

The potential role of extracellular regulatory kinase in the survival of patients with early stage adenocarcinoma

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Background: Lung cancer is among the most common types of neoplasias, and adenocarcinoma is the most frequent histological type. There is currently an extensive search for prognostic biomarkers of nonsmall cell lung cancer (NSCLC).

Methods: We analyzed the correlation of clinical data and patient survival with the levels of activated extracellular regulatory kinase (ERK) in histological samples of surgically resected early stage lung adenocarcinoma. We randomly selected 36 patients with stage I or II lung adenocarcinoma who underwent pulmonary lobectomy between 1998 and 2004. Patients were divided into the following two groups according to immunohistochemical profile: a group with <15% ERK-positive tumor cells and a group with ≥15% ERK-positive tumor cells. For data comparison, an enrichment analysis of a microarray database was performed (GSE29016, n=72).

Results: Activated ERK levels were ≥15% and <15% in 21 (58%) and 15 (42%) patients, respectively. There were no statistically significant differences in age, sex, smoking history, and body mass index (BMI) among the groups stratified by ERK levels. The survival rate was lower in the ERK ≥15% group than in the ERK <15% group (P=0.045). Enrichment analyses showed no correlation between variations in gene expression of ERK in patients with adenocarcinoma and survival rates in patients with stage I and combined stage II + III disease.

Conclusions: Our findings suggest that high ERK positivity in cells from biological samples of lung adenocarcinoma is related with tumor aggressiveness and a poorer prognosis.

Keywords: Lung cancer; adenocarcinoma; extracellular regulatory kinase (ERK); survival

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Introduction

Lung cancer is among the most common types of neoplasms and is the leading cause of death in the United States and the second major cause of death in Brazil (1-3). Unfortunately, the disease is usually advanced at the time of diagnosis: thus, precluding surgical treatment and restricting patients

to chemotherapy and/or radiation therapy with a minimal chance of cure (4). Due to this aggressive behavior of lung cancer, it is necessary to identify molecules, proteins, or signaling pathways related to tumor growth, which have an influence on the outcome. The study of these prognostic factors could stimulate the development of new potential

therapies targeting specific molecules (5).

A persistent search for prognostic biomarkers of nonsmall cell lung cancer (NSCLC) is currently ongoing (5). In this context, the epithelial growth factor receptor epidermal growth factor receptor (EGFR)-dependent RAS/RAF/MEK/ERK signaling cascade is under intensive investigation to identify new prognostic factors of lung cancer because they regulate signals for cell growth and survival and are involved in cell cycle regulation, angiogenesis, cell proliferation, and migration (6-8). A third of all forms of cancers are associated with enhanced activity of this cascade (9). For this reason, many EGFR (10,11), RAS, RAF, and MEK inhibitors have been developed as potential blockers of the activity or proliferation of different components of the extracellular signal-regulated kinase (ERK) signaling pathway (12-14). Therefore, the potential of overactive RAS, RAF, MEK, or ERK as prognostic factors for lung adenocarcinoma has become an interesting area of research.

Most of the studies searching for prognostic factors on NSCLC encompass all its types but do not discriminate in a specific cell type or samples from a particular stage, which makes it difficult to draw more detailed conclusions. Thus, it is essential to have a more specific population with minimal confusing factors to study potential prognostic factors in NSCLC. Based on these concerns, we selected only those patients who had been surgically treated for early lung adenocarcinoma to study the relationship of ERK and prognosis in this particular population.

We hypothesized that increased levels of activated ERK in patients with lung adenocarcinoma were associated with a poorer prognosis or decreased patient survival. Therefore, we conducted an immunohistochemical study using tumor samples from patients surgically treated for lung adenocarcinoma and performed a microarray analysis of experiments from different databases that included a population having a profile similar to that of our samples.

Materials and methods

We selected patients with lung adenocarcinoma who underwent pulmonary resection between 1998 and 2004 and were included in a previous study by Sánchez *et al.* (15). The present work was approved by the local Research Ethics Committee under protocol number 1852/08.

Surgical staging was determined according to the TNM classification system (16,17). All data related to preoperative evaluation, surgical techniques, and postoperative results are

described in the article by Sánchez *et al.* (15). We randomly selected 36 patients with postoperative stage I or II disease and with available survival data and immunohistochemical samples.

Analysis of gene expression

Gene expression analyses were performed using microarray data from five different studies (18-22). However, we present data from a single study by Staaf *et al.*, a database that included 39 adenocarcinoma patients with 72 available patients (GSE29016, Illumina HT-12 V3.0) (18). GSE29016 was retrieved from the gene expression database Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>), considering it was the only experiment that included all parameters analyzed in our study. To perform gene expression enrichment analysis, Gene Set Enrichment Analysis (GSEA) software, which requires a gene set, the parameters to be analyzed, and gene expression data for data processing was used. We used the Kyoto Encyclopedia of Genes and Genomes (KEGG) to obtain the gene set to be analyzed. Subsequently, we employed the online tool string to confirm interactions among the 261 genes of the group. The following parameters were analyzed: smoking status (8 nonsmokers and 21 smokers), TNM stage (30 with stage I and 9 with combined stage II + III disease, respectively), and sex (18 males and 21 females).

After the enrichment analysis was performed using GSEA as described by Mootha *et al.* (23), a survival curve for each of the parameters versus gene expression was plotted using GraphPad Prism 5.

Samples

Tissue samples for histopathological studies were obtained from surgical specimens of primary adenocarcinoma lesions from patients with surgical stage I or II. All samples were fixed in 10% buffered formalin and embedded in paraffin tissue blocks, which were then processed for immunohistochemical analyses. A tissue section was stained with hematoxylin and eosin (H&E) and analyzed by a pathologist to confirm the presence of adenocarcinoma in the sample.

Immunohistochemistry

Tissue sections measuring 4 cm in thickness were prepared on silanized slides. According to a previously standardized

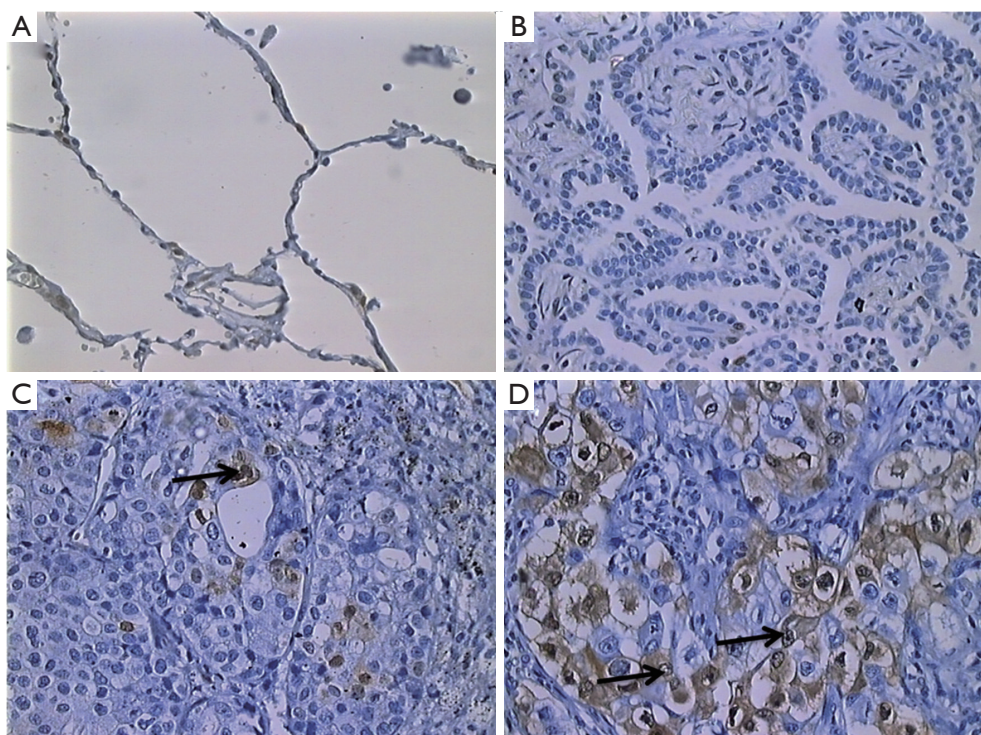


Figure 1 Immunohistochemical reaction for ERK, extracellular regulatory kinase detection in normal lung tissue. (A) Lung adenocarcinoma without ERK expression; (B) positive cases with low ERK expression; (C) high ERK expression; (D) nuclear and cytoplasmic staining is indicated by arrows. ERK, extracellular regulatory kinase.

protocol, the following procedures were performed after deparaffinization and rehydration: heat-mediated antigen retrieval with sodium citrate buffer, blocking of endogenous peroxidase in a 5% H₂O₂ solution in methanol, and blocking of nonspecific binding in 1% bovine serum albumin (BSA) solution. Subsequently, the slides were incubated overnight at 4 °C with rabbit polyclonal antibody specific for the double phosphorylated form of ERK1/2 (Thr202/Tyr204; Cell Signaling, Beverly, MA, USA) diluted in a 1:300 ratio in 1% BSA. Plate washing, incubation, and color reaction were performed using the HRP-labeled conjugated polymer kit (Invitrogen®). Sections were then counterstained with hematoxylin. Negative controls were obtained using the same protocol, but without the primary antibody.

Analysis of immunohistochemical reactions

Immunohistochemical analyses were performed by counting ERK-positive cells/1,000 cells in consecutive microscopic fields, taking into account only tumor cells. Cell count was performed by two independent observers. A maximum

interobserver discrepancy of 30% was considered or a third observer was included. Cell counting was performed using a Zeiss® Imager microscope coupled to the Image Pro Plus® 6.1 software. For statistical analysis, patients were divided into the following two groups based on a previous study: a group with <15% ERK-positive tumor cells and a group with ≥15% ERK-positive tumor cells (24) (*Figure 1*).

Statistical analyses

Mean values were compared using Student's *t*-test, whereas categorical variables were compared using Fisher's exact test. In addition, for survival analyses, we used Kaplan-Meier curves and Cox regression analyses in univariate and multivariate survival platforms. Statistical data were processed and analyzed using SPSS software, version 18.0 (IBM, USA, Chicago, 2009).

Results

Activated ERK levels were ≥15% and <15% in 21 (58%)

Table 1 Characteristics of lung adenocarcinoma patients according to positivity for ERK, extracellular regulatory kinase in tumor cells

Characteristics	Cellular positivity for ERK [%]			P
	Total n=36 [100]	<15 n=15 [42]	≥15 n=21 [58]	
Age (in years)	64±8	63±8	65±9	0.37 ^a
Males, number [%]	22 [61]	8 [53]	14 [67]	0.50 ^b
Staging, number [%]				0.171 ^b
I A	7 [19]	2 [13]	5 [24]	
I B	16 [44]	10 [67]	6 [29]	
II A	6 [17]	1 [7]	5 [24]	
II B	7 [19]	2 [13]	5 [24]	
Smokers, number [%]	26 [72]	9 [60]	17 [81]	0.26 ^b
FEV1, %	82±22	77±24	87±19	0.174 ^a
BMI, kg/m ²	25.5±5.5	25.3±5.5	25.6±5.6	0.84 ^a

Data are presented as means ± standard deviation or cell count (percentage). FEV1%, forced expiratory volume in the first second; BMI, body mass index; P, statistical significance; ^a, Student's *t*-test; ^b, Fisher's exact test.

Table 2 Selected characteristics and their relation with survival in patients with lung adenocarcinoma

Characteristic	N	Event number [%]	Multivariate analysis	
			HR (95% CI)	P
ERK, per/1,000 cells				
≥15	21	11 [52]	4.2 (1.0-17.1)	0.045
<15	15	3 [20]	1.0	
Age (in years)	36	14 [39]	1.04 (0.97-1.12)	0.32
Sex				
Male	22	9 [41]	1.0	0.90
Female	14	5 [36]	1.1 (0.3-3.9)	
BMI, kg/m ²	36	14 [39]	0.91 (0.81-1.03)	0.139
FEV1%	36	14 [39]	0.99 (0.96-1.02)	0.324
Smoking history				
Present	26	10 [39]	0.4 (0.1-1.5)	0.40
Absent	10	4 [40]	1.0	

ERK, extracellular regulatory kinase.

and 15 (43%) patients, respectively (*Figure 1*). There were no statistically significant differences in age, sex, smoking history, and body mass index (BMI) between the groups stratified by ERK (*Table 1*). When survival was compared between the ERK ≥15% and ERK <15% groups, the former group showed lower survival than the latter during the study period (*Table 2*). We also performed statistical analyses using a cutoff threshold above and below 45% ERK positivity, and the results were very similar to those obtained when the cutoff threshold of above and below

15% were used.

By using the multivariate Cox model after adjusting for age, sex, BMI, forced expiratory volume, and smoking history, the difference in survival between the ERK ≥15% and <15% groups was not only maintained but also statistically significant (P=0.045). This difference is shown graphically in *Figure 2*.

Enrichment analysis showed no correlation of variations in ERK gene expression in the 39 adenocarcinoma patients with stage I and combined stage II + III disease. Likewise,

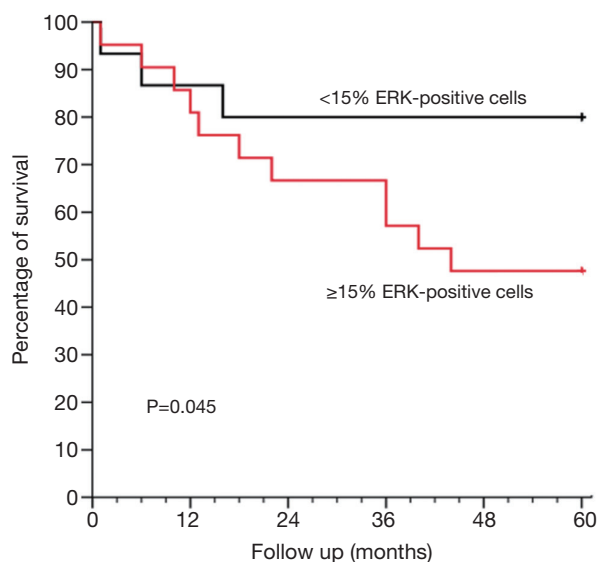


Figure 2 Kaplan-Meier mortality curves show that ERK, extracellular regulatory kinase quantification allows for discrimination between good and poor prognosis. The black line represents cases with <15% ERK-positive cells and the red line indicates cases with $\geq 15\%$ ERK-positive cells. The gray line represents the cumulative survival for all patients throughout the 5-year evaluation period. ERK, extracellular regulatory kinase.

gender and smoking did not show a positive correlation with variations in ERK gene expression.

Discussion

Cancer is the result of the deregulation of multiple signaling pathways, and the inhibition of a single pathway may not be sufficient to trigger apoptosis or growth inhibition (6,25-27). ERK activation is involved in multiple cellular functions such as motility, proliferation, differentiation, and apoptosis (28,29). ERK activation influences the development and activation of normal cells as well as the abnormal proliferation of tumor cells (30,31). There is little evidence on the specific role of ERK activation in the development of NSCLC (24,32). By studying 111 patients with NSCLC, Vicent *et al.* demonstrated a positive correlation between ERK activation and later stages of the disease (24). Our study only examined patients with early stage adenocarcinoma who underwent surgical treatment: thus, constituting a population different from that described by Vicent *et al.* (24), which included patients with advanced stages and different histological types of lung

carcinoma. Moreover, Vicent *et al.* separately analyzed ERK activation in the cytoplasm and nucleus and showed that there were no significant differences between these cell compartments (24). Therefore, we decided to collectively analyze these parameters in our study. However, Harding *et al.* suggested that receptor location, either in the plasma membrane or cytosol, may lead to different responses in the MAPK cascade activation (33). This finding is of great importance in the search for new therapies with ERK, and not MEK, as a target (34). It is believed that signal transduction is stronger in the plasma membrane and weaker in the cytosol (35). Therefore, activation of plasma membrane receptors would trigger a stronger cellular response, whereas ERK activation in the cytoplasm would affect only a subpopulation of cells, leading to an intermediate response (35). For this reason, future therapies would need to address differences in ERK cascade activation on the basis of receptor location. Furthermore, overexpression of this pathway may play an important role in the pathogenesis and progression of breast cancer and other cancers, making their components potential therapeutic targets (36-38).

We performed enrichment analysis using data from five different experiments (18-22), but because they yielded similar results, the dataset GSE29016 was selected because it was the only dataset containing all our study parameters. Our enrichment analysis had some limitations, in particular with regard to the number of patients diagnosed with adenocarcinoma. Only 39 patients were selected, and most of them were in stage I as verified in our dataset. Moreover, this experiment included few nonsmokers, which is expected in this disease group. Finally, our dataset contained more male patients, as opposed to the female predominance in the other datasets.

Our study demonstrated that high ERK levels are inversely correlated with survival. However, this was not revealed by the enrichment analysis, probably because of the sample type assayed (mRNA *vs.* protein) as well as the different patient profiles. In the literature, the presence of high ERK levels has been studied mainly in association with tumor aggressiveness and as potential therapeutic targets, and survival analyses have been restricted to only a few studies (39).

This study has several limitations that should be considered before interpreting the results. Our study included a small number of patients; however, they formed a specific group of individuals who did not show advanced stage disease and underwent surgical treatment. This

also explains the limited number of patients with stage II disease. However, this makes the study population more homogeneous and helps in avoiding bias associated with the inclusion of more patients with an advanced stages disease, which tend to show higher ERK expression.

Conclusions

Taken together, our results suggest that higher ERK positivity in cells from biological samples of patients with lung adenocarcinoma patients is associated with tumor aggressiveness and poorer prognosis. Despite its limitations, this study demonstrates the importance of the role of ERK in lung adenocarcinomas and reinforces the need for additional studies to confirm our findings.

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Authors' contribution: S.L.M. conceived the study, collected the data, participated in the analysis of the samples and drafted the manuscript. C.B.M, R.T.M, M.C.F, R.M. collected the data, participated in the analysis of the samples and drafted the manuscript. M.B.S. participated in the microarray analysis. F.K, C.F.A. conceived the study, drafted and approved the manuscript final version.

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