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Flow cytometric evaluation of transferrin receptor in transitional cell carcinoma

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Abstract We evaluated flow cytometric (FCM) analysis of transferrin receptor (TFR) expression as a marker for the malignant potential in transitional cell carcinoma (TCC). TCCs from 55 patients were analyzed by FCM using an anti-TFR monoclonal antibody (CD71) and a TCC-specific monoclonal antibody (EH14), which recognizes most TCC cells irrespective of the grade. The cells were divided into subpopulations according to DNA ploidy determined simultaneously. TFR expression correlated well with the grade and the stage of the tumors. TFR expression of the aneuploid tumors was significantly higher than that of the euploid tumors in all subpopulations. EH14 expression did not correlate with the grade or the stage of the tumors. EH14 expression of the aneuploid tumors was significantly higher than that of the euploid tumors in the whole cell population but not in the subpopulations. In moderately differentiated tumors or in T1 tumors, TFR expression was higher in multiple or recurrent tumors than in simple tumors. The cell size or shape were not the primary reasons for the enhanced expression of TFR in the high-grade or the high-stage tumors; instead, overproduction of TFR may take place in these tumors. Clinically, many of the TCC tumors are grouped into G2 or T1 tumors, some of which will be invasive cancers. Quantitative analysis of TFR expression using FCM may be useful to predict the prognosis of these tumors.

Key words Transitional cell carcinoma · Flow cytometry · Transferrin receptor · EH-14 · DNA ploidy

Introduction

Transferrin receptor (TFR) is a 190 kDa glycoprotein expressed on the cytoplasmic membrane of a wide variety of cells. One of its key roles is control of cell growth through iron uptake. In the 1980s, enhanced expression of TFR was reported in various malignant tissues [6]. In transitional cell carcinoma (TCC), immunohistological expression of TFR was reported to correlate with grade or stage [2, 12]. Although the mechanism or significance of these observations remains unclear, several recent reports have stressed the importance of TFR expression on malignant cells. Barabas and Faulk [1] suggested an association of TFR expression with drug resistance in cancer cells. Kemp et al. [7] reported an inhibitory effect of anti-TFR antibodies on the growth of neoplastic cells. One antibody was used in a phase Ia trial for advanced refractory cancer [3].

Using flow cytometry (FCM), Chin et al. [4] reported that DNA ploidy of cancer tissue correlated well with the histological malignancy of TCC. Because FCM can be used as a quantitative method, we intended to analyze TFR expression with FCM. The size or shape of the cells may influence the FCM data of surface antigens such as TFR because a larger cell with a more complicated surface would be more easily detected than a smaller one with a smoother surface. If a tumor contains more of these large cells, the antigen expression may be elevated on FCM. We have developed a TCC-specific monoclonal antibody (EH14) which stains most TCCs equally, regardless of the grades on immunohistochemistry [13]. We think that this antibody may be used as a control antibody which expresses cell size and shape on FCM. We therefore compared TFR expression and EH14 expression of TCCs which were divided into subpopulations by DNA ploidy in order to know whether TFR expression on FCM can be a quantitative marker of TCC.

Material and methods

Tissue specimens were obtained during transurethral or open surgery from 55 patients with TCC, 41 males and 14 females with a mean age of 73 years. (range 50–86 years). Nineteen of the

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55 patients had well differentiated (G1) tumors, 28 had moderately differentiated (G2), and eight had poorly differentiated tumors (G3). The tumors were T1 in 32 patients, T2 in 17 patients, and T3 in six patients. The specimens were minced in RPMI medium, rinsed with phosphate-buffered saline (PBS), filtered through a nylon mesh (200 μm), frozen in RPMI medium containing 10% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO), and stored at −80°C until use. The cells were again suspended in PBS and divided into three portions: one for TFR analysis using an anti-TFR monoclonal antibody (CD71, Dako), another for EH14, and the other for control. EH14, which we developed, is a TCC-specific monoclonal antibody that recognizes most TCC cells irrespective of the grade but does not recognize normal transitional epithelium [13].

First, cells of each portion were incubated for 1 h with diluted CD71 (×500), EH14 (×250), or normal mouse serum(×100) as control. Washed twice with PBS containing 0.02% EDTA and 4% skim milk by centrifuging at 800 *g* for 5 min, the cells were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated rabbit antimouse immunoglobulin. Washed again, the cells were fixed for 2 h with 5 ml of 70% cold ethanol (−20°C). After the cells were treated with ribonuclease (0.25 mg/ml) for 30 min at 37°C, DNA was stained with propidium iodide (50 μg/ml) (Funakoshi, Tokyo, Japan). The cells were analyzed by a flow cytometer (Epics V, Coulter) after filtration through a nylon mesh.

DNA content was measured with an argon laser (488 nm). DNA ploidy was determined in each sample according to Murphy's method [9]. Briefly, a euploid tumor was defined as a tumor with more than 83% of 2C cells and an aneuploid tumor was defined as one with less than or equal to 83% of 2C cells. Regions of interest were determined on a scattergram of DNA content in order to obtain three subpopulations of cells: diploid (2C), intermediate ploidy (IM), and tetraploid (4C). A euploid tumor contains some IM or 4C cells and an aneuploid tumor contains many diploid cells. These variations may be derived from heterogeneity of the tumor or from stages of the cell cycle. To avoid confusion, we called a tumor with a euploid pattern on histogram as "a euploid tumor" and we called cells gated on the 2C region of the scattergram as "2C subpopulation". Expression of TFR and EH14 were measured according to the method for T-antigen as we reported previously [10]. Briefly, a standard line was determined so that the percentage of the positive cells were approximately 1% of the control sample. Then the percentage of the positive cells was recorded on the stained sample (Fig. 1). Positive cell ratios were obtained either from the whole cell population or from each subpopulation.

The data were statistically analyzed using Mann-Whitney's *U*-test.

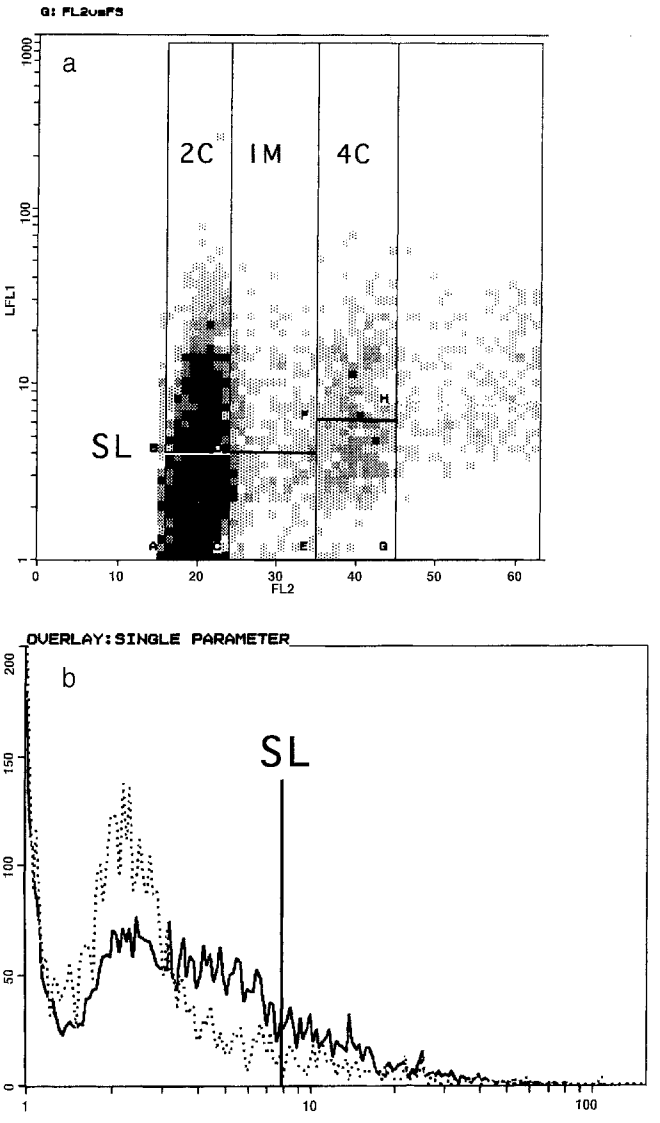


Fig. 1 On a scattergram (*above*), regions of interest were obtained on diploid (*2C*), intermediate (*IM*), and tetraploid (*4C*) subpopulations, respectively. Standard lines (*SL*) were individually determined on a histogram (*below*). The standard line was determined so that the percentage of the positive cells was approximately 1% of the control sample (*dotted line*). Then the percentage of the positive cells was recorded on the stained sample (*continuous line*)

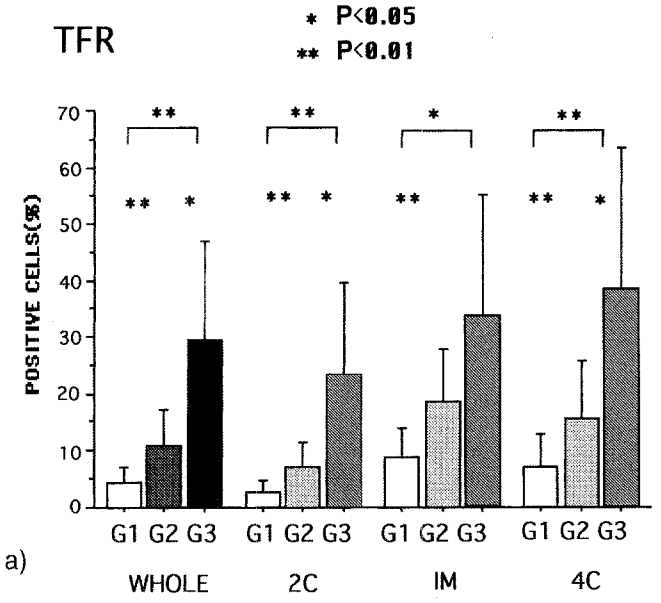
Results

Of the 55 tumors, 26 were diagnosed on the DNA histogram as euploid and the other 29 as aneuploid. TFR expression correlated well with the histological grade either in the whole cell population or in any subpopulation (Fig. 2A), but EH 14 expression did not (Fig. 2B). TFR expression also correlated well with the stage either in the whole cell population or in the subpopulations (Fig. 3A), but EH 14 expression did not (Fig. 3B).

TFR expression of the whole cell population was significantly higher in aneuploid tumors than in euploid ones. The difference was also significant in the subpopulations of cells (Fig. 4A). The difference in EH14 expression between aneuploid and euploid tumors was significant in the whole cell population, but not significant in the subpopulations (Fig. 4B). In G2 tumors or in T1 tumors TFR expression was higher in multiple or recurrent tumors than in simple tumors (Fig. 5).

Discussion

Histological grading is routinely used for evaluation of malignant potential in TCC. However, histological grading alone does not always correlate with the clinical course of the patients. Immunohistological staining of ABO isoantigen [8], T-antigen [8], Tn-antigen [11], TFR [2, 12], or other growth factor receptors [5] may be useful. Because this method is a qualitative analysis, the results depend largely on examiners. Although the DNA ploidy analysis using FCM is also a qualitative examination, FCM can be used quantitatively as we reported previously using a monoclonal antibody against T-an-



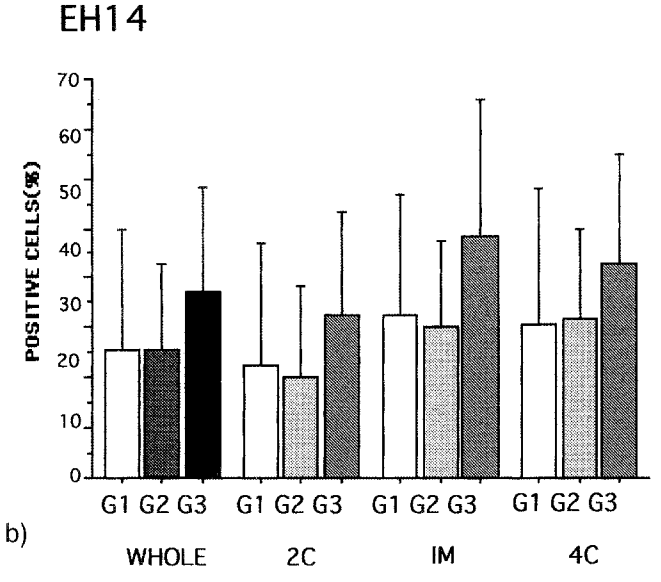
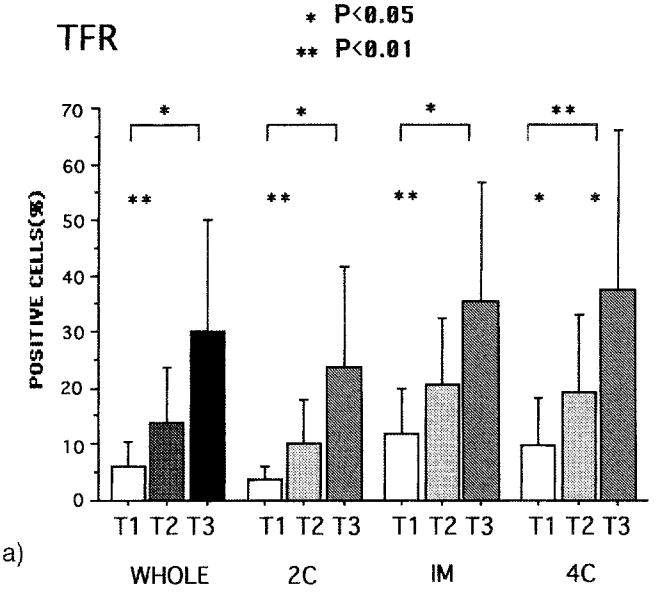


Fig. 2 **a** Transferrin receptor (TFR) expression correlated well with the histological grade. **b** The TCC-spcific monoclonal antibody (EH14) expression did not correlate with the grade either in the whole cell population or in the subpopulations



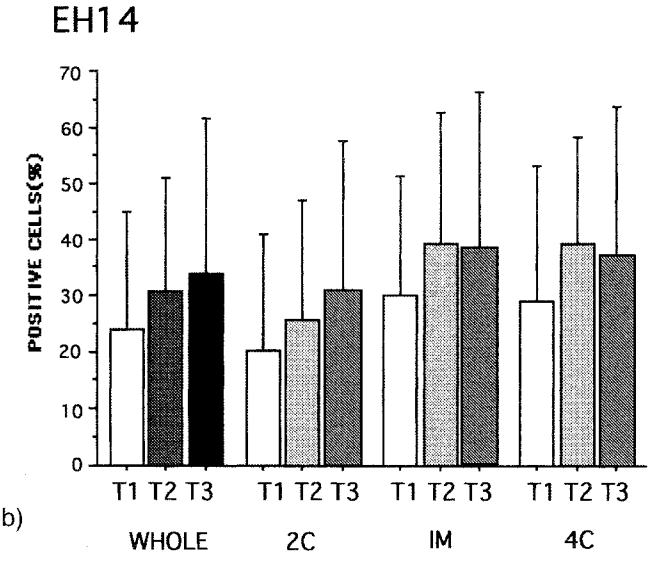


Fig. 3 **a** TFR expression correlated well with the stage. **b** EH14 expression did not correlate with the stage in the whole cell population or in the subpopulations

tigen [10]. This technique is, however, complicated and requires many cells because T-antigen and cryptic T-antigen must be discriminated by neuraminidase treatment. Some investigators reported that TFR expression correlates well with histological grades using immunohistochemistry [2, 12].

We intended to evaluate TFR expression by FCM in order to obtain more objective results than those obtained by the conventional immunohistochemical method. A TCC may contain heterogeneous cells. In addition, the size or shape of the cells may influence the result of FCM for surface antigens such as TFR because a larger cell with a more complicated surface would be more easily detected by FCM than a smaller one with a smoother surface. In order to examine the effect of the cell size or shape on flow cytometric antigen expression, we compared TFR with a TCC-specific monoclonal antibody (EH14), dividing the cells into subpopulations by DNA ploidy. EH14 stains most TCCs equally, regardless of the grades on immunohistochemistry [13]. In fact, EH 14 expression did not correlate with the grade or the stage in the whole cell population or in the subpopulations. On the other hand, EH 14 expression showed a significant difference between aneuploid and euploid tumors in the whole population, but not in the subpopulations. The IM or 4C cells may be larger and may have a more complicated surface than the 2C cells. An aneuploid tumor contains more IM and 4C cells than

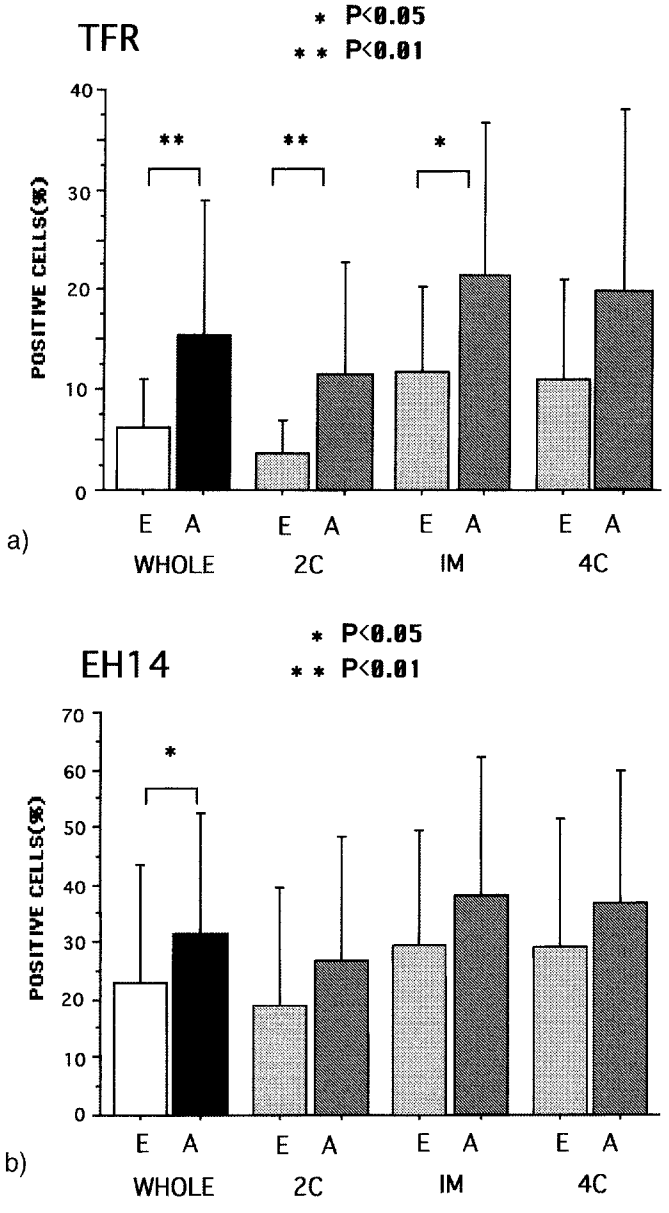


Fig. 4 **a** TFR expression was significantly higher in the aneuploid (*A*) tumors than in the euploid (*E*) ones in the whole cell population and in the subpopulations. **b** EH14 expression was significantly higher in the aneuploid tumors than in the euploid ones in the whole cell population but the difference was not significant in the subpopulations

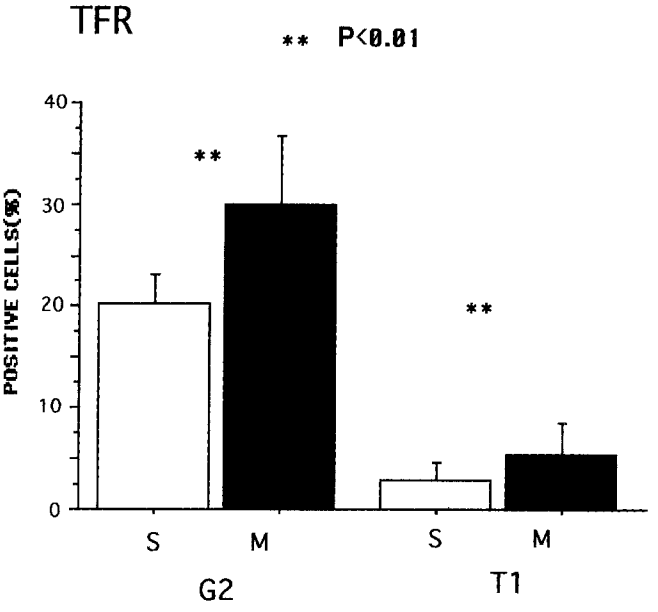


Fig. 5 TFR expression was significantly higher in multiple or recurrent tumors (*M*) than in simple tumors (*S*) both in G2 and in T1 tumors

a euploid tumor does, probably because the former contains more proliferating cells and also because it contains more heterogeneous cell populations than the latter. This can explain why EH14 expression showed a significant difference between the aneuploid and the euploid tumors in the whole population, but not in the subpopulations. On the other hand, TFR expression correlated well with the grade, the stage, and the DNA ploidy in the whole population and in the subpopulations. The enhanced TFR expression in the aneuploid tumors can not be explained solely by the cell size or shape, because the difference was significant in the subpopulations. These results suggests that high-grade or high-stage tumors produce more TFR than other tumors do.

On conventional pathological grading, a large proportion of tumors are liable to be diagnosed as G2, limiting help for therapeutic decision making. In fact, 28 of 55 tumors in our series were G2 tumors. In addition, 32 of the 55 cases were T1 tumors and some of them may be invasive later. On FCM, the recurrent or multiple tumors showed significantly higher TFR expression than the simple and nonrecurrent tumors of G2 or T1. Therefore, the quantitative measurement of TFR expression seems especially useful in these equivocal cases.

Because the number of samples in this study was limited and because flow cytometric evaluation of TFR expression required fresh tissue samples, the correlation between TFR expression and long-term prognosis of the patients remains to be clarified. However, we believe that the quantitative measurement of TFR using FCM is a valuable method to estimate the malignant potential of TCC, especially in patients with G2 or T1 tumors on conventional histological examination.

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