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Epidermal growth factor receptor expression in squamous cell lung carcinomas: an immunohistochemical and gene analysis in formalin-fixed, paraffin-embedded material

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Abstract. Epidermal growth factor (EGF) and its receptor (EGFr) constitute an important and well-characterized mitogenic system in various ectodermal tissues. We evaluated the expression of EGFr and examined possible EGFr gene alterations in 18 formalin-fixed, paraffin-embedded squamous cell lung carcinomas (SCLC) by an immunohistochemical assay, Southern blotting and differential polymerase chain reaction (DPCR). The immunohistochemical study employing the F_4 and EGF-R1 monoclonal antibodies, directed against the intra- and extra-cellular portion of the receptor respectively, showed EGFr over-expression in 89% of the SCLC cases examined. All cases showed positive immunostaining for both antibodies, thus excluding the possibility of truncated receptors. In addition, analysis of the EGFr gene was carried out by Southern blotting and DPCR on paraffin extracted DNA from the same carcinoma cases. We found amplification of the EGFr gene in 5/18 (27%) SCLCs. All 5 positive cases showed EGFr over-expression, suggesting a possible correlation between the presence of EGFr gene amplification and over-expression of receptor protein. No correlation was observed among EGFr staining, EGFr gene amplification and differentiation of carcinomas. In addition, Southern blot analysis with HER-A2, a probe which hybridizes a sequence of the receptor's intracellular domain, revealed three novel *Eco*RI restriction fragment patterns. We suggest that these patterns correspond to *Eco*RI polymorphic sites of the receptor's tyrosine kinase domain.

Key words: Epidermal growth factor receptor – Squamous cell lung carcinoma – Southern blot – Differential polymerase chain reaction – Immunohistochemistry

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Introduction

Several changes occurring at the level of DNA are thought to be required for malignant transformation. One family of molecules known to be capable of influencing this process are the growth factor receptors. Much evidence has emerged for changes in the gene copy number, gene structure and level of expression of a subset of these in certain types of human cancers. The type 1 growth factor receptors, epidermal growth factor receptor (EGFr) and the c-*erb*B-2 protein are often over-expressed, predominantly in squamous cell carcinomas and adenocarcinomas (Ozanne et al. 1986; Gullick and Venter 1989). No comprehensive reports have emerged on the normal distribution of expression of the c-*erb*-B3 protein, the new member of the type 1 growth factor receptors. EGFr has an external domain which binds epidermal growth factor (EGF), transforming growth factor-a, Vaccinia growth factor and amphiregulin, a transmembrane section and a cytoplasmic part which contains a tyrosine specific protein kinase (Todderud and Carpenter 1989). Stimulation of the receptor by any of the ligands mentioned above activates the receptor which in turn triggers cellular mechanisms that regulate cell growth (Fisher and Lakshamanan 1990). The gene encoding the EGFr has been demonstrated on the short arm of chromosome 7 (Hunter 1984); in normal human cells this encodes mRNA molecules of 10.1 and 5.8 kb (Shimizu et al. 1984; Ullrich et al. 1984). EGFr is expressed at particularly high levels on epithelia (Ullrich et al. 1984). Increased activity is most often due not to a structural abnormality, but rather to over-expression of normal receptors (Wynford-Thomas 1991).

Several reports based on immunohistochemistry, autoradiography, Western blotting and radioimmunoassay have demonstrated elevated levels of EGFr in lung tu-

mours, particularly in squamous cell lung carcinomas (SCLC), as a consequence of gene amplification and increased mRNA expression (Gullick 1991). This study was carried out in order to evaluate the expression of EGFr, detect possible EGFr gene alterations in SCLCs and evaluate the efficiency of molecular biology techniques in archival tissue which forms the basis of traditional clinicopathological correlation in surgical pathology. For this purpose we collected 18 formalin-fixed, paraffin-embedded cases and analysed them by an immunohistochemical assay, Southern blotting and differential polymerase chain reaction (DPCR).

Materials and methods

Eighteen cases of surgically removed, formalin-fixed and paraffin-embedded SCLCs retrieved from the files of "Sotiria" and 401 Army General Hospital, were studied. The specimens were selected on the basis that tumour cells comprised more than 90% of the tissue block. Histologically they consisted of 6 well, 6 moderately and 6 poorly differentiated carcinomas.

Immunohistochemical staining was performed using the biotin streptavidin detection kit (Biomakor, Biodata Hellas). Briefly, after dewaxing, hydrating and washing in phosphate buffered saline (PBS), the sections were treated with 0.1% trypsin (Sigma, St. Louis, Mo., USA) for 25 min at 37° C when the antibody F_4 was used and without trypsinization in the case of EGF-R1. After washing for 5 min in PBS, the sections were incubated for 10 min in normal horse serum (Vector, Calif., USA) followed by overnight incubation with F_4 (Biomakor) and EGF-R1 (Amersham, UK) monoclonal antibodies. The mouse monoclonal antibody F_4 is raised against a synthetic intracellular 12aa peptide corresponding to residues 985–996 of the intracellular domain of the human EGFr. The EGF-R1 antibody is directed against the extracellular portion of the receptor. EGF-R1 is an IgG_{2b} murine mAb (Waterfield et al. 1982). The hybridoma which produces EGF-R1 was grown in cell culture and the antibodies were isolated from serum-free medium by affinity chromatography on protein A. Optimal dilutions were 1:700 for F_4 and 1:50 for EGF-R1 (2.5 mg/ml). Sections were incubated overnight at 4° C. The second and third steps were carried out using a goat-anti-mouse Ig biotin streptavidin detection kit (Biomakor). After washing in PBS, the slides were incubated with 0.05% diaminobenzidine (BDH, UK) and counterstained with Mayer's haematoxylin. Skin biopsies were used as positive control. Stained sections were examined microscopically and scored as negative (−), variably positive (+) and consistently positive (++).

DNA was extracted from paraffin-embedded material by a modification of the method of Mies (Mies et al. 1991). Sections (10 μm) cut from a paraffin block were placed in nylon mesh tissue biopsy bag, and deparaffinized in xylene heated at 65° C for 10 min. After that, the sections were transferred in xylene at room temperature for 20 min and then rinsed twice with 100% alcohol for 10 min. Then, the tissue sections removed from the bags and transferred to 20 ml polypropylene Falcon tubes containing 4 to 20 ml of lysis solution consisted of: 0.05 M TRIS HCl pH 8.0, 0.15 M sodium chloride, 5 mM EDTA, containing 1% SDS and proteinase K (Boehringer Mannheim, Ind., USA) at a final concentration of 500 mg/ml. Lysis was carried out in a 45° C water-bath, under gentle agitation. Additional proteinase K (250 mg/ml) was added on each successive day of lysis, until most or all of the tissue was dissolved. After that, the samples were centrifuged at 2500 rpm for 10 min, the supernatant was extracted three times with an equal volume of phenol and the aqueous phase was treated with RNAse A (Boehringer Mannheim) at a final concentration of 100 μg/ml at 37° C for 30 min. DNA was further extracted three times with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Mixing of the organic and aqueous phases was done by hand gently, for each 10 min extraction. After organic extraction the aqueous phase was dialysed exhaustively against 10 mM TRIS HCl, 1 mM EDTA, pH 8.0 at 4° C. DNA concentration was measured with the standard spectrophotometric methods. The quality of DNA was assessed electrophoretically.

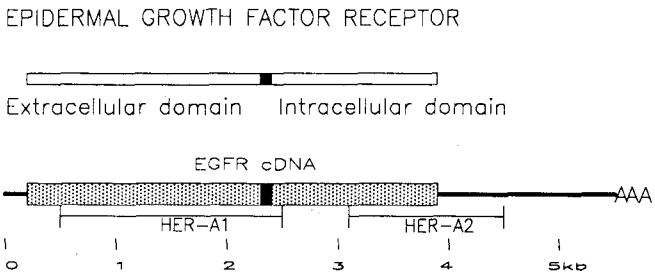

Fig. 1. Schematic representation of *Eco*RI subclones of the epidermal growth factor receptor (EGFr)cDNA. HER-A2 corresponds to the intracellular domain while HER-A1 to most of the external and transmembrane domains (*dotted area*)

DNA for Southern blotting was extracted from the same tumour cases, digested with *Eco*RI and *Hin*dIII restriction enzymes, separated on a 0.8% agarose gel and transferred to Hybond-N (Amersham) membranes. The blots were probed with P^{32} radiolabelled HER-A2 and HER-A1 cDNA fragments of the EGFr that correspond to segments of the intra- and extra-cellular domain (Fig. 1). The blots were autoradiographed using Kodak XAR-5 film and intensifying screens. Equal amount of DNA from the cell line A431 was used as a positive control.

To confirm the results obtained by Southern blot analysis, we employed a sensitive PCR technique, which can detect variations in gene dosage, using miniscule amounts of tumour DNA. The basis of DPCR is the co-amplification of a target gene and a reference gene in the same reaction vessel (Grabbe et al. 1992; Neubauer et al. 1992). 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. As reference gene we used a 150 bp fragment of interferon-γ (IFN-γ). This method is sensitive if certain factors are taken into consideration: These include a) DNA degradation and b) the amount of tissue. DNA fragments less than 300 bp influence the results of DPCR because fewer higher molecular weight fragments are available for PCR amplification. If the target gene gives a shorter PCR product than the reference gene, analysis of severely fragmented DNA would result in a false positive determination for gene amplification. To avoid the fragmentation of DNA, we have used the modified DNA extraction method mentioned above. This method produces good quality of DNA. Sections less than 10 μm in thickness and 3 mm in diameter produce weak signals and sections more than 10 μm in thickness and 15 mm in diameter were associated with higher background, probably because of non-specific PCR amplification. Therefore, sectioning of the paraffin blocks into 5 to 10 μm slices appeared to be important for optimal results.

Specific DNA sequences (Table 1) were amplified in a PCR mixture containing 1 μg of the target DNA sample, 50 mM potassium chloride, 10 mM TRIS-HCl pH 8.3, 1.5 mM magnesium chlo-

Table 1. Amplified sequences

Gene	Length of PCR-amplified segment	Sequence region
IFN-γ	150 base pairs	4582–4731
EGFr	110 base pairs	3901–4010

Table 2. Oligonucleotide primers

	Primer sequence
IFN-γ	5′-CTC TTT TCT TTC CCG ATA GGT-3′ 3′-CTG GGA TGC TCT TCG ACC TCG-3′
EGFr	5′-AGC CAT GCC CGC ATT AGC TC-3′ 3′-CAA AGG AAT GCA ACT TCC CA-5′

ride, 0.25 mM of each dNTP, 20 pmol/μl of each amplimer and 2.5 units of AmpliTaq polymerase (Perkin Elmer Cetus, Calif., USA) in 100 μl. The reaction was overlaid with two drops of mineral oil (Sigma, St. Louis, Mo., USA) and placed into a DNA thermocycler 480 (Perkin Elmer Cetus). The following protocol was used: first cycle 10 min at 94° C and 72° C, 2–40 cycles 1 min at 94° C, 50° C and 72° C respectively and last cycle 10 min at 92° C and 72° C. Ten percent of each PCR reaction product was electrophoresed for 2 h 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 densitometry was performed on a video image analysis system. The results are expressed as the ratio of:

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

The oligonucleotides used as primers (Table 2) were synthesized on a Cyclone Plus synthesizer (Milligan Bioresearch, Mass., USA), by phosphoramidite chemistry, and deprotected by treatment with ammonium hydroxide overnight at 55° C and OPC Cartsidge purification.

Statistical analysis was done by Chi-square test with Yate's correction.

Results

The expression of EGFr was studied immunohistochemically in paraffin sections of 18 SCLC using two different monoclonal antibodies, F_4 and EGF-R1, directed against the intra- and extra-cellular portion of the receptor respectively. The immunohistochemical data (Table 3) revealed that 16/18 (89%) of the carcinomas had EGFr over-expressed ($p<0.001$). All positive cases showed membrane and/or cytoplasmic immunoreactivity for EGFr. All carcinomas showed positive staining for both antibodies, excluding the possibility of truncated receptors. Heterogeneity of tumour staining was present in most of the samples examined. In some specimens, a clear difference was observed in the immunostaining of EGFr between central and peripheral tumour cells with peripheral cells more often positive. In other cases no difference in the number of cells that showed positivity to EGFr was observed between the central and peripheral areas of the tumour. The heterogeneity of EGFr staining had no obvious correlation to the tumour morphology as assessed by routine H&E staining. This may indicate clonal expansion of cells with EGFr over-expression. The predominant cell type immunoreactive for the receptor was of moderate size and was well-differentiated. Representative patterns of immunoreactivity are shown in Figs. 2–4. Table 4 shows the staining intensity in the tissues examined. F_4 mAb expressed particularly strong signal in 16/18 cases, instead of EGF-R1 who showed strong staining pattern in only 6/18 cases. Recent

Table 3. Immunohistochemical results

Number of cases	Differentiation	EGFr staining pattern					
		F4			EGFR1		
		++	+	+/−	++	+	+/−
6	Poorly differentiated	5	1	−	−	6	−
6	Moderate differentiated	6	−	−	3	3	−
6	Well differentiated	5	1	−	3	3	−
Total 18		16	2	−	6	12	−

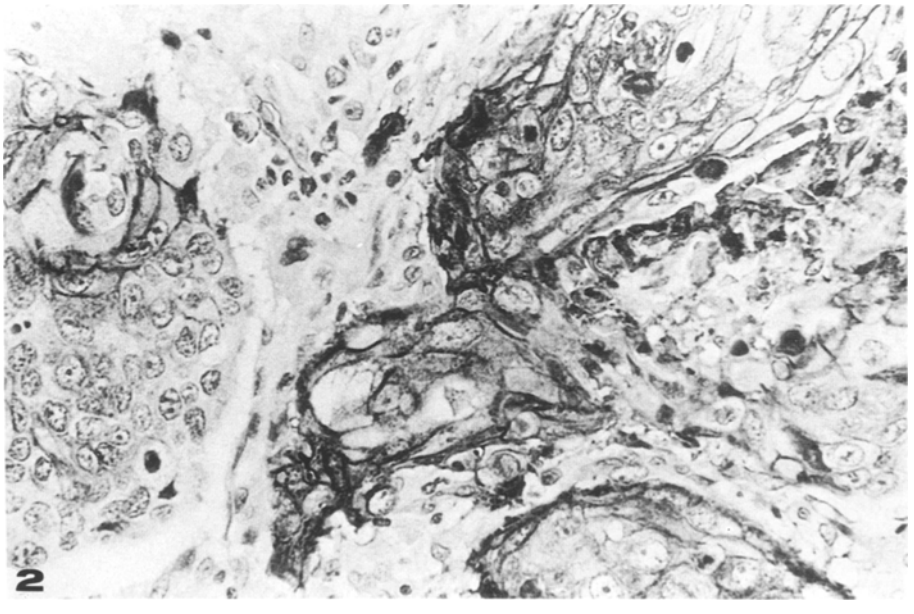

Fig. 2. Squamous cell lung carcinoma, moderately differentiated. Intense positive (++) immunostaining against F_4 antibody. Immunoperoxidase method, H&E counterstaining, ×400

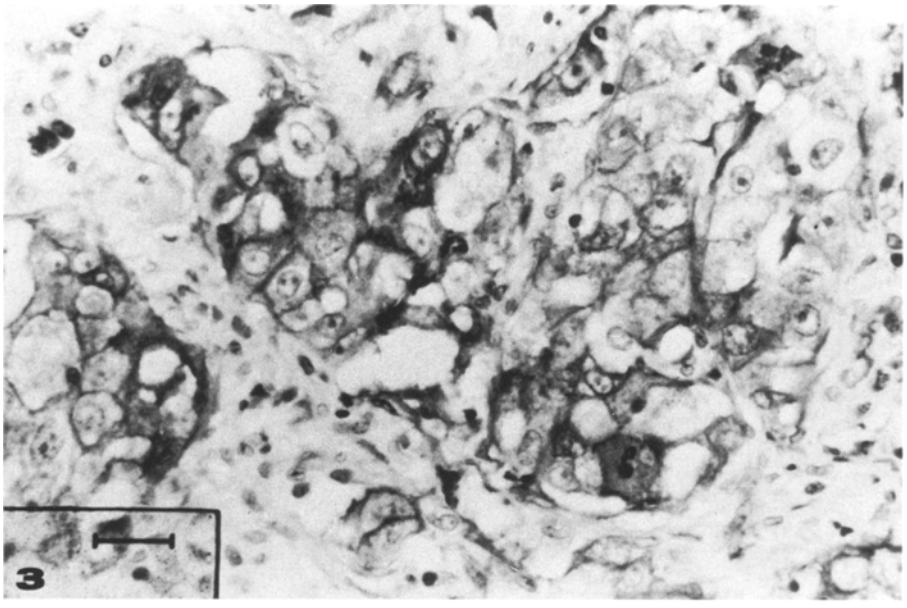

Fig. 3. Squamous cell lung carcinoma, moderately differentiated. Intense positive (+ +) immunostaining for EGF-R1 antibody. Immunoperoxidase method, H&E counterstaining, ×400

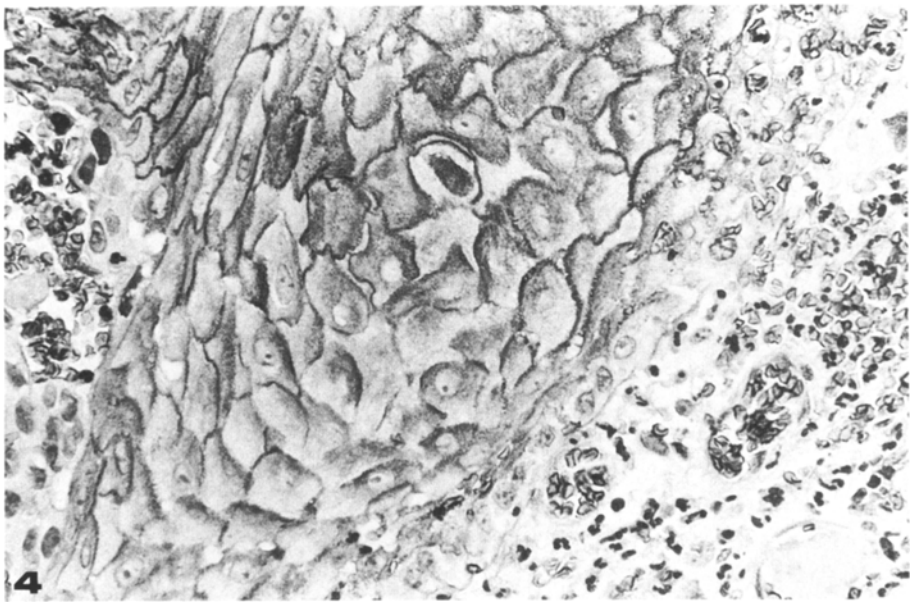

Fig. 4. Squamous cell lung carcinoma, well-differentiated. Intense positive (+ +) immunostaining for EGF-R1 antibody. Immunoperoxidase method, H&E counterstaining, ×400

studies (Gusterson et al. 1985; Cerny et al. 1986; Tuzi et al. 1990) showed EGF-R1 immunoreactivity only on frozen sections. In our assay we used different antibody concentrations with or without trypsinization, but when the sections were treated proteolytically, the staining was consistently negative. A possible explanation is that trypsin cleaves the sequence at arginine and lysine residues thus truncating the receptor. By increasing the concentration of the antibody and omitting the trypsinization step, interpretable signal was revealed in most of the cases. Staining intensity for EGF-R1 in most of the cases (12/18) was moderate (+). Interestingly, we also noticed staining differences in the positive control we used; F_4 revealed moderate immunostaining intensity, while EGF-R1 was negative or equivocal. The different immunohistochemical results could reflect alterations of the extracellular domain epitope due to the fixation procedure or that the sensitivity of EGFRL antibody is lower compared with that of F_4.

The Southern hybridization data are summarized in Tables 4 and 5. Of the 18 SCLCs evaluated, 5 i.e. a proportion of 27%, showed EGFr gene amplification (Figs. 5, 6). A 431 DNA and DNA from samples 1, 2, 6, 7, 8, 11, 12 and 14 showed the same restriction fragment pattern while samples 3–5, 9, 10, 13, and 15–18 expressed a different restriction pattern. More specifically 1, 9 and 10, 4 and 18, 13 and 15, 5, 16 and 17 showed similar restriction pattern respectively. To exclude the possibility of partial enzyme digestion, the reactions were repeated twice and we obtained the same results. This finding indicates either gene rearrangement or *Eco*RI polymorphic sites. To determine possible gene rearrangements, we digested DNA samples 1–12 with *Hin*dIII and 2, 5–8, 10, 11, 13, 14, 16 with *Eco*RI and

Table 4. Results of EGFr immunostaining pattern and gene amplification in squamous cell carcinomas of the lung

Specimen	Differentiation state	EGFr staining pattern		EGFr gene amplification
		F4	EGFR1	
C1	Poorly differentiated	++	+	−
C2	Moderate differentiated	++	+	−
C3	Well differentiated	++	++	−
C4	Moderate differentiated	++	++	−
C5	Moderate differentiated	++	++	+
C6	Well differentiated	++	+	−
C7	Moderate differentiated	++	++	−
C8	Moderate differentiated	++	+	−
C9	Well differentiated	++	+	−
C10	Poorly differentiated	++	+	−
C11	Well differentiated	++	+	+/−
C12	Well differentiated	+	++	+
C13	Well differentiated	++	++	−
C14	Poorly differentiated	+	+	−
C15	Poorly differentiated	++	+	−
C16	Poorly differentiated	++	+	+
C17	Poorly differentiated	++	+	+
C18	Moderate differentiated	++	+	+

Table 5. Relationship between histological differentiation of squamous cell carcinomas of the lung, EGFr (F4) staining intensity and EGFr gene amplification

Differentiation	EGFr (F4) staining intensity			EGFr gene amplification	
	++	+	−	+	−
Poorly differentiated	5	1	–	2	4
Medium differentiated	6	–	–	2	4
Well differentiated	5	1	–	2	4
Total	16	2	–	6	12

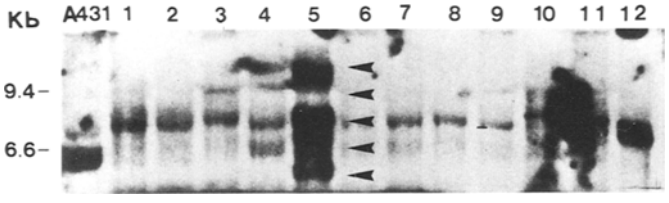

Fig. 5. Southern blot analysis of EGFr gene in squamous cell lung carcinoma specimens. Number 1–12 corresponds to specimen numbers from Table 4. The first lane contains DNA from A431 cell line which is known to have amplified sequences of the EGFr gene. Specimens 5 and 12 have EGFr gene amplified. Additional restriction fragments indicating possible *Eco*RI polymorphic sites are observed in lanes 3–5 and 9–10 (*arrowheads*, see Results)

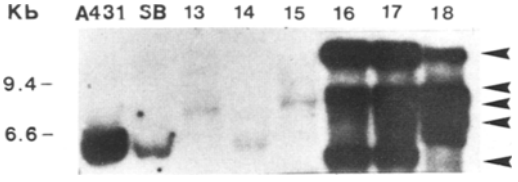

Fig. 6. Southern blot analysis of EGFr gene in squamous cell lung carcinoma specimens. Numbers 13–18 correspond to specimen numbers from Table 4. Lane SB contains DNA from SB cell line which has chromosome 7 diploidy. Specimens 16–18 have EGFr amplified. Lanes 13, and 15–18 show additional restriction fragments (*arrowheads*), indicating possible *Eco*RI polymorphic sites (see Results)

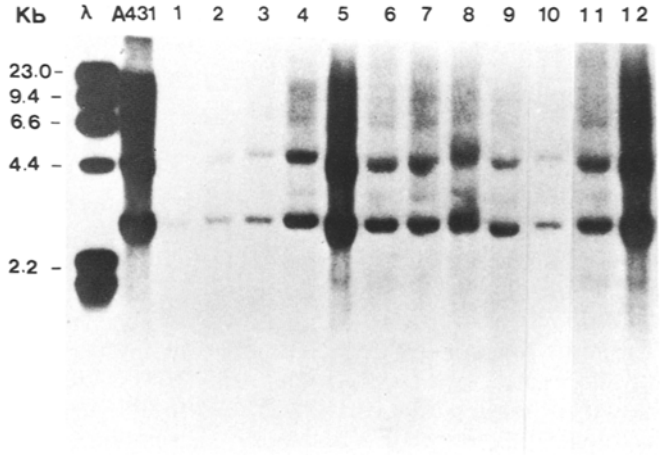

Fig. 7. Southern blot analysis of specimens 1–12. The DNA samples were digested with *Hin*dIII and the blots were hybridized with HER-A2 probe. Specimens 5 and 12 have EGFr gene amplified

hybridized them with HER-A2 and HER-A1 respectively. In both of the cases the results were the same (Figs. 7, 8). These findings suggest that the region hybridized with HER-A2 contained polymorphic sites, although the possibility of rearrangements cannot be excluded. Eight cases were excluded due to insufficient amount of DNA.

In order to verify the EGFr gene amplification results we observed by Southern blot analysis, we examined DNA from the same samples, using the DPCR technique. After 41 cycles of amplification using EGFr and IFN-γ specific amplimers, we found no difference in the intensity of the bands among the samples we examined. However, when EGFr gene was co-amplified with amplimers designed specifically for the single copy reference

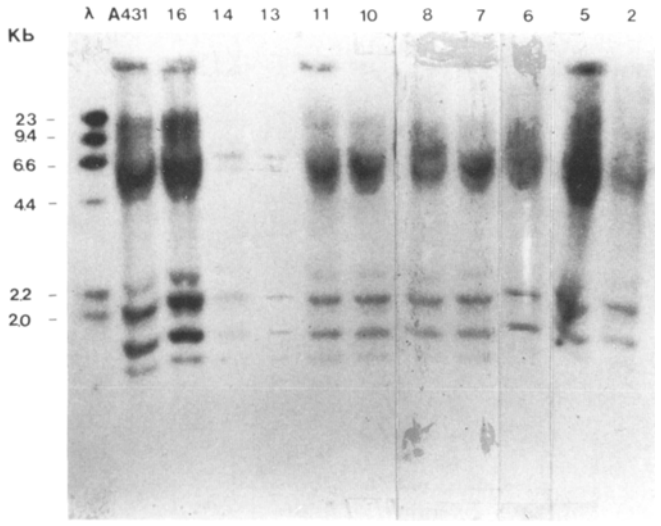

Fig. 8. Southern blot analysis of specimens 2, 5–8, 10, 11, 13, 14 and 16. The DNA samples were digested with *Eco*RI and the blots were hybridized with HER-A1 probe. Specimens 5 and 16 show EGFr gene amplification

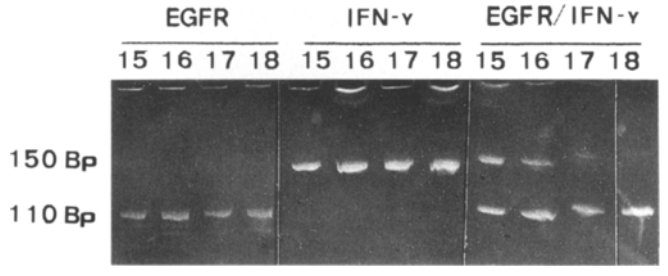

Fig. 9. Analysis of EGFr gene amplification by differential PCR from formalin-fixed, paraffin-embedded squamous cell carcinoma of the lung specimens. Lanes 1–8: Primers used for the PCR were for EGFr (*lower band*) and IFN-γ (*upper band*). Lanes 9–12: PCR products of EGFr and IFNγ indicating amplification of EGFr gene in samples 16–18 (see Results)

gene, the ratio of relative intensity of target gene sample to relative intensity of reference gene sample was increased in specimens 5, 12, 16, 17 and 18. We also observed a reduction of IFN-γ band among the same specimens, as shown in Fig. 9. Video image analysis revealed a 1.4, 1.7, 1.6, 2.2 and 2.1 fold reduction respectively. These findings are in accordance with those observed by Southern blot analysis ($p < 0.01$). We did not observed any difference in the rest of the samples (Fig. 9). As shown in Table 4, no correlation was found between histological differentiation and EGFr staining intensity/EGFr amplification.

Discussion

In this study we examined 18 SCLC specimens, in order to evaluate EGFr expression and possible EGFr gene alteration in archival, formalin-fixed, paraffin-embedded material. The semi-quantitative immunohistochemical assay we performed, revealed that 16/18 (89%) SCLCs had EGFr over-expression. Using Southern blotting and a DPCR technique, 5/18 (27%) of the examined SCLCs showed EGFr gene amplification. Southern blot analysis also revealed 3 novel restriction fragment patterns.

EGF is a small 54 amino acid polypeptide with a molecular weight of 6.54 kDa which promotes the growth of a wide range of cell types. Cells correspond to EGF by expressing a receptor for EGF, a 170 kDa phosphoglycoprotein which spans once the cell membrane. When EGF binds to the N-terminal extracellular domain of the receptor, it stimulates the tyrosine specific protein kinase activity associated with the intracellular domain of the receptor. This results in EGF autophosphorylation, oligomerization and finally phosphorylation of several target proteins which play a crucial role in cell growth and differentiation (Carpenter and Zendegul 1986).

EGFr is implicated in oncogenesis through autocrine, paracrine and heterocrine stimulation and over-expression. Over-expression of EGFr may be a consequence either of gene amplification or of increased transcription (which is a more frequent event) and is observed in a wide range of carcinomas and sarcomas, but most commonly in squamous cell carcinomas (Gullick 1991). The A431 cell line with 2.6×10^6 receptors per cell, has the highest reported number of EGF receptors (Todderud and Carpenter 1989).

Although there are several papers which address the concept of EGFr status in non-small cell carcinomas of the lung, most of them are based on frozen material. In this and in a previous study we have examined EGFr immunoexpression on paraffin sections employing the two monoclonal antibodies F_4 and EGF-R1, the latter being stained on paraffin sections for the first time (Gorgoulis et al. 1992). These antibodies are directed against the intra- and extra-cellular domain of the receptor respectively. All cases positive for EGFr showed immunoreactivity for both antibodies, thus excluding the possibility of truncated receptors. The staining pattern in both antibodies was membranous and/or cytoplasmic.

The intracellular localization of the receptor has been described previously in normal and neoplastic tissues as a result of internalization process of EGF/EGFr complex (Gusterson et al. 1984; Damjanov et al. 1986; Sugiyama et al. 1989). The receptor – ligand complex has been found to be located on the cell membrane at first, and then rapidly internalized into the cytoplasm (Carpenter and Cohen 1976; van't Hof et al. 1989). In addition, it has been argued that proliferating cells display membranous EGFr staining while post-mitotic cells reveal intracellular localization (Damjanov et al. 1986). Therefore, it is likely that immunolocalization of EGFr in carcinomas could be related to the cell cycle. In our study, all cases that showed immunoreactivity for EGFr, displayed a striking intra-tumour heterogeneity of expression. The predominant pattern of protein distribution was characterized by a combination of areas with a high percentage of positive cells and of virtually or completely EGFr-negative tumour components. This expression pattern would be compatible with a clonal ex-

pansion model of EGFr positive neoplastic cells, during the progression stage of carcinoma development (Agosti et al. 1992). The different degree of immunohistochemical staining could reflect alterations of the extracellular domain epitopes due to the fixation or binding of the extracellular domain by EGF or transforming growth factor-a, which would prevent the antibody connection.

The next step in our study was to examine the possibility of EGFr gene amplification and gene alterations. Gene amplification is an important mechanism leading to over-production of the encoded protein. Human tumours often show evidence of DNA amplification, events in which cellular oncogenes are increased sometimes by as much as 1000 fold. In addition, DNA amplification is usually responsible for the high frequency of drug resistance following in vitro selection of many human cell lines. It was first observed in cells selected for resistance to cytotoxic drugs in tissue culture and led to the hypothesis that in some tumour cells, amplification of specific genes may give them a selective growth advantage during chemotherapy. The mechanism underlying such DNA amplification is unknown, although one possibility could be multiple, successive, unequal cross-over events. It has been suggested that the levels of amplification of these genes can correlate with progression to a more malignant phenotype. EGFr gene amplification was first observed in the cell line A431 which was derived from an epidermoid carcinoma of the vulva (Ullrich et al. 1984). Since then, EGFr gene amplification has been detected in frozen material of various primary tumours of the brain (Libermann et al. 1985) and in squamous cell carcinomas (Ozanne et al. 1986). Our analysis was performed on DNA extracted from paraffin blocks and the DNA samples were analysed by Southern blot and DPCR. DPCR is a simple and sensitive method for detecting quantitative alterations of genes (Grabbe et al. 1992; Neubauer et al. 1992) if certain factors are taken into consideration (see Materials and methods). It appears that at some point during the DPCR there is a competition between the two co-amplified sequences for some limiting factors and the amplified gene uses more of those, excluding the single copy reference gene from full PCR amplification. Using both methods we found that 27% of the examined SCLCs had EGFr gene amplified. This finding is in keeping with that reported previously by Berger (Berger et al. 1987a) who estimated that the frequency of EGFr gene amplification in SCLCs is 20%. All cases with EGFr gene amplification had also EGFr over-expression. Therefore, it seems that in these specimens increased EGFr production may be a consequence either of gene amplification or amplification with some degree of increased transcription. Since extraction of RNA from formalin-fixed, paraffin-embedded material for Northern blot analysis is not yet possible due to RNA degradation, we cannot clarify the origin of EGFr over-production in these cases, and mRNA in situ hybridization is not yet a well-established method for quantitation.

We have also observed the amplification of EGFr gene in 3 out of 6 bladder carcinomas cases examined (unpublished data). In contrast, Berger et al. 1987b found EGFr amplification in bladder carcinomas in only 1 case out of 29.

In addition, DNA analysis of the SCLCs revealed three novel restriction fragment patterns. This finding indicates possible *Eco*RI polymorphic sites rather than gene rearrangement, since digestion with restriction endonuclease *Hin*dIII, hybridization with HER-A2 and further digestion with *Eco*RI and hybridization with HER-A1, revealed identical restriction fragment patterns. It is quite interesting that these novel cleavage sites may indicate point mutations in the exons and intervening sequences which correspond to the tyrosine kinase domain of the receptor. It is note worthy that the novel restriction fragments were observed in cases with and without gene amplification.

Karyotypic abnormalities including translocations and in some cases increased copies of the chromosome 7 have been documented in A431 cell line (Shimizu et al. 1980). A study of Shimizu (Shimizu et al. 1984) has shown that the overproduction of EGFr in A431 cell lines is generally linked to chromosome 7 translocation. Unlike translocation of oncogenes in other tumours, the recipient chromosome is not specific (Leder et al. 1983). In human tumours EGFr gene rearrangement with or without amplification has been described in glioblastomas (Brown 1991) and one carcinosarcoma of the lung (Berger et al. 1987a). Since the v-*erb*B oncogene may transform cell through expression of a truncated receptor, the observation of amplification and rearrangement of the receptor gene in addition with abnormal transcription in certain human tumours could be involved in the progression and behaviour of neoplasia (Libermann et al. 1985). Further investigation is under way in our laboratories in order to clarify whether the novel restriction fragment patterns found in the present study, correspond to rearrangements or *Eco*RI polymorphic sites of the EGFr gene.

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