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Evaluation of flow-cytometric three-parameter analysis for EGFR quantification and DNA assessment in human bladder carcinomas

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Abstract Flow-cytometric multi-parameter staining is an excellent method for defining tumour subpopulations. This provides further understanding of tumour heterogeneity and defines the biological relevance of tumour subpopulations. A method of quantifying the epidermal growth factor receptor (EGFR) in parallel with DNA staining, which was previously established in bladder carcinoma cell lines, was applied to twenty-five biopsies of urothelium and urothelial neoplasms. Uro5, a surface glycoprotein, was used to identify urothelial cells. Objective quantification of receptor content via flow cytometry was achieved with beads of defined numbers of antigen-binding sites, and receptor numbers obtained from urothelial and nonurothelial cells were compared with staining intensity in a three-step immunoperoxidase detection of the EGFR. The data obtained matched the immunohistochemical findings and were more sensitive in the low range (ca. 5×10^3) of receptors. Parallel definition of the proliferative fraction and DNA-ploidy of tumour cells means that this method satisfies the requirements of objective quantification for oncological diagnosis.

Key words Multiparameter flow cytometry · DNA measurements · EGFR-quantification · Immunohistochemistry

Introduction

In human bladder tumours upregulation of the epidermal growth factor receptor (EGFR) is correlated with increasing malignancy [15–17], and an objective quantification is of diagnostic interest. Some quantitative methods for the EGFR, including radioligand assays to measure specific protein levels [31, 36, 37] or Northern blots to measure mRNA [21, 23, 24, 33, 34], use whole tumour tissue as source for quantification. Since this is a very variable mixture of tumour cells and inflammatory and mesenchymal stromal cells, objective quantification of tumour cell properties is hampered.

Flow cytometers are apt instruments for objective quantification. While routine measurements are comparative, there is now an emphasis on extending quantification [4]. In previous in vitro studies we have demonstrated that flow-cytometric quantification with Quantum-beads is sensitive in the range of 4×10^4 receptors per cell and have correlated this method with ligand-binding assays [2]. Furthermore, post-transcriptional EGFR regulation of two bladder cancer cell lines was monitored in two- and three-dimensional culture systems and correlated with S-phase fraction (SPF) [3]. The well-differentiated cell line, RT4, showed good correlation of EGFR content and SPF (down-regulation of both EGFR and SPF in three-dimensional spheroid culture), whereas the poorly differentiated cell line, J82, did not (down-regulation of EGFR in spheroids, but no reduction of SPF).

Bladder cancer diagnosis might also be improved by the definition of DNA-ploidy and the proliferative fraction [35], which can be further improved by tumour cell selection with an epithelium-specific antigen such as cytokeratin 18 [19, 22, 39]. In contrast to a flow-cytometric one-parameter DNA analysis, DNA-aneuploid tumours will be detected more easily by a multiparameter technique [9, 11, 22, 29]. The recognition of DNA-aneuploid subpopulations by multiparameter analysis can be utilized as an important diagnostic tool [12].

To combine the advantages of objective EGFR quantification with parallel DNA measurements for DNA-ploidy and proliferation assessment, a three-parameter fluorescence staining was applied to dissociated bladder tumours; this included the use of microbeads with defined numbers of antigenic sites for objective receptor quantification. Measurements were performed on a single laser flow cytometer to maintain easy standardization for clinical use. Since it is technically easier to

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Table 1 Data of EGFR-quantification and DNA analysis of twenty-five primary bladder tumors. Epidermal growth factor receptor (*EGFR*) content of all cells, stromal cells, and cells after Uro5-gating are listed. S-phase fraction (*SPF*) and DNA index (*DI*) of both, the main stem line and the additional stem line are presented. (*SD* standard deviation, *np* not present, *na* not analysed, *EGFR* epidermal growth factor receptor)

Serial no.	Pathol. staging	Pathol. grading	EGFR content (all cells)	EGFR content (non-urothelial cells)	EGFR content (urothelial cells)	EGFR content (urothelial cells±SD)	SPF (urothelial cells)	DI (urothelial cells)	SPF (additional stem line)	DI (additional stem line)
1	pTis	G3	2.94×10^4	17.2×10^4	32.7×10^4	11.8×10^4	27.5	1.7	np	np
2	pTa	G1	1.69×10^4	6.90×10^3	19.4×10^4	4.50×10^3	3.4	1.0	20.7	1.8
3	pTa	G1	2.15×10^4	6.30×10^3	22.5×10^4	4.80×10^3	2.7	1.0	np	np
4	pTa	G1	2.04×10^4	6.30×10^3	24.5×10^4	7.00×10^3	9.5	1.0	np	np
5	pTa	G1	1.84×10^4	2.30×10^3	25.5×10^4	7.20×10^3	2.6	1.0	np	np
6	pTa	G1	4.10×10^3	np	4.10×10^3	1.20×10^3	3.0	1.0	np	np
7	pTa	G2	3.50×10^4	10.2×10^4	38.5×10^3	9.10×10^3	2.9	1.0	np	np
8	pTa	G2	30.6×10^4	8.50×10^3	32.7×10^4	7.80×10^3	8.7	1.0	np	np
9	pTa	G2	3.11×10^4	26.3×10^4	32.0×10^4	2.60×10^3	6.3	1.0	np	np
10	pTa	G3	3.22×10^4	6.30×10^3	39.6×10^4	3.80×10^3	1.5	1.0	np	np
11	pT1	G1	3.84×10^4	33.5×10^4	43.2×10^4	22.9×10^4	10.9	1.0	35.6	1.5
12	pT1	G2	29.3×10^4	n.v.	29.9×10^4	7.10×10^3	4.5	1.0	np	np
13	pT1	G2	20.3×10^4	2.60×10^3	33.5×10^4	9.40×10^3	14.1	1.0	np	np
14	pT1	G2	43.7×10^4	27.3×10^4	72.4×10^4	20.7×10^4	28.0	1.0	32.8	1.7
15	pT1	G2	39.7×10^4	0	49.0×10^4	11.1×10^4	24.0	1.0	np	np
16	pT1	G3	83.0×10^4	27.1×10^4	10.16×10^5	27.0×10^3	7.2	1.0	np	np
17	pT1	G3	22.7×10^4	11.0×10^4	23.8×10^4	10.6×10^4	23.3	1.8	10.7	3.1
18	pT1	G3	96.9×10^4	np	96.9×10^4	25.0×10^4	16.5	1.0	np	np
19	>pT2	G2	10.9×10^4	3.60×10^3	17.5×10^4	4.80×10^3	13.2	1.0	np	np
20	>pT2	G3	27.0×10^4	np	27.0×10^4	16.2×10^4	25.3	1.9	np	np
21	>pT2	G3	83.2×10^4	22.0×10^4	14.30×10^5	44.2×10^4	na	na	np	np
22	>pT2	G3	13.3×10^4	0.50×10^3	16.7×10^4	5.20×10^3	39.8	1.0	42.1	1.7
23	>pT2	G3	9.80×10^3	2.00×10^3	19.0×10^4	6.20×10^4	26.0	1.6	np	np
24	pT3b	G3	71.6×10^4	20.8×10^4	86.3×10^4	25.7×10^4	27.8	1.5	36.6	3.1
25	pT4	G3	7.20×10^3	0	17.8×10^4	7.20×10^3	na	na	np	np

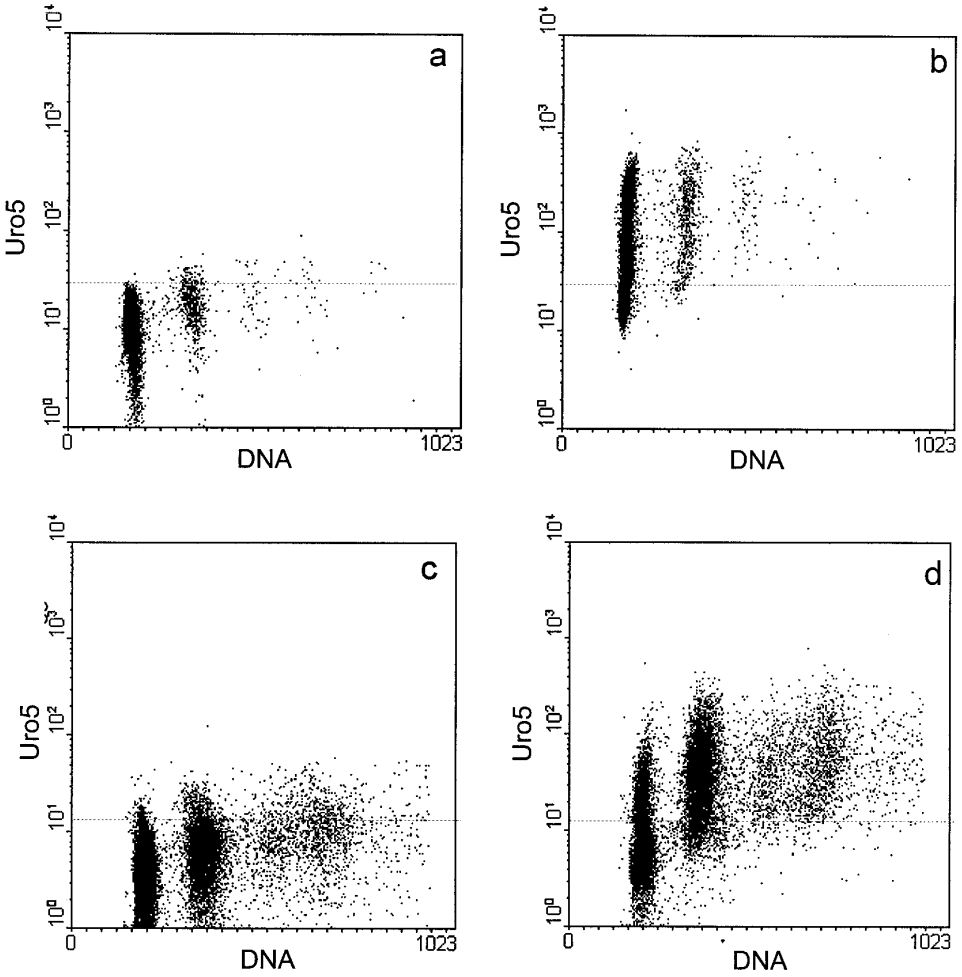


Fig. 1a–d Dot plots of flow-cytometric measurements of two bladder tumours with different proportions of nonurothelial cells. **a**, **c** control measurements; **b, d** Uro5-stained samples. The first example (**a, b**) of a papillary, DNA-diploid tumour contains a tiny proportion of stromal cells, so most of the cells are Uro5 positive. The other case **c, d** demonstrates a DNA-aneuploid bladder tumour with nearly all cells Uro5 positive. Additionally, some DNA-diploid cells are Uro5 stained, indicating a DNA-diploid stem line. The high amount of Uro5-negative, nonurothelial cells is typical of a stroma-invasive tumour. Uro5-positive cells can be gated (*dotted line*) and EGFR quantification can be performed selectively for cancer cells

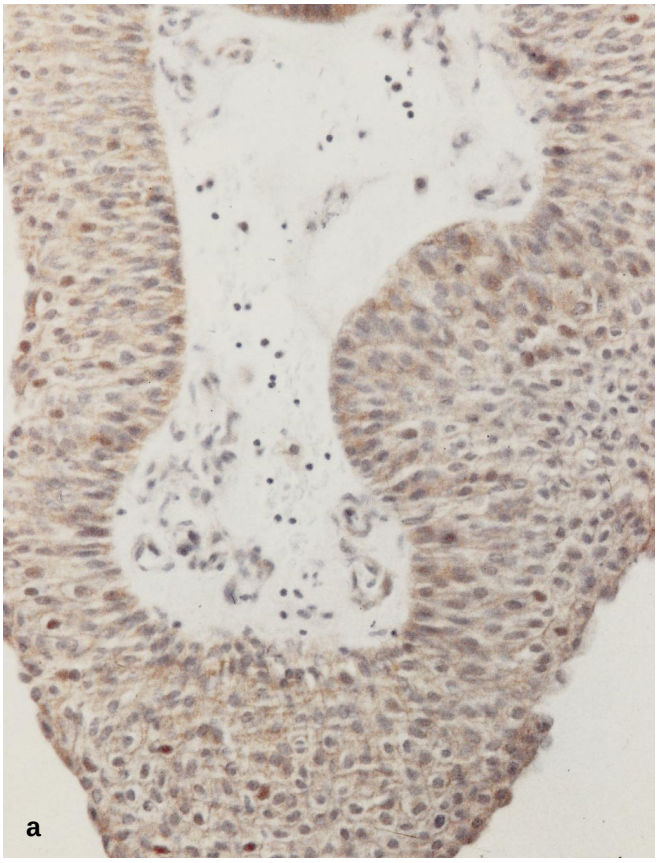
work with cell surface molecules, the antibody Uro5, directed against a cell surface glycoprotein with high specificity for both normal and malignant urothelial cells [7], was used to differ urothelial from nonurothelial cells, and visualized together with the antibody against EGFR. Comparison of the resulting data with immunohistochemical data from reference sections of the same tissue showed that quantification of EGFR was reliable and more sensitive with this technique. In addition, the option of relating EGFR content to the DNA-aneuploid or DNA-diploid tumour stem lines will help validate the often presumed prognostic relevance of the EGFR [13, 17, 18].

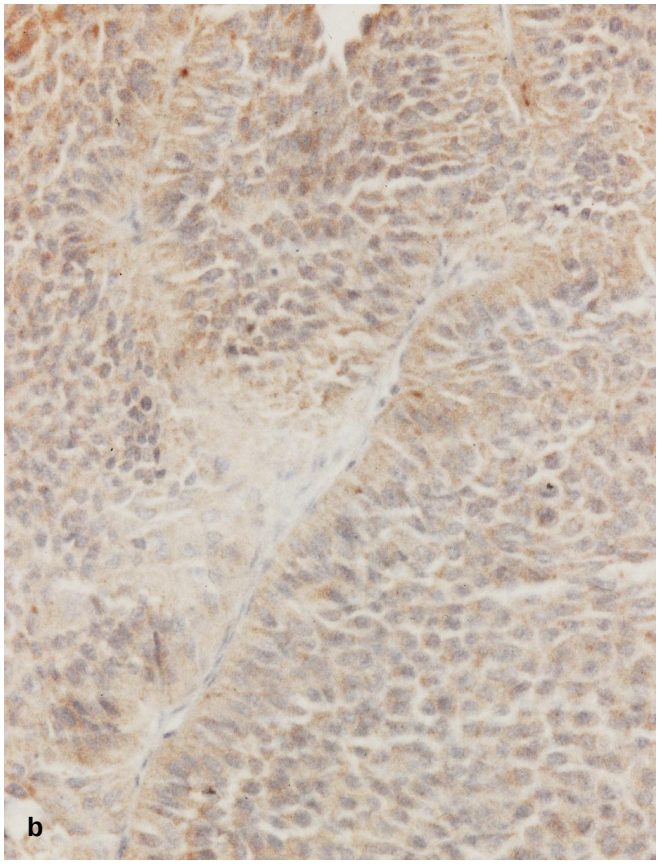
Materials and methods

Specimens of bladder neoplasms, obtained by transurethral resection (TUR) in the Department of Urology (St. Josef Hospital, Regensburg, Germany) were put into ice-cold PBS immediately after removal and directly transferred to the Institute of Pathology. Tumour tissue was inspected by a pathologist, and samples were split for routine pathology with paraffin embedding, for the frozen tumour bank, and direct dissociation for flow-cytometric measurements. Mechanical dissociation of tumour samples was performed with crossed scalpels in petri dishes. The material was then suspended in ice-cold PBS, scraped and needled with a 40-µm syringe, and filtered through a 40-µm nylon mesh. Tumour samples were either resuspended in PBS containing 5% DMSO/2% sucrose (1×10^6 cells per ml) and stored at –20°C, or cells were stained and analysed immediately.

An indirect immunocytochemical staining technique with different antibody subclasses (IgG1 and IgG2b) was applied for simultaneous EGFR and Uro5 staining (A3007, CIS/Uro5, Signet). Concentrations of first antibodies were 5 µg/ml for the EGFR antibody and 2 µg/ml for the Uro5 antibody. Subclass specific rabbit anti-mouse IgG1 (200 µg/ml) and IgG2b (100 µg/ml) antibodies, labelled with fluoresceine-isothiocyanate (FITC) or phycoerythrine (R-PE) respectively, (SBA, Heidelberg, Germany) were used as second-step reagents; neither antibody showed crossreactivity to other IgG subclasses (data not shown). For each measurement, unspecific antibodies of each subclass (IgG1 and IgG2b/Dako, Hamburg, Germany) were carried in parallel samples. After cell permeabilization with 70% MeOH for 1 h at room temperature, propidiumiodide (PI) was chosen for DNA staining in a working dilution of 50 µg/ml. Sections (5 µm) from paraffin blocks were stained with a sensitive three-step immunoperoxidase technique (Kit K0492, Dako) the monoclonal primary antibody Clone Ab-1 (Oncogene Science, Hamburg, Germany) being used in a concentration of 5 µg/ml and DAB as substrate, followed by short counterstaining with haematoxylin. Semiquantitative assessment of staining intensity was applied in tumour and stromal areas of the section separately. As a positive control, sections of the same tumour were included in all measurements to allow comparison of the staining intensities. As a negative control, a nonspecific isotype IgG (Dako) antibody was used for each tumour.

To measure three-parameter fluorescence, cells were gathered on a FACScan flow cytometer (Becton Dickinson) and instrument





Flow-cytometric EGFR quantification (receptor number/cell)

All cells	Nonurothelial cells	Urothelial cells
44000	27000	72000

Flow-cytometric EGFR quantification (receptor number/cell)

All cells	Nonurothelial cells	Urothelial cells
38000	34000	43000

Fig. 2 a, b EGFR stained section of a papillary bladder tumour. Nonurothelial cells 8endothelial cells) show lower EGFR expression: **b** The specimen shows a small number of nonurothelial cells with moderate EGFR expression. **c** Specimen of a well-differentiated papillary bladder tumour. There is hardly any difference in EGFR expression between tumour cells and stromal cells. **d** EGFR stained section of an invasive bladder tumour. Urothelial cells show strong EGFR positivity. However, the large number of nonurothelial cells show few EGFR-positive cells

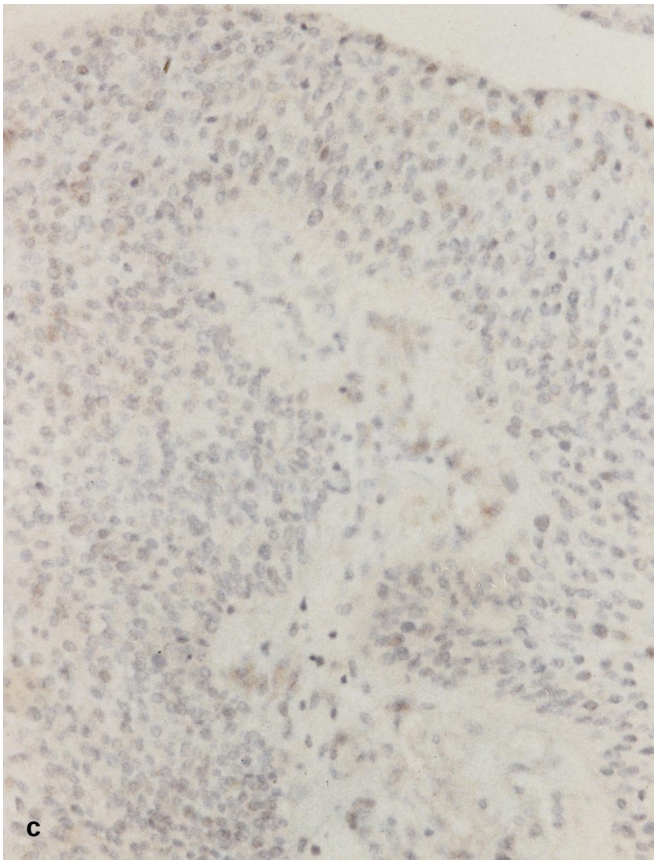
settings were determined for each fluorescence dye individually with the help of an established protocol [3]. All specimens were stained with the DNA dye PI, and three samples/tumour were prepared: cells incubated with unspecific immunglobulins were run as control; a second sample, stained with anti-Uro5 (R-PE) and anti-EGFR (FITC); and a sample, containing microbeads, just stained with EGFR antibodies. As list-mode data, 1×10^5 events/measurement were gathered. Microbeads (Quantum Simply Cellular, FCSE, Leiden, The Netherlands) with defined antigenic binding sites [38] were used for absolute quantification. The batches of beads used were lots nos. 061093, 41693, 120192, and A-0519996. Cells and beads were stained together as one suspension. Instrument settings and data evaluation were carried out as previously described [2, 3].

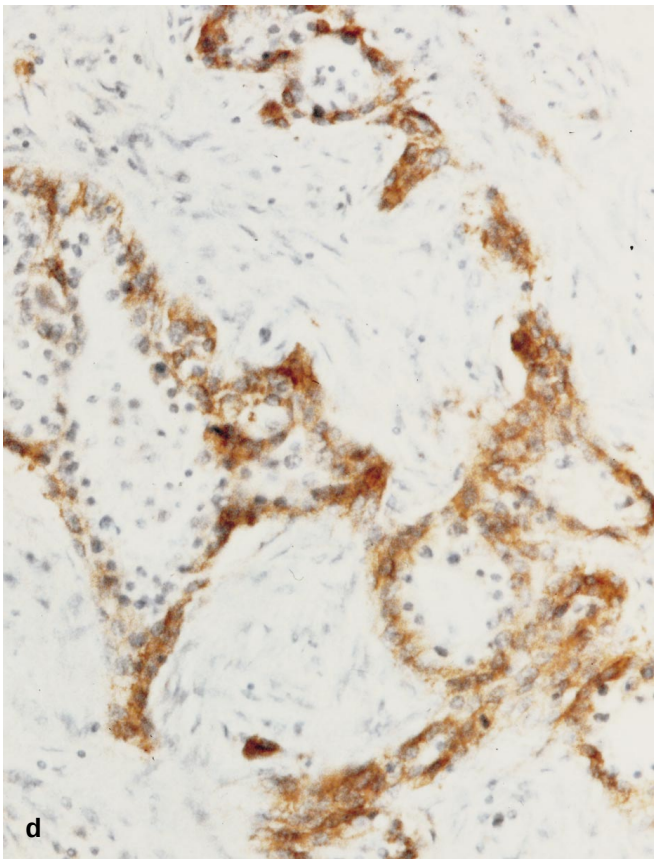
For each measurement a regression curve was calculated by plotting the mean fluorescence intensity for each bead population against the corresponding number of binding sites (Microsoft, Excel 5.0). In all cases the coefficient of correlation was over 0.99. The Mann–Whitney test was used to evaluate differences in EGFR expression for urothelial cells vs all cells. Further subgroups according to grade and stage of the tumour were compared with the Wilcoxon test. Significant differences were assumed at $P < 0.05$. The dispersion of EGFR content of the Uro5-positive cell population was calculated as standard deviation of the mean (Table 1).

Cell cycle analysis and determination of SPF were calculated with the Multi-Plus Software (Phoenix Flow System, Tucson, USA). After rough gating of doublets and debris on a dot plot of the PI signals area versus pulse width, Uro5-positive tumour cells were selected. Then, cell cycle phases were determined using the analysis model of order zero, including the option of further debris and aggregate exclusion [20]. From each tumour sample, Uro5-stained cells were chosen for DNA analysis. Uro5-negative cells served as internal DNA-diploid standard.

Results

Staining of tumour samples and assessment of EGFR quantities were identical in quality to measurements perfomed with three-dimensional tissue cultures [2, 3]. Urothelial cell selection as a basis for EGFR quantification is shown in Fig. 1. Flow-cytometric measurements of two bladder tumours with different amounts of nonurothelial cells are depicted. Tumour one (Fig. 1a, b) is an example of a papillary, DNA-diploid tumour with a low proportion of stromal cells. In Fig. 1a the IgG isotype control staining is shown, which determines the fluorescence intensity of unspecific staining (the control level is shown by the





Flow-cytometric EGFR quantification (receptor number/cell)

All cells	Nonurothelial cells	Urothelial cells
15000	6000	18000

Flow-cytometric EGFR quantification (receptor number/cell)

All cells	Nonurothelial cells	Urothelial cells
83000	22000	1430000

Fig. 2 c, d

dotted line). In Fig. 1b Uro5 staining has been performed: most of the cells are positive for the urothelial marker protein. Figure 1c and d demonstrates a measurement of an DNA-aneuploid tumour. Nearly all DNA-aneuploid cells are Uro5 positive. Additionally, a DNA-diploid Uro5-positive stemline was detected. The large proportion of stromal cells is typical for an invasive tumour. The DNA-diploid stromal cells are Uro5 negative. Nonurothelial cells were discriminated by Uro5-gating.

Table 1 summarizes data relating to EGFR quantification and DNA analysis. The EGFR content is listed separately for tumour cells, stromal cells, and all cells measured. In addition, the dispersion in the EGFR content of tumour cells is indicated. SPF and DI are listed, for both DNA-diploid and DNA-aneuploid subpopulations. Most of the tumours analysed contained mesenchymal cells that were negative for Uro5 but also expressed EGFR. The EGFR content of these cells was determinable after tumour cell selection.

Our data confirm the correlation of tumour grade and EGFR content: poorly differentiated tumours have a higher EGFR content than well-differentiated carcinomas. Tumours with an EGFR content over 7×10^4 per cell are poorly differentiated G3.

In all cases, the EGFR content of nonurothelial cells was found to be lower than the EGFR content of tumour cells. Consequently, the quantified receptor content of all measured cells was always lower than the EGFR content of tumour cells, according to the ratio of stromal and urothelial cells. Overall, we see a significantly higher EGFR content after the selection of urothelial cells than in the total cell population ($P = 0.001$). If we look at a subgroup, such as exclusively invasive tumours (≥ pT1), the tumour cells show a significantly higher receptor content after tumour cell gating ($P = 0.0076$). In non invasive tumours no significant differences in EGFR expression were detected after urothelial cell selection.

SPF of over 20% were determined for invasive tumours (serial nos. 20, 22, 23, 24). All DNA-aneuploid tumours with a DI over 1 are poorly differentiated G3 tumours. Two tumours with a subpopulation DI >3 were found. Both tumours are poorly differentiated G3.

In Fig. 2a–d, four examples of flow-cytometric EGFR quantification in comparison with immunohistochemical

EGFR staining are given. Figure 2a shows a well-differentiated papillary tumour. A higher EGFR content was quantified for urothelial cells than for nonurothelial cells. In Fig. 2b the stromal portion of the specimen is low. The EGFR content for tumour cells is slightly higher than that of the whole cell fraction. Figure 2c is an example of a papillary tumour with very low EGFR expression. Immunohistochemical EGFR staining shows very low intensity for both the tumour portion and the stromal component. Flow-cytometric quantification makes differences in receptor expression become obvious: tumour cells have a slightly higher EGFR expression, with 1.8×10^4 receptors per cell, than do stromal cells, with 6×10^3 EGFR per cell. Figure 2d shows an example of an invasive tumour which demonstrates that after Uro5 gating the EGFR of tumour cells is much higher than in all cells without tumour cell selection. Tumour cells express 14.3×10^5 EGFR per cell, as opposed to the stromal component, with only 2.2×10^4 receptors per cell.

Discussion

Although the EGFR content of cells derived from urothelial tumours is considered to be an indicator of malignancy [13, 17], measurement of the EGFR is not applied as a diagnostic or prognostic tool in clinical practice. We have established a flow-cytometric method for selective EGFR quantification in bladder cancer cell lines [3] and found that a three-parameter staining technique on a single laser instrument is reliable for EGFR quantification and DNA analysis after tumour cell selection. Uro5 was chosen because it is a panurothelial membrane glycoprotein, is well preserved in normal urothelium and urothelial neoplasms, and shows excellent specificity [7]. In vitro studies demonstrate the possibility of simultaneous, flow-cytometric EGFR quantification and cell-cycle analysis on a single laser flow cytometer. Detection of EGFR regulation has been sensitive in the range of 1×10^4 to 4×10^4 receptors per cell and can be correlated with ligand-binding assays [2]. In this paper, we describe the first application of this method to primary tumour specimens and compare EGFR quantification with immunohistochemical staining patterns.

Twenty-five biopsy specimens of bladder tumours were analysed for EGFR quantification and DNA analysis. Receptors were stained with FITC by an immunochemical two-step technique. DNA staining was performed with PI, and the DNA-ploidy and SPF of each specimen was determined. The urothelial-specific protein Uro5 was chosen for cell selection and immunochemically detected with R-PE.

Though the number of samples used in this study is not sufficient for a prospective clinical evaluation, the report shows the practicality and advantages of the method. EGFR expression was found to be unambiguously higher in invasive tumours than in papillary, noninfiltrative tumours that were more highly differentiated. This is in accordance with results from other research groups [15, 17]. After tumour cell gating the difference was more pronounced than without gating. The significant difference found was due mainly to the change in receptor numbers in infiltrative tumours, explained by the fact that papillary tumours commonly have much less stroma than invasive tumours.

More conventional methods, including ligand-binding assays, ELISAs and one- or two-parametric flow-cytometric analysis, also yield quantitative data on EGFR expression, but these techniques are susceptible to error owing to contamination by stromal or inflammatory cells.

While our own immunohistochemical data did not show much intratumour heterogeneity of EGFR in tumour cells, tumours do show inter- and intratumoural variation of the relative amount of stromal cells, which cannot be discriminated by methods using whole tumour tissue. Furthermore, the required cell number in primary tumour samples is often insufficient for radio-ligand binding assays.

Because inflammatory and/or mesenchymal cells can be excluded from the analysis with this method, direct correlations can be made between EGFR expression and DNA content of the cells. DNA-aneuploid subpopulations, mostly related to invasive growth, were found with high proliferation index and excessive EGFR expression. The prognostic value of the DNA-content of solid tumours has been recognized and is documented in several publications [1, 5, 8, 14, 32]. A benefit of multiparametric measurements is the option of direct correlation of two important prognostic markers: DNA-content and antigen (e. g. EGFR) expression.

Figure 1 gives an example for two bladder tumours, which differ in their proportions of nonurothelial cells. Figure 1a and b (first tumour) demonstrates that most of the cells are Uro5 positive. EGFR quantification will be accurate in both two- and three-colour measurements. The second tumour (Fig. 1c, d) contains a high percentage of nonurothelial cells. These cells could be discriminated only with the three-parameter technique, and estimation of EGFR expression will be performed exclusively for tumor cells. While these are the first data, including receptor quantification, similar three-parametric measurements have been tried with carcinoma cell lines [6], and it has been pointed out that tumour subpopulations can be defined. While the overall number of papers on solid tumours is low, the definition and quantification of tumour subpopulations is well established in haematology, and the routine use of these applications in haematological malignancies in increasing [28]. The method demonstrated here is applicable to the investigation of other types of tumours and the analysis of oncoproteins. The technique requires preservation of whole cells and the integrity of both the marker protein and the antigens analysed. Tissue preparation and cell disaggregation have to be adapted to the material of interest and typical tumour growth patterns (medullary or scirrhous) have to be considered. Depending on the tissue type to be analysed, an appropriate marker protein has to be chosen.

Good results regarding cell selection can also be achieved by cytokeratin staining [38]. This antigen is expressed in large amounts of epithelial cells and is resistant to mechanical tissue disaggregation [22].

Immunohistochemistry is a valuable tool in pathology and allows the identification of tumours. Immunohistochemical staining can be related to morphology, but objective quantification is confined mainly to nuclear signals [10] and is still carried out mostly as a semiquantitative score [25–27]. Comparison of an indirect immunofluorescence technique measured with flow cytometry with a three-step peroxidase technique quantified by eye showed that flow-cytometric antigen quantification was more sensitive in the low receptor range. Figure 2c shows an example where differences in EGFR expression between the stromal and the urothelial portions are hardly visible. Here flow-cytometric EGFR quantification of the same tumour resulted in 6×10^3 and 1.8×10^4 receptors for mesenchymal and urothelial cell types respectively. In addition, double staining is hardly used for routine immunohistochemistry; however, it allows rapid identification of several cellular properties within one flow-cytometric measurement.

The data presented indicate a useful method for definition and quantification of subpopulations of solid tumours, which has to be validated in prospective studies and which may well be applicable for determination of tumour prognosis or for monitoring the results of therapy, for example in haematological oncology.

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