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Original Article

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Carcinoma epidermóide do pulmão: Polissomia e amplificação do cromossoma 7 e do gene EGRF com forma *wild type* nos exões 19 e 21

Polysomy and amplification of chromosome 7 defined for EGFR gene in squamous cell carcinoma of the lung together with exons 19 and 21 wild type

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Resumo

Objectivo: O receptor do factor de crescimento epidérmico (EGFR) está sobreexpresso na maioria dos carcinomas do pulmão de não pequenas células (CPNPC) e é um dos principais alvos específicos dos inibidores da tirosina cinase (TKI) utilizados para o tratamento do CPNPC avançado. Apesar disto, há um considerável número de factores biológicos que também estão associados à resposta dos EGFR-TKIs. Este estudo teve como principal objectivo a pesquisa de mutações somáticas e amplificação do *EGFR* em casos de carcinoma epidermóide do pulmão. **Material e métodos:** Secções

Abstract

Purpose: The epidermal growth factor receptor (EGFR) is overexpressed in the majority of non-small-cell lung cancers (NSCLC) and is a major target specific EGFR tyrosine kinase inhibitors (TKIs) developed and used for the treatment of advanced NSCLC. A number of biological factors are also associated with EGFR-TKIs responsiveness. This study was focused on EGFR somatic mutations and amplifications in squamous cell lung cancer. **Material and methods:** Representative sections of squamous cell carcinoma were selected from 54 surgical specimens

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representativas de carcinoma epidermóide foram seleccionadas de 54 casos em que o tecido estava fixado em formal e incluído em parafina, sendo depois submetidos à construção de TMA. A determinação da expressão proteica do EGFR foi feita por imunoistoquímica (IHQ) (Zymed, laboratórios). A hibridização *in situ* de fluorescência (FISH) foi realizada com a sonda EGFR LSI / CEP 7 (Vysis; Abbott Molecular, EUA). O ADN genómico foi extraído de 48 casos, amplificado por reacção em cadeia da polimerase (PCR) para pesquisa de mutações nos exões 19 (deleções) e 21 (mutações pontuais). Todos os casos expressaram positividade para a citoqueratina de alto peso molecular e foi observada negatividade para CK7, CD56 e cromogranina. **Resultados:** A sobreexpressão proteica do EGFR foi identificada em 49 casos, pela aplicação do *score* de Hirsh/ Cappuzzo (2005). A pesquisa de alterações génicas no cromossoma 7 e do gene *EGFR* foram analisadas por FISH e de acordo com o método de Cappuzzo (2005), foi identificada alta polissomia em 31 casos e amplificação em 7 casos. Por electroforese capilar, foram detectadas no exão 19 do *EGFR*: deleções em heterozigotia em 3 dos 48 casos estudados e o exão 21 apresentou-se sempre na sua forma *wild-type*, quando estudado por enzimas de restrição. **Conclusões:** A detecção de deleções e mutações pontuais no *EGFR* mostrou ser um evento raro no carcinoma epidermóide do pulmão. Apesar de a presença de mutações no *EGFR* ser um indicador molecular e de sensibilidade eficaz em doentes com CPNPC avançado, submetidos ao tratamento com EGFR-TKIs, é a determinação de amplificações e de polissomias no gene *EGFR* que melhor traduz a eficácia do tratamento nos doentes com carcinoma epidermóide, quando isolado do grupo de CPNPC.

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Palavras-chave: Carcinoma do pulmão, carcinoma epidermóide, tecido incluído em parafina, EGFR, amplificação, polissomia.

from formalin-fixed paraffin-embedded tissues and submitted to TMA construction. Determination of EGFR protein expression was done by immunohistochemistry(IHC) (Zymed, Laboratories). Fluorescence *in situ* hybridization (FISH) was performed with LSI EGFR/CEP 7 (*Vysis*; Abbott Molecular, USA). Genomic DNA was extracted from 48 cases and exon 19 was amplified by polymerase chain reaction (PCR) for search deletions and point mutations for exon 21. All cases expressed high weigh cytokeratin and were observed negativity for CK7, CD56 and chromogranin. **Results:** EGFR protein overexpression was identified in 49 cases, by the application of Hirsh's scoring system. The chromosome 7 and EGFR gene were analyzed by FISH and scored according to Cappuzzo's method that showed high polysomy in 31 cases and amplification in 7 cases. Deletion in exon 19 of EGFR was detected in 3 cases of 48 samples; the exon 21 of EGFR was expressed in its wild type by RFLP in all cases. **Conclusions:** Detection of common EGFR deletion and mutation showed to be a rare event in Squamous cell carcinoma of the lung. While EGFR mutation is the most effective molecular predictor or sensitivity in patients with advanced NSCLC submitted to EGFR-TKIs treatment, amplification and polysomy is the most effective molecular predictor for EGFR-TKIs responsiveness in squamous cell carcinoma, when validated isolated from the group of NSCLC.

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Key-words: Lung cancer, epidermoid carcinoma, paraffin-embedded tissue, EGFR, gene amplification, gene polysomy.

Introduction

Lung cancer is one of the most common human cancers and the leading cause of cancer death world-wide[1,2].

Non-small cell lung cancer (NSCLC) comprises approximately 85% of all cases divided into squamous-cell carcinoma (SCC), adenocarcinoma (AC), large cells carcinomas and others[3-5].

The treatment of lung cancer is based on the stage of the cancer, and on patients performance status: for patients with early stage disease (stage I or II) surgical resection is considered the primary therapeutic choice. However, that majority of NSCLC cases have reached locally advanced (stage III) or metastatic stages (stage IV) at the time of diagnosis and chemotherapy is usually recommended as first line therapy.

Surgical resection is considered to be a curative treatment during earlier stage disease, but 5-year survival after surgical resection remains less than optimal, ranging from 67% for pT1N0 patients to 23% for patients with ipsilateral mediastinal lymph node involvement[3-6].

Receptor and non-receptor tyrosine and serine/threonine kinases have emerged as promising targets for specific drug development because overexpression or aberrant activation of these kinases often play an important role in the molecular pathogenesis of solid tumours. Further, the structural heterogeneity of these kinases has permitted development of small compounds which is a specific way inhibit their activity.

Epidermal growth factor receptor (EGFR), a 170 kDa tyrosine kinase (TK), is a member of the human epidermal receptor (HER) family that consists of four transmembrane tyrosine kinase receptors, EGFR (HER1, erbB-1), HER2 (erbB-2, HER/neu), HER3 and HER4. Upon ligands binding, the receptors homo or hetero-dimerize in between or either other growth factors. Subsequently, activation of receptor intrinsic tyrosine kinase activity occurs together either downstream signalling cascades, mainly including Ras-Raf-MAP-kinase pathway, PI3K-Akt pathway, and STAT pathway. All these have strong stimulatory effect on cell proliferation, differentiation, survival, angiogenesis and migration[7,8].

EGFR has emerged as a critical tumorigenic factor in the development and progression of NSCLC. EGFR and erbB-2 are expressed in many solid tumours and their overexpression is associated with poor prognosis.

Activating mutations of EGFR are related to increased response rate and survival in patients treated with EGFR tyrosine kinase inhibitors (TKIs)[9]. Specific EGFR tyrosine kinase inhibitors (TKIs) have been developed and used clinically in the treatment of advanced NSCLC. They disrupt EGFR signalling by competing with adenosine triphosphate (ATP) for binding sites at tyrosine kinase domain, and thus inhibiting the phosphorylation and activation of EGFRs and the downstream signalling network[10].

Tyrosine kinase inhibitors after first line chemotherapy are used to complement conventional chemotherapy and several studies are required to define molecular characteristics, different in adenocarcinoma and squamous cell carcinoma, to predict the response to those drugs[11].

Somatic mutation is the mutation that occurs only in somatic cells, which are in contrast to germ cells. A large number of somatic mutations have been identified in the EGFR gene in NSCLC. In general, these

mutations can be included into three major types: in-frame deletion, insertion, and missense mutation. Most of these mutations are located in the tyrosine kinase coding domain (exons 18-21) of the EGFR gene. The amino acids 746-750 encoded by exon 19 and amino acid 858 encoded by exon 21 are two mutation hotspots, which accounts for over 80% of all the detected mutations[12,15].
A considerable number of retrospective studies have reported that two activating mutations, small in-frame deletion in exon 19 and substitution of leucine for arginine at amino acid 858 in exon 21 (L858R), have a striking correlation with EGFR-TKI sensivity.
Both activating mutations are able to enhance kinase activity of EGFR and the activation of its downstream signaling, and play a pivotal role in supporting NSCLC cell survival. When the specific EGFR-TKIs are applied, the excessive survival signals that cancer cells are "addicted to" are counteratcted and a clear apoptosis occurs[14].
The present study intended to clarify the status of EGFR gene by FISH, PCR and its immunohistochemical expression in squamous cell carcinoma of the lung.

Material and methods

Sample selection

A cohort of 54 formalin-fixed paraffin-embedded samples, adequate for analysis were included in this retrospective study, concerning sections of squamous cell carcinoma selected from surgical specimens. In Table I is resumed the age and gender of the patients.
Slides of tumor samples stained with hematoxylin-eosin were independently reviewed by two pathologists and representative areas were marked. Core tissue biopsy speciemens (3mm in diameter) in triplicate were obtained from individual paraffin-embedded samples (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a punch-extractor pen (Histopat, Histopathology, Ltd.). Each tissue array block contain 12 specimens, which allowed all 162 specimens (triplicate speciemens of 54 cases) to be contained in 13 array blocks. This TMA construction was used for FISH and immunohistochemical analysis.

Table I – Patients characteristics

	Female (7)	Male (47)
Age (years) median	65,8	66,9
Age (years) range	54-73	48-84

Immumohistochemistry

Three µm sections of TMA were placed on coated slides and were allowed to dry overnight. After deparaffinization and rehydration, antigen unmasking was performed using pronase E for 10 minutes. Endogenous peroxidase activity was quenched using 15 minutes incubation in 3% diluted hydrogen peroxide (H_2O_2). For blocking nonspecific binding of secondary antibody, Ultra V Block (Ultra Vision Kit; TP-015-HL; Lab-Vision) was applied to the sections and then the sections were incubated at room temperature with primary anitbodies against EGFR (clone 31G7; Zymed Laboratories) at a dilution of 1:20 for 30 minutes. After washing with phosphate-buffered saline (PBS) the slides were incubated with biotin-labeled secondary antibody for 30 minutes. Primary antibody biding was located in tis-

sues using peroxidase-conjugated streptavidin (LabVision). 3,3-diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen, according to manufacturer's instructions. The slides were counterstained with hematoxylin, dehydrated and mounted. In parallel we used known positive and negative controls.

IHC staining for EGFR antibody was scored according to Hirsch: intensity and percentage of cells. The staining was read as follow:

Grade +	Score	Intensity/positivity
0	<10 +	IHC negative
1 50% cells	10-100	1 – Weak → IHC low positive
2 75% cells	101-200	2 – Moderate → IHC moderate positive
3 >75% cells	201-300	3 – Strong → IHC intense positive

Fluorescence *in situ* hybridization

In situ hybridization is a sensitive method that is generally used to detect specific gene sequences in tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest.

Dual-color FISH analysis was performed on paraffin-embedded tissue blocks on the cases studied, using the Vysis LSI EGFR/CEP 7 probe assay (Vysis; Abbott Molecular, USA). In brief, sections of 4 µm thickness from the tissue microarray blocks were used, they were baked overnight at 56°C, deparaffinized in xylol, rehydrated in 100%, 70% ethanol and bidestilled water. Slides were then submitted to a pre-treatment in a pressure cooker with 10mM citric acid-trisodium salt buffer pH6 for 4 minutes. Washed in 2× SSC salts (sodium chloride and sodium citrate) pH 7 for 5 minutes at room temperature. Slides were immersed for 15 minutes in proteinase K solution at 37°C, they were then rinsed in 2x SSC pH7 for 5 minutes at room temperature. The slides were then dehydrated in 70%, 90% and 100% ethanol then air dried. Ten microliters of probe mixture were applied on the target areas and a 22mmx22mm glass coverslip was placed over the probe.

Coverslips were sealed with rubber cement and codenatured at 83°C for 5 minutes and incubated overnight at 37°C in a humidity chamber. Post-hybridization washes in buffer (50% formamide 2x SSC pH7) at 46°C and washed with 2x SSC pH7. Slides were air-dried in the dark and counterstained with DAPI.

Analysis was performed using Nikon Eclipse 80i and DXM 1200F Workstation equipped with Nikon filter set with single-band exitors for rhodamine, fluorescein isothiocyanate, DAPI.

Overlapping cells were excluded from analysis. Two signals were counted as adjacent or fused only if they were separated by less than one domain.

One hundred spindel cells interphase nuclei with strong and well delineated signals were examined by two different individuals. A split signal in 5% or more of spindle cells was required for a result to be classified as positive.

DNA preparation and molecular analysis of the EGFR gene

Polymerase Chain Reaction (PCR) – is a method that allows logarithmic amplification of short DNA sequences within a lon-

ger DNA molecule. Briefly, genomic DNA was extracted from serial 5-µm archival paraffin-embeded tissue, containing a representative portion of each tumor block, using the QIAmp DNA Mini Kit (Qiagen, IZASA, Portugal). One hundred nanograms (ng) of DNA were amplified in a 50 µl reaction solution containing 5 µl of 10x buffer (Roche, Portugal), 2,5 mM $MgCl_2$, 0,2 µM of each complementary primer, 200 µM deoxynucleoside triphosphate and 1 unit of DNA polymerase (Roche, Portugal). Amplifications were performed using a 5 minutes initial denaturation at 95ºC; follwed by 40 cycles 30 seconds at 95ºC, 1 minute at 60ºC 1 minute at 72ºC and a 10 minutes of final extension at 72ºC. EGFR gene (exons 19 and 21) mutations were determinated using the intron-based primers according to a published method[6]. We performed mutational analysis of exon 19 deletion and L858R point mutation of the EGFR gene, as previously described[7]. Briefly, exon 19 deletion was determinated by common fragment analysis using PCR with an FAM-labeled primer set, and the products were electrophoresed on ABI PRISM 3100 (Applied Biosystems) and all electropherograms were reanalysed by visual inspection to check for the mutations. The L858R mutation was performed using also MJ MiniOpticon (Bio-Rad), and its products were then detected by restriction fragment length polymorphism (RFLP) analysis. The restriction enzyme MscI was used to digest the TGGCCA sequence in the amplicon of the wild-type allele. In contrast, mutant type (L858R) was not digested because of the base substitution of T to G at the first base of TGGCCA. On the other hand, a new FauI restriction site, CCCGC, that can be used to distinguish L858R mutant allele from wild-type. The PCR products were digested with the restriction enzymes MscI and FauI and run on 3% agarose gel, and the existence of this mutation was accessed. The Fig. 4 presents the predicted gel-electrophoresis patterns for PCR-RFLP samples.

Statistical analysis

Statistical analyses of categorical variables were performed using bilateral Chi-squared tests or Fisher exact test as appropriate. To compare percentage of markers expression ANOVA test was applied.

Results

Patient characteristics

This study included 47 male and 7 female patients with a median age of 66 years. All of them had a history of smoking. Stages were diagnosed I (52.8%), II (31.6%) and III (15.6%) (Table I).

EGFR expression by immunohistochemistry

Immunohistochemical EGFR protein overexpression was identified in 49 cases, by the application of Hirsh's scoring system (Table II).

Table II – EGFR protein expression detected by IHC in squamous cell carcinoma of the lung

Results	Immunohistochemistry		
IHC +	Low	Mod	Int
	14	14	17
IHC -		9	

Table III – Comparision of detected EGFR copy number using FISH and IHC in Squamous cell carcinoma of the lung

TNM status	Patients		EGFR protein expression IMC		EGFR gene expression FISH Cappuzzo	
	N	%	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)
T1NOMx	14	26%	11 (79%)	3 (21%)	7 (50%)	7 (50%)
T1N1Mx	4	7%	4 (100%)	0	4 (100%)	0
T2N0Mx	19	35%	16 (84%)	3 (16%)	16 (84%)	3 (16%)
T2N1Mx	4	7%	4 (199%)	0	1 (25%)	3 (75%)
T2N2Mx	2	4%	1 (50%)	1 (50%)	1 (50%)	1 (50%)
T3N0Mx	8	15%	7 (100%)	1 (50%)	4 (50%)	4 (50%)
T3N1Mx	2	4%	1 (50%)	1 (50%)	2 (100%)	0
T4N0Mx	1	2%	1 (100%)	0	1 (100%)	0

Table IV – EGFR molecular alterations scored according Cappuzzo *et al*, 2005 criteria

Results	FISH					
	Disomy	Low trisomy	High trisomy	Low polysomy	High Polysomy	Ampli cation EGFR / CEP 7
IHC +	5	1	1	6	25	7
IHC -	1	1	1	–	6	–
TOTAL	6	2	2	6	31	7

EGFR mutations determinated by FISH and PCR

The chromosome 7 and EGFR gene were analyzed by FISH and scored according to Cappuzzo's (2005) method that showed in 31 cases high polysomy and in 7 cases amplification (Table III and Fig. 5).

Through capillary electrophoresis, deletion in exon 19 of EGFR was detected in 3 cases of 48 samples; the exon 21 of *EGFR* was expressed in its wild type by RFLP in all cases (Figs. 1-4).

Correlation between EGFR expression and EGFR mutations

(Table V).

Table V – Relationship between the results obtained in IHC and FISH of 54 screened patients

Results	FISH NEG	FISH POS	TOTAL
IHC NEG	3	6	9
IHC LOW POS	8	7	15
IHC MOD POS	4	10	14
IHC INT POS	3	13	16
TOTAL	18: 3 / 15	36: 6 / 30	54

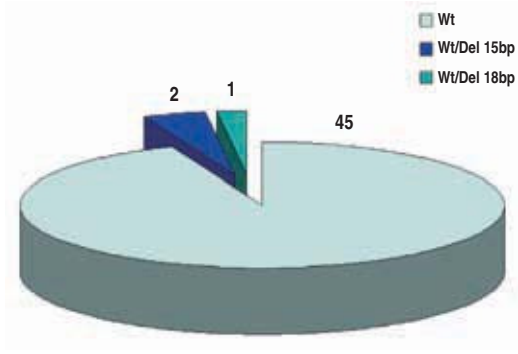

Fig. 1 – Capillary electrophoresis – Exon 19 of EGFR

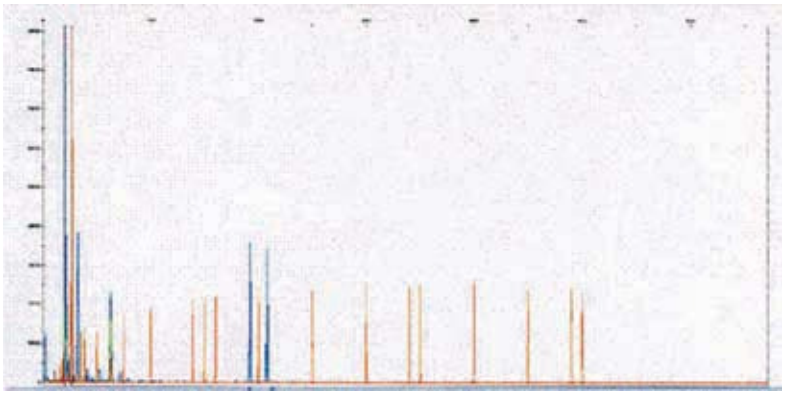

Fig. 2 – In–frame deletion of exon 19 (del E746-A750): Representative nucleotide sequence of the EGFR gene in tumor speciemens, the nucleotide sequence of heterozygous in–frame deletions in exon 19 by direct sequencing (double peaks)

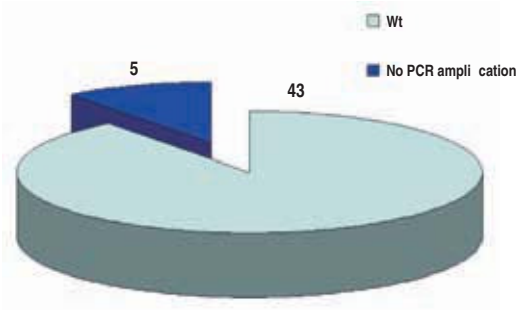

Fig. 3 – RFLP – Exon 21 of EGFR

Fig. 4 – Agarose gel – Exon 21 of EGFR

Discussion/Conclusions

EGFR mutation screening could be number one test to provide the most direct and valuable information to help clinicians to make treatment decision when dealing with NSCLC in general[1].

Meanwhile somatic mutations of EGFR gene are found in about 2-8% of USA non-small-cell lung cancer patients because of considering together all histological types of NSCLC. In the current study – all smokers: exon 19 deletion was found in 3/48 (6.25%) of Portuguese derived lung epidermoid carcinomas and in exon 21 – any L858R mutation in the 48 cases studied was identified. In-frame deletions in exon 19 and in exon 21 mutations L858R are the EGFR mutations most commonly identified and most clearly associated with TKI responsiveness.

Low number of EGFR gene copies per cell, including disomic and trisomic patterns, did not influence the level of protein expression, whereas a high gene copy number, clustered amplification or high polysomy, correlated with protein level.

EGFR protein overexpression in all tumours with gene amplification is the mechanism for overexpression. Balanced disomy for chromosome 7 and EGFR gene while expressing high protein expression might be controlled by other mechanisms, the complex interaction between gene and protein levels.

Identifying a panel of predictive markers is very important for selection of advanced NSCLC patients for EGFR-TKI therapy. Although several important demographic and clinical factors are associated with treatment response, EGFR somatic mutations are still the most effective predictor for EGFR-TKI sensitivity in lung cancer histological types.

Our study is the first that concerning only cases of squamous cell carcinoma of the lung in surgical stages, after reviewing the published medical literature which only includes cases of non-small-cell lung Cancer in advanced stages with higher expressions of the parameters here included.

However, the results obtained showed that squamous cell lung carcinoma in surgical stages expressed EGFR protein and high polysomy of chromosome 7 in 46.3% and amplification in 12.9% of the cases. Only in 3 cases we have detected deletions in exon 19.

The presence of high polysomy, amplification and deletions in exon 19 of EGFR gene have implications on tumorigenesis process due to his increased enzymatic activity become a TKIs therapeutic target.

These conclusions have to be correlated with follow-up of these patients to define potential recurrence related with the results obtained.

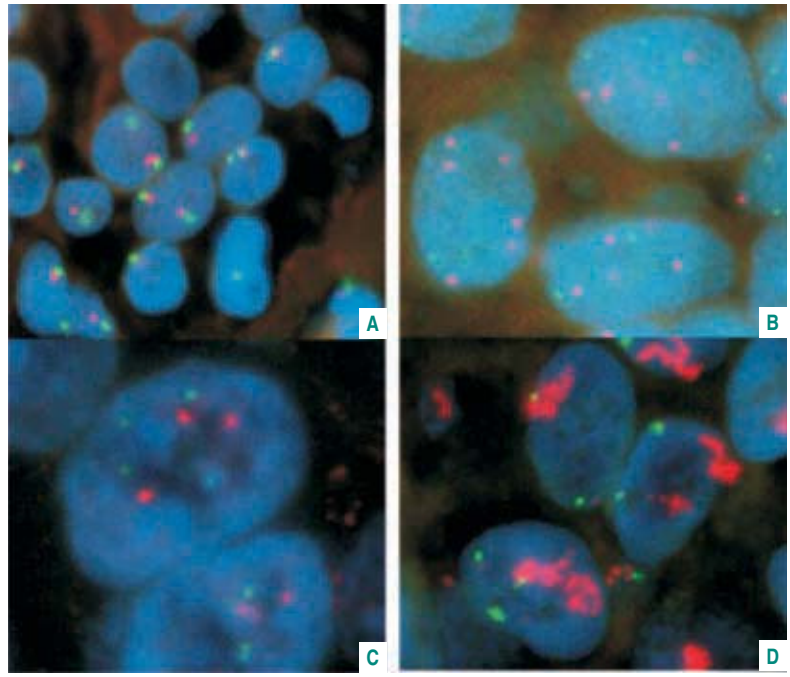

Fig. 5 – FISH analysis for EGFR (7p12/CEP7) for squamous cell carcinoma of the lung (A) – Disomy, (B) – Polysomy, (C) – Trisomy, and (D) - Ampli cation

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