GENETIC ALTERATIONS OF THE EPIDERMAL GROWTH FACTOR RECEPTOR PATHWAY IN A SERIES OF NON-SMALL CELL LUNG CANCER CHARACTERIZED BY MORPHOLOGY AND IMMUNOPHENOTYPE.

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BACKGROUND

- The World Health Organization (WHO) classification of non-small cell lung cancer (NSCLC) includes adenocarcinoma (AC), squamous cell carcinoma (SCC), adenosquamous carcinoma (ASC) and large cell undifferentiated carcinoma (LCUC). Histologically, ACs can show four main different patterns: acinar, papillary, bronchioloalveolar (BAC) or solid with mucin. SCCs show keratinization, pearl formation and/or intercellular bridges, features that vary with degree of differentiation. Some cases can exhibit components of both AC and SCC, and they are classified as adenosquamous carcinoma (ASC). LCUCs, the least common of all NSCLC, are poorly differentiated tumors coming from diagnosis of exclusion after ruling out the evidence of squamous or glandular differentiation.
- Stage for stage, AC is associated with worse prognosis than SCC, with the exception of T1 N0 M0 tumors. It is important to differentiate between AC and SSC because they can be candidates for different treatments, especially in advanced stages.
- Activating mutations and/or increased copy number of the *EGFR* gene have been reported in tumors from patients with NSCLC with increased response rate to some tyrosine kinase inhibitors (TKIs).
- *EGFR* mutations occurred most frequently in women, in non smokers and in patients with adenocarcinomas rather than those with other histologic types, in agreement with profile of TKI-responders.

We analyzed the expression of six tumor markers to typify NSCLC and to correlate morphology and immunophenotype to the *EGFR* status (immunohistochemical expression, gene mutations and copy number), and *KRAS* mutations in a series of NSCLC collected in the area of Barcelona, Spain.

DESIGN

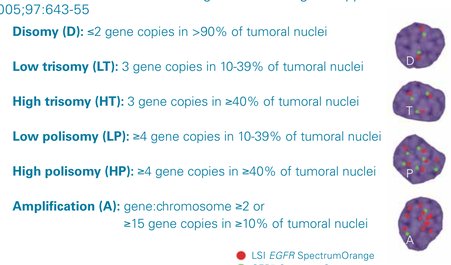
The study was performed on formalin-fixed, paraffin-embedded specimens from surgically excised tumors. Representative hematoxylin and eosin stained (H&E) sections of each case were examined microscopically and classified according to the latest WHO classification of lung tumors. The cohort included 90 NSCLC patients (78 men and 12 female); 40 cases were diagnosed as AC (including 6 BAC), 38 as SCC, 2 as ASC and 10 as LCUC.

A panel of six monoclonal antibodies (CK7, CK20, CK5/6, CK903, TTF-1 and P63) was used to discern glandular versus squamous immunophenotypic profile. The IHC staining was considered as positive when it was found on more than 10% of tumors cells. CK20 was included on the panel to exclude possible metastasis from colon adenocarcinoma.

EGFR expression was assessed by IHC using the antibody clone 31G7, and tumors were considered positive when displaying more than 10% immunoreactive cells.

EGFR gene copy number was assessed by dual-color FISH using both a centromeric probe (CEP7 Spectrum Green) and a locus specific probe (*EGFR* LSI Spectrum Orange), and tumors were classified into six categories according to Cappuzzo's criteria (**Figure 1**).

Figure 1. Criteria for the six FISH categories according to Cappuzo et al. *J Natl Cancer Inst* 2005;97:643-55



Mutational study of exons 18, 19 and 21 *EGFR*, and exon 1 *KRAS* was conducted by PCR followed by bidirectional sequencing, using the ABI PRISM® BigDye Terminator v1.1 Cycle Sequencing Kit.

Statistical Analyses of *EGFR* expression, *EGFR* mutations and *EGFR* gene copy number were investigated for their association with clinicopathological features by Fisher's exact test and p-values were considered statistically significant when less than 0.05.

Figure 2. Large cell undifferentiated carcinoma (A: H&E x 200) showing adenocarcinoma type immunoprofile: TTF-1 nuclear positivity (B), CK7 expression (C) and P63 negative (D)

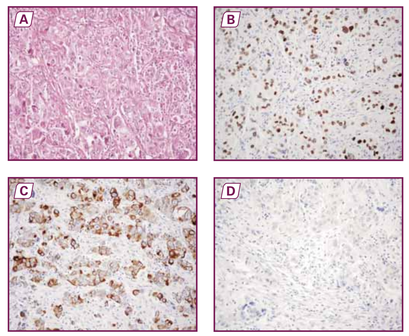


Figure 3. Large cell undifferentiated carcinoma (A: H&E x 200) showing squamous cell type immunoprofile: P63 nuclear positivity (B), CK5/6 expression (C) and TTF-1 negative (D)

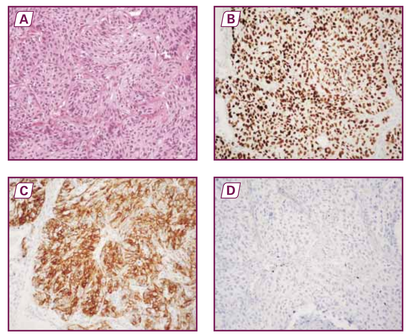


Figure 4. EGFR immunohistochemical positivity in NSCLC (clone 31G7 x 400)

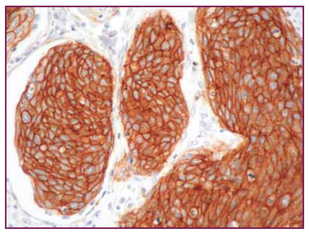


Figure 5. FISH-positive tumors exhibiting different patterns of gain in the *EGFR* gene copy number: (A) Amplification in tight gene clusters with ≥ 15 gene copies per tumoral nucleus (B) Amplification with a gene:chromosome ratio ≥ 2

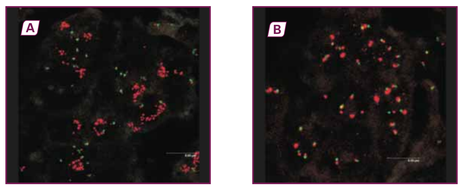
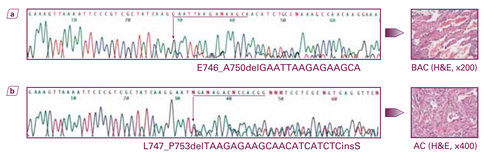


Figure 6. Exon 19 mutations of the *EGFR* gene found in the present study. (a) Bronchioloalveolar carcinoma with a 15 nt deletion; (b) Adenocarcinoma with 21 nt deletion



RESULTS

Immunophenotypic profile. Based on the results obtained with the panel of six antibodies applied in the different histologic categories (**Table 1, 2**), immunohistochemistry (IHC) profiles were defined as follows: squamous type when P63 positive and TTF-1 negative. The expression for CK5/6 and CK903 strengthen this profile. Glandular type when P63 negative and TTF-1 positive. The CK7 expression reinforced this profile. Undetermined and mixed type profiles were assigned in the cases having P63 and TTF-1 negative, and P63 and TTF-1 positive, respectively.
Table 3 illustrates the classification of our series based both in morphology and IHC patterns. Interestingly, 8 out of 10 tumors (80%) previously classified as LCUC were reassigned as AC (n=5) or SCC (n=3) according to their IHC profile (**Figures 2, 3**).

***EGFR* Expression.** IHC could be performed in 82 out of 84 specimens (98%). Sixty eight cases (83%) were found to be positive (**Figure 4**), while 24 were negative. Thirty three out of 68 positive cases corresponded to AC, and 35 to SCC. Neither histologic type (AC including BAC versus other types) nor gender significantly correlated with *EGFR* expression.

***EGFR* Gene Copy Number Status.** Seventy six out of 84 specimens (90%) could be assessed by FISH. Forty three cases (57%) were found to carry high *EGFR* copy number (ratio LSI/CEP7 ≥2 or gene copies ≥4 per tumoral nuclei). Of those, 9 cases (21%) exhibited amplification (**Figure 5**) while 34 (79%) exhibited high polysomy. Out of the 33 cases with low gene copy number, 12 (36%) had disomy, 6 (18%) had low trisomy, 8 (24%) had high trisomy, and the other 7 (21%) had low polysomy.
Increased *EGFR* copy number by FISH histologically corresponded to 23 AC and 20 SCC. Neither histologic type (AC including BAC versus other types) nor gender significantly correlated with *EGFR* gene copy number.

***EGFR* Mutational Status.** By sequencing, 76 out of 84 specimens (90%) could be evaluated for exons 18, 19 and 21. Among these cases, 3 mutations (4%) were detected corresponding to large *EGFR* exon 19 deletions previously described (2 cases with delE746-A750 and one case with delL747-P753insS). Mutational analysis of paired normal tissue (lymph nodes) was done to confirm somatic origin for these mutations. A germinal 2754C>T nucleotide substitution previously reported in exon 21 was detected in one case that did not trigger aminoacid change (R836).
Mutations were found in 2 AC (including one LCUC reassigned to AC) and 1 BAC, all cases from patients who carried high *EGFR* gene copy number (2 with high polysomy and one with amplification) (**Figure 6**). Histologic type (AC including BAC versus other types) not significantly correlated with *EGFR* mutations. The 3 mutations were detected in tumors from women, which implies that 30% of female patients in our series carried *EGFR* mutations. Thus, *EGFR* mutations tended to occur in women rather than men (Fisher's exact test; p=0.002).

***KRAS* Mutational Status.** Sequencing was carried out in 81of 84 specimens (96%). Among these cases, 8 mutations (10%) were detected corresponding to 5 G12C, 2 G12V, and one G13D. All of these cases were AC (including 2 LCUC reassigned to AC) without *EGFR* alterations. Seven patients carried high *EGFR* gene copy number, specifically high polysomy, and one with disomy. Neither of them showed *EGFR* mutations.

CONCLUSIONS

- Immunophenotypic profile and morphology together, enabled the classification as AC or SCC of the majority of cases (80%) which were previously considered as LCUC using morphological patterns alone.
- A panel including P63, TTF-1 and citokeratins (CK903, CK5/6, and CK7) is recommended in order to classify NSCLC, contributing to the accurate correlation between histologic type, molecular status and treatment response.
- The rate of *EGFR* mutation in our series of NSCLC is very low (4%), in agreement with previous results obtained from other Western populations.
- The type of *EGFR* mutations found in our series as well as its clinicopathologic context (histological type and gender) is also in agreement with previous published results obtained from similar series.
- In our series, FISH seems to be a more appropriate technique to find alterations in the *EGFR* status.

Table 1. IHC expression versus histologic diagnosis.

	Nº cases (Σ=90)	CK7	CK20	CK5/6	CK903	TTF-1	P63
AC *	40 *	**37 ***	0	2	3	**31 ***	2
SCC	38	9	2	**32**	**26**	1	**34**
LCUC	10	7	0	3	1	5	3
ASC	2	1	0	2	0	0	2

Results expressed refer to positive cases (more than 10% of tumoral cells positive).
*BAC included.

Table 2. Ability of different antibodies to distinguish between AC and SCC.

	CK7	CK903	CK5/6	TTF-1	P63
Accuracy	85	81	90	**87**	**92**
Specificity	76	93	95	**97**	**95**
Sensibility	93	68	84	**78**	**89**
Positive Predictive Value	80	90	94	**97**	**94**
Negative Predictive Value	91	76	90	**80**	**90**

Association criteria:
1. CK7 and TTF-1 expression attribute to AC.
2. CK903, CK5/6 and P63 expression attribute to SCC.
Results expressed in percentages.

Table 3. Histologic type versus IHC profile.

	AC*	SCC	LCUC	ASC	Total
Glandular type	31 *	0	5	0	36 *
Squamous type	1	33	3	2	39
Undetermined	8	4	2	0	14
Mixed type	0	1	0	0	1
Total	40 *	38	10	2	90

*BAC included.

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