Detection of epidermal growth factor receptor and c-*erb*B-2 gene amplification in transitional cell bladder carcinoma using the differential PCR technique

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Abstract. Amplification of genes associated with cell control and differentiation is found in many human tumours and its detection may have important value in predicting tumour progression. In this study we examined 72 DNA samples extracted from paraffin-fixed formalin-embedded transitional cell carcinomas with a novel differential PCR technique that can detect variations in gene dosage using small amounts of tumour DNA. We have observed that this technique under certain conditions has many advantages over traditional gene analysis techniques. Our study revealed EGF-r and c-*erb*B-2 gene amplification in 2/72 (3%) and 11/72 (15%) bladder carcinomas, respectively. EGF-r gene was amplified in 2/24 (8%) grade III carcinomas while c-*erb*B-2 was amplified in 2/25 (8%) and 9/24 (37.5%) grade II and grade III carcinomas, respectively. All cases with EGF-r and c-*erb*B-2 gene increased copy number were classified as invasive on the basis of muscularis propia invasion. The association between c-*erb*B-2 amplification and tumour grade as well as stage of the carcinomas was statistically significant indicating direct linkage to bladder carcinoma progression, while the relationship between EGF-r gene amplification and the above mentioned parameters did not reach significance.

Introduction

Epidermal growth factor receptor and c-*erb*B-2 genes are located on chromosomes 7 locus p22-24ter and 17 locus q21, respectively. They encode transmembrane phosphoglycoproteins characterized by an extracellular domain which interacts with various growth factors, a transmembrane section and an intracellular domain with tyrosine kinase activity. EGF-r is stimulated by EGF, TGF-α, and amphiregulin (1-5). This stimulation results in activation of cell growth regulation mechanisms (6). On the other hand the ligand which activates c-*erb*B-2 has not yet been identified (7,8), although recently a rat ligand has been identified (9).

EGF-r and c-*erb*B-2 are expressed on cells derived from all three germ layers and expressed at particularly high levels on epithelia (10,11). Considering their wide tissue distribution, it is not surprising that EGF-r and c-*erb*B-2 are frequently expressed in human tumours. Interestingly, increased activity is most often due not to a structural abnormality but rather to overexpression of normal receptors (12).

Overexpession of EGF-r and c-*erb*B-2 has been observed in squamous cell carcinomas and adenocarcinomas (10,13-16). One important mechanism of protein overproduction is gene amplification. EGF-r and c-*erb*B-2 gene amplification has been observed in human malignancies (13,19-21) and in certain cases related with adverse prognosis.

The information regarding EGF-r and c-*erb*B-2 gene amplification in bladder carcinomas is limited. In order to evaluate the amplification of these genes in transitional cell bladder carcinomas (TCC) we analysed the DNA from 72 carcinoma specimens with a novel PCR technique and correlated our findings with pathological features of the tumours.

Materials and methods

Tissue samples. Seventy-two cases of surgically removed and biopsy specimens formalin-fixed and paraffin-embedded transitional carcinoma of the bladder retrieved from the files of Red Cross Hospital, Athens were studied. Histologically they consisted of 23 grade I, 25 grade II and 24 grade III carcinoma cases (WHO classification 1973). Forty- four had superficial tumours (pTa and pT1) and 28 had muscle invasive disease. In addition, 29 samples from adjusted normal urothelium were examined.

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DNA extraction from paraffin. The DNA extraction was performed on 5 μm sections of the paraffin-embedded tumour tissue. Serial 5 μm sections were processed and the first section was stained with hematoxylin and eosin to visualise the extent of the tumour cells within each sample. The boundaries of the malignant tissue were delineated microscopically and excess stromal tissue was excised from the paraffin sections on the slide using sterile surgical blades, in order to ensure that only neoplastic cells were included. The remaining neoplastic tissue was scraped with a sterile blade and wrapped with gauze, tied, placed into xylene and put in a 65°C waterbath for 10 min followed by 20 min in xylene at RT and then rinsed with 100% alcohol. Tissue sections were transferred to 20 ml polypropylene in Falcon tubes containing 4-12 ml of a lysis solution (as determined from the number of sections cut where the solution should cover the section completely).

The lysis solution consistsed of 0.5% sodium dodecyl sulfate (SDS) and proteinase K (Boehringer), at a final concentration of 500 μg/ml from a 20 μg/ml stock in ddH_2O. The solution was buffered with TNE (0.05 M Tris HCl pH 8.0, 0.15 M NaCl, 5 mM EDTA). Lysis was carried out in a 45°C waterbath with very gentle agitation. This procedure was carried out for 72 h with the addition of new proteinase K (250 μg/ml) on each successive day of lysis. The samples were then centrifuged (2.500 rpm, 10 min) in a bench Beckman centrifuge. The supernatant was extracted 3 times with equal volume of phenol. Then the aqueous phase was treated with RNAse A (Boehringer) at final concentration of 100 μg/ml at 37°C for 30 min. The extraction continued with 3 additional extractions with equal volumes of phenol: chloroform:isoamyl alcohol (25:24:1) or until no middle-phase was visible. After each addition of the organic solvent, the organic and aqueous phases were mixed with gentle inversion (samples were never mixed in the vortex to avoid mechanical stress) and were centrifuged (3.000 rpm, 10 min, RT) in a bench centrifuge. The upper (aqueous) phase was removed using a 1 ml blue tip, cut almost at the middle and placed in a new 50 ml sterile Falcon tube.

After organic extraction the aqueous phase was transferred to dialysis membranes (Sigma) in order to remove most of the proteins with MW of 12 KDa or greater. The samples were dialyzed versus TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) in a 10^3 times larger volume, for 3 successive changes (2.3 and 16 h, respectively). The dialysis was carried out at 4°C under continuous stirring. Following dialysis, the DNA in the TE solution was measured at OD_{260}. The ratio OD_{260}/OD_{280} was greater than 1.8.

Differential polymerase chain reaction (DPCR). EGF-r and c-*erb*B-2 gene amplification was studied with differential PCR (17). The basis of differential PCR is the co-amplification by PCR of a target gene and a reference gene in the same reaction vessel. If the target gene copy number is greater than that of the reference gene, the PCR product of the target gene will be over-represented and the PCR amplification of the reference gene will be suppressed. This method is reliable if certain factors are taken into consideration: (i) DNA degradation: DNA fragments less than 300 bp in size influence the results of DPCR (18). To avoid the fragmentation of DNA, we have used the modified DNA extraction method mentioned above. This method produces good quality of DNA (ii) Amount of tissue (17).

Furthermore, we used an analytical algorithm to effectively eliminate any false-positive determination of gene amplification due to DNA fragmentation and the changing dynamics of the PCR itself (Fig. 1).

The following analytical algorithm for the detection of gene amplification was used. First DPCR with two sets of primers specific for different exons of the same γ-IFN gene were co-amplified in the same reaction vessel, one set of amplimers resulting in a PCR product of 150 bp and the other set giving a 82 bp band. Imbalances in the PCR amplification of the γIFN150 and the γIFN82 fragments should not be caused by the presence of gene amplification and therefore can be used as an indirect measure of the quality of the target genomic DNA. Thus, for our studies paraffin sections giving γIFN82/γIFN150 ratios equal to 1 indicated that the sample could be further analyzed. Second, a DPCR with the predicted target gene product smaller than the reference gene product: EGF-r/γIFN150 and c-*erb*B-2/γIFN150. Positive samples from this reaction should then be analyzed by a DPCR, giving a reference gene product that is smaller than the target gene EGF-r/γIFN85 and c-*erb*B-2/γIFN85. If this critical reaction is positive, indicating the presence of gene amplification, verification using each target gene as reference gene was employed (EGF-r/c-*erb*B-2).

Specific DNA sequences (Table I) were amplified in a PCR mixture containing 1 μg of the target DNA sample, 50 mM KCl, 10 mm Tris-HCl pH 8.3, 1.5 mm $MgCl_2$, 0.25 mm of each dNTP, 20 pmol/μl of each amplimer and 2 Units of AmpliTaq polymerase (Perkin Elmer Cetus, Ca) in 100 μl. The reaction was overlaid with two drops of mineral oil (Sigma, St. Louis, MO) and placed into a DNA thermocycler 480 (Perkin Elmer Cetus, Ca). The following protocol was used: 1st cycle 10 min at 94°C, 2-40 cycles 1 min at 94°C, 50°C and 72°C respectively and last cycle 10 min at 72°C. Ten percent of each PCR reaction product was electrophoresed for 2 hours in a 12% polyacrylamide gel and stained with ethidium bromide. The relative intensity of the bands was quantified using photograph negatives of the ethidium stained gels and densiotometry was performed on a video image analysis system. The results are expressed as the ratio of:

$$\frac{\text{Relative intensity of target gene sample}}{\text{Relative intensity of reference gene sample}}$$

The oligonucleotides used as primers (Table II) were synthesized on a cyclone Plus Synthesizer (Milligan Bioresearch Mass, USA) by phosphoramidite chemistry and deprotected by treatment with NH_4O_4 O/N at 55°C and OPC cartridge purification.

Controls for DPCR. Cell lines A431 and MDA 361 which carry 15-20 fold EGF-r gene and 2-4 fold c-*erb*B-2 gene amplification, respectively were used as controls.

Statistical analysis. Statistical analysis was carried out by chi-square test with Yale's correction and additional Fisher's exact test in certain correlations.

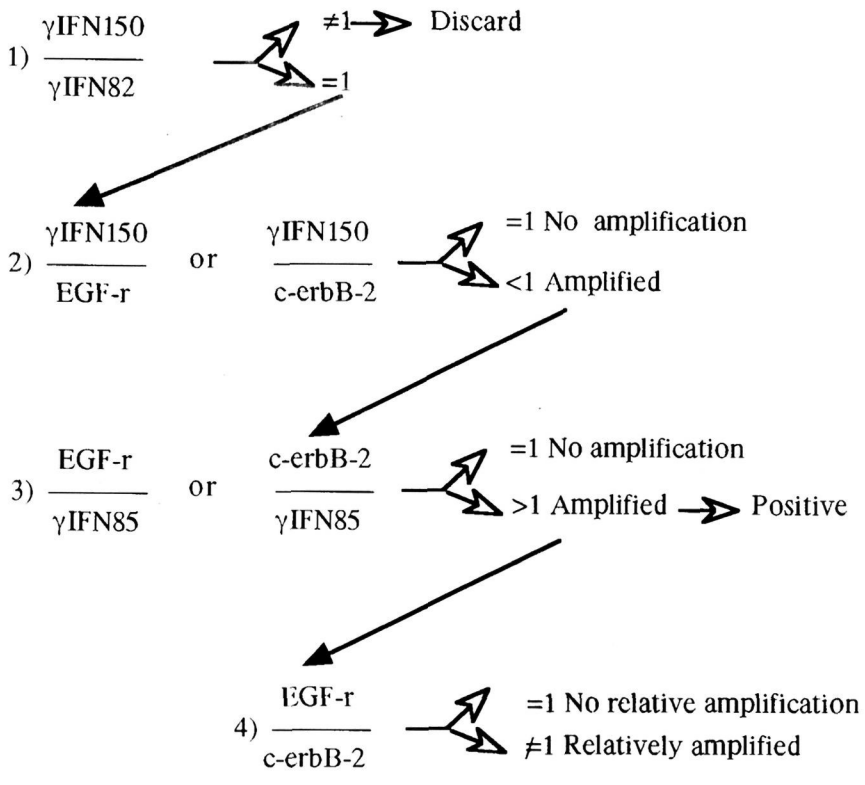

Figure 1. An analytical algorithm for the detection of EGF-r and c-*erb*B-2 gene amplification in archival tissue.

Table I. Amplified sequences.

Gene	Length of PCR-amplified sequence	Sequence region	Ref. no.
IFNγ	150 bp	4582-4731	37
IFNγ	82 bp	2012-2093	37
IFNγ	85 bp	4647-4731	37
EGF-r	110 bp	3801-4010	38
c-*erb*B-2	98 bp	2122-2213	39

Table II. Oligonucleotide primers.

	Primer sequence
IFNγ (150)	5'-CTC TTT TCT TTC CCG ATA GGT-3'
	3'-CTG GGA TGC TCT TCG ACC TCG-5'
IFNγ (82)	5'-GCA GAG CCA AAT TGT CTC CT-3'
	3'-GGT CTC CAC ACT CTT TTG GA-5'
IFN (85)	5'-AGT GAT GGC TGA ACT GTC GC-3'
	3'-CTG GGA TGC TCT TCG ACC TC-5'
EGF-r	5'-AGC CAT GCC CGC ATT AGC TC-3'
	3'-CAA AGG AAT GCA ACT TCC CA-5'
c-*erb*B-2	5'-CCT CTG ACG TCC ATC ATC TC-3'
	3'-ATC TTC TGC TGC CGT CGC TT-5'

Results

The DNA obtained from the majority of the formalin-fixed samples (72 out of 75), was of high quality as assessed electrophoretically. The quality of DNA was also verified from the first PCR reaction of the algorithm: γIFN-150/γIFN-82 which showed in most of the cases a ratio between 0.8 and 1 (Fig. 2A,B).

The DNA was then analysed with the differential PCR technique which revealed EGF-r and c-*erb*B-2 gene amplification in 2/72 (3%) and 11/72 (15%) bladder carcinomas respectively (Fig. 2D-H). EGF-r gene was amplified in 2/24 (8%) grade III carcinomas while c-*erb*B-2 was amplified in 2/25 (8%) and 9/24 (37.5%) grade II and III transitional cell bladder carcinomas, respectively (Table III and IV). In addition, all specimens with EGF-r and c-*erb*B-2 gene amplification were tumours classified as invasive on the basis of muscularis propia invasion (Table V and VI). Statistical analysis revealed a strong correlation between c-*erb*B-2 gene amplification and grade as well as stage of the carcinomas ($P=7.7\times10^{-4}$ and $P=2.5\times10^{-6}$ with Fisher's exact test respectively). The relationship between EGF-r gene amplification and grade as well as stage of the disease did not reach significance ($P=0.11$ and $P=0.127$ with Fisher's exact test respectively).

Comparison between the positive cell lines (A431 and MDA 361) and the carcinomas with amplification showed a 6 and 9 fold amplification for EGF-r gene and a 5- to 8 fold

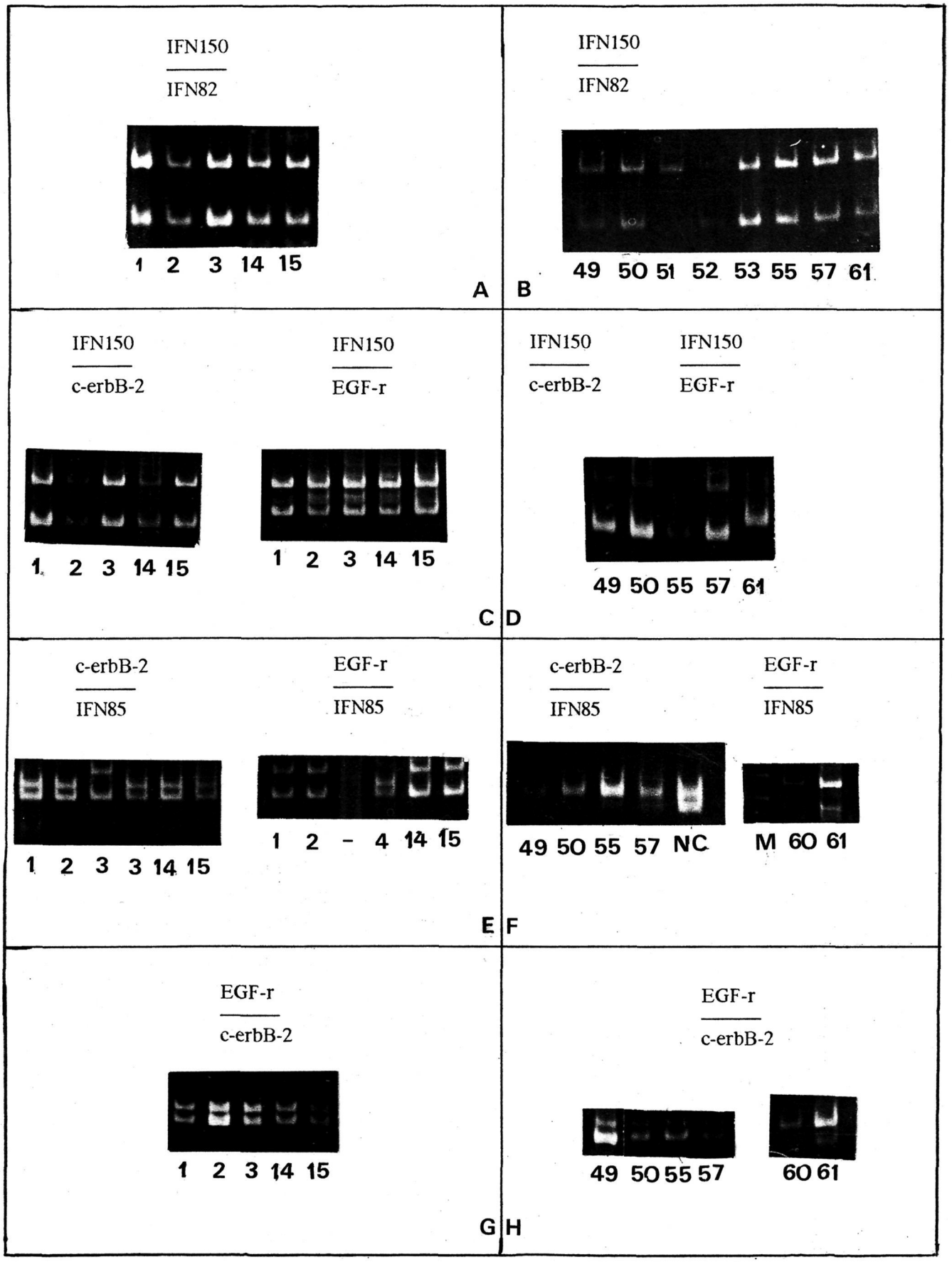

Figure 2. Analysis of EGF-r and c-*erb*B-2 gene amplification by differential polymerase chain reaction (DPCR). A, C, E, G: Samples No 1, 2, 3, 14, 15 have no indication of EGF-r or c-*erb*B-2 gene amplification. In Fig. 2E the reactions c-*erb*B-2/ IFN85 and EGF-r/ IFN85 in sample 3 are shown side by side. B, D, F, H: Samples No 49, 50, 55, 57 with c-*erb*B-2 gene amplification and samples No 60 and 61 with the EGF-r gene amplified. Samples No 51 and 52 were discarded due to imbalances in the quality control reaction. NC = Negative Control (Normal Urothelium); M = Marker.

Table III. Relationship between EGF-r gene amplification and grade of the carcinomas.

EFG-r gene amplification	Grade I	Grade II	Grade III
+	-	-	2 (8%)
-	23	25	22 (92%)

Table IV. Relationship between c-*erb*B-2 gene amplification and grade of the carcinomas.

c-*erb*B-2 gene amplification	Grade I	Grade II	Grade III
+	-	2 (8%)	9 (38%)
-	23	23 (92%)	15 (62%)

Table V. Relationship between EGF-r gene amplification and grading of muscle invasive and superficial tumours.

EGF-r gene amplification	Grade I	Grade II	Grade III	Grade I	Grade II	Grade III
	Superficial			Invasive		
+	-	-	-	-	-	2 (8%)
-	23	23 (92%)	-	-	2 (8%)	22 (92%)

Table VI. Relationship between c-*erb*B-2 gene amplification and grading of muscle invasive and superficial tumours.

c-*erb*B-2 gene amplification	Grade I	Grade II	Grade III	Grade I	Grade II	Grade III
	Superficial			Invasive		
+	-	-	-	-	2 (8%)	9 (38%)
-	23	23 (92%)	-	-	-	15 (62%)

for c-*erb*B-2 gene. The association between the degree of c-*erb*B-2 gene amplification, tumour grade and stage was also statistically significant ($P<0.01$). In addition no adjacent normal urothelium showed EGF-r or c-*erb*B-2 gene amplification.

Discussion

Quantitative and/or qualitative changes in the expression of oncogenes and their products are implicated in malignant transformation. There are several mechanisms which affect the function or expression of genes that control cell proliferation and differentiation (22).

Gene amplification represents an important mechanism of genetic alteration leading to over-production of proteins encoded by these genes. It was first observed in cells selected for resistance to cytotoxic drugs in tissue culture. This led to the hypothesis that in some tumour cells amplification of specific genes may give them a selective growth advantage during chemotherapy. Direct evidence that this can occur, came from analysis of tumour cells taken from patients treated with methotrexate (23). Another set of genes often amplified in tumour samples are oncogenes. It has been proposed that the levels of amplification of these genes can correlate with progression to more malignant phenotypes and with patients prognosis (24).

Type I growth factor receptors (EGF-r, c-*erb*B-2 and c-*erb*B-3) are a closely related group of molecules which are expressed widely in normal tissues. Deregulation of their expression seems to be involved in oncogenesis. The EGF-r is overexpressed and its gene is amplified in a wide range of carcinomas and sarcomas. Its expression has been shown to be of prognostic importance in predicting the biological behaviour of certain type of carcinomas (10,19,25,26). Overexpression of c-*erb*B-2 protein in human malignancies, a feature usually associated with c-*erb*B-2 amplification, has been related with adverse prognosis in breast and ovarian carcinomas (13,20,21). In this work we examined EGF-r and c-*erb*B-2 gene amplification in 72 transitional cell carcinoma of the urinary bladder and correlated the results with pathologic features of the tumours.

For this purpose we analyzed the extracted DNA samples from our formalin fixed paraffin embedded samples with the differential PCR technique and a diagnostic algorithm. In a previous study we used the DPCR method and observed that under certain conditions, its sensitivity is comparable with that of Southern blot analysis (27). The advantages of DPCR over traditional gene analysis methods are: (i) the capability to examine larger numbers of samples in a shorter time period (maximum 2 days); (ii) the method is non-radioactive and (iii) using the appropriate controls, DPCR estimate can semi-quantitative gene dosages.

By performing the DPCR technique on our bladder samples we found EGF-r and c-*erb*B-2 gene amplification in 2/72 (3%) and 11/72 (15%) cases, respectively. The majority of the specimens with increased EGF-r and c-*erb*B-2 copy numbers were high grade and invasive (see Tables III-VI). In addition, degree of amplification was also higher in high grade and invasive carcinomas. These findings are in accordance with those of Berger *et al*, who observed a 8-10 fold EGF-r gene amplification in 1/29 (3.5%) bladder carcinomas (28) and Coombs *et al*, who found that 12 out of 86 (14%) transitional cell tumours had an amplified c-*erb*B-2 gene (29). The latter study also indicated an association between degree of amplification and tumour grade.

We also observed immunohistochemically a close relation between EGF-r and c-*erb*B-2 overexpression, grade and tumours stage. It is notable, that video image analysis showed in all cases where EGF-r and c-*erb*B-2 gene was amplified an increase of 10% for EGF-r and 25% for c-*erb*B-2 of staining signal compared to the other specimens with overexpression of these molecules (unpublished data). It seems that c-*erb*B-2 gene amplification represents a frequent

and important mechanism of c-*erb*B-2 overexpression in transitional cell bladder carcinoma and is directly linked to tumour grade and progression. On the other hand EGF-r gene amplification is a less frequent event and possibly EGF-r overexpression in bladder tumours is a consequence of increased transcription. The latter hypothesis cannot be substantiated since our study was performed in formalin-fixed material where mRNA analysis is not feasible.

There are several reports which address the concept of various proto-oncogenes in the pathogenesis and prognosis of transitional cell bladder carcinoma, but their role remains still unclear (30-32). Recently, Dalbagni *et al* proposed a sequence of genetic events which lead to bladder tumour progression and are correlated with pathological indices of poor clinical outcome (33). It is possible that these genetic events may lead to secondary deregulation of growth factor receptor expression. The deregulation of growth factor receptor expression (overexpression, autocrine stimulation) in conditional renewal tissues is sufficient for tumour initiation, but in renewal tissue such as urothelium, molecular lesions which simply shorten cell cycle time would not be adequate to initiate tumorigenesis (34). In renewal tissue the expected initial molecular lesion is one which inactivates the decycling/terminal differentiation mechanism, for example loss of anti-oncogene p53 activity rather than positive autocrine growth signals. Therefore EGF-r and c-*erb*B-2 overexpression and gene amplification possibly represent late events in bladder oncogenesis although there are reports on morphologically normal and hyperplastic cells from the bladder overexpressing the c-*erb*B-2 product (29). Furthermore, the simultaneous expression pattern of EGF-r and c-*erb*B-2 in the majority of the specimens we examined (unpublished data) could indicate that c-*erb*B-1 (EGF-r) and c-*erb*B-2 form heterodimeric receptors, a phenomenon observed *in vitro* (35). These heterodimeric receptors have particularly high affinity for EGF, which is found in very high concentrations in urine (80 ng/ml) (36).

Further evaluation is required to clarify these hypotheses which may allow us to predict prognosis of bladder cancer more accurately.

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