, G2 and G3 cancer cell populations.

Group	Height	Number	Cancer population				
A	<6.9mm	11 (35.5%)	G1 (72.7%)	G1(65%)+G2(35%) (18.2%)			G1(40%)+G2(40%)+G3(20%) (9.1%)
B	<6.9mm	9 (29.0%)	G2(60%)+G3 (40%) (44.5%)	G2 (33.3%)			G3 (22.2%)
C	>7mm	11 (35.5%)	G1(27%)+G2(53%)+G3(20%) (45.5%)	G1(30%)+G2(70%) (,272%)	G2 (9.1%)	G3 (9.1%)	G2(70%)+G3 (30%) (9.1%)

Chi-square test. A p value less than 0.05 denoted the presence of a significant statistical difference.

Results

Histopathology

Histopathological assessments of normal urothelial mucosa, simple

hyperplasia, dysplasia, pTa G1, G2 and G3, CIS G2 and G3 and invasive carcinoma were made based upon the criteria reported by Epstein *et al.* (1998) and Simon *et al.* (2004). Flat urothelial hyperplasia consists of a markedly thickened mucosa without cytological atypia. Dysplasia or low-grade intraepithelial neoplasia has appreciable cytologic and architectural changes felt to be pre-neoplastic, yet falling short of the diagnostic threshold for transitional cell CIS.

It was found that some papillary non-invasive TCC lesions (pTa tumors) were exclusively composed of G1 cancer cells, but others were intermingled with various fractions of G2 and G3 cancer cells (Table 1). In pTa lesions with the peak-heights (the maximum height as measured from the base of a papillary TCC lesion to the top in the section cut along the long axis) shorter than 6.9 mm, two apparently different groups of tumors were identified. One group (Group A) was mainly composed of G1 cancer cells, although it sometimes contained also G2 and G3 cancer cells, and another (Group B) was composed of only G2 and G3 cancer cells without Gl cancer cells. In 11 papillary cancer lesions belonging to the former group, 8 (72.7%) were composed of exclusively G1 cancer cells, 2 (l8.2%) were composed of G1 and G2, and l (9.1%) was composed of G1, G2 and G3 cancer cells, and the percent cell numbers of Gl cancer cells were predominantly high even in the lesions mixed with G2 and G3 cancer cells (Table 1).

In 9 papillary cancer lesions belonging to the latter group, 3 (33.3%) were composed of exclusively G2 cancer cells, 4 (44.5%) were composed of G2 and G3 cancer cells, and 2 (22.2%) were composed exclusively of G3 cancer cells.

In 11 papillary cancer lesions belonging to the group of pTa lesions with the peak-heights longer than 7 mm (Group C), no case was composed of exclusively Gl cancer cells and 3 (27.3%) were composed of G1 and G2 cancer cells, l (9.1%) was composed of exclusively G2 cancer cells, 5 (45.5%) were composed of G1, G2 and G3 cancer cells, 1 (9.1%) was composed of G2 and G3 cancer cells without containing G1 cancer cells, and l (9.1%) was composed of exclusively G3 cancer cells, and the percent cell numbers of G1 cancer cells were markedly decreased even in the lesions still containing these cancer cells. All these results are summarized in Table 1.

In the papillary tumor lesions composed of different sub-populations, G1, G2 and G3, no specific localizations of them in papillary carcinoma lesions were identified, and the distribution of them seemed to be random.

Furthermore, the flat urothelial mucosa existing at both sides, or *sleeves*, of the tumor stalks in continuity to papillary cancer lesions were also examined histopathologically and summarized in Table 2. Moreover, 45.5%, 44.4%, and l00% of Group A, B, and C papillary cancer lesions, respectively, showed invasions into the stalks or into the lamina propria, and all cells showing invasions were G2 and/or G3 cancer cells without Gl cancer cells (Table 2). As the results show, all papillary cancer lesions mainly composed of G1 cancer cells belonging to group A had apparently normal urothelial mucosa, hyperplastic or dysplastic mucosa at both side *sleeves*, except 1 (4.5%) case with a CIS urothelium-*sleeve*, but those composed of G2 and G3 cancer cells without Gl cancer cells belonging to group B also often had G2 and G3 cancer cells,

Table 2: The frequencies of apparently normal urothelium and hyperplastic and dysplastic urothelial lesions existing at the flat mucosae continuous to the papillary carcinoma lesions classified into 3 groups, with the ratio of invasions and fractions of constituting GI and G2, and G3 cancer populations in them.

Groupe	Mucosae at the papillary carcinoma *sleeves*				Invasive lesions
	Nomal	Hyperplasia	Dysplasia	CIS	
A	8/22	10/22	3/22	1/22	5/11 (45.5%)
n=11	(36.4.%)	(45.5%)	(13.6%)	(4.5%)	(G2: 3/11: 27.3%) (G3: 2/11: 18.2%)
B	4/18	0/18	0	14/18	5/9 (44.4.%)
n=9	(22.2%)	(77.8%)		(G2: 2/9: 22.2%)	(G3: 2/9: 22.2%)
C	7/22	5/22	5/22	5/22	11/11 (100%)
n=11	(31.8%)	(22.7%)	(22.7%)	(22.7%)	(G2: 7/11: 63.6%) (G3: 4/11: 36.4%)

namely CIS lesions, at both sleeves. On the other hand, the papillary cancer lesions belonging to group C also often had apparently normal, hyperplastic or dysplastic mucosa at both *sleeves*, although a few had G2 or G3 cancer cells, CIS on both *sleeves*.

Immunohistochemical staining

All results of immunohistochemical stainings are summarized in Table3.

(a) DNA-instability test

After hydrolysis with 2N HCl at 30°C for 20 min, all cancer cells in papillary non-invasive carcinoma G1, G2 and G3, CIS G2 and G3, and invasive carcinoma were positively stained, without exception, with anti-cytidine antibody, indicating increased DNA instability reflecting their malignancy, whereas the vascular endothelial cells, fibroblasts and inflammatory cells in the same slides were all neg-

ative (Figure 1a-d).

On the other hand, apparently normal urothelium, and hyperplastic and dysplastic lesions also showed areas with clones positively stained with the DNA-instability testing, and the percent numbers of positive areas in them were 28.3%, 37.7%, and 61.5%, respectively (Table 3). Furthermore, almost all apparently normal urothelial mucosa, hyperplastic and dysplastic urothelial mucosae existing at the *sleeves* continuous to papillary tumors were positively stained with the DNA-instability test (Figure 2).

The results of histopathological assessments together with the results of DNA-instability testing on all specimens in one cystectomized bladder bearing carcinomas are shown in an overview illustration (Figure 3).

(b) PCNA immunohistochemistry

The distribution of cycling cells (positively stained

Table 3. Summarized results of immunohistochemical stainings for DNA- instability testing, PCNA, DFF45, and VEGF. The percent numbers of PCNA-positive cells, and percent numbers of positive areas for p53, DFF45 and VEGF were listed in both DNA-instability testing positive and negative areas.

	DNA-instability test: (%)	PCNA (mean±S.D.)	p53 (%)	DFF45 (%)	VEGF (%)
Normal (n=60)	positive 28.3 (17/60)	60.69±28.99**	100** (17/17)	82.3** (14/17)	88.2** (15/17)
	negative 71.7 (43/60)	13.52±12.20	9.30 (4/43)	16.28 (7/43)	16.28 (7/43)
Hyperplasia (n=85)	positive 37.7 (32/85)	51.36±30.62**	100** (32/32)	84.4** (27/32)	81.3** (26/32)
	negative 62.4 (53/85)	20.30±24.46	17.0 (9/53)	22.6 (12/53)	15.1** (8/53)
dysplasia (n=65)	positive 61.5 (40/65)	59.52±22.36**	100** (40/40)	90.9** (40/40)	88.6** (39/44)
	negative 38.5 (26/65)	11.50±9.81	20.0 (5/25)	24.0 (6/25)	24.0 (6/25)
Carcinoma (n=35)	100 (n=35)	69.44±30.54	100 (n=35)	100 (n=35)	94.3 (n=33)

(**), statistically significant difference between the values for positive and negative DNA-instability testing
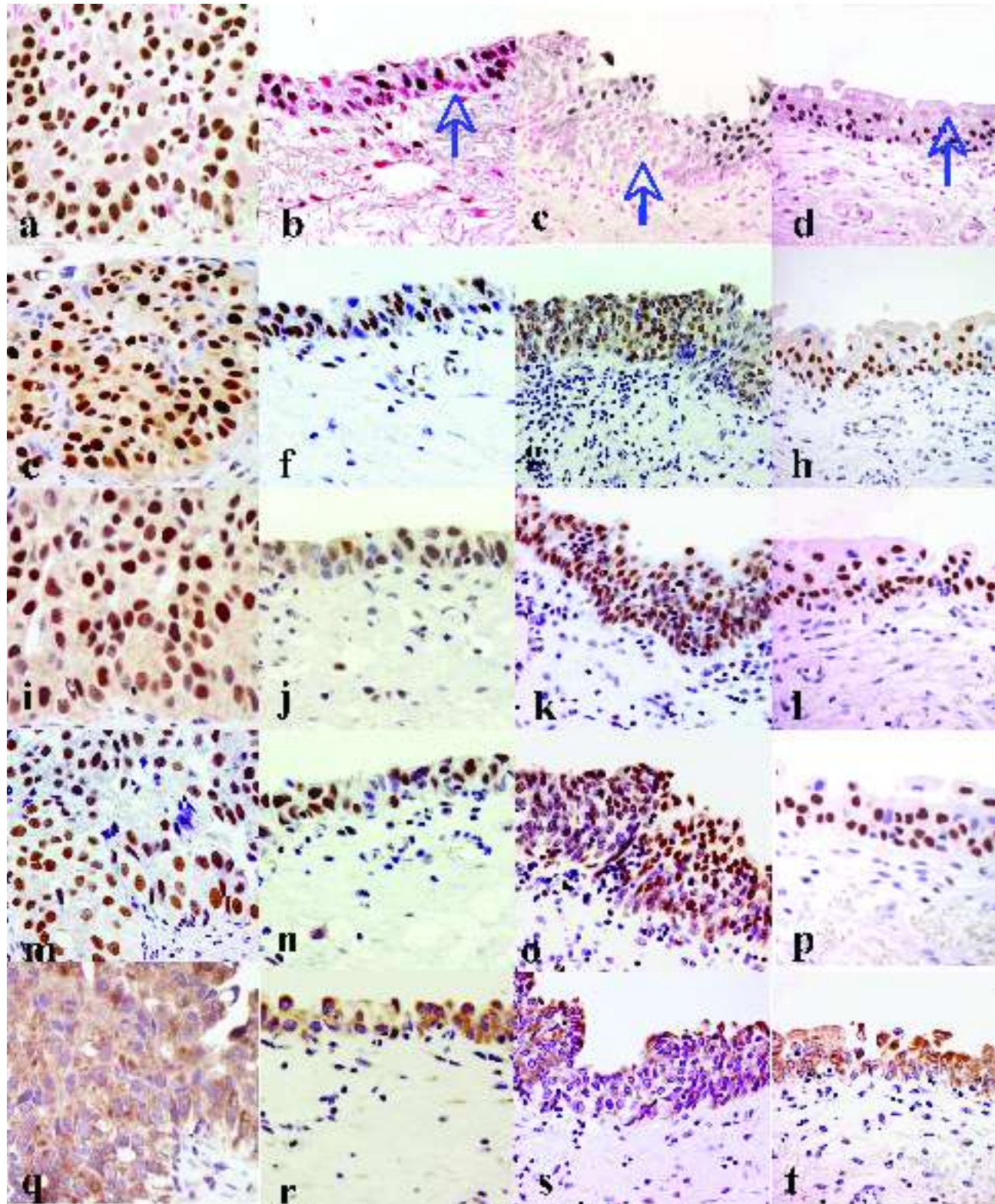
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Figure 1. Summarized results of immunohistochemical stainings for DNA-instability testing (a-d), PCNA (e-h), p53 (i-l), DFF45 (m-p), and VEGF(q-t), in urothelial carcinoma (a, e, i, m, q), dysplastic urothelial lesions (b, f, j, n, r), hyperplastic urothelial lesions (c, g, k, o, s) and apparently normal urothelium (d, h, l, p, t). Arrows indicate the borders of positive and negative DNA-instability testing.
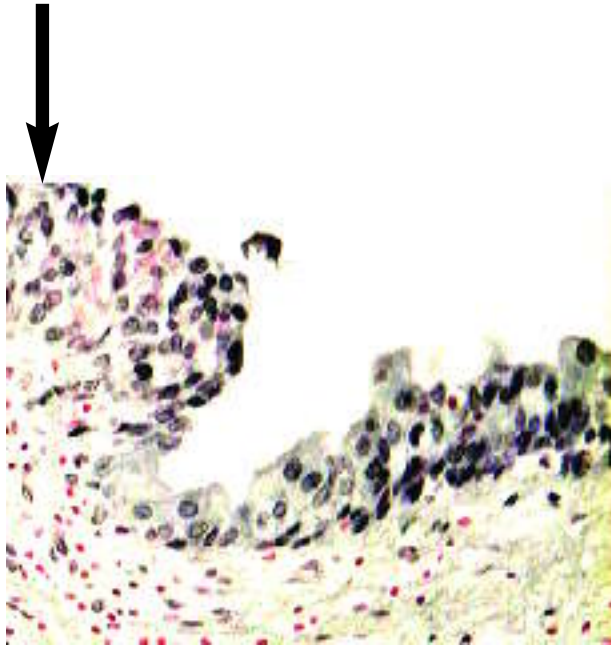
Figure 2. A flat apparently normal urothelium continuous to a small papillary non-invasive carcinoma G1 (indicated by an arrow), positively stained with DNA-instability testing.

for PCNA) in apparently normal urothelial areas which negatively stained with the DNA-instability test was well defined in the basal layer with preserved polarity of cell proliferation and differentiation (PCNA-index, 33.52%), but it was markedly disturbed in the areas positively stained with DNA-instability testing, and even cells in upper layers were also often positively stained for PCNA (PCNA-index, 60.69 %) (Table 3, Figue 1 e, h).

The values for the areas negatively stained with DNA-instability testing in hyperplastic and dysplastic urothelial lesions were 20.30% and 11.50%, and those for the areas in the same lesions positively stained with DNA-instability testing were 5l.36% and 59.52%; the polarity of PCNA-positive cell distributions in dysplastic urothelial lesions were further disturbed, reflecting the progressed loss of polarity of cell proliferation and differentiation. The values of PCNA-index for cancer lesions, were 50.08% for pTa G1, 53.91% for pTa G2, 88.21%for pTa G3, and 60.67% for CIS G2 and 89.33% for CIS G3 (mean value 69.44%); PCNA-positive cells were distributed in the entire cancer lesions with complete loss of polarity. No difference was found statistically, however, among the values for the areas positively stained with DNA-instability testing in apparently normal urothelium, and hyperplastic, dysplastic and cancer lesions (Figure 4, Figure 1 e, h).

(c) p53 immunohistochemistry

All areas positively stained with DNA-instability testing in apparently normal urothelium, hyperplastic and dysplastic urothelial lesions, as well as all cancer lesions including pTa and CIS were also positively stained for p53 (Figure 1 i, l). Whereas among 43 apparently normal urothelium, 53 hyperplastic and 25 dysplastic urothelial lesions with negative DNA-instability testing, only 4 (9.3%), 9 (17%) and 5 (20%) areas expressed p53 positivity, respectively (Table 3). The proportions of p53-positive areas with positive DNA -instability testing were statistically higher than those with negative DNA-instability testing ($p<0.01$) (Table 3). Moreover G2 and G3 cancer cells were stained much more strongly than G1 cancer cells. Furthermore, the percent numbers of p53-positive areas in the DNA-instability test-positive area were comparable to that of cancer lesions (Figure 4).

(d) DFF45 immunohistochemistry

As shown in Table 3, the proportion of DFF45-positive areas with positive DNA-instability testing were 82.4%, 84.4%, and 90.9% in apparently normal urothelium, hyperplastic and dysplastic urothelial lesions, respectively, and the values were significantly higher than those in the areas with negative DNA-instability testing, in which the proportion of DFF45-positive areas were 16.3%, 22.6% and 24%, respectively. All cancer lesions were positive for DFF45, and the percent numbers of DFF45-positive areas in DNA-instability test-positive areas in apparently normal urothelium, and hyperplastic and dysplastic urothelial lesions were comparable to those of cancer lesions (Figure l, m, p, Figure 4).

(e) VEGF immunohistochemistry

The proportion of VEGF-positive areas with positive DNA-instability testing were 88.2%, 81.2%, and 88.6% in apparently normal urothelium, hyperplastic and dysplastic urothelial lesions, respectively, and the values were significantly higher than those with negative DNA-instability testing (Figure 1, q, t), in which the proportion of VEGF-positive areas were 16.3%, 15.1% and 24.0%, respectively as shown in Table 3. In cancer lesions, the proportion of VEGF-positive areas was 94.3%,
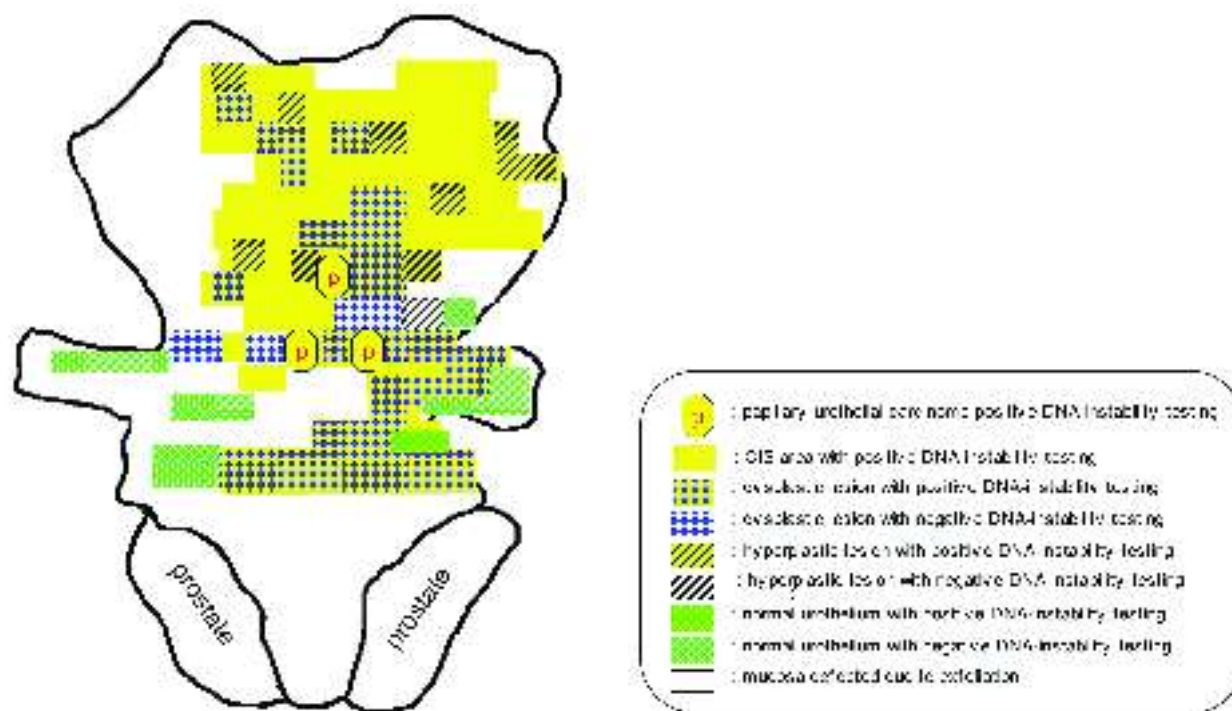
Figure 3. An overview illustration of a surgically resected bladder-bearing cancer lesion cut into blocks covering the entire mucosal surface as shown from the histopathological diagnosis and the results of DNA-instability testing.

and the percent numbers of VEGF-positive areas in DNA-instability test-positive areas in apparently normal urothelium, and hyperplastic and dysplastic urothelial lesions were comparable to that of cancer lesions (Figure 4).

Discussion

The DNA-instability test is based on the finding that the DNA molecule in cancer cells is much more unstable than in normal cells, and is more prone to denaturing by HCl hydrolysis (Fukuda *et al.*, 1986, 1993), reflecting its accumulated genome-wide alterations (Lengauer *et al.*, 1998).

In the present study, all urothelial carcinomas including pTa G1, G2 and G3, and CIS G2 and G3 showed, without exception, diffusely-stained positivity with the DNA-instability test (Fig 1a, Table 3). Furthermore, clones positive to the DNA-instability testing were already detected in apparently normal urothelium, as well as in hyperplastic and dysplastic urothelial lesions, and the percent numbers of areas positively stained with DNA-instability testing in the clones were, 28.3%, 37.7%, and 61.5%, respectively, showing a steady increase in them by clonal expansion (Table 3, Fig 1 b, c, d). These clones were also immuno-positive for p53, DFF45 and VEGF, and the total percent numbers of positively stained areas for these cancer markers in the areas positively stained by DNA-instability testing were comparable to those in cancer lesions, with no statistical differences between them (Figure 4). The percent numbers of PCNA-positive cells in apparently normal urothelium, and in hyperplastic and dysplastic urothelial lesions positively stained with the DNA-instability testing also showed no statistical differences with respect to those in cancer lesions (Figure 4). This suggests that the clones positively stained with the DNA-instability test should be identified as cancer clones at an early stage of cancer progression.

In previous reports (Hirai *et al.*, 2001; Iwasa *et al.*, 2001; Sun *et al.*, 2003), a new concept of *procancer* clone was proposed to designate such early cancer clones. A *procancer* clone shows abnormal phenotypic expressions upon DNA-instability testing, and shows positivity other cancer markers such as PCNA, p53, DFF45 and VEGF, albeit in the absence of the morphological atypia which is commonly used as the basis for cancer diagnosis. These
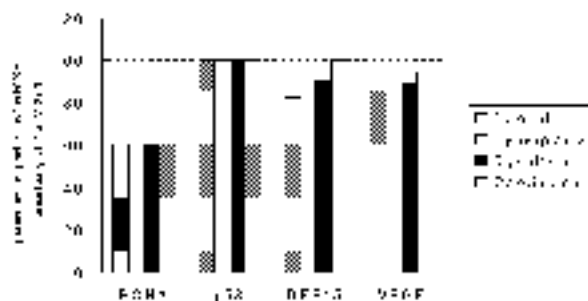
Figure 4. Summarized percent numbers of areas positive for PCNA, p53, DFF45 and VEGF in the areas positive to DNA-instability testing. No differences were found statistically between the values of the clones with positive DNA-instability testing in apparently normal urothelium, and hyperplastic and dysplastic urothelial lesions and those in apparent carcinoma lesions.

functional alterations were referred to as *functional atypia* as opposed to morphological atypia.

The most seriously challenged pitfall in the WHO classification of urothelial neoplasias (Epstein *et al.*, l998; Simon *et al.*, 2004) seems to be that it does not contain a definition of flat non-invasive G1 cancer lesion. Flat urothelial carcinomas without invasion, defined as CIS, contains cytologically identifiable malignant cells with intensified cellular and structural atypia, namely G2 and/or G3 cancer cells. According to this concept, CIS Gl is absent, although it is pointed out that dysplasia shares morphological and genetic features with CIS (Hofstäder *et al.*, 1986). As described in the Introduction, the genetic changes in pTa Gl and G2 cancers were demonstrated to be different from those in CIS G2 and G3, and pTa G2 and G3 should be produced by cancer progression from pTa G1, thereby ruling out the possibility that pTa G2 and/or G3 could be the direct precursor of CIS G2 and/or G3.

The *procancer* clones with high DNA instability would produce multiple sub-clones by enhanced proliferative activity as revealed by increased PCNA-positivity, resulting in clonal expansion. Mutation of the p53 gene would also enhance proliferation and reduce the likelihood of apoptotic cell death of *procancer* clones, and the induction of DFF45 would also make them escape from apoptosis. Paracrine secretion of VEGF would induce the commencing of papillary growth above the flat level of *procancer* clones. The proportion of VEGF-positive areas with positive DNA-instability testing in apparently normal urothelium, and hyperplastic and dysplastic urothelial lesions were 88.2%, 8l.3%, and 88.6%, respectively, as shown in Table

3, and the remaining 11.8%, 18.2%, and 11.4%, respectively, were the *procancer* clones without VEGF secretion. These latter cancer clones will not form papillary structures but will show cancer progression to flat CIS G2 cancers (Figure 5).

The peak-height of a papillary carcinoma should be proportional to the length of its life history after commencing papillary elevation of a pre-existing *procancer* clone due to the stimulated proliferation of dermal vascular endothelial cells by its paracrine secretion of VEGF. The small papillary carcinoma group with peak-heights shorter than 6.9 mm contained two different sub-groups: Group A containing Gl, Gl+G2, or Gl+G2+G3, and Group B composed of G2, G2+G3, or G3 without Gl (Table 1). The group B papillary carcinomas containing high-grade cancer clones with a presumably short life history could not be derived from papillary Gl cancer, because the possibility of so rapid cancer progression of G1 with its complete abolition would be minimum; and they are regarded as having been produced directly from pre-existing flat high-grade cancer lesions, CIS G2 and/or G3 by additional induction of VEGF. The papillary carcinomas belonging to Group A should have been originally derived from papillary G1 cancers by cancer progression during the life history, because Gl cancer clones still remained in large proportions. In Group C papillary carcinomas with a longer life history, two pathways through papillary Gl and through flat CIS G2 and/or G3 may exist (Figure 5). The difference in the genetic alterations observed in non-invasive papillary Gl and G2 cancer lesions and high-grade CIS G2 and G3 cancer lesions again supports the existence of two different pathways in cancer progressions (Stadler *et al.*, 2001).

The fact that no papillary carcinomas composed of Gl+G3 were found indicates that the cancer progression occurs stepwise from G1 to G2, and from G2 to G3, and the direct progression from Gl to G3 does not seem to exist (Table 1).

As shown in Table 2, the mucosae existing at both sides of the stalk, *sleeves* of papillary carcinomas belonging to Group A all showing positive DNA-instability testing, mainly consisted of *procancer* clones in apparently normal, hyperplastic or dysplastic urothelium. And those composed of CIS G2 and G3 comprised only 4.5%, which again indicates that Group A papillary carcinomas were largely derived from *procancer* clones in apparently normal urothelium, and in hyperplastic and dys-
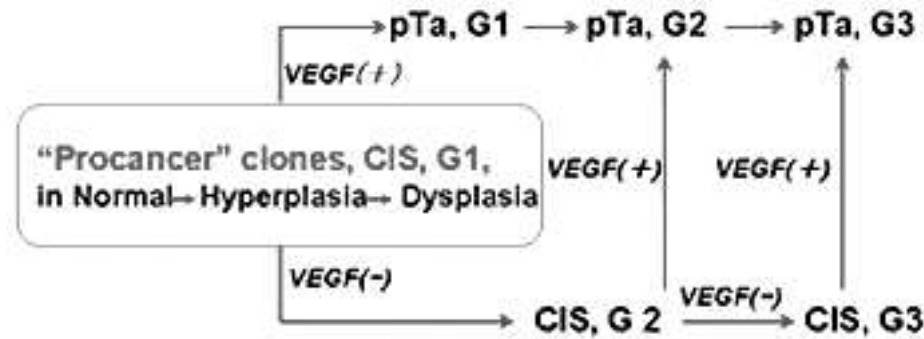
Figure 5. Two different pathways of cancer progression from the "procancer" clones existing in apparently normal urothelium, and hyperplastic and dysplastic urothelial lesions, to pTa GI due to paracrine secretion of VEGF, and to flat CIS G2 without paracrine secretion of VEGF.

plastic urothelial lesions. On the other hand, 77.8% of the *sleeve* were composed of CIS G2 or G3 cancer lesions in Group B papillary carcinomas, indicating that the majority of them were derived from preceding flat G2 and/or G3, CIS lesions. Group C papillary carcinomas had the *sleeves* composed of *procancer* clones in apparently normal, hyperplastic, or dysplastic urothelium positively stained with DNA-instability testing, or in high-grade CIS lesions, indicating again the presence of two different ways of forming papillary carcinomas through *procancer* clones and flat high-grade CIS lesions (Figure 5).

An overview illustration of all specimens contained in one resected tumor-bearing bladder shown in Figure 3 revealed widely spread *procancer* and cancer clones showing multiple continuities. As shown in this paper, many *procancer* clones are found even in apparently normal urothelium, and hyperplastic and dysplastic urothelial lesions, and these phenomena may be specific for the cancer-bearing bladder. This is in accordance with the observations herein reported that urothelial hyperplasia may be seen in flat mucosa adjacent to low-grade papillary urothelial lesions, and dysplastic lesions are typically seen in bladder with urothelial neoplasia and are uncommon in patients without it (Farrow *et al.*, 1976; Koss *et al.*, 1986; Rübben *et al.*, 1988; Epstein *et al.*, 1988). Expansion of the *procancer* clones in apparently normal urothelium by induced proliferative potency with loss of polarity of cytodifferentiation will induce the morphological changes of the urothelium to hyperplastic urothelium, and then to dysplastic urothelium. Morphological changes of normal urothelium to hyperplastic and dysplastic urothelial lesions may, vice versa, indicate the presence of occult *procancer* clones in them, although certain reactive responses might be also included among these changes. Certain carcinogens dissolved in vesical urine could evoke carcinogenesis widely over the entire surface mucosa and presumably produce multiple *procancer* clones, as was seen in experimentally induced epidermal squamous cell carcinoma by topical application of 20-methylcholantrene (Hirai *et al.*, 2001). The multicentric procancer clones will show widespread intra-mucosal infiltration covering wide mucosal areas, partly because cell-to-cell contacts of urothelial mucosa are much looser in comparison to those of squamous cells, possibly allowing *procancer* cells to invade into the adjacent mucosa more easily. The fact that all cells showing invasion into the stalks of papillary carcinomas or into the lamina propria were G2 and/or G3 cells (Table 2) indicates that the acquisition of invasive potency by cancer cells requires additional steps of cancer progression from G1 to G2 and then to G3 stages.

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