

Expression of ABC transporters, p53, Bax, Bcl-2 in an archival sample of non-small cell lung cancer bearing a deletion in the EGFR gene

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Abstract. EGFR mutations have been correlated to responsiveness to treatment with tyrosine kinase inhibitors. These drugs are themselves substrates for ABC transporters. In the present work we describe the immunohistochemical profile of an archival sample from a male Brazilian patient with no Asian ancestry and never smoker, diagnosed with non-small cell lung cancer. This tumor was found to contain an in-frame hemi- or homozygous deletion, E746-A750 in exon 19 of the EGFR gene. Immunohistochemistry revealed a relatively weak staining for the ABC transporter subfamily ABCC1 and strongly for ABCB1. The cytoplasm stained positively for Bax and the nucleus stained for p53, but was negative for Bcl-2. Antibody against acetylated lysine revealed staining in both, cytoplasm and nucleus of tumor cells in contrast to normal cells which were essentially negative. The overall immunohistochemistry pattern obtained for this sample indicates that the del E746-A750 mutation may have down-regulated the expression of ABCC1. The results also suggest that the NSCLC analyzed displayed a transcriptionally active chromatin as judged by the results obtained with the anti-acetylated lysine antibody.

Introduction

Lung cancer is the most prevalent of all cancers and has a very poor clinical outcome. The two main groups of lung cancer, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) present intrinsic and acquired resistance to chemotherapy, a feature that stems from the activity of multidrug resistance proteins that effectively act as pumps increasing the efflux of drugs from the tumor cells. These transporters are part of a family of proteins characterized by the presence of the ATP binding cassette (ABC), such as ABCC1 (multidrug resistance related protein 1 or MRP1), as well as ABCB1 (P glycoprotein or Pgp) and ABCG2 (breast cancer resistance protein or BCRP) (1). Some of the recent therapeutic strategies for the treatment of NSCLC included the use of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), such as Iressa® (AstraZeneca). However, this drug proved to be more effective only in a small percentage of certain populations, namely of Asian descent, bearing a deletion in exon 19, E746-A750, corresponding to the amino acid residues ELREA (2). This in-frame deletion is clustered around the catalytic site of the tyrosine kinase domain of the EGFR receptor. The resistance to treatment in other populations with NSCLC, or even in the originally susceptible group results from the appearance of secondary mutations in the EGFR gene. These mutations prevent drug binding to EGFR. In addition, drug resistance in these solid tumors is connected to their extrusion by ABC transporters ABCB1 and ABCG2 (3,4). In spite of the clear correlation between drug resistance and ABCB1 expression for certain types of cancer, this situation is not so obvious with solid tumors such as those in NSCLC. Such a phenotypic marker would be desirable as an indicator of patient prognosis and establishment of therapeutic strategies.

To our knowledge there is no information on EGFR mutations in Brazilian patients with NSCLC. In the present study 70 paraffin-embedded biopsies of patients with NSCLC were examined. Of these, 64 were analyzed for the occurrence

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of mutations in exon 19 of the EGFR gene. In this population one NSCLC tumor biopsy bearing the E746-A750 deletion was found. Thus, it became relevant to enquire about the effect that such mutation may have had on the expression of some of the EGFR downstream signaling cascade. Among the different effectors involved, transporters participating in the resistance process and the general profile of genes that promote the DNA damage response typical of transformed cells, that is, key elements that mediate the intrinsic pathway of apoptosis were obvious choices. Accordingly, the expression of major classes of regulators of apoptosis such as p53, Bcl-2 and Bax were investigated in the EGFR mutated archival sample. In addition, as an attempt to verify whether the signaling pathway would extend to epigenetic modifications involving acetylation-dependent chromatin remodeling, the general pattern of protein acetylation of the tumor cells was also studied.

Materials and methods

Patients. A retrospective survey was conducted with a local population consisting of patients attending the Federal University of Rio de Janeiro Hospital Medical School diagnosed with NSCLC. The protocol was approved by the Ethics in Research Committee of the Clementino Fraga Filho University Hospital of the Federal University of Rio de Janeiro.

Histology. Samples of normal or tumor pulmonary tissue were obtained surgically or from biopsies. These samples were fixed in 10% formaldehyde solution, dehydrated and placed in paraffin for the histological analysis. Formalin-fixed lung 5- μ m-thick sections were stained with haematoxylin and eosin for the evaluation of tumor type. All histological analyses were performed by two independent observers. For the immunohistochemical study slides with 5- μ m-thick sections were prepared and were mounted on poly-L-lysine coated slides. For mutation analysis the paraffin-embedded tissues from the biopsies were cut in 10- μ m-thick sections which were subsequently deparaffinized with xylene.

Analysis of mutations. The paraffin-embedded tissue from biopsies or surgical samples was cut in 10- μ m-thick sections which were subsequently deparaffinized with xylene. DNA extraction was carried out using the DNeasy tissue kit (Qiagen), according to the manufacturer's recommendations. The extracted DNA was then amplified by PCR. Primer sequences for exon 19 (an exon within the tyrosine kinase domain that revealed a mutation) were: forward: 5'-GGTGC ATCGCTGGTAACATCC-3' and reverse: 5'-TGGGCC TGAGGTTTCAGA-3'. Amplification was performed with 35 cycles using an annealing temperature of 55°C. The amplified products were purified using GFX™ PCR DNA (GE Healthcare). DNA sequencing was performed using the dideoxy dye terminator cycle sequencing method. All DNA sequences were carried out in both, sense and antisense chains. The DNA sequence of the mutation was confirmed by analysis of an independent DNA extraction. Alignment of the deduced amino acid sequence was generated by using Vector NTI software (Invitrogen).

Immunohistochemistry. For this set of experiments the indirect immunoperoxidase technique was used. Only 16 samples, including the one with the mutation, were analyzed with all antibodies. Immunohistochemical staining was carried out using the following primary human monoclonal antibodies: anti-EGFR (Santa-Cruz Biotechnology), anti-Acetylated Lysine (Cell Signalling Tech., USA) anti-MRP, anti-Pgp, anti-Bcl-2, anti-Bax, anti-P53 (Dako A/S, Glostrup, Denmark). Briefly, frozen sections were immersed in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. After being rinsed in phosphate-buffered saline (PBS) containing 0.5% Tween-20 for 10 min, tissue sections were incubated with non-immune horse serum for 30 min and, subsequently, with the respective monoclonal antibody in a humidified chamber overnight, at room temperature. Two sections from each sample were incubated with either PBS alone or mouse monoclonal IgG1 (concentration-matched) (Dako A/S) and served as negative controls. After being rinsed in PBS for 10 min, all tissue sections were incubated for 30 min with a goat anti-mouse peroxidase conjugate (1:200) (Zymed Laboratories, Inc., San Francisco, CA, USA). After being rinsed in PBS, all sections were developed with a solution containing hydrogen peroxide and diaminobenzidine. Preparations were lightly counterstained in Harris's haematoxylin, dehydrated and mounted in Permunt (Fisher Scientific, Pittsburgh, PA, USA).

Results

EGFR mutations. Among the archival samples analyzed at exon 19, one referred to as patient 02 revealed the deletion ELREA. Patient 02 was a 44-year-old, male, Caucasian, with no record of Asian ancestry, a non-smoker who presented with progressive lumbar pain with bilateral lower limbs radiation and weakness prior to admission. Magnetic resonance imaging disclosed a 5-cm paravertebral mass at L4-L5 growing through the intervertebral foramina. Chest radiograph revealed a 4-cm right upper lobe mass. Bronchofiberscopy did not show endoscopic lesion and the bronchoalveolar lavage was negative for malignant cells and microorganisms. Decompression and biopsy of L5 was carried out and non-small cell metastatic carcinoma was diagnosed. The patient was treated with local radiotherapy 30 Gy and chemotherapy consisting of etoposide-platinum 4 cycles as first line and gemcitabine 2 cycles as second line. This produced relief of symptoms and a partial radiographic response.

DNA sequence analysis revealed a deletion comprising amino acids ELREA at positions E746-A750. This is shown in Fig. 1A, in which the tumor biopsy and the wild-type sequences are compared. Inspection of Fig. 1B highlights the absence of the wild-type sequence within the region encompassing the deletion. This result was consistent with either a hemi- or a homozygous del E746-A750 in the tumor biopsy.

Immunohistochemistry of this sample with anti-EGFR revealed a strong reaction, mainly located on the cell membranes as shown in Fig. 2D. This result is consistent with the over-expression of EGFR reported for several types of tumors, especially NSCLC (5).

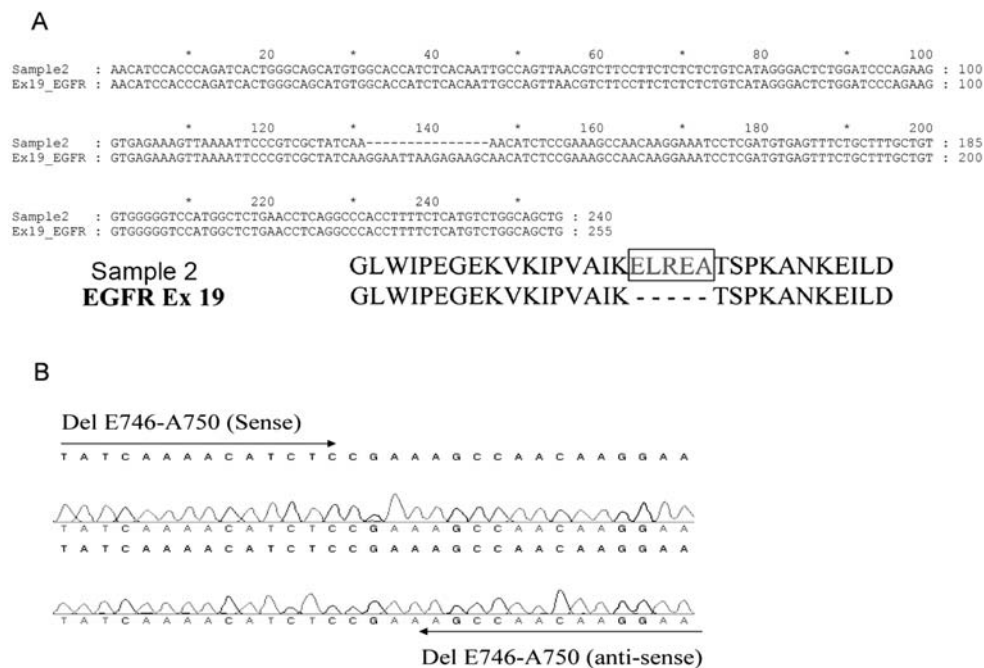


Figure 1. Nucleotide and amino acid sequence of the exon 19 region containing the ELREA deletion. (A) Alignment of nucleotide sequences of exon 19 of patient 2 and the wild-type sequence. The gap shown in the sequence for patient 2 corresponds to the deletion. The deduced amino acid sequence corresponding to the deletion is also shown. (B) Electrophoretogram that generated the sequences in (A). The diagram shows that the nucleotide sequences corresponding to the deletion (wild-type) are absent in both chains.

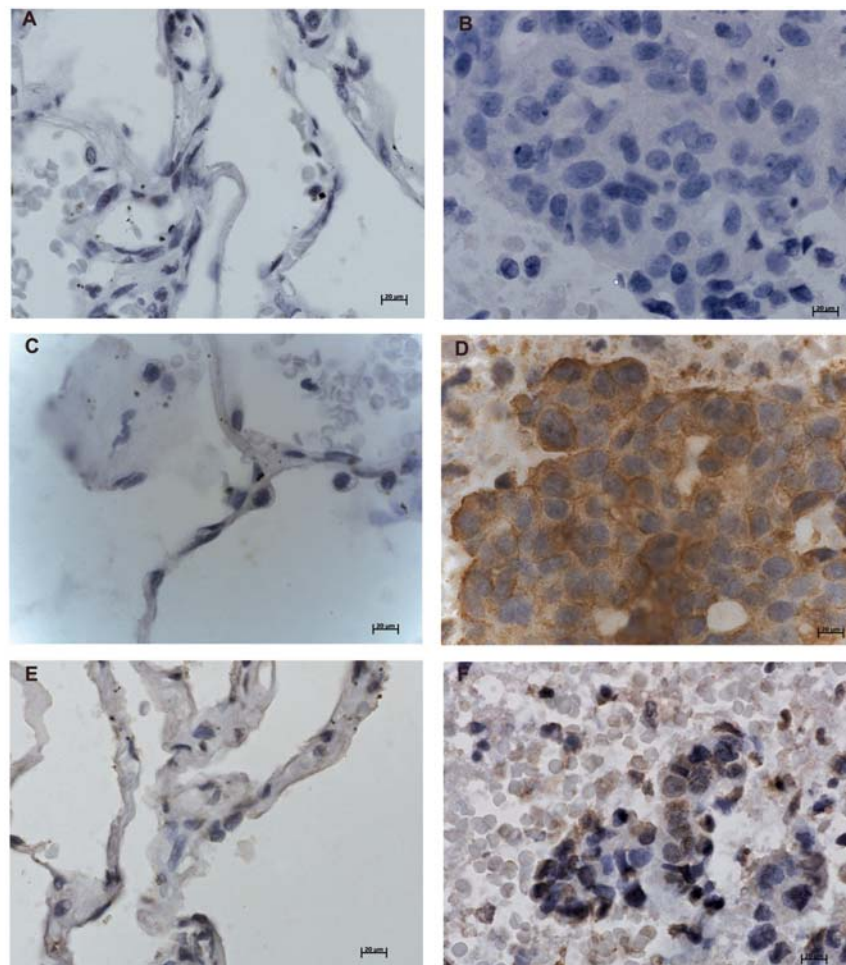


Figure 2. Immunohistochemical staining of EGFR and histone acetylation expression. (A) Normal lung negative control. (B) Adenocarcinoma negative control. (C) Absence of EGFR expression in normal lung. (D) EGFR expression on the tumor cell membrane. (E) Faint expression of acetylated lysine in the normal lung. (F) Acetylated lysine expression in the nucleus and faintly in the cytoplasm of tumor cells.

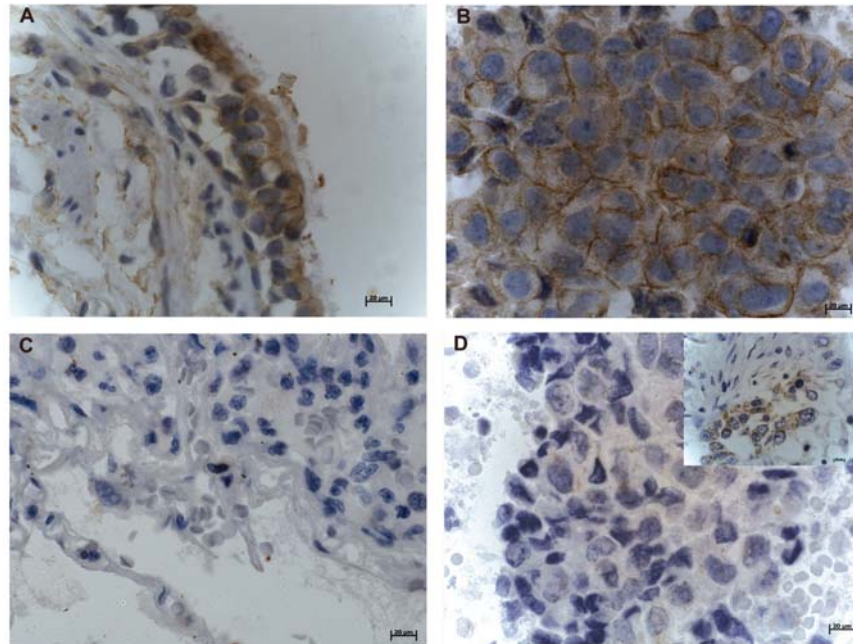


Figure 3. Immunohistochemical staining of ATP-binding cassette proteins in human lung adenocarcinoma. (A) ABCB1 expression in normal lung on endothelial cells, pneumocytes and alveolar cells. (B) ABCB1 expression in tumor cells containing the mutation displaying strong positivity on the cell membrane. (C) Absence of ABCC1 expression in normal lung. (D) Absence of ABCC1 expression in tumor cells containing the mutation. For comparison an inset with another adenocarcinoma positive for ABCC1 has been added.

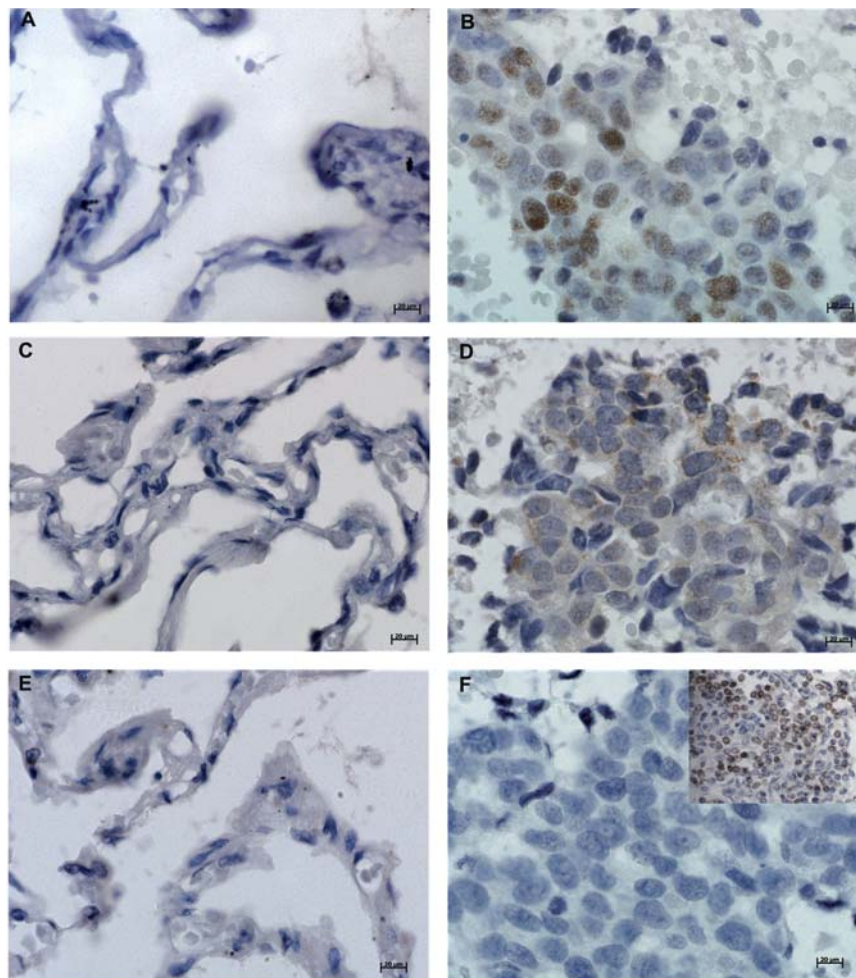


Figure 4. Immunohistochemical staining of proteins involved in the apoptotic process in human lung adenocarcinoma. (A) Absence of p53 expression in normal lung. (B) p53 expression in tumor cells containing the mutation displaying positive nuclear staining. (C) Absence of Bax expression in normal lung. (D) Bax expression on the tumor cell showed a faint cytoplasmic positivity. (E) Absence of Bcl-2 expression in normal lung. (F) Absence of Bcl-2 expression in tumor cells containing the mutation. For comparison an inset with a lymphocyte inflammatory infiltrate of another adenocarcinoma positive for Bcl-2 has been added.



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acetylation. Acetylated lysine could be clearly in the nucleus and faintly in the cytoplasm of the biopsy containing the mutation (Fig. 2F). In contrast, normal lung tissue displayed only residual staining (Fig. 2E). This result showed that there seems to be a definite connection between activation of EGFR and protein acetylation, presumably histones. The occurrence of acetylated histones would be consistent with a transcriptionally active chromatin. However, the question whether the mutation in the EGFR gene affected this pattern remains to be investigated.

ABC transporters expression. ABCB1 could be detected in the biopsy with the homozygous deletion. ABCB1 expression was also studied in other adenocarcinomas samples not containing the mutation and in the normal lung. As shown in Fig. 3, ABCB1 was present in the normal lung on endothelial cells, pneumocytes and alveolar cells (Fig. 3A). In the tumor biopsy presenting the mutation, tumor cells displayed a strong positivity for ABCB1 on the cell membrane (Fig. 3B). Sixteen other adenocarcinomas were analyzed for comparison and 14 were ABCB1 positive.

The ABCC1 transporter, on the other hand, could not be detected in the normal lung or tumor biopsy containing the mutation (Fig. 3C and D). For comparison an inset with the biopsy of another adenocarcinoma positive for ABCC1 has been added. Only 4 out of other 16 adenocarcinomas analyzed were positive for ABCC1.

Proteins involved in the apoptotic process. The normal lung was negative for p53, Bax or Bcl-2 (Fig. 4). The neoplastic cells from the biopsy containing the mutation, on the other hand, showed a faint cytoplasmic positivity for Bax and a positive nuclear staining for p53. No positivity for Bcl-2 was observed. For comparison an inset of an inflammatory infiltrate positive for Bcl-2 has been added. Among the 16 adenocarcinomas used for comparison 14 were positive for Bax, 10 for p53 and only one for Bcl-2.

Discussion

Because of the relatively high prevalence of EGFR mutations in east Asians diagnosed with NSCLC, it has been suggested that ethnicity and geographical location may both influence the occurrence of mutations in the EGFR gene and hence, the establishment of NSCLC. However, the results of a recent multi-centre study supported a genetic, rather than geographic factor, as a determinant of NSCLC (6). In order to contribute to the ongoing discussion within the context of genetic versus geographical influence, a retrospective survey was conducted with Brazilian patients diagnosed with NSCLC. Only one biopsy, showed a mutation at exon 19 of the EGFR gene. This result is somewhat different from other studies that have shown that ~10-20% of NSCLC patients display a mutation in exon 19 of the EGFR gene (7-9). Whether this low prevalence (~1.5%) is characteristic of the Brazilian population will require further confirmation.

The present report, the first one involving a Brazilian population, has evidenced the deletion ELREA in exon 19 of EGFR gene. This deletion is normally prevalent in patients with pulmonary adenocarcinoma, who tend to belong to a

population of never-smokers, females and of east-Asian ethnic origin (6,10,11). In the present case, however, the patient was male and Caucasian, which in itself underscores an interesting population peculiarity. In addition, the results showed that a concomitant EGFR wild-type sequence did not occur within the deletion tract. This could be interpreted as amplification of the deleted allele from a single copy gene, i.e., cells may have been reduced to hemizygosity. Alternatively, a statistically significant increase in the frequency of homozygosity of the allele considered may have occurred. Again this is atypical since most somatic EGFR mutations have been reported to be heterozygous (12). Notwithstanding, it is known that as a result of genome instability in NSCLC, there are widespread numerical and structural changes in the chromosomes. The main changes include losses of chromosomes 9 and 13 and gain of chromosome 7, where the EGFR gene is located. The chromosome arms most frequently involved in gains are 7p and 7q, a fact that supports an increased copy number of EGFR. Indeed, trisomy of chromosome 7 has been proposed to be one of the earliest changes in NSCLC, those having been found in premalignant lung tissue of patients (13). By taking into account the relatively high probability of trisomy of chromosome 7 in the biopsy cells or gene gain, it is conceivable that Del E746-A750 observed consisted of a homozygous mutation.

In the present study, neoplastic cells with the EGFR mutation depicted a strong positivity for ABCB1 at the cell membrane. ABCB1 positivity in adenocarcinomas has been observed in 87% of our specimens. Other authors (14-16) also described the expression of ABCB1 in adenocarcinomas, and it has been suggested that ABCB1 expression might be related to higher grading (17) and to smoking habits (18). However, the patient bearing the adenocarcinoma with the mutation was a non-smoker and the high positivity to ABCB1 might be due to the advanced stage.

The expression of ABCC1 has been described in NSCLC (19). No positivity was seen for ABCC1 in the tumor presenting the EGFR mutation despite being present in four other adenocarcinomas not bearing the mutation. Other authors (15) have also found that ABCC1 is expressed less often in adenocarcinomas and, it has also been reported, that ABCC1 levels are highest in TNM stage I and decline with advanced stage (17), the biopsy under study was a metastasis and this could also explain the absence of ABCC1 expression.

The possibility existed that the mutation induced other differences as a result of epigenetic mechanisms. For example, there could be a modulation of pro- and anti-apoptotic proteins making these cells more susceptible to the apoptotic process and to the effect of tyrosine kinase inhibitors. Other authors observed positivity for p53 in around 60% of lung cancers (20) and the present case was positive for p53 as well as 75% other adenocarcinoma specimens studied by us. Similarly, the positive, albeit weak, expression of the pro-apoptotic protein Bax and the negative expression of the anti-apoptotic protein Bcl-2 were in accordance to that previously reported for adenocarcinomas, where the expression of Bax has been reported and the frequency of Bcl-2 is low (20,21).

In conclusion, the present study analyzed whether mutations in the EGFR could be detected retrospectively

among a sample of Brazilian patients suffering from lung cancer, and if this mutation could, indirectly, modify proteins involved with the apoptotic process or with the permanence of chemotherapeutic inside the neoplastic cell, therefore affecting its efficacy. Among the specimens of NSCLC, it was possible to describe one mutation, comprising a deletion in exon 19, del E746-A750 in the EGFR gene, that was homozygous, rather than hemi- or heterozygous. At least in the case under study, none of the parameters observed (ABCB1, ABCC1, p53, Bax, Bcl-2) markedly differed from other adenocarcinomas.

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