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Protein networking in bladder cancer: Immunoreactivity for FGFR3, EGFR, ERBB2, KAI1, PTEN, and RAS in normal and malignant urothelium

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Summary. A panel of markers, selected for the suspected bladder cancer relevance of their corresponding genes, were explored for their expression and subcellular location in urinary bladder tissue. The expression in normal urothelium, in non-metastasised transitional cell carcinomas (TCC), and in primary metastasised TCC with corresponding metastases was mapped. Potential associations between the proteins were identified. The observations were then combined in a set of hypotheses aimed at further hypothesis testing.

Membranous ERBB4 and cytoplasmic p21RAS were downregulated in carcinoma cells compared with normal urothelium cells. FGFR3 was translocated from the cytoplasm to the nucleus. ERBB2 was translocated to the membrane and seemingly upregulated in one subgroup and conversely downregulated in another. EGFR, KAI1 and possibly PTEN revealed increased membranous immunoreactivity in non-metastasised tumours. The metastases showed decreased nuclear FGFR3 and membranous PTEN staining compared with corresponding primary tumours. EGFR expression was positively correlated with the expression of PTEN and FGFR3. The expression of ERBB2 was negatively correlated with p21RAS expression.

According to our results, bladder carcinogenesis comprises FGFR3 translocation to the nucleus, upregulation of EGFR, ERBB2, KAI1 and PTEN; downregulation of p21RAS; and translocation of EGFR, ERBB2, and possibly PTEN to the membrane. Our results support the hypotheses regarding PTEN and KAI1 functioning as tumour suppressors in bladder cancer. EGFR and KAI1 may discriminate between non-metastasised and metastasised cancers. A complex network of associations between the factors is suggested.

Key words: Bladder, Carcinoma, Immunohistochemistry, FGFR3, KAI1, PTEN

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Introduction

Many genetic alterations that likely contribute to the development and progression of transitional cell carcinoma (TCC) of the urinary bladder has been reported. Biochemical and biophysical studies have provided information about the individual gene products; structures, functions, and interactions with other cellular constituents. The challenge is now to understand the reciprocal interplay between these factors.

A frequently used strategy for selection of markers to investigate is, at present, to study proteins with some structural relationship, or proteins known to constitute a signalling pathway. We have in stead chosen to investigate proteins that together contribute to a certain cellular process -development of bladder cancer- and search for interactions between them. Therefore, with a basis in recent reviews of genes currently believed to be important for bladder cancer development and progression (Brandau and Bohle, 2001; Knowles, 2001), and our own recent findings (Røtterud et al., 2004), we selected six of the corresponding proteins and examined their expression in a) normal bladder tissue, in b) metastasised and non-metastasised bladder carcinomas, and c) in primary tumours and their corresponding metastases. By comparing protein expression in these groups and exploring interactions, we aimed to discover

new associations that would be of interest for further confirmatory studies.

The proteins studied:

FGFR3

FGFR3 protein is a type IV tyrosine kinase growth factor receptor (Kim et al., 2001). Binding of a ligand induces dimerisation and activation of downstream signaling pathways, among them the mitogen-activated protein kinase (RAS/MAPK/ERK) pathway and the phospholipase Cγ (PLCγ) pathway. FGFR3 is involved in the regulation of proliferation, differentiation, and apoptosis in a cell type specific manner (reviewed in L'Hôte and Knowles, 2005).

The *FGFR3* gene is mutated in 30-40% of bladder carcinomas (Cappellen et al., 1999; Sibley et al., 2001). Mutations are more frequent in benign bladder tumours and bladder carcinomas of low stage and grade (Billerey et al., 2001; van Rhijn et al., 2002), as compared with those of higher stages/grades (Kimura et al., 2001). FGFR family members are widely expressed in normal epithelium and mesenchyme of multiple human tissues (Hughes, 1997). The FGFR3 protein was not detected in normal urothelium but frequently in bladder carcinomas (Gómez-Román 2005).

EGFR

EGFR is one of the type I tyrosine kinase growth factor receptors. Following ligand binding and activation, the receptor is rapidly internalised and subsequently degraded or recycled. Members of the EGFR family have frequently been implicated in various forms of human cancers and serve both as prognostic markers and therapeutic targets (reviewed in Prenzel et al., 2001). Several signal transduction pathways are involved in EGFR signaling, among them the mitogen-activated protein kinase (MAPK) and the phosphatidyl inositol 3-kinase (PI3K) pathways (Bjorge et al., 1990; Rosen and Greenberg, 1996). EGFR protein has previously been detected in normal human urothelium (Gusterson et al., 1984). Amplifications and rearrangements in the EGFR gene are rarely detected in bladder cancer (Wood et al., 1992), but it is still considered to be one of the most important oncogenes in bladder cancer development (Brandau and Bohle, 2001). We have previously reported that membranous EGFR staining is upregulated in non-metastasised bladder cancer as compared with metastasised cancer and normal urothelium (Røtterud et al., 2004).

ERBB2

ERBB2 is another type I tyrosine kinase growth factor receptor and believed to be the preferred dimerisation partner for the other EGFR family members (reviewed in Rubin and Yarden, 2001). Detectable membrane staining was reported in frozen samples of normal urothelium (Press et al., 1990). The ERBB2 overexpression rates reported in bladder cancer have varied considerably, from 14 to over 70% (Moch et al., 1993; Sauter et al., 1993; Ravery et al., 1997; Ioachim et al., 2000; Chow et al., 2001; Jimenez et al., 2001; Menard et al., 2001). In a previous study, we did find that membranous ERBB2 expression was increased in about 40% of the studied bladder carcinomas, with no significant difference between non-metastasised and metastasised tumours (Røtterud et al., 2004).

KAI1

The KAI1 protein (also called CD82, R2, C33, IA4, or 4F9) belongs to the tetraspanin family of cell surface glycoproteins, initially detected as a tumour metastasis suppressor in prostate cancer (Dong et al., 1995). KAI1 inhibits cell migration (Zhang et al., 2003), and attenuates EGF signaling by accelerating EGFR endocytosis (Odintsova et al., 2000). Although expressed in various normal tissues, KAI1 was not detected by immunohistochemistry (IHC) in normal urothelium (Huang et al., 1997). However, White et al. detected KAI1 protein in frozen normal bladder tissue by western blotting (White et al., 1998). KAI1 protein was reported to be downregulated with the development of several cancers (Guo et al., 1996; Lombardi et al., 1999; Uchida et al., 1999) including bladder carcinomas (Ow et al., 2000), and KAI1 mRNA expression was lost in invasive and high-grade bladder cancers (Yu et al., 1997).

PTEN

PTEN is a dual protein and lipid phosphatase. PTEN antagonises the effect of PI3K through dephosphorylation of phosphatidyl inositol-3,4,5-triphosphate, and dephosphorylates serine, threonine, and tyrosine residues on protein substrates like FAK (Focal Adhesion Kinase) and SHC (SH2 containing protein). Thus, PTEN induces cell cycle arrest and apoptosis and is therefore believed to function as a tumour suppressor (reviewed in Simpson and Parsons, 2001). PTEN is one of the most common mutation targets in human cancers (Cantley and Neel, 1999). Deletions or mutations are reported in more than 20% of bladder cancers, but often affecting one allele only (Cairns et al., 1998; Aveyard et al., 1999). Using Western blotting, Koksal et al. found reduced PTEN protein expression in 4/18 TCC patients. Three of these had muscle-invasive bladder cancer (Koksal et al., 2005).

p21RAS

The p21RAS proteins HRAS, KRAS, and NRAS belong to the RAS subfamily of small GTPases (reviewed in Ehrhardt et al., 2002). In their activated

GTP-bound state, the p21RAS proteins function as signal transducers. Several signal transduction pathways are involved in p21RAS signaling, among them the MAPK pathway, the PI3K pathway, and the PLC pathway (reviewed in Adjei, 2001). p21RAS proteins are widely expressed in normal tissues, with significant variation among cell types (Furth et al., 1987; Chesa et al., 1987). In normal urothelium, p21RAS expression is reported in from 0% to 100% of the cases (Viola et al., 1985; Chesa et al., 1987; Furth et al., 1987; Dunn et al., 1988; Agnantis et al., 1990; Miao et al., 1991). Also, in malignant urothelium the protein expression is reported with considerable variation (Dunn et al., 1988; Ye et al., 1993). The incidence of mutations in the RAS genes varies strongly among different tumour types (Bos, 1989). In bladder carcinoma, the frequency of HRAS mutations is reported from 1 to 30% (mean, 13%) (Knowles and Williamson, 1993; Levesque et al., 1993; Burchill et al., 1994; Saito et al., 1997; Cattan et al., 2000).

Materials and methods

Patients and normal tissue donors

Tissue samples from normal bladders, primary bladder tumours and metastases were obtained from three groups. The "normal urothelium group" (NU; n=15) consisted of bladder biopsies from autopsy cases with no cancer diagnosis. The material was obtained within 24 hours after death, and only samples with intact urothelium were included. The two cohorts of bladder cancer patients were constructed by tracing the hospital registry for all cases referred to The Norwegian Radium Hospital in the period 1985 to 1996. Patients with invasive TCC with histologically verified metastases (the "metastasised cancer group", MC; n=51) and cases with muscle-invasive TCC who did not develop distant metastases during a follow-up period of more than five years (the "non-metastasised group", NMC; n=19) comprised the two groups. All eligible cases with accessible material were included in the study. Tumours with squamous cell carcinoma or adenocarcinoma differentiation were excluded. All samples from the NMC group were TURB biopsies. The primary tumour samples from the MC group were either TURB biopsies (n=38) or cystectomy samples from patients with no pre-operative treatment (n=13). The metastasis samples were either biopsies (n=23), resections (n=25) or autopsies (n=3). Equal handling of all samples regarding fixation and embedding procedures was ensured.

The patients are described previously (Røtterud et al., 2004). Demographic data of the patients are given in Table 1. Altogether, 24 women and 61 men were included. The mean age was 62 years in the NU group (range: 15-87), 62 years in the NMC group (range: 42-76), and 64 years in the MC group (range: 38-81). The gender and age distributions were not significantly different between the three groups (data not shown).

All transitional cell carcinomas showed invasive growth, and the T-categories (Hermanek and Sobin, 1987) were distributed as follows: T1, 6 tumours (all had metastasised); T2, 17 tumours; T3, 28 tumours; and T4, 19 tumours. Two tumours were of WHO grade 1, 15 were of grade 2 and 53 were of grade 3. Metastases were localized in lymph nodes (12, of which 7 were regional); skeleton (3); skin (2); brain (2); lung (6); breast (1); gastrointestinal tract (5); liver (5); kidney (1); vaginal wall (3); penis (1); and other locations (9). The median observation time for patients in the metastasised cancer group was 32 months (range: 1-181), and for those in the non-metastasised cancer group, 112 months (range: 67-164). In patients with metastasised cancer, the median interval between specimen sampling from primary tumour and metastasis was 5.5 months (range: 0-77). T-category and WHO grade (Mostofi, 1973) were settled at the time of cystectomy or biopsy in patients not undergoing cystectomy.

Tissue microarray

The tissue microarray was assembled by transferring two punch cores of 0.6 mm diameter from each donor

Table 1. Patient demographics.

	Normal urothelium, (n=15)	Non-metastasised cancer (n=19)	Metastasised cancer (n=51)
Gender			
female	4	5	15
male	11	14	36
Age			
mean (range)	62 (15-87)	62 (42-76)	64 (38-81)
T-category			
T1	-	0	6
T2	-	9	8
T3	-	9	19
T4	-	1	18
WHO grade			
1	-	0	2
2	-	1	14
3	-	18	35
Treatment*			
1	-	1	20
2	-	17	29
3	-	1	2
Status at last observation			
Alive	-	16	3
Dead of TCC	-	0	48
Dead of benign illness	-	3	0

* Treatment against primary tumour: 1, no curatively intended treatment; 2, cystectomy with or without radiation and/or chemotherapy; 3, curatively intended radiotherapy.

archival tissue block to an empty recipient paraffin block by a manual tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA). Representative areas for tissue punching were selected by careful inspection of hematoxylin-eosin stained sections by a surgical pathologist (JMN). All available blocks from each specimen were evaluated.

Immunohistochemistry (IHC)

From the tissue microarray block, 4 µm sections were immunostained, using either the StrAviGen MultiLink kit (BioGenex, San Ramon, CA, USA) on a BioGenex OptiMax Plus 2.0 automated immunostainer (for FGFR3, EGFR, ERBB2, and PTEN) or the DAKO EnVision + System (K4007, DAKO Corporation, CA, USA) on a Dakoautostainer (for KAI1 and p21RAS). Deparaffinised and rehydrated sections were pre-treated to unmask epitopes, and stained according to the manufacturer's recommendations and data specified in Table 2.

StrAviGen: Briefly, sections were washed in PBS and blocked with 1% hydrogen peroxide (H_2O_2) in ethanol for 10 min at room temperature. After incubation with primary antibodies for 30 min at room temperature, detection of the resulting immune complex was carried out according to manufacturer's instructions. The slides were incubated with biotinylated secondary antibodies (1:30) and horseradish peroxidase-conjugated streptavidin (1:30) for 20 minutes each, before development with 0.07% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Saint Louis, Missouri, USA) freshly prepared in 0.05M tris(hydroxymethyl)aminomethane (Tris-) buffer at pH 7.6 containing 0.02% H_2O_2, and counterstaining with haematoxylin.

EnVision: Sections were treated with 0.03% H_2O_2 for 5 min at room temperature to block endogenous peroxidase activity. After incubation with primary antibodies for 30 min at room temperature, the sections were incubated with peroxidase labelled polymer conjugated to goat anti-mouse secondary antibody for 30 min before development for 10 min with DAB, and then counterstained with haematoxylin.

Positive control slides were processed in parallel with each batch of staining. The materials used were as follows: FGFR3, small intestine; EGFR, squamous cell carcinoma of the cervix; ERBB2 and p21RAS, breast carcinoma; KAI1, tonsil; PTEN, normal skin. Negative controls included replacement of the primary antibody with mouse myeloma protein of the same subclass and concentration.

IHC scoring

The IHC scoring was performed by an experienced pathologist (JMN). The percentage of tumour cells (or for normal urothelium, transitional epithelium cells) showing staining in the plasma membrane (denoted as membranous frequency) and in the cytoplasm (denoted as cytoplasmic frequency), was evaluated semiquantitatively and categorized in four levels as follows: 0, 0%; +, >0 - ≥10%; ++, >10 - ≥50%; and +++, >50%. The overall intensity of cytoplasmic staining (denoted as cytoplasmic intensity) was evaluated semiquantitatively and categorized in four levels as follows: 0, absent; +, weak; ++, moderate; and +++, strong. The evaluation of the intensity of the membranous staining was evaluated but finally omitted, since it was found to be strongly influenced by the cytoplasmic intensity (the two scores did not differ sufficiently to trust them to be independently assessed). For FGFR3, membranous staining was omitted and the frequency of nuclear staining (denoted as nuclear frequency) was scored in stead. The intensity of nuclear staining was not registered.

The p21RAS antibody applied in our study binds

Table 2. Antibodies used in the study.

Antigen	Clone	Clonality	Pre-treatment procedure	Dilution	Name and address of purchaser
FGFR3	C-15	polyclonal (rabbit)	Microwave 2x5min at 850W in 10mM citrate buffer, pH 6.0	1:75,60 min., ambient temp.	Santa Cruz Biotechnology, Santa Cruz, California
EGFR	H11	monoclonal (mouse)	pronase treatment for 10 min	1:100, over night at ambient temp.	Dako AS, Glostrup, Denmark
ERBB2	CB11	monoclonal (mouse)	Microwave 2x5min at 850W in 10mM citrate buffer, pH 6.0	1:200, 30 min., ambient temp.	Novocastra laboratories Ltd., Newcastle upon Tyne, UK
KAI1	5B5	monoclonal (mouse)	Microwave 5min at 850W, then 15min at 350W in Tris-EDTA, pH 9.1	1:25, 30 min., ambient temp.	Novocastra laboratories Ltd., Newcastle upon Tyne, UK
PTEN	17.A	monoclonal (mouse)	Microwave 2x5min at 850W in 10mM citrate buffer, pH 6.0	1:200 over night at ambient temp.	NeoMarkers, Fremont, CA, USA
p21RAS	NCC-RAS-001	monoclonal (mouse)	No pre-treatment	1:50, 30 min., ambient temp.	Dako AS, Glostrup, Denmark

HRAS most strongly, but a crossreaction with other RAS proteins cannot be excluded. The detected protein is therefore referred to unspecifically as "p21RAS".

If the scores from a pair of cores from one specimen differed by one level, the highest level was made valid. If the scores from a pair of cores differed by two levels or more, full-scale sections were stained. If scores from tissue microarray cores were substantially different (difference in level of two or more) from the full-scale section, all samples were revised. If the difference was sustained, the result from the full-scale section was made valid. Samples with one informative core were deemed as valid.

Statistical analysis

Differences between the groups were evaluated by the Kruskal-Wallis test (for all groups together) and the Mann-Whitney test (pairwise comparisons between the groups). Expression in primary tumours and corresponding metastases were evaluated by a) Wilcoxon test for differences between the pairs, and by the b) "agreement rate" (AR), the number of pairs having identical scores in both primary tumour and corresponding metastasis, divided by the total number of informative pairs for that parameter. Associations between all parameters were evaluated with Chi squared test for trend and Spearman's rank correlation. Both methods gave similar results for all combinations tested, so only the correlation results and rho statistics are presented. The observation time ranged from the date the primary tumour sample was obtained, until the date of death or, for living patients, February 25th. 2003. The statistical program SPSS (release 10.1) was used for all calculations. Since the results from this study were only exploratory and meant for generating new hypotheses, no correction for multiple p-values was done.

Results

Alterations in expression level and subcellular location

FGFR3

In normal urothelium, FGFR3 appeared to be constitutively expressed in the cytoplasm, detected as weak to moderate staining intensity in all cells of all NU samples (see Table 3, FGFR3 C_{int} for normal urothelium: "+" = 47%; "++" = 53%; and C_{fr}: "+++" = 100%. See also Fig. 1a). Only one sample showed nuclear staining (Table 3: N_{fr} "++" = 6%).

In the carcinomas, nuclear staining was detected in nearly all samples (Fig. 1b and Table 3: only 5% (=NMC) and 2% (=MC) had score "0" for N_{fr}). In Fig. 2 we present the p-values for comparisons between the groups, one triangle for each parameter. See upper left triangle: the dotted line between MC and NMC and (p =) 0.12 indicates no calculated difference for FGFR3 N_{fr} between the MC and NMC groups. According to the calculations presented in Fig. 2 for cytoplasmic frequency and intensity (FGFR3 C_{fr} and FGFR3 C_{int}), no upregulation of the FGFR3 protein level was detected in the carcinoma groups compared with the level in normal urothelium (dotted lines between NU and MC or NMC, respectively). However, 10 of 70 carcinomas (16% of NMCs and 14% of MCs, Table 3), but no normal samples, did display strong cytoplasmic intensity (C_{int} at "+++"), indicating a "within-group" shift in expression level distribution from NUs to the carcinomas.

EGFR

The normal samples seemed to have an "EGFR pool" in the cytoplasm: weak to moderate staining intensity in the cytoplasm of all cells, but "usually" (73%) no staining in the plasma membrane (Table 3). Also the frequency of cytoplasmic staining was highest in the normal tissue (Fig. 2). Going from NUs to NMCs, the EGFR protein seemed to be mobilised from cytoplasm to membrane: As previously reported, the non-metastasised cancers displayed more membranous EGFR than both MCs and NUs (Røtterud et al., 2004). Membranous EGFR was scored as "++" or "+++" in nearly 70% of the NMCs, and in 40% of the MCs. But once established, membranous expression in carcinomas was withheld in the metastases: Comparing primary MC samples and corresponding metastases, more than half of the pairs had identical scores for membranous EGFR expression (Table 4, line 4: AR=0.56).

ERBB2

The normal samples showed no membranous ERBB2 staining, whereas nearly all displayed some cytoplasmic immunoreactivity in at least some cells (Table 3: only 13% were completely negative in the cytoplasm). Among the carcinoma samples, membranous staining was frequently seen (Table 3). Cytoplasmic ERBB2 was lost in half of the NMCs, half of the MCs, and also half of the metastases. Six of nineteen NMCs (32%) and 22/51 MCs (43%) displayed no detectable ERBB2 staining at all; the others expressed ERBB2 in the cytoplasm only, in the membrane only, or at both places (data not shown). ERBB2 expression in the primary MC tumours seemed to be withheld in the corresponding metastases (Table 4).

KAI1

KAI1 expression was barely seen among the normal cases: four samples had weak cytoplasmic expression and some or no membranous expression in less than half of the cells (Table 3; Fig. 1c), the other samples had none. The NMC tumours had higher frequency and intensity of cytoplasmic KAI1 staining than NUs and MCs, whereas the frequency of membrane staining was not different between the three groups (Fig. 2, p=0.17). Despite the differences in cytoplasmic staining, all three

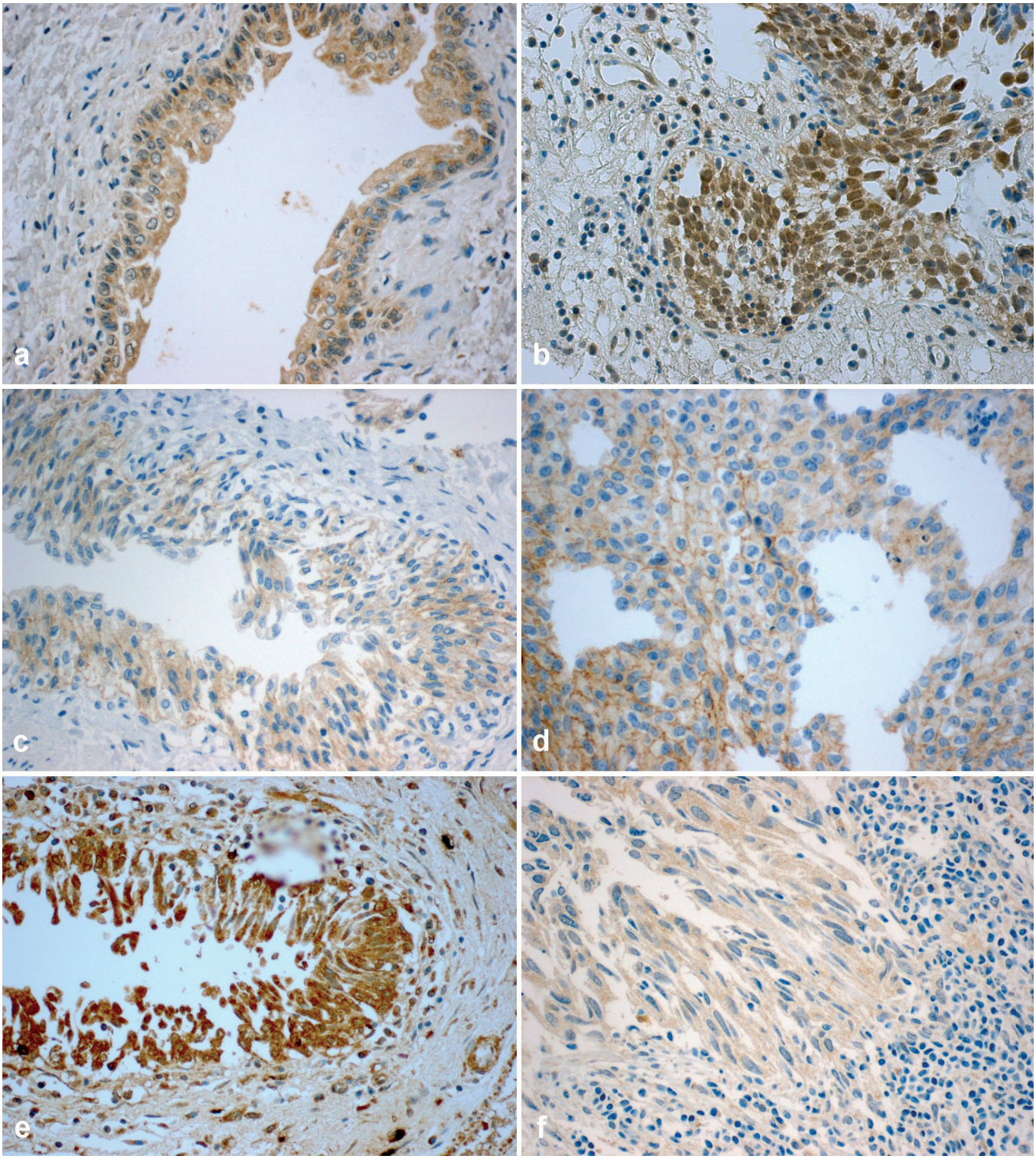

Fig. 1. Some typical staining results: **a)** FGFR3 staining in NU sample; **b)** FGFR3 staining in tumour from NMC patient; **c)** KAI1 staining in NU sample; **d)** KAI1 staining in tumour from MC patient; **e)** p21RAS staining in NU sample; **f)** p21RAS staining in tumour from MC patient. x 200

KAI1 parameters (M_{fr}, C_{fr}, and C_{int}) correlated well with each other (Table 5), interpreted as absence of cytoplasmic pooling of KAI1 protein in substantial amounts: Only three MC samples and one NMC sample displayed moderate (that is, "anything more than weak") cytoplasmic staining and concomitantly no membrane staining. An example of membrane staining in a MC tumour is shown in Fig. 1d. Among the metastases, KAI1 was absent from the plasma membranes in 81% (39/48) of informative samples (Table 3).

PTEN

In the normal samples, PTEN was expressed at low or moderate levels in the cytoplasm but only occationally in the membrane (Table 3). Nearly half of the NMC cases showed membranous PTEN expression in virtually all cells, accompanied by a slight increase in cytoplasmic intensity (Table 3). As expected from a potential tumour suppressor, membranous PTEN expression was reduced from "+++" and "++" in the primary tumour to "absent" in the corresponding metastasis in 9 of 51 patients (18%). Only one patient showed the opposite change (data not shown and Wilcoxon, Table 4). Altogether, nearly 90% of the metastases were devoid of membranous PTEN (Table 3), and more than half of these samples had no or weak cytoplasmic intensity (Table 3).

p21RAS

p21RAS was strongly expressed in the cytoplasm of virtually all normal urothelium cells (Fig. 1e), but infrequently at the membrane. Both cytoplasmic frequency and intensity of p21RAS were less prominent in NMCs and MCs compared with NUs (Fig. 1f, 2). As

Table 3. IHC score in percent of informative cases.

		Normal urothelium (n=15)			Non-metastasised primary cancer (n=19)			Metastasised primary cancer (n=51)			Metastases (n=51)		
		N_{fr}	C_{fr}	C_{int}	N_{fr}	C_{fr}	C_{int}	N_{fr}	C_{fr}	C_{int}	N_{fr}	C_{fr}	C_{int}
FGFR3:	0	93	0	0	5	0	0	2	0	0	19	4	4
	+	0	0	47	11	0	47	6	0	41	2	9	49
	++	6	0	53	26	16	37	16	10	45	19	6	38
	+++	0	100	0	58	84	16	76	90	14	60	81	9
		M_{fr}	C_{fr}	C_{int}	M_{fr}	C_{fr}	C_{int}	M_{fr}	C_{fr}	C_{int}	M_{fr}	C_{fr}	C_{int}
EGFR:	0	73	0	0	26	37	37	57	22	22	61	33	33
	+	13	6	67	5	0	37	4	0	47	2	2	39
	++	13	0	33	21	5	16	16	12	24	12	8	20
	+++	0	93	0	47	58	11	24	67	8	24	55	6
ERBB2:	0	100	13	13	47	53	53	61	53	53	69	49	49
	+	0	40	80	5	0	26	4	0	27	0	2	33
	++	0	27	7	5	11	16	14	10	16	14	14	14
	+++	0	20	0	42	37	5	22	37	4	18	35	4
KAI1:	0	80	73	73	53	32	32	67	57	57	81	67	67
	+	6	0	27	11	5	32	6	6	25	4	2	13
	++	13	27	0	11	0	16	14	10	10	6	8	19
	+++	0	0	0	26	63	21	14	27	8	8	23	2
PTEN:	0	85	0	0	3	11	11	68	10	10	89	27	27
	+	0	0	14	0	0	11	6	0	22	4	2	27
	++	7	7	85	5	11	32	10	8	40	0	4	36
	+++	7	93	0	42	79	47	16	82	28	7	67	11
p21RAS:	0	73	0	0	58	42	42	74	32	32	78	30	30
	+	6	0	0	11	5	11	6	0	12	9	0	11
	++	0	0	0	11	11	21	8	12	28	7	17	30
	+++	20	100	100	21	42	26	12	56	28	7	52	28

For each protein, its immunoreactivity in two subcellular locations and the intensity of eventual cytoplasmic expression was scored at four levels in one sample from each tumour, and repeated at least once. Samples were allocated to correct group and the distribution registered. N_{fr}: nuclear frequency; M_{fr}: membranous frequency; C_{fr}: cytoplasmic frequency; C_{int}: cytoplasmic intensity. An example of how to read the table (p21RAS, lower left corner): "Among the normal urothelium samples, 73% of them showed no membranous staining (M_{fr}: 73% at score 0), whereas all the samples concomitantly displayed cytoplasmic staining in >50% of the tumour cells ($C_{fr:}$ 100% at score +++) and at strong intensity (C_{int}: 100% at score +++), of p21RAS."

many as 33% of the carcinomas (23/70) showed no detectable p21RAS expression at all (data not shown). Infrequently we observed membranous p21RAS expression in the metastases (Table 3).

Interactions between the proteins

KAI1 and EGFR

EGFR frequency and intensity in cytoplasm were associated with KAI1 frequency in the membrane. Membranous EGFR was associated with all three KAI1 parameters (Table 5). As many as 28 of 51 MC tumours expressed no KAI1 at all (data not shown). Nineteen of these did neither express EGFR in the membrane (data not shown).

ERBB2 and PTEN

The frequency of ERBB2 immunoreactivity in the membrane was positively correlated with the frequency of cytoplasmic PTEN expression (Table 5).

PTEN and p21RAS

Membranous PTEN was rather strongly correlated with membranous p21RAS (Table 5). However, 57% of the cases were "doubly negative" for both proteins in the membrane.

Other interactions

Cytoplasmic expression (both frequency and intensity) of ERBB2 was negatively correlated with cytoplasmic expression of p21RAS (Table 5).

Membranous EGFR frequency was correlated with cytoplasmic intensity of PTEN (Table 5).

Cytoplasmic intensity of FGFR3 correlated with membranous frequency of EGFR expression and cytoplasmic frequency and intensity of PTEN, whereas the cytoplasmic frequency of FGFR3 also correlated with cytoplasmic frequency of PTEN (Table 5). Furthermore, membranous frequency of EGFR

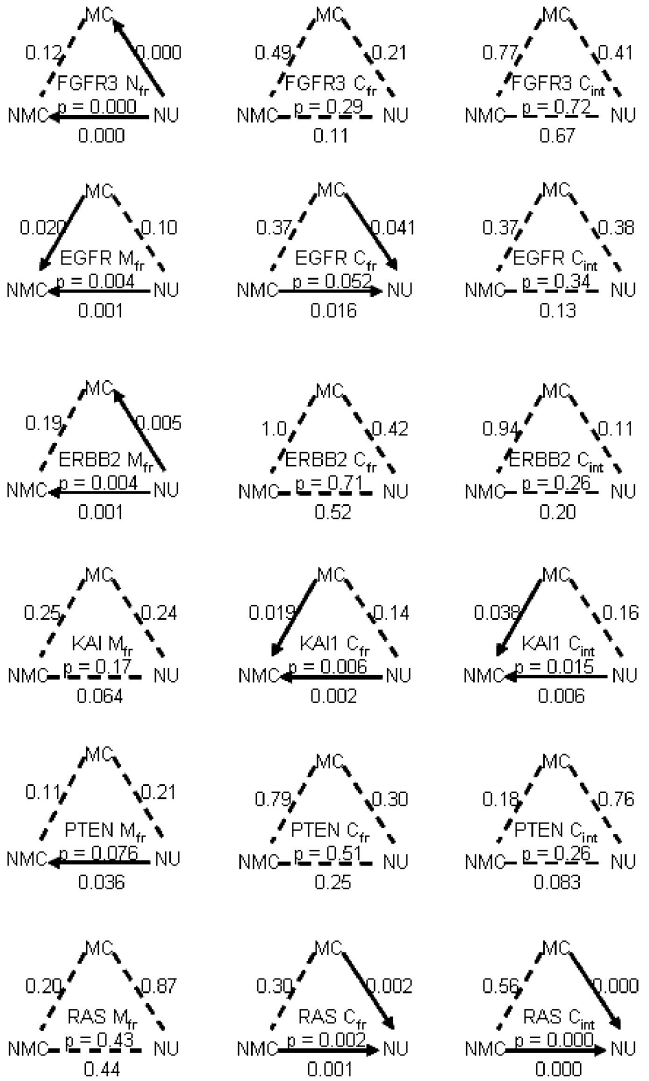

Fig. 2. Comparisons of protein expressions between all groups, both overall (all groups together) and pairwise between each of the groups. Numbers within each triangle specify p-values from the overall comparisons. Numbers along each side in the triangles denotes p-value from pairwise comparisons. Solid lines denote significant differences according to a set level of 0.05; dotted lines denote a p-value above 0.05. Arrowheads indicate an increase in the expression.

Table 4. Comparing protein expression in primary tumour and corresponding metastasis.

Variable	Wilcoxon, p=	AR
FGFR3 C_{fr}	0.05	0.79
FGFR3 C_{int}	0.23	0.45
FGFR3 N_{fr}	0.020*	0.51
EGFR M_{fr}	0.79	0.56
EGFR C_{fr}	0.092	0.54
EGFR C_{int}	0.36	0.38
ERBB2 M_{fr}	0.28	0.55
ERBB2 C_{fr}	0.74	0.47
ERBB2 C_{int}	0.90	0.37
KAI1 M_{fr}	0.24	0.63
KAI1 C_{fr}	0.50	0.50
KAI1 C_{int}	0.65	0.44
PTEN M_{fr}	0.021*	0.70
PTEN C_{fr}	0.10	0.55
PTEN C_{int}	0.05	0.27
p21RAS M_{fr}	0.30	0.67
p21RAS C_{fr}	0.99	0.33
p21RAS C_{int}	0.93	0.33

*Primary tumours > metastases

Table 5. Spearman's rho correlations between all reported expression parameters, calculated for primary metastasised tumours only.

		FGRF3			EGFR			ERBB2			KAI1			PTEN			RAS		
		N_{fr}	C_{fr}	C_{int}	M_{fr}	C_{fr}	C_{int}	M_{rf}	C_{rf}	C_{int}	M_{rf}	C_{rf}	C_{int}	M_{rf}	C_{rf}	C_{int}	M_{rf}	C_{rf}	C_{int}
FGFR3	N_{fr}	-																	
	C_{fr}	0.56* 0.000	-																
	C_{int}	0.20& 0.15	0.15 0.29	-															
EGFR	M_{fr}	-0.14 0.32	0.17 0.23	0.45 0.001	-														
	C_{fr}	H#	H	0.19 0.18	0.35 0.013	-													
	C_{int}	H	0.14 0.32	0.30 0.031	0.45 0.001	0.77 0.000	-												
ERBB2	M_{fr}	H	H	H	H	0.12 0.41	0.15 0.31	-											
	C_{fr}	0.12 0.41	-0.14 0.33	H	-0.15 0.28	H	H	0.42 0.002	-										
	C_{int}	H	-0.16 0.26	H	-0.21 0.14	H	H	0.48 0.000	0.94 0.000	-									
KAI1	M_{fr}	-0.22 0.12	H	H	0.33 0.018	0.28 0.047	0.35 0.015	H	-0.23 0.10	-0.16 0.27	-								
	C_{fr}	-0.18 0.24	H	H	0.30 0.036	H	H	H	-0.25 0.074	-0.25 0.074	0.66 0.000	-							
	C_{int}	-0.19 0.18	H	H	0.29 0.038	0.17 0.25	0.18 0.20	H	-0.26 0.067	-0.23 0.102	0.82 0.000	0.96 0.000	-						
PTEN	M_{fr}	H	0.22 0.12	0.20 0.17	0.16 0.26	H	H	0.12 0.42	H	H	H	0.099 0.49	0.11 0.44	-					
	C_{fr}	0.18 0.21	0.50 0.000	0.36 0.10	0.18 0.21	H	H	0.29 0.39	0.16 0.27	0.20 0.17	H	0.13 0.37	H	0.24 0.092	-				
	C_{int}	H	0.233 0.10	0.40 0.004	0.37 0.009	H	H	H	H	H	H	H	H	0.11 0.47	0.49 0.000	-			
p21RAS	M_{fr}	H	0.17 0.23	0.11 0.43	0.13 0.35	H	-0.13 0.37	-0.10 0.48	-0.27 0.054	-0.31 0.027	-0.12 0.48	H	H	0.46 0.001	-0.10 0.48	-0.20 0.17	-		
	C_{fr}	H	0.33 0.02	H	H	0.18 0.21	0.14 0.35	-0.10 0.49	-0.32 0.025	-0.32 0.024	0.13 0.37	H	0.10 0.48	H	H	-0.25 0.080	0.39 0.005	-	
	C_{int}	0.15 0.29	0.30 0.032	H	H	H	H	-0.16 0.28	-0.32 0.026	-0.32 0.026	H	H	H	H	H	-0.30 0.036	0.55 0.000	0.85 0.000	-

* Uppermost number denotes the rho statistic, the lowermost number denotes the p-value. Only rho statistics for p-values below 0.5 are specified. & Statistics obtained with a probability between 0.05 and 0.5 are shown in red colour. # H denotes p>0.5 (no rho value given). N_{fr}: nuclear frequency; M_{fr}: membranous frequency; C_{fr}: cytoplasmic frequency; C_{int}: cytoplasmic intensity.

correlated with cytoplasmic intensity of PTEN. All correlations were positive.

Discussion

Our results indicate that during the development from normal urothelium to bladder cancer, FGFR3 is translocated from the cytoplasm to the nucleus. EGFR is translocated from the cytoplasm to the cell membrane. ERBB2 expression increases in some tumours but the protein is also subject to cytoplasmic sequestration in some. KAI1 is upregulated in NMC samples compared with the MC and NU groups, whereas the results for PTEN "points in the same direction" without reaching the 0.05 level. p21RAS is downregulated with the carcinogenic progression.

FGFR3

Nuclear FGFR3 staining has been reported previously. Reid et al. observed FGFR3 in nuclei of choroid plexus cells in murine embryos (Reid and Ferretti, 2003). Zammit et al. detected FGFR3 in the nuclei of both normal and malignant breast epithelium (Zammit et al., 2001). No hints are given to its function in the nucleus, but its "close relative" FGFR1 has been reported to regulate the expression of c-Jun and cyclin D1 after being translocated to the fibroblast nucleus by Importin ß (Reilly and Maher, 2001). A role as a transcription factor could be in accordance with FGFR3's proposed function as an oncogene in bladder cancer (Cappellen et al., 1999). The difference between normal urothelium and non-metastasised tumours indicates a diagnostic role for nuclear FGFR3 expression.

The observation that 10 of 70 carcinomas displayed strong cytoplasmic intensity, well above what was seen in any normal sample (Table 3), indicates that FGFR3 protein may be upregulated in at least some tumours. The lack of membranous FGFR3 was puzzling, so we performed an additional search for membranous FGFR3 in full-scale sections. Among 23 randomly chosen samples (19 tumours, 4 normal samples), we did find 4 positive cases (data not shown). Two of them had a score of +, one had ++ and one had +++. None of these were normal samples. However, with this frequency of membranous FGFR3 expression, our series was too small to provide any significant information. Our results do not support recent findings reported by Gómez-Román et al: they found no FGFR3 expression in normal urothelium, whereas half of their T2 carcinomas expressed FGFR3 in the plasma membrane (Gomez-Roman et al., 2005). Gómez-Román and coworkers did not report cytoplasmic or nuclear immunoreactivity. The antibodies and the techniques used were different in the two studies.

EGFR

The NMCs had a higher frequency of membranous EGFR expression than both MCs and NUs. If this was due to a translocation from cytoplasm to cell membrane when the epithelial cells are becoming neoplastic, we could expect to see a reduction in cytoplasmic intensity from the NUs to the NMCs, but this was not observed (Fig. 2). However, a translocation may well proceed without reducing the cytoplasmic pool in detectable amounts. The observed reduction in membranous EGFR from NMC samples to MC samples does not support the previously suggested role of EGFR as a tumour promoter in urothelium (Brandau and Bohle, 2001).

More than half of the pairs of primary MC samples and corresponding metastases had identical scores for membranous EGFR expression, indicating a stable regulation and location mechanism in at least some tumours.

The cytoplasmic restriction seen in the normal samples may reflect a pool of constitutively expressed EGFR, retained in the endoplasmic reticulum until "called upon". The four normal cases displaying membranous EGFR may be interpreted as showing a response to some benign condition like inflammation: EGFR is known to be involved in various inflammatory conditions like inflammatory skin disorders (Mascia et al., 2003), asthmatic or plugged airways (Lee et al., 2000; Hamilton et al., 2003), and in inflammatory duodenal ulcer (Shi and Zhang, 1997). On the other hand, in inflammatory urothelium EGFR was reported to be more frequently seen in the cytoplasm than in the cell membrane (van Velzen et al., 1996). Another such condition is smoking: Increased EGFR expression was reported in bronchial cells of smokers (Kurie et al., 1996). Smoking is a known risk factor for bladder cancer, but EGFR expression status in the bladder urothelium of smokers has not been reported.

The EGFR antibody applied in our study binds an epitope in the extracellular part of the receptor, so we cannot provide information about a potential translocation of the intracellular part of the EGFR receptor to the cell nucleus, as previously reported by others (Lipponen and Eskelinen, 1994).

ERBB2

The change from being restricted to the cytoplasm in normal samples to be frequently detected in the plasma membrane in carcinomas, implies a translocation of ERBB2 with the carcinogenic transformation. This presumed translocation did not lead to an overall reduction in cytoplasmic staining in the MC or NMC groups as compared with the NU group (Fig. 2). However, since cytoplasmic ERBB2 was indeed lost in half of the NMCs, half of the MCs, and also half of the metastases, a counterbalancing upregulation may have occurred in other samples (Table 3).

We also observed that 30-40% of the carcinomas lost all ERBB2 staining. The remainders expressed ERBB2 restricted to the cytoplasm, to the the membrane, or in some samples expressed at both places (data not shown).

These two aspects are in accordance with regulation of ERBB2 at two levels; i) increased protein expression and ii) translocation of protein from the cytoplasm to the membrane. The moderate to strong cytoplasmic intensity seen in a few carcinomas may be due to increased protein levels in those tumours, whereas translocation may have depleted the cytoplasmic reservoir and increased the membrane expression in others. Both mechanisms may have been active in the remaining samples. Thus, our results indicate that increased membranous ERBB2 expression seen in carcinomas does not mirror an increased activity of the ERBB2 gene.

The stable expression of ERBB2 in primary MC tumours and metastases, together with the high frequency of membranous expression in carcinoma

samples compared with the normal samples, fits well with its proposed role as a tumour promoter (Rubin and Yarden, 2001).

KAI1

Our results from the cytoplasmic staining indicate an increased protein level in the non-metastasised tumours, as compared with normal urothelium and metastasised tumours. A low expression in MCs and metastases, the upregulation seen from normal cells to non-metastasised tumours, and the absence of membranous KAI1 in 81% of the metastases, goes well with its proposed function as a metastasis inhibitor (Dong et al., 1995).

A correlation between cytoplasmic and membranous staining for a certain protein indicates a free distribution of this protein between the two locations (no sequestering in the cytoplasm). An apparent discordance exists within our results for KAI1: the membranous staining was similar for the groups (Fig. 2), and all three KAI1 parameters correlated well with each other (Table 5) whereas a difference (between the groups) was detected for the cytoplasmic staining (Fig. 2). A closer inspection of the data showed that the membranous and cytoplasmic staining was concordant with few exceptions: Only three MC samples and one NMC sample displayed at least moderate cytoplasmic staining and concomitantly no membrane staining. The existence of a mechanism for sequestering KAI1 in the cytoplasm is therefore not totally excluded, but it seems to be rare in normal urothelium and primary tumours. Since KAI1 is barely detectable in normal samples, its increased expression in NMCs may also be exploited for diagnostic purposes.

PTEN

Our observations in normal and NMC samples suggest an increased expression level and a frequently occurring but not compulsory translocation mechanism activated in neoplastic cells.

The difference between the three groups regarding PTEN expression in the membrane or cytoplasm did not, in this study, reach the frequently used significance level of 0.05 (Fig. 2, a difference between all three groups was detected for membranous frequency with a level of significance of 0.076. A difference detected in the pairwise comparison between the NU and NMC groups is therefore accordingly probable), but the low p-value obtained in the pairwise comparison of the rather few samples of normal urothelium and non-metastasised cancers may justify a closer look at the translocation mechanism and the regulation of the expression level.

The marked reduction of PTEN expression seen in the metastases compared with the corresponding primary tumours, and the frequently observed lack of membranous staining in the metastases, often combined with no or low cytoplasmic expression, are supportive of its suggested role as a metastasis inhibitor (Hwang et al., 2001; Davies et al., 2002).

p21RAS

Both cytoplasmic frequency and intensity was reduced from the NU group to the NMC and MC groups (Fig. 2). This seems to be directly related to the expression level of detectable protein, since membranous staining of p21RAS was similar in all three groups. Furthermore, one third of all carcinomas lost all detectable p21RAS expression, supporting the impression that p21RAS was downregulated from normal samples to carcinomas. The *HRAS* gene located on the short arm of chromosome 11, is frequently involved in deletions or loss of heterozygosity in bladder cancer (Fearon et al., 1985; Knowles et al., 1994). However, the *HRAS* gene locus itself was not found to be encompassed by these alterations (Shaw and Knowles, 1995), suggesting another mechanism for downregulation of the p21RAS protein.

The oncogenicity of p21RAS has been connected with its hyperactivity in transformed cells (DeFeo et al., 1981; Chang et al., 1982). Whether p21RAS protein was overexpressed in carcinomas compared with the expression in normal urothelium, could not be assessed by our method, so this aspect could not be evaluated. The consequence of a downregulation, though, could be of biological relevance. The low expression of membranous p21RAS in the metastases (Table 3) indicates that membranous p21RAS is not important for promoting most metastatic bladder carcinomas. Since normal urothelium possesses a uniform expression pattern of p21RAS, downregulation of cytoplasmic p21RAS can be of diagnostic significance.

Protein interactions

KAI1 and EGFR

The extensive correlating pattern seen between EGFR and KAI1 parameters may be due to the fact that all three KAI1 parameters (M_{fr}, Cfr, and C_{int}) correlated strongly with each other. The statistically detected association between the two proteins may be influenced by as many as 19 cases that were negative for both KAI1 and EGFR.

A correlated expression of KAI1 with EGFR (and ERBB2) is nevertheless in accordance with several known functions of these proteins: EGFR and ERBB2 may possibly increase KAI1 expression by signaling through the RAS pathway, activating the AP1 transcription factor that has a binding site in the KAI1 promoter (Marreiros et al., 2003). Furthermore, KAI1 physically associates with EGFR and ERBB2; it regulates compartmentalisation of EGFR, possibly also ERBB2, and dimerisation of EGFR (Odintsova et al., 2003). This leads to attenuation of the EGF receptor signaling (Odintsova et al., 2000). Besides, EGFR activates the focal adhesion kinase (FAK) in focal

complexes, which subsequently activates or recruits the Src, CAS and Crk proteins (relevant for cell migration). KAI1 downregulates CAS, thus counteracting the motility-promoting activity of EGFR (Zhang et al., 2003).

ERBB2 and PTEN

The frequency of ERBB2 immunoreactivity in the membrane was positively correlated with the frequency of cytoplasmic PTEN expression (Table 5). However, when a parameter is equally expressed in all samples, it presents as a constant and will not be suitable for calculating correlations. For PTEN we observed that 45 of 50 cases were scored as ++ or +++. It is therefore debatable whether this parameter should be viewed as a constant and therefore not relevant for this calculation.

PTEN and p21RAS

PTEN and p21RAS are functionally associated: The p21RAS pathway activates the PI3K pathway, which is inhibited by PTEN (Wu et al., 1998; Chan et al., 2002). Besides, PTEN inhibits SHC, which activates p21RAS (Gu et al., 1998). However, more than half of the cases were negative for both proteins in the membrane so the absence, more than the presence, of these proteins could be the basis for the detected association. A closer inspection of the data supports this: among the samples being positive for one or the other ("not doubly negative"), we did see all expression profiles represented (PTEN only, p21RAS only, or both expressed simultaneously). This positive correlation we detected is therefore not due to a mechanism where the presence of one of them induce expression of the other.

An association based on lack of membranous PTEN and p21RAS may be caused by translocation of these proteins from the membrane, or by downregulation of the expression level. The mechanisms by which PTEN and p21RAS localise to the cell membrane are quite different (electrostatic attraction and farnesylation, respectively) and joint translocation is thus not very likely. A downregulation is not plausible either since nearly all samples that were doubly negative for PTEN and p21RAS in the membrane expressed cytoplasmic PTEN, and most of them (17/28) expressed p21RAS in the cytoplasm. Thus, we suggest that the association is based on two coexisting but otherwise unrelated events leading to loss of PTEN and p21RAS from the membrane of carcinoma cells, but taking place simultaneously.

ERBB2 and p21RAS

p21RAS is a signal transducer downstream of ERBB2, but the existence of a negative correlation between their cytoplasmic pools is not previously known.

FGFR3, EGFR and PTEN

The observed correlations between all three proteins indicates a common regulatory factor or process, not yet identified.

Deduced hypotheses

Based on this, the following hypotheses were generated, aimed for further hypothesis testing:
- FGFR3 is translocated from the cytoplasm to the nucleus during carcinogenesis
- EGFR is translocated from the cytoplasm to the membrane during carcinogenesis
- High membranous EGFR expression indicates tumours of low metastatic potential
- ERBB2 is translocated from the cytoplasm to the membrane during carcinogenesis
- Increased ERBB2 protein levels designate a subgroup of tumours with potential implications for treatment and survival
- Membranous KAI1 expression inhibits metastasis
- PTEN is translocated from the cytoplasm to the membrane during carcinogenesis
- Membranous PTEN expression denotes tumours of low carcinogenic and/or metastatic potential
- p21RAS protein is downregulated during carcinogenesis (in cells with no amplified RAS gene)
- Membranous p21RAS expression is not necessary for the metastatic process
- ERBB2 protein may downregulate p21RAS expression
- EGFR, PTEN and FGFR3 expressions are regulated by a common factor or process

Conclusions

The staining detected in normal urothelium is in accordance with a constitutive expression of FGFR3, EGFR, PTEN, and p21RAS in the cytoplasm. ERBB2 was detected in the cytoplasm in most of the samples, but at low intensity. Membranous expression was rarely seen for any of the proteins, as was KAI1 in any location.

In the carcinoma samples, FGFR3 was located in the nucleus. A variable number of tumours lacked cytoplasmic staining of EGFR, ERBB2, PTEN, and p21RAS. An increase in membranous expression was most apparent for ERBB2, but also EGFR and PTEN, and to a lesser extent KAI1, was expressed in the plasma membrane in a variable number of cases. Membranous EGFR (and cytoplasmic KAI1) was most frequently seen among the non-metastasised cancers.

Comparing expression profiles of the different proteins in the three groups and in the collection of metastases, KAI1 and PTEN expressions were in accordance with a role as metastasis suppressors. Surprisingly, so were also the expression of EGFR and p21RAS, while FGFR3 and ERBB2 presented in accordance with a tumour-promoting role.

The complex network of protein interactions indicated by our results may reflect cellular responses, signaling pathways or cellular mechanisms affecting the interconnected proteins simultaneously.

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