

# Chromosome 7 Multiplication in EGFR-positive Lung Carcinomas Based on Tissue Microarray Analysis

EVANGELOS TSIAMBAS<sup>1,2\*</sup>, NICHOLAS S. MASTRONIKOLIS<sup>3\*</sup>, ALICIA Y. LEFAS<sup>4\*</sup>,  
STAVROS N. GEORGIANNOS<sup>5</sup>, VASILEIOS RAGOS<sup>6\*</sup>, PANAGIOTIS P. FOTIADES<sup>7</sup>,  
NIKOLAOS TSOUKALAS<sup>8</sup>, NIKOLAOS KAVANTZAS<sup>2</sup>, ANDREAS KARAMERIS<sup>9</sup>,  
DIMITRIOS PESCHOS<sup>10\*</sup>, EFSTRATIOS PATSOURIS<sup>2</sup> and KONSTANTINOS SYRIGOS<sup>11</sup>

<sup>1</sup>Department of IHC & Molecular Biology, 401 GAH, Athens, Greece;

<sup>2</sup>Department of Pathology, Medical School, University of Athens, Athens, Greece;

<sup>3</sup>Department of ENT, Head and Neck Surgery, Medical School, University of Patras, Patras, Greece;

<sup>4</sup>Faculty of Medicine, University of Southampton, Southampton, U.K.;

<sup>5</sup>Department of Breast Surgery, Blue Cross Hospital Breast Cancer Action Fund, London, U.K.;

<sup>6</sup>Department of Maxillofacial, School of Medicine, University of Ioannina, Ioannina, Greece;

<sup>7</sup>Department of Surgery, Leicester General Hospital, Leicester, U.K.;

<sup>8</sup>Department of Oncology, 417 VA Hospital (NIMTS), Athens, Greece;

<sup>9</sup>Department of Pathology, 417 VA Hospital (NIMTS), Athens, Greece;

<sup>10</sup>Department of Physiology, Medical School, University of Ioannina, Ioannina, Greece;

<sup>11</sup>3rd Department of Medicine, Athens School of Medicine, "Sotiria" General Hospital, Athens, Greece

**Abstract.** *Background/Aim:* Epidermal growth factor receptor (EGFR) over-activation is observed in significant proportions of non-small cell lung carcinomas (NSCLC). Our aim was to investigate the role of chromosome 7 multiplication with regard to its influence in EGFR expression, combined or not with gene amplification. *Materials and Methods:* Using tissue microarray technology, fifty (n=50) primary NSCLCs were cored and re-embedded into the final recipient block. Immunohistochemistry (IHC) and also chromogenic in situ hybridization (CISH) were performed. *Results:* EGFR expression at any level was detected in 40/50 (80%) cores. Over-expression was observed in 23/40 (57.5%) cases. Gene amplification was identified in 11/50 (22%) cases whereas chromosome 7 polysomy in 8/50 (16%) cases. Pure chromosome 7 multiplication alone led to low or moderate levels of

expression. Overall EGFR expression was correlated with gene ( $p=0.001$ ) and interestingly with chromosome 7 centromere numerical imbalances ( $p=0.004$ ). *Conclusion:* EGFR expression is associated not only with amplification, but also with chromosome 7 centromere multiple copies. Chromosome 7 multiplication –due to centromere region amplification or true polysomy– is critical for applying monoclonal antibody targeted therapeutic strategies excluding the pure non-amplified cases.

Critical genes for regulation of signal transduction to the nucleus are located on chromosome 7. Among them, Epidermal growth factor receptor (EGFR-gene locus: 7p12, exons: 30), MET proto-oncogene, receptor tyrosine kinase (c MET-gene locus: 7q31, exons: 24), and V-raf murine sarcoma viral oncogene homolog *B1B-Raf* proto-oncogene, serine/threonine kinase (*BRAF*-gene locus: 7q34, exons: 22) are frequently deregulated in solid malignancies including lung, colon and head and neck carcinomas (1-3). Especially in NSCLC, those genes are correlated with established criteria for applying targeted-therapeutic strategies in subgroups of patients characterized predominantly by specific mutations or amplification (4, 5). Chromosome 7 deregulation due to aneuploidy/polysomy/monosomy or single/complex gene abnormalities (mutation/amplification) plays a crucial role in those malignancies thus increasing their biological aggressive behavior (6, 7). The discovery of novel agents such as glucocorticoid receptor, which regulates

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\*These Authors contributed equally to this study.

*Correspondence to:* Dr. Evangelos Tsiambas, 17 Patriarchou Grigoriou E Street, Ag. Paraskevi, 153 41 Athens, Greece. Fax: +30 210 6526259, e-mail: tsiambasecyto@yahoo.gr

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mitotic progression and its reduced expression is detected in a panel of human liver, lung, prostate, colon, and breast cancers, improves our molecular knowledge in understanding mechanisms that induce chromosomal instability (8).

EGFR gene deregulation mechanisms in lung carcinomas –mainly in adenocarcinoma (LAC) and Squamous cell carcinoma (LSCC) - include mutations and gene amplification in a subset of patients. In addition, lung adenocarcinoma tissues harbor a broad spectrum of genomic rearrangements including predominantly somatic mutations in several oncogenes (9-11). Somatic mutations in exons 18, 20 and 21 are based on classical amino acid changes, whereas in exon 19 small in-frame deletions and insertions have also been detected. Concerning *EGFR* numerical alterations detected by fluorescent *in situ* hybridization (FISH) in NSCLC, high gene copy numbers are found in almost 60% of the patients (12, 13). Moreover, gene amplification leads to EGFR protein over-expression in the corresponding specimens (40-80%) detected by Immunohistochemistry (IHC) (14, 15). Although anti-EGFR monoclonal antibody (mAbs) inhibition strategies in NSCLC patients are under consideration, the gene amplification mechanism is the critical molecular event as it happens in *HER2* gene amplified depended breast cancers cases (16-18).

In the current study we explored the role of gene and chromosome 7 numerical alterations in LAC and LSCCs associating with the EGFR protein expression in them.

## Materials and Methods

**Patients and corresponding tissues.** We obtained, for the purposes of our study, fifty (n=50) formalin-fixed paraffin-embedded archival tissue samples of histologically proven NSCLC including fifteen (n=15) lung adenocarcinomas (LAC) and thirty-five (n=35) Lung squamous cell carcinomas (LSCC). The Department of Pathology, 417 VA Hospital, Athens, Greece provided the corresponding tissue blocks for specific protein and molecular analyses. The current research on human paraffin-embedded tissues complied with the principles laid down in the Declaration of Helsinki. Most of them were initially diagnosed by the performance of CT guided Fine needle aspiration (FNA), using conventional and liquid-based cytological methods. All corresponding Hematoxylin and Eosin (H&E) stained slides were reviewed by two pathologists for confirmation of diagnosis and classified according to World Health Organization (WHO) grading and staging (TNM system) criteria for lung cancer (revised 2014). The tissue samples correspond to 41 male (Range=47-67 years) and 9 female (Range=51-72 years) patients. Clinicopathological data are demonstrated in Table I.

**Tissue microarray construction.** Areas of interest were identified in H&E stained slides by light microscopy (Olympus BX-50, Melville, NY, USA). Selection of those areas was performed on the basis of tumor sufficiency and accurate histological confirmation, avoiding sites of necrosis or bleeding. The corresponding paraffin blocks were obtained for the construction of one TMA block. Using ATA-100 apparatus (Chemicon International, Temecula, CA, USA), the

entire source blocks were cored one or two times (in order to secure the presence of each case in the final block) and 1-mm diameter tissue cylindrical cores were transferred from each conventional donor block to the recipient block. The final constructed TMA block contained 65 cores of tissue cylindrical specimens, including five cores of the control group (normal appearing lung epithelia). After 3 mm microtome sectioning and H&E staining, we observed microscopically that all examined cases were represented by at least one (37 cases) or two tissue spot (13 cases)-confirmation of the adequacy of specimens (Figure 1).

**Antibodies and probes.** Ready-to-use EGFR monoclonal mouse antibody (clone 31G7-Zymed/Invitrogen, San Francisco, USA) recognizing predominantly the extracellular domain of EGFR protein, not reacting with other erbB receptors was used. *EGFR* gene status was determined by applying the ready to use SPOT LIGHT EGFR DNA Probe (Zymed/Invitrogen, San Francisco, USA). This digoxigenin-labeled probe is located on 7p12 and covers the entire *EGFR* gene area. Chromosome 7 status was determined by the ready to use biotin labeled chromosome 7 centromeric probe (Zymed/Invitrogen, San Francisco, USA) recognizing the specific repetitive centromeric DNA sequences known as satellite DNA.

**Immunohistochemistry (IHC).** As described above, the IHC procedure for EGFR antigen was carried out on 3 µm serial paraffin sections of TMA block. The corresponding slide was deparaffinized and rehydrated. It was also enzyme digested (proteinase K) for 10 min at 37 °C. The NBA kit (Zymed/Invitrogen, San Francisco, U.S.A) was used for the following detection steps. Blocking solution was applied to the slides for 10 min, followed by incubation for 1 h using the EGFR monoclonal antibody (dilution 1:10) at room temperature. Following incubation with the secondary antibody for 10 min, diaminobenzidine tetrahydrochloride (DAB) (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. For negative control slides, the primary antibody was omitted. IHC protocol was implemented by the use of an automated staining system (I 6000 – Biogenex, San Ramon, CA, USA). Membranous and sub-membranous cytoplasmic staining was considered acceptable for the marker, according to manufacturer's data sheet (Figure 2). Colon cancer tissue sections overexpressing EGFR and normal appearing colon and lung epithelia were used as positive and negative controls respectively. EGFR protein expression levels were evaluated semi-quantitatively by using Zymed's Evaluation Guidelines. According to the scoring guidelines, the examined cases were classified as follows: Score 0: no staining or membrane staining in <10% of tumor cells; Score 1+: faint membrane staining in >10% of tumor cells; Score 2+: weak or moderate complete membrane staining in >10% of tumor cells and Score 3+: strong, complete membrane staining in >10% of tumor cells. Scores of 0 and 1+ were considered as negative for EGFR expression while Scores 2 + and 3 + as positive (over expression).

**Chromogenic *in situ* hybridization (CISH).** CISH SPOT-Light Chromogenic ISH Detection Kit was applied (Zymed/Invitrogen, San Francisco, USA). CISH for chromosome 7 status and *EGFR* gene analysis was performed on 5 µm thick paraffin serial sections of the TMA block described above. Two slides were incubated at 37°C overnight followed by a 2 h incubation at 60°C. They were then

deparaffinized in xylene for 5 min twice, and in ethanol for 3 min thrice. The slides were rinsed in deionised water and then placed in a coplin jar containing CISH FFPE Pretreatment Buffer (CISH Tissue Pre-treatment Kit, Zymed/Invitrogen, San Francisco, USA). For heat pre-treatment, the coplin jar was capped, loosely screwed, placed in a pressure cooker and timed for 10 min after the pressure built up. The slides were then immediately washed in deionised water followed by enzyme digestion, which was performed by covering the sections with pepsin (CISH Tissue Pre-treatment Kit, Zymed/Invitrogen, San Francisco, USA) for 5 min at 37°C. The slides were washed with deionised water, dehydrated with graded ethanol and air-dried. Ready to use dig-labeled *EGFR* gene and a biotin-labeled chromosome 7 centromere probe was applied to each section, respectively. Twenty (n=20) microliters of probe were applied to each of the TMA sections. The CISH Polymer and the Horseradish (HRP) Detection Kit (Zymed/Invitrogen, San Francisco, USA) - containing similar steps to IHC - were used. Shortly, afterwards TMA sections were placed in 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and diluted with methanol for 10 min to block endogenous peroxidase. To block unspecific staining, Cas BlockTM (Zymed/Invitrogen, San Francisco, USA) was applied and incubated for 10 min. TMA sections were lightly counterstained with hematoxylin and dehydrated in graded ethanol. At the end of the process, CISH centromere signals or gene copies were easily visualized as dark brown/blue scatters or in small clustered dots, using a conventional, bright-field microscopy (Figure 1b). Interpretation of *EGFR* gene and chromosome 7 centromere signal results was based on Zymed's Evaluation Chart for CISH. According to this guide, two gene copies per nucleus demonstrate normal *EGFR* gene pattern, whereas 6-10 or small clusters characterize low-level gene amplification. In this case, chromosome 7 status must be evaluated to exclude aneuploidy (3-5 centromeric signals per nucleus; diploid pattern demonstrates normal chromosome status). Finally, high gene amplification level is confirmed by the presence of more than 10 gene copies or large clusters of them per nucleus in more than 50% of the examined cells (Figure 3).

**Statistical analysis.** Associations between variables including protein expression levels, chromosome and gene signals and pathological parameters were performed by the application of Chi-square ( $\chi^2$ ) test and Kendall's tau-b coefficient. Two-tailed *p*-values  $\leq 0.05$  were considered statistically significant. Results and correlations (*p*-values) are described in Table II.

## Results

IHC and CISH results were successfully obtained from all analyzed NSCLC cases. *EGFR* protein expression at any level was detected in 40/50 (80%) cores. *EGFR* overexpression (score: 2+/3+) was observed in 23/40 (57%) cases. The rest (n=17/40) demonstrated low (score: 1+) expression. Concerning histological type, protein overexpression was observed in 9/15 LACs, and in 14/50 LSCCs. Ten out of the fifty (n=10/50) cases were negative (score: 0) for the marker. Gene amplification was identified in 11/50 (22%) cases whilst chromosome 7 centromeric multiple copies were detected in 8/50 (16%) cases. In three tissue cores (n=3), gene amplification was also co-detected. Concerning the rest of the

samples (n=5), pure chromosome 7 multiplication alone led to low or moderate levels of expression (2+,1+). *EGFR* overall expression was significantly correlated with gene and interestingly also marginally with centromeric chromosome 7 numerical imbalances ( $p=0.001$ ,  $p=0.004$ , respectively). No other statistically significant associations were identified regarding IHC/CISH analyses and histological type, gender or tumor localization. The combined IHC and CISH results are presented in Table II and also in the histogrammic demonstrations (Figure 4).

## Discussion

The molecular landscape and also epidemiology of lung cancer is a very promising field of research in the era of targeted therapeutic strategies (19). Genetic susceptibility to *EGFR* mutations seems to be correlated with different genetic polymorphisms. There are at least three polymorphisms within the *EGFR* gene. CA-SSR1 (CA simple sequence repeat 1) is located in intron 1 and the number of CA repeats ranges from 14 to 22, with 16 being the most common number of repeats (20). East Asians tend to have longer repeats. Shorter repeats are associated with increased transcription and protein expression (21, 22). Two single nucleotide polymorphisms (SNPs) in the promoter region with effects on increased transcription and expression of *EGFR* mRNA have also been identified. However, both of these SNPs (-216G/T, -191C/A) are less common in East Asians (23). Similarly, another crucial agent is tobacco consumption and its influence in activating the carcinogenetic mechanism leading to lung cancer. But in contrast to this, *EGFR* mutations are more frequent among non-smokers. In fact, many studies have shown that the risk of the aforementioned resulting lung cancer is independent of smoking as exemplified by those with *EGFR* mutations not being affected by smoking dose, and the fact that East Asians have a higher risk for this type of lung cancer than Caucasians. In contrast, smoking related lung cancer increases with smoking dose (24-26). Caucasians are more susceptible to this type of lung cancer based on epidemiological data.

Expression of *EGFR* is deregulated in a variety of solid tumors and has been correlated with disease progression and poor survival (27). In 34-84% of NSCLC patients, *EGFR* overexpression is also detectable; an increased expression of *EGFR* is proposed to be of prognostic and also of potential predictive relevance (28). High *EGFR* gene copy numbers are found in almost 60% of the patients (29). Based on its central role in cellular tumor growth, *EGFR* is intended as a favored drug target for the development of specific anti-NSCLC treatments (30). A great number of *EGFR* specific therapeutics has been developed and tested in clinical trials; including specific antibodies such as cetuximab and

necitumumab, as well as small-molecule tyrosine kinase inhibitors (TKI) like erlotinib, afatinib, and gefitinib (31). The identification of patients who might benefit from these selective drugs is of tremendous interest. Although EGFR targeted therapies have been approved, there exists no general consensus concerning the evaluation of EGFR expression patterns in NSCLC (32).

In the current study, we analyzed the EGFR at the protein (IHC) and also at the gene (CISH) level combined with chromosome 7 numerical imbalances. According to our findings, EGFR overexpression (score: 2+/3+) was observed in a significant proportion of the examined cases. Gene amplification (low to high) and chromosome 7 multiplicity was detected in specific subgroups of examined cases. In three cases (n=3), gene amplification was also detected. Overall EGFR expression was significantly correlated with gene and interestingly also with chromosome 7 centromere numerical imbalances. Our results indicate that EGFR expression levels are associated not only with amplification, but also with chromosome 7 multiple signals in some low or overexpressed cases. Several protein and molecular studies have shown that EGFR expression is related to the gene amplification mechanism, but the role of chromosome 7 multiplication is under consideration (33). The significance of the assessment of *EGFR* gene copy number and EGFR protein expression as biomarkers to predict therapy responders as well as the selection of patients who might potentially benefit from EGFR targeted therapies was demonstrated by different studies (34, 35). In fact, data for the relationship between EGFR expression on the protein level and response to EGFR specific therapies is inconsistent (36). Nevertheless, an increased *EGFR* gene copy number has recently been proposed as a predictor of anti-EGFR targeted therapies in lung cancer patients (37). The evaluation of *EGFR* gene status by FISH is delicate: *EGFR* gene variations in tumor cells are focal and low levels of EGFR amplification are difficult to visualize. Based on this technique there are controversial results regarding the frequency of EGFR FISH-positive and also over expressed cases in LAC and LSCC cases. In some studies, FISH positivity occurred more frequently in LAC than in LSCC, but differences did not reach statistical significance combined with c MET activation (38). In another study EGFR over-expression occurred most frequently in the acinar subtype, followed by the papillary and solid subtypes (39).

In addition to *EGFR* gene numerical imbalances, - especially amplification- chromosome 7 multiplication or true polysomy based not only on FISH/CISH but also on comparative genomic hybridization (CGH) or similar molecular methods – is also critical for handling patients with lung cancer by applying monoclonal antibody targeted therapeutic strategies. With the embedded knowledge and experience regarding breast cancer Chromosome 17

Table I. Clinicopathological data of the examined cases.

	n=50
Gender	
Male	41
Female	9
Histology	
LAC	15
LSCC	35
Grade	
1	8
2	29
3	13
Stage	
I	8
II	11
III	23
IV	8
Smoking status	
Non	6
Active	37
Former	7

LAC: Lung adenocarcinoma; LSCC: Lung squamous cell carcinoma.

instability, some molecular studies suggest that in growth factor receptors (*i.e.* HER2, EGFR, c-MET) protein overexpression is mainly correlated with gene amplification but in a subset of cases also with polysomy or multiplication (40). Polysomy implies that the number of a particular chromosome is greater than diploid and it has been represented by  $\geq 3$  signals in FISH assays with a probe targeted to the centromeric area of the particular chromosome. Recent studies reported that chromosome 17 polysomic cases defined by multiplication of CEP17 in FISH assays were frequently related to 17q gain involving centromeres or amplification of the centromeric region rather than whole chromosome multiplication (true chromosome 17 polysomy). A study group reported that only one of 18 CEP17 polysomic cases (increased copy number of CEP17 by FISH) was true chromosome 17 polysomy by microarray-based comparative genomic hybridization and FISH for *HER2* (17q12), *CEP17*, *SMS* (17p11.2), and *RARA* (17q21.2). Therefore, CEP17 multiplication by *in situ* hybridization does not indicate true chromosomal 17 polysomy in all cases (41, 42).

## Conclusion

In conclusion, EGFR over-expression occurs frequently in NSCLC patients. Based on our results, EGFR expression levels are associated not only with amplification, but marginally also with chromosome 7 centromere multiple

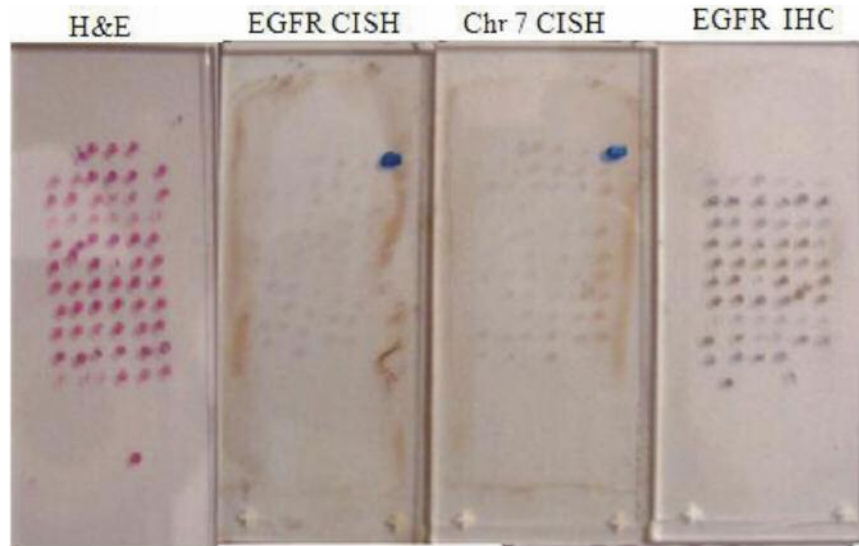


Figure 1. The set of slides including the tissue cores of the examined lung carcinoma cases.

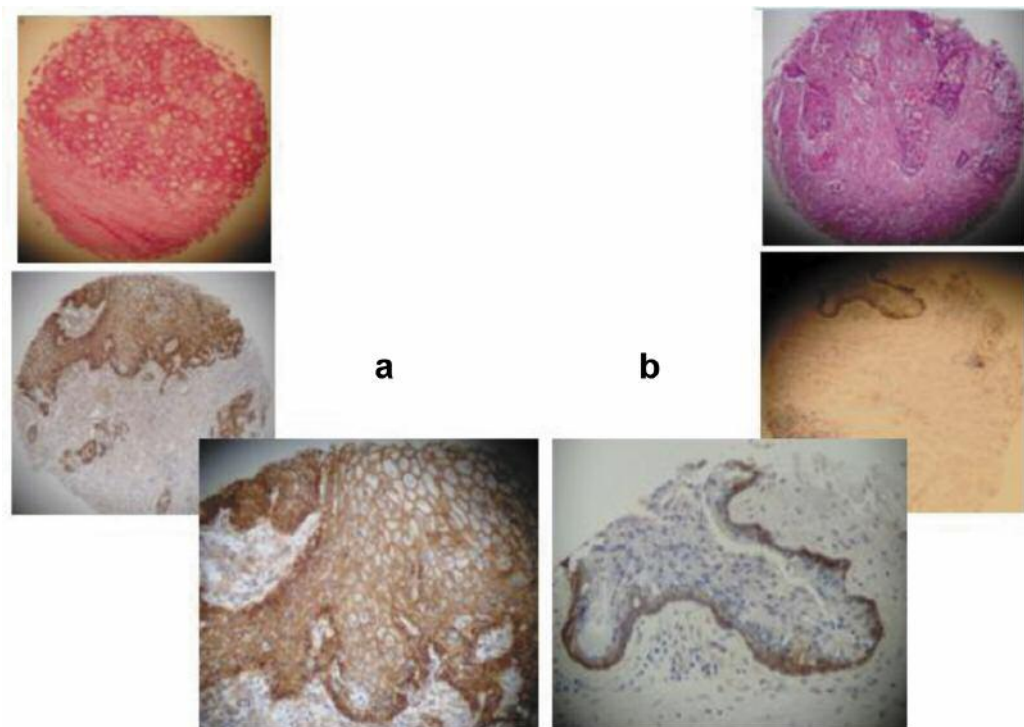


Figure 2. *EGFR* protein over-expression of two cases: left LSCC and right LAC. Note the dense brown staining pattern in the corresponding cancerous epithelia (H&E, *EGFR* DAB staining, original magnification 100 $\times$ , 400 $\times$ ).

signals. The predominant mechanism in the development of LAC *EGFR* overexpression is gene amplification. Regarding lung cancer, chromosome 7 numerical imbalances as true

polysomy or CEP 7 multiplication are crucial genetic events that modify the protein expression of *EGFR*, in some cases independently of *EGFR* gene amplification (43).

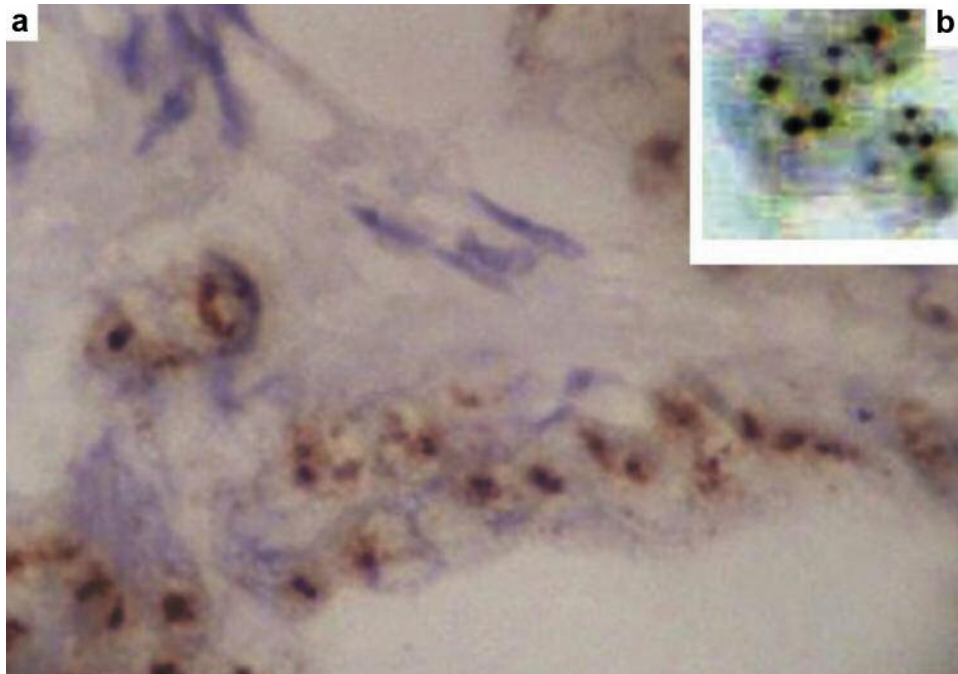


Figure 3. CISH analysis in lung carcinoma cores a. EGFR gene amplification, b. chromosome 7 polysomy. Note multiple gene copies as clusters and also chromosome copies inside the corresponding nuclei, respectively (original magnification 400×).

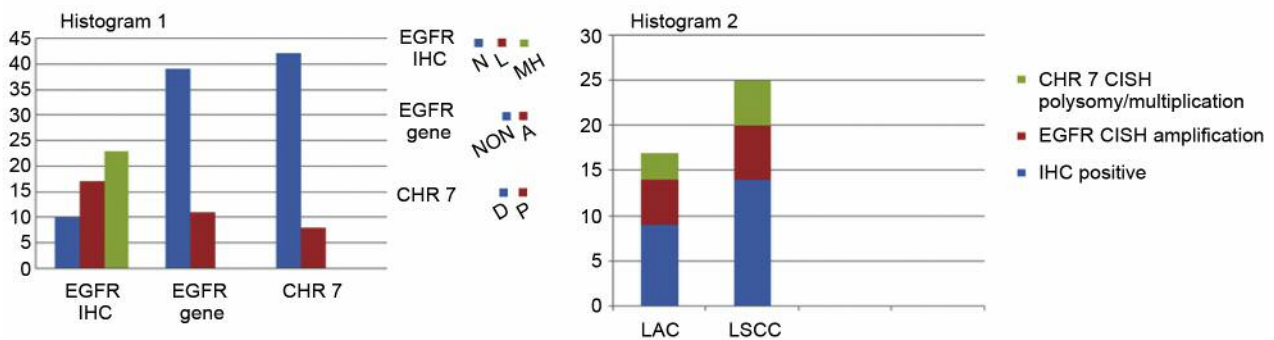


Figure 4. Histogram 1. Combined EGFR IHC/CISH and Chr 7 CISH results in the examined carcinomas, Histogram 2. Comparative combined EGFR IHC/CISH and Chr 7 CISH results in the examined LACs and LSCCs.

Table II. Combined EGFR IHC and EGFR/Chromosome 7 CISH results.

	EGFR CISH		p-Value	Chr 7 CISH		p-Value
	Amplified	Non-amplified		Diploid	Multiple copies	
EGFR IHC			0.001			0.004
0	0	10		10	0	
1+	0	17		17	2	
2+/3+	11	12		15	6	

CISH: Chromogenic *in situ* hybridization; IHC: immunohistochemistry; 2+/3+: EGFR protein over-expression (9/15 LAC, 14/50 LSCC). Multiple copies in chromosome 7 analysis: due to pure polysomy or centromere region amplification.

## Conflicts of Interest

The authors declare no conflicts of interest.

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