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Flow cytometric evaluation of Thomsen-Friedenreich antigen on transitional cell cancer using monoclonal antibody

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Summary. In 31 transitional cell cancer (TCC) tissues and 5 normal bladder mucosae (NBM), we compared the results of flow cytometry (FCM) and immunohistochemical examination in evaluating the expression of Thomsen-Friedenreich antigen (T-Ag) using a monoclonal antibody. On immunohistochemical examination, 14 (45%) cancer tissues showed T-Ag, while 7 (23%) cancer tissues and all NBM showed only cryptic T-Ag, which was detected only after neuraminidase treatment. Ten (32%) high grade cancer tissues showed neither T-Ag nor cryptic T-Ag. On FCM the T-Ag positive cells (TPC) and the T-Ag positive cells after neuraminidase treatment (nTPC) were counted in fresh cell suspensions. FCM was more sensitive than immunohistochemical study in detecting T-Ag. Additionally, FCM revealed that some tumors had both T-Ag and cryptic T-Ag at the same time. The ratio of nTPC to TPC was well correlated with the stage or grade of the tumor and may be a more reliable marker of TCC than the expression of T-Ag assessed by immunohistochemical techniques.

Key words: Transitional cell cancer – Flow cytometry – T antigen – Monoclonal antibody

Bladder cancer accounts for a considerable number of deaths among urological tumors. Although the prognosis for patients with superficial bladder cancer is relatively good, about 25 percent of these tumors ultimately become invasive [12]. Many tumor markers such as chromosomal analysis [2, 7], blood type antigens [1, 4, 5], transferrin [10] and other enzymatic proteins [6] have been reported to be useful in predicting the prognosis. However, more sensitive and specific tumor markers would be of help to physicians in deciding the most appropriate therapeutic strategies.

Thomsen-Friedenreich antigen (T-Ag), which is a precursor of MN blood group antigen and contains the specific carbohydrate chain Gal-β-GalNac, is a known marker of transitional cell cancer (TCC). In most previous reports [3, 4, 13], peanut agglutinin (PNA) was used to detect T-Ag on tissue. We have produced conventional and monoclonal antibodies (mAb) for T-Ag, with which we examined the bladder cancer tissues immunohistologically, as reported in previous papers [8, 9, 14]. The mAb was more sensitive and specific than the polyclonal one or PNA. In normal mucosa, T-Ag is not expressed or it is only detectable after neuraminidase treatment (T-Ag(−), cryptic T-Ag(+); T(−) cT(+)). Many TCC tissues have naked T-Ag (T(+)) on the surface. As reported by many investigators [3, 14], the expression of T- or cT-Ag is correlated with the grade of the tumor; T(−) cT(+) tumors seem to be less malignant while T(−) cT(−) tumors usually have poorer prognoses. On the other hand, T(+) tumors, which comprise nearly half of the cases, exhibit a wide variety of malignant potential. This limits the usefulness of T-Ag as a marker of TCC. In this study, we quantitatively analyzed T-Ag positive cells (TPC) and TPC after neuraminidase treatment (nTPC) with flow cytometry (FCM). The nTPC consisted of TPC and cryptic TPC. We compared the results obtained with FCM to the expression of T- and cT-Ag in TCC obtained using immunohistochemical study.

Materials and methods

Preparation of mAb 3B9 against T-Ag

Glycoprotein was isolated from ghosts of pooled human erythrocytes of types O, NN, by the lithium diiodosalicylatephenol method. It was desialated by 1 N HCl and absorbed on a column of PNA conjugated gel (PNA-GEL, EY Laboratories). After extensive washing, the column was eluted with 0.2 M D-galactose in Tris-buffered saline containing 0.2% Triton X-100 at pH 7.4. The eluant contains T-Ag. BALB/c mice were immunized with 0.5 mg of T-Ag with adjuvant complete Freund. Spleen cells from an immunized mouse were fused with sp2/0 myeloma cells. The surpernatants were screened by the hemagglutination test with desialated red blood cells and ELISA with purified T-Ag. The immunogloblin class of mAb 3B9 was IgG1, as determined with a mouse mAb isotyping kit

(Amersham International plc.). The mAb was purified on a protein A Sepharose column (Pharmacia, Uppsala, Sweden).

Tissue preparation

Fresh surgical TCC tissues from 31 patients (G1; 6 patients, G2; 10, G3; 15) and 5 normal bladder mucosae (NBM) specimens obtained at autopsy within one hour after death were analyzed. A part of the tissue in each patient was fixed with buffered formalin, embedded in paraffin and cut to 5 μm thickness. After deparaffinizing, hydrating and washing thoroughly in phosphate buffered saline (PBS) (pH 7.4), sections were used for immunohistochemical examination. The remaining tissue was minced with scissors in medium RPMI 1640 immediately after resection, and filtrated through a 200 μm nylon filter to remove connective tissues and cell clusters. The cells were counted, centrifuged at 800 G for 5 min and resuspended to the concentration of 10^6 per ml.

Immunohistochemistry

After the endogenous peroxidase was blocked with methanol containing 0.3% H_2O_2 the sections were incubated with normal rabbit serum to prevent nonspecific reactions. Then they were labelled with mAb 3B9 as the first antibody. The sections were stained using the indirect immunoperoxidase method with a Vecstain ABC kit for mouse IgG (Vector Lab., Burlingam, CA). As negative controls the mAb was replaced by normal mouse sera routinely.

FCM

The cells from each patient were divided into four parts (samples 1–4). Samples 1 and 2 were reserved for the TPC evaluation. Samples 3 and 4 used for the nTPC evaluation were treated with neuraminidase type VIII (0.2 units/ml in pH 6.5 PBS, Sigma, West Germany) for one hour at 37°C and washed once with 10 mM PBS of pH 7.2 and centrifugated at 800 G for 5 min. The cells of all four samples were then incubated with PBS containing 10% goat serum to block any nonspecific reactions. Sample 2 for TPC and Sample 4 for nTPC were incubated for 30 min at room temperature with mAb 3B9 solution (1 μg/ml) diluted by PBS. Samples 1 and 3 were incubated with isotype mouse Ig (mouse monoclonal anti-rat IgGl, ZYMED Laboratories, Inc.) at the same concentration and were used as controls for samples 2 and 4, respectively. All four samples were then washed with PBS, centrifuged at 800 G for 5 min and incubated with fluorescein conjugated rabbit anti-mouse Ig (2 μg/ml, Seikagaku Kogyou, Japan) for 30 min at room temperature. Then the cells were washed again, centrifuged and resuspended with PBS at an appropriate concentration (2×10^5 cells/ml).

Epics C flow cytometer was used for cell analysis. The laser power selected was 200 KV. More than 4,000 cells per sample were analyzed. The photomultiplier was arranged to maintain the percentages of positive cells in the control samples (samples 1 and 3) at about 2%. The percentages of TPC in sample 2 or nTPC in sample 4 were then recorded.

Results

On immunohistochemical examination, 14 (45%) of the thirty-one tumors were classified as T(+), 7 (23%) tumors and 5 NBM were T(−) cT(+) and 10 tumors were T(−) cT(−). On T(+) tumors, cells were usually stained diffusely, as shown in Fig. 1.

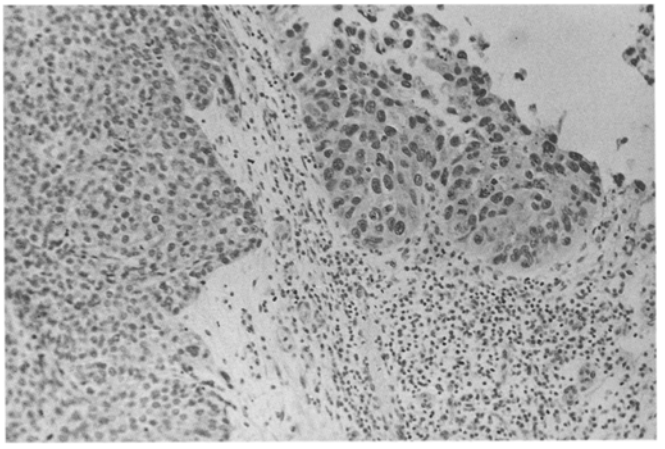

Fig. 1. Immunoperoxidase staining of a deparaffinized tumor (G2). The tumor cells are stained diffusely in comparison to the connective tissues

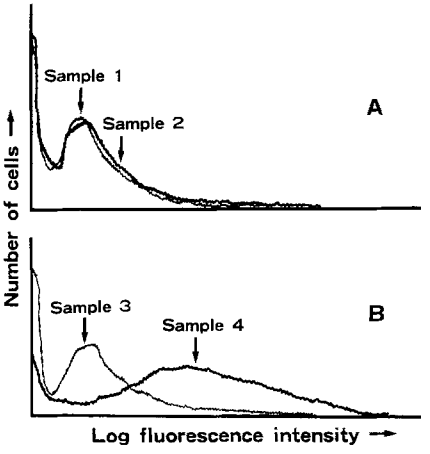

Fig. 2A and B. Expression of T-Ag in NBM. T-Ag were fluorescently labelled with mouse anti-T mAb 3B9 and rabbit anti-mouse IgG labelled with FITC. Samples 1 and 2 were analyzed without neuraminidase treatment (**A**), Samples 3 and 4 were analyzed after neuraminidase treatment (**B**). Samples 1 and 3 were incubated with mouse isotype IgG for the primary antibody, giving a control value of about 2.0%. TPC in sample 2 is 3.2% and nTPC in sample 4 is 30.2%. The nTPC/TPC ratio is 9.5

Figure 2 shows the flow cytograms of NBM. The histogram represents the integral green fluorescence on a decade log scale on the X axis and the cell number on the Y axis. Without neuraminidase treatment, NBM contained no TPC (Fig. 2 A) comparing sample 2 to sample 1, whereas TPC increased markedly after neuraminidase treatment (Fig. 2 B). The flow cytograms of a G2 pT1 tumor are shown in Fig. 3. TPC account for nearly half of the cells in this tumor which did not increase after neuraminidase treatment. In other words, this tumor contains few cryptic TPC. Figure 4 shows the flow cytograms of a G1 pTa tumor. This tumor contains not only TPC but also cryptic TPC, because the number of positive cells increased after neuraminidase treatment.

As shown in Fig. 5, the percentage of TPC in NBM was significantly lower than that in any grade of TCC

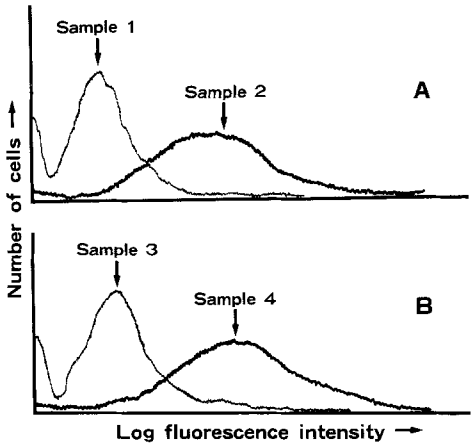

Fig. 3A and B. Expression of T-Ag in TCC (G2, pT1). TPC in sample 2 is 41.6% and nTPC in sample 4 is 45.5%. This tumor has no cryptic T-Ag and the nTPC/TPC ratio is 1.09

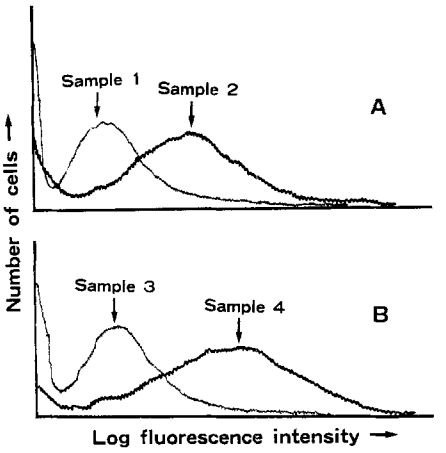

Fig. 4A and B. Expression of T-Ag in TCC (G1, pTa). TPC in sample 2 is 17.4% and nTPC in sample 4 is 37.8%. This tumor has both T-Ag and cryptic T-Ag, and the nTPC/TPC ratio is 2.17

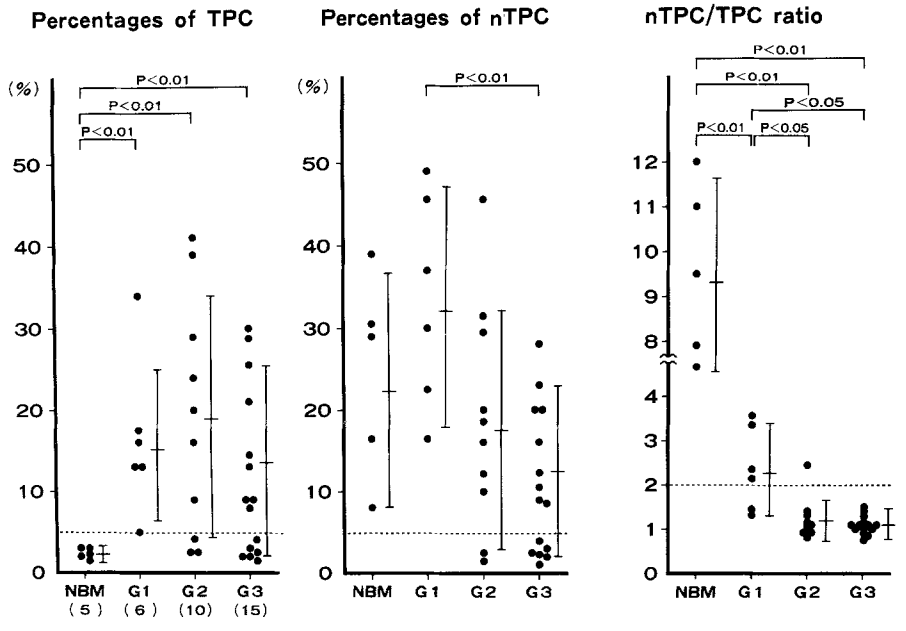

Fig. 5. TPC, nTPC and the nTPC/TPC ratio in the grades of TCC are summarized. The number of cases is shown in parentheses

($p<0.01$), whereas there was no significant difference among the grades of TCC. The percentage of nTPC did not differ appreciably among the groups. The only significant difference was that nTPC in G1 were more frequent than those in G3 ($p<0.01$). The nTPC/TPC ratio was highest in NBM, followed by that in G1 ($p<0.05$) and G2 or G3 tumors. Regarding the tumor stage, the percentage of TPC in NBM was lower than that in any stage of TCC. The percentage of TPC in Ta+T1 was higher than that in T3 tumors. The percentage of nTPC did not differ much among the groups, although Ta+T1 showed a significantly higher percentage of nTPC than T3. The nTPC/TPC ratios was highest in NBM. Among the TCC groups, Ta+T1 showed a higher nTPC/TPC than T2 or T3 (Fig. 6).

Table 1 shows the relation between the tumor grade and the nTPC/TPC ratio in T(+) tumors on immunohistochemical examination. Of 14 tumors, 9 had low nTPC/PC ratios (<2) and consisted of 4 G2 cases and 5 G3 cases. On the other hand, 5 tumors showed high nTPC/TPC ratios (≧2) and consisted of 4 G1 cases and one G2 case. The difference was significant on the X square test.

Table 1. Relation between grade and nTPC/TPC ratio in T-Ag positive tumors on immunohistochemical examination

nTPC/TPC	No. of tumors	G1	G2	G3
<2	9	0	4	5
≧2	5	4	1	0
	14	4	5	5
		χ^2 : P < 0.05		

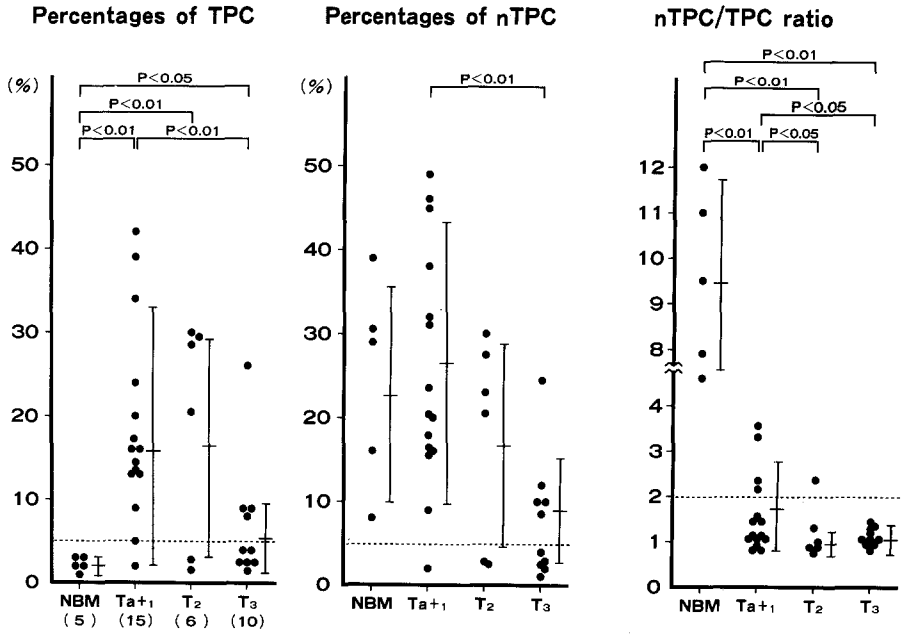

Fig. 6. TPC, nTPC and the nTPC/TPC ratio in the stages of TCC are summarized. The number of cases is shown in parentheses

Discussion

Recently a number of investigators have reported that T-Ag is expressed on several cancer tissues including bladder cancer [11]. Coon et al. [3] found a correlation between the grade and T-Ag expression of TCC. T-Ag is always cryptic or detectable only after neuraminidase treatment on NBM. TCC having cryptic T-Ag are usually less malignant than those with naked T-Ag. Absence of T-Ag even after the neuraminidase treatment or negative cT-Ag is often an ominous prognostic sign. There is, however, a problem in that nearly half of the TCC have T-Ag and they exhibit a wide spectrum of malignant potential [3, 14].

In most previous investigations [3, 4, 13], PNA was used for the detection of T-Ag. We have developed polyclonal and monoclonal antibodies against T-Ag, which was purified from human erythrocytes. These antibodies, especially mAb 3B9, are more specific and sensitive than PNA [8, 14]. In this study, using mAb 3B9, we applied an indirect staining technique on FCM and the ABC method for immunohistochemical study. NBM had only nTPC on flow cytometrical examination, and some T(−) tumors had a low percentageof TPC or nTPC with FCM. On the other hand, 8 of 14 T(+) tumors increased their fluorescence intensities after neuraminidase treatment, as shown in Fig. 4. This shows the presence of both T-Ag and cryptic T-Ag on the same tumor. The nTPC/TPC ratio correlated well with the tumor grade and may be a good tumor marker for malignancy, especially in cases with T-Ag on immunohistochemical examination.

Antigenic heterogeneity with T and cryptic T-Ag can be confirmed only by quantitative analysis using FCM. Additionally, flow cytometric evaluation of T-Ag, unlike histologic examination, does not depend on the experience of the examiners. We could show that 22 of 31 tumors have TPC on FCM (the borderline is 5%, as shown in Fig. 5), although only 14 tumors have T-Ag on immunohistochemical examination. FCM was more sensitive than immunohistochemical examination in detecting T-Ag, and the ratio of nTPC to TPC correlated with the grade and stage of the tumors. As shown in Table 1, T(+) tumors on immunohistochemical examination could be divided into groups with a high and low ratio of nTPC/TPC; the former was less malignant than the latter. The nTPC/TPC ratio is highest in NBM and decreases as the tumor grade rises. Thus, it may be a good marker of TCC, especially in immunohistochemically T(+) tumors.

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