

Spectrum of somatic *EGFR*, *KRAS*, *BRAF*, *PTEN* mutations and *TTF-1* expression in Brazilian lung cancer patients

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Summary

Lung cancer is the leading global cause of cancer-related mortality. Inter-individual variability in treatment response and prognosis has been associated with genetic polymorphisms in specific genes: *EGFR*, *KRAS*, *BRAF*, *PTEN* and *TTF-1*. Somatic mutations in *EGFR* and *KRAS* genes are reported at rates of 15–40% in non-small cell lung cancer (NSCLC) in ethnically diverse populations. *BRAF* and *PTEN* are commonly mutated genes in various cancer types, including NSCLC, with *PTEN* mutations exerting an effect on the therapeutic response of *EGFR/AKT/PI3K* pathway inhibitors. TTF-1 is expressed in approximately 80% of lung adenocarcinomas and its positivity correlates with higher prevalence of *EGFR* mutation in this cancer type. To determine molecular markers for lung cancer in Brazilian patients, the rate of the predominant *EGFR*, *KRAS*, *BRAF* and *PTEN* mutations, as well as TTF-1 expression, was assessed in 88 Brazilian NSCLC patients. *EGFR* exon 19 deletions (del746–750) were detected in 3/88 (3.4%) patients. Activating *KRAS* mutations in codons 12 and 61 were noted in five (5.7%) and two (2.3%) patients, respectively. None of the common somatic mutations were detected in either the *BRAF* or *PTEN* genes. TTF-1 was overexpressed in 40.7% of squamous-cell carcinoma (SCC). Our findings add to a growing body of data that highlights the genetic heterogeneity of the abnormal *EGFR* pathway in lung cancer among ethnically diverse populations.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, stemming in part from the lack of effective early detection schemes impacting survival (Kadara *et al.*, 2011). There is an urgent need for novel biomarkers that could be clinically applied as prognostic factors and somatic mutations are obvious candidates for being used as prognostic markers (Li *et al.*, 2013; Travis *et al.*, 2013).

Epidermal growth factor receptor (EGFR) plays an important role in cell proliferation and survival

(Reungwetwattana *et al.*, 2012). The frequency of activating mutations of the *EGFR* gene in non-small cell lung cancer (NSCLC) varies according to ethnicity and is noted in 15% of NSCLC diagnosed in Caucasians, 40% in Asians and 33.3% in Latin Americans mostly of Spanish origin (Paez *et al.*, 2004; Leidner *et al.*, 2009; Rosell *et al.*, 2009; Arrieta *et al.*, 2011; Cote *et al.*, 2011).

Activating *KRAS* mutations that predominantly cluster to either codons 12 and 13, and rarely in codon 61 (Suda *et al.*, 2010) are encountered with differing rates in NSCLC diagnosed in different ethnic groups: 30% in Caucasians, 10% in East Asians and 16.6% in Latin Americans (Hunt *et al.*, 2002; Riely *et al.*, 2008; Arrieta *et al.*, 2011). Mutations in *KRAS* and *EGFR* that appear to be mutually exclusive are currently being used as molecular biomarkers

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for determining both prognosis and therapeutic targets in NSCLC (Murray *et al.*, 2006; Imer *et al.*, 2007; Mok *et al.*, 2009; Brevet *et al.*, 2011; Heigener & Reck, 2011). Tyrosine kinase inhibitors (TKIs) are widely used as an adjuvant treatment to chemotherapy in advanced stage NSCLC cases that specifically display activating *EGFR* mutations (Keedy *et al.*, 2011). NSCLC harbouring activating *KRAS* mutations, are resistant to *EGFR*-TKIs treatment and patients have shorter survival and response rates (Pao *et al.*, 2005; Borràs *et al.*, 2011; Gaughan & Costa, 2011).

BRAF is mutated in a wide range of human cancers, including lung cancers (Xing, 2005; Dhomen & Marais, 2009; Gaughan & Costa, 2011). Ninety per cent of *BRAF* mutations are represented by a single somatic mutation *BRAF*^{V600E} (Cantwell-Dorris, 2011; Gaughan & Costa, 2011) and approximately 50% of lung adenocarcinomas harbour the recurrent somatic oncogenic mutations in *EGFR*, *KRAS* and *BRAF* (Girard, 2013; Oxnard *et al.*, 2013).

Mutations in other genes are involved in modulating therapeutic response to inhibitors of the *EGFR/PI3K/AKT* pathway (Su *et al.*, 2011). *PTEN* is a dual specificity phosphatase which directly antagonizes the phosphatidylinositol-3 kinase (PI3K) signalling pathway (Endersby & Baker, 2008; Tang *et al.*, 2011). *PTEN* is a tumour suppressor gene that is frequently mutated in human cancer, with most mutations leading to an inactivation of the gene (Cantley & Neel, 1999; Simpson & Parsons, 2001; Pandolfi, 2008). Although inactivating *PTEN* mutations are present in approximately 10% in NSCLC, *PTEN* expression is diminished in a larger proportion of these tumours (almost 70%) possibly by epigenetic mechanisms (Tang *et al.*, 2006; Li *et al.*, 2012).

TTF-1 is a DNA-binding protein found to be expressed in lung cells, regulates the activity of proliferating cells and also plays a role in angiogenesis (Berghmans *et al.*, 2008; Wislez *et al.*, 2010). TTF-1 was reportedly over-expressed in approximately 80% of lung adenocarcinomas (Maeshima *et al.*, 2008). An activating *EGFR* mutation is associated with TTF-1 over-expression in lung adenocarcinoma (Yatabe *et al.*, 2005; Tapia *et al.*, 2009) and the pattern of *EGFR*(+)/TTF-1(−) seems to be an exclusive signature for NSCLC, especially squamous-cell carcinoma (SCC) subtype (Berghmans *et al.*, 2008).

Ethnicity has been shown to affect risk for developing lung cancer. African Americans have higher incidence rates for lung cancer and family history of lung cancer, compared with pack/year smokers matched Caucasians (Cote *et al.*, 2005; Haiman *et al.*, 2006). Moreover, for lung cancer patients, being of Japanese ethnicity and never-smoker are independent favourable prognostic factors for overall

survival compared with Caucasians (Kawaguchi *et al.*, 2010). These ethnic differences are in all likelihood the result of the combined differences in the rate of germline and somatic sequence alterations in genes involved in NSCLC pathogenesis.

The spectrum of somatic *EGFR*, *KRAS*, *BRAF*, *PTEN* mutations and TTF-1 expression and its potential associations in genetically heterogeneous Brazilian lung cancer patients has not been previously reported, and that was the focus of this study.

2. Materials and methods

(i) Subjects

The study cohort encompassed 88 patients diagnosed with NSCLC who were eligible for surgery, with no previous history of chemotherapy or radiotherapy. Patients were recruited from a referral centre of thoracic surgery (Hospital Julia Kubitschek – FHEMIG, Belo Horizonte, Brazil) between 1 January 2006 and 31 December 2011. Controls were 28 healthy individuals older than 55 years with no previous personal or family history of cancer, randomly recruited from the outpatient clinic in the same medical centre during the same time period. A group of 96 healthy Brazilian individuals, representative of Southeastern Brazil, were used as controls for genomic ancestry.

(ii) Ethics statement

The Ethics Committee of Universidade Federal de Minas Gerais (Comitê de Ética em Pesquisa da UFMG, # 373-05) approved the study protocol and all participants signed a written informed consent.

(iii) *EGFR/KRAS/BRAF/PTEN* genotyping

Genomic DNA of participating NSCLC patients was isolated from fresh tumour tissue samples as well as from peripheral blood, according to a proteinase K-based standard protocol (Miller *et al.*, 1988). Peripheral blood was collected in vacuum tubes and genomic DNA was isolated using the high salt method of Lahiri and Nurnberger (Lahiri & Nurnberger, 1991) and was extracted from all study participants – NSCLC cases and controls. Genotyping for germline and somatic alterations was carried out for the following genes and mutations: exons 18 (G719S), 19 (746_750del, D761Y and L747S), 20 (insertions and T790M) and 21 (L858R and L861Q) of *EGFR*, exons 2 and 3 of *KRAS* (codons 12, 13 and 61), exon 15 of *BRAF* (*BRAF*^{V600E}) and all nine exons of *PTEN* (to evaluate any inactivating mutation in the entire coding regions of the gene) were amplified by PCR with primers specific for each region

Table 1. Characteristics of PCR amplification of EGFR and KRAS

Gene	Exon	Sequence (forward/reverse)	T _m ^a (°C)	Product size (bp)
<i>BRAF</i>	15	5'-TCATAATGCTTGCTGTGATAGGA-3'/5'-GGCCAAAAATTTAATCAGTGGA-3'	55	179
<i>EGFR</i>	18	5'-GGCGTACATTTGTCCTCCC-3'/5'-TGCCTTTGGTCTGTGAA-3'	55	505
	19	5'-CCCAGCAATATCAGCCTT-3'/5'-GCCTCCATTTCTTCATCAA-3'	58	590
	20	5'-CTCTCCCCTGCATCTGTCA-3'/5'-TATCTCCCCTCCCCGTATCT-3'	56	421
	21	5'-TTCAAGCCCAGGTCTCAACT-3'/5'-CAGCTGTGACCTTTCCCAAT-3'	56	675
<i>KRAS</i>	2	5'-GTGTGACATGTTCTAATATAGTCA-3'/5'-GAATGGTCTGCACCAGTAA-3'	55	170
	3	5'-CCAGACTGTGTTTCTCCCTC-3'/5'-TGCATGGCATTAGCAAAGAC-3'	55	245
<i>PTEN</i>	1	5'-GCAGCTTCTGCCATCTCTCT-3'/5'-TTTTCGCATCCGCTACTCC-3'	55	206
	2	5'-TTTGATTGCTGCATATTCAGA-3'/ 5'-CATCACAAAGTATCTTTTTCTGTGG-3'	55	239
	3	5'-GGTGGCTTTTTGTTTGTGG-3'/5'-CAATGCTCTTGGACTTCTTGA-3'	55	231
	4	5'-AAAGATTCAAGCAATGTTTGTG-3'/5'-TCTCACTCGATAATCTGGTGAC-3'	55	235
	5	5'-TGAGGTTATCTTTTTACCACAG-3'/5'-GGAAAGGAAAAACATCAAAA-3'	55	291
	6	5'-TTTTTCAATTTGGCTTCTTTTT-3'/5'-TGTTCCAATACATGGAAGGATG-3'	55	220
	7	5'-AAAGGCATTCTGTGAAATAA-3'/5'-TTTGGATATTTCTCCCAATGAA-3'	55	250
	8	5'-GTCATTTTCTTTTTCTTTTTCTTTT-3'/5'-CAACAACCCCAAAATGT-3'	52	300
	9	5'-TGGGTTTTCAATTTAAATTTTCTTTC-3'/ 5'-CATGGTGTTTTATCCCTCTTGA-3'	55	250

^aT_m: annealing temperature.

(Table 1). For PCR reactions 2 µl of DNA at 30 ng/µl were mixed with 2.5 µl of 10X IIB buffer (40 mM NaCl; 10 mM Tris-HCl pH 8.4; 0.1% Triton X-100; 1.5 mM MgCl₂), 2.5 µl of 0.2 mM dNTP, 0.5 µl of each primer at 10 pmol/µl and 0.25 µl of Taq polymerase (Invitrogen, Brazil) 0.625 U, on a final volume of 25 µl. Samples were placed on an Eppendorf Mastercycler[®] (Hamburg, Germany) at 94 °C for 3 min and then 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension time at 72 °C for 5 min. PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, São Paulo, Brazil) following manufacturer's protocol and visualized on a silver-stained 6.5% polyacrylamide gel. To improve the sensitivity of the *KRAS* mutation detection we used the COLD-PCR method as previously described (Zuo *et al.*, 2009).

Sequences were obtained on ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Bi-directional sequence data were analysed using Sequencer 4.9 software (<http://genecodes.com>). Positive findings for *EGFR* exon 19 deletions were also confirmed by fragment analysis. The genomic fragment including all exon 19 was amplified in 10 µl final PCR volume of the following: 1X PCR buffer (10 mM Tris-HCl pH 8.3 or pH 9.2, 75 mM KCl, 3.5 mM MgCl₂), 200 µM dNTPs, 1.0 U of Platinum Taq DNA polymerase (Life Technologies, São Paulo, Brazil), 20 ng of genomic DNA, 1.5 µM of M13-40 forward primer labelled with the FAM dye, 1.5 µM of each unlabelled reverse primer and 0.1 µM of each unlabelled forward primer.

(iv) Immunohistochemistry of TTF-1

Tissue sections from 27 samples previously diagnosed by pathological report as SCCs, three adenocarcinomas and four diagnosed as NSCLC poorly differentiated carcinoma were stained with TTF-1 antiserum. Briefly, 4 µm paraffin-embedded sections were dewaxed in xylene and hydrated with graded ethanol. Endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 min. Heat-induced epitope retrieval was performed with 1 mM EDTA buffer pH 8.0 for 30 min in a steamer at 96 °C. Primary polyclonal rabbit antiserum was used at 1:100 for 18 h at 4 °C. This was followed by incubation with the labelled streptavidin-biotin kit NovoLinkTM Max Polymer (Novocastra, UK). Peroxidase activity was developed with DAB (Sigma, St Louis, MO) with timed monitoring using a positive control sample. The sections were then counterstained with haematoxylin, dehydrated and mounted. All slides were examined under light microscopy and staining for TTF-1 was evaluated according to the presence or not of the protein by two pathologists who were blinded to the clinical course of the patient.

(v) Genomic ancestry analysis

Germline DNA of all 88 lung cancer patients and 96 ethnically diverse Brazilian controls were genotyped with a set of 40 biallelic short insertion/deletion polymorphisms (In/Dels), as previously described (Bastos-Rodrigues *et al.*, 2006). Amplicons were size fractionated using an ABI 3130 DNA sequencer (Applied Biosystems) and analysed using the

GeneMapper® Software (version 3.7). To estimate the proportion of European, African and Amerindian biogeographical ancestry of each individual we used the Structure program, version 2.3 (<http://pritch.bsd.uchicago.edu/structure.html>).

(vi) Statistical analysis

The proportion of European, African and Amerindian bio-geographical ancestry of each individual was used for stratifying statistical analysis. For statistical comparisons between cases and controls, the two-tailed Mann–Whitney *U* test was used. Single-marker allelic and genotypic association tests were performed using the Unphased software package version 3.0.12 (www.mrcbsu.cam.ac.uk/personal/frank/software/unphased/). A value of $P \leq 0.05$ was considered statistically significant. Odds ratios and 95% confidence interval were calculated.

3. Results

(i) Sample characteristics

Demographics and relevant clinical and pathological data of all 88 NSCLC cases are shown in Table 2. The control group (for *EGFR* germline mutation genotyping) encompassed 28 healthy individuals, comprising 64% women and 36% men with a mean age of 72.8 ± 9.15 years (range 55–89 years).

(ii) *EGFR/KRAS/BRAF/PTEN* mutation status

EGFR gene genotyping showed the presence of the 746_750del (LREA domain) in 3/88 patients (3.4%), and was exclusively detected in 3/45 (6.6%) of patients with adenocarcinoma. No sequence alterations, especially G719S, were noted in exon 18 of the *EGFR* gene. Only one patient diagnosed with adenocarcinoma (1.5%) showed the L585R variation in exon 21 of *EGFR*. In addition to these clearly pathogenic mutations in the *EGFR* gene, two previously reported polymorphisms were also identified in DNA of cases and controls: a silent base substitution (CAG>CAA) at c.2538 position (corresponding to *rs1050171*) was detected in 61.3% of the cases ($n=54$) and a substitution at IVS-60T>C position (*rs10241451*) was noted in 20.4% of the cases ($n=18$). The *rs10241451* did not show a significant association with the disease both by allele and genotype frequency ($P=0.29$ and $P=0.32$, respectively) when compared with controls, whereas a significant association between *rs1050171* and lung cancer compared with cancer-free controls was noted only by allele and not by genotype frequency ($P=0.04$ and $P=0.11$, respectively) (Table 3).

Codon 12 *KRAS* mutations were noted in five male patients (5.7%) diagnosed with adenocarcinoma: three harboured the Gly12Cys (c.34G>T) mutation and

Table 2. Clinical data of non-small cell lung cancer (NSCLC) patients

Variants	N	%
Age		
Median	59	
Range	32–83	
Sex		
Men	57	64.8
Women	31	35.2
Histological subtype		
ADC	45	51.1
SCC	28	31.8
NSCLC-favour adenocarcinoma ^a	15	17.1
Smoking history		
Smokers	73	82.9
Never smokers	15	17.1
Tumour stage		
IA	2	2.2
IB	17	19.3
IIA	21	23.9
IIB	16	18.2
IIIA	24	27.3
IIIB	5	5.7
IV	3	3.4

^a Tumours positive for TTF-1 marker (Travis *et al.*, 2013). ADC, adenocarcinoma; SCC, squamous-cell carcinoma.

two the Gly12Asp (c.35G>A) mutation. No mutations in codon 13 of *KRAS* were found. Two female smokers patients (3%) diagnosed with SCC at ages 60 and 63 years old showed a previously reported missense mutation (*rs17851045*) in codon 61 (c.182A>T), which leads to histidine for glutamine change (H61Q). No sequence alterations in exons 1–9 of the *PTEN* gene were noted and the *BRAF*^{V600E} mutation was not detected in any of the samples analysed.

(iii) TTF-1 immunohistochemistry

All three adenocarcinoma samples, which served as positive controls for TTF-1 expression, demonstrated positivity, as expected. Eleven of the 27 SCCs analysed displayed positive TTF-1 expression and in 16 tumours no expression was present (Table 2). Four tumours previously described as poorly differentiated carcinoma also showed positive TTF-1 expression. All positive tumours for TTF-1 expression ($n=15$) were considered NSCLC-favour adenocarcinoma.

(iv) Genomic ancestry analysis

We genotyped germline DNA from all 88 lung cancer patient samples and 96 controls, for 40 polymorphic In/Del loci which form a powerful ancestry informative test battery (Bastos-Rodrigues *et al.*,

Table 3. Allele and genotype frequencies of rs1050171 and rs10241451 in patients and controls

	Patients		Controls		P	OR	95% CI
	N	%	N	%			
rs1050171/G2538A							
Genotype							
GG	28	31.8	15	53.6	0.11	1.0	0.92–9.06
GA	27	30.6	5	17.8		2.89	
AA	33	37.6	8	28.6		2.21	
Allele							
G	83	47.2	35	62.5	0.04	1.0	1–3.46
A	93	52.8	21	37.5		1.86	
rs10241451/2284–60T>C							
Genotype							
TT	71	80.7	24	85.7	0.32	1.0	0.32–3.69
TC	13	14.8	4	14.3		1.09	
CC	4	4.5	0	0		2.52×10^8	
Allele							
T	155	88.0	52	92.8	0.29	1.0	0.57–5.38
C	21	12	4	7.2		1.76	

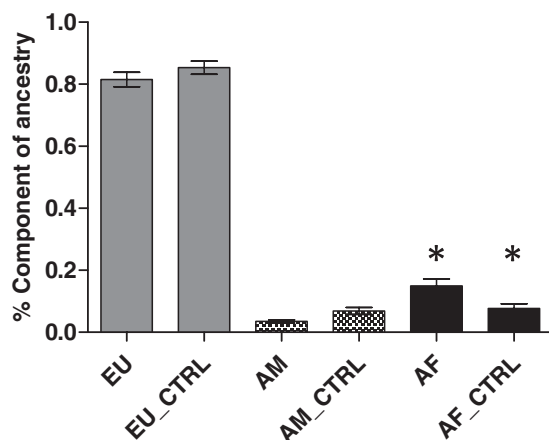


Fig. 1. Analysis of genomic ancestry of patients with lung cancer. EU, Europeans; AF, African; AM, Amerindians. Significant difference was found between Africans when compared with control group ($P=0.004$).

2006). For the case group, the proportions of European, African and Amerindian ancestry were 0.87 ± 0.02 (mean \pm SE), 0.09 ± 0.02 and 0.04 ± 0.007 , respectively, whereas for the control group, the results were 0.85 ± 0.02 , 0.08 ± 0.01 and 0.07 ± 0.001 , respectively. The proportion of European, African and Amerindian ancestry in each group indicated that an African component was more prevalent in lung cancer patients than controls ($P=0.03$) (Fig. 1).

4. Discussion

In the present study that focused on Brazilian lung cancer patients, the *EGFR* 746_750del, a mutation that is associated with a better response of NSCLC

patients to TKIs, such as Gefitinib and Erlotinib (Han *et al.*, 2006; Riely *et al.*, 2006; Irmer *et al.*, 2007) was detected somatically in only three of 88 tumour samples (3.4%). This is a significantly lower rate than the rates reported for ethnically diverse populations worldwide, with rates ranging from 15 to 40% of NSCLC analysed, primarily adenocarcinomas (Irmer *et al.*, 2007; Matsuo *et al.*, 2007; Leidner *et al.*, 2009; Tapia *et al.*, 2009; Vlastos *et al.*, 2010). A previous study of Latin American patients from biopsies taken from Argentinean, Colombian, Peruvian and Mexican lung cancer patients (Arrieta *et al.*, 2011) reported the same deletion in 48.4% patients (185/382 cases) and this was in agreement with data from Asian (60%) and European populations (62.2%) (Rosell *et al.*, 2009). A previous study also detected a low incidence of mutations in a Caucasian population (12%) and suggested that the specific lung cancer subtype analysed as well as the technique used could explain the wide range of somatic mutations reported (Wislez *et al.*, 2010). The L858R *EGFR* somatic mutation was found in only one patient and other mutations such as L861Q and G719S were not noted in the present study. These results are in line with data reported by Vlastos *et al.* (2010) that analysed Caucasian population, but contrasts other studies reporting high rates of these *EGFR* mutations in NSCLC in patients of Asian (40%) and European (37.8%) descent (Han *et al.*, 2006; Murray *et al.*, 2006; Riely *et al.*, 2006; Irmer *et al.*, 2007; Matsuo *et al.*, 2007; Rosell *et al.*, 2009). Specifically, the L858R mutation that has been reportedly detected in 12.5 to 45% of NSCLC-associated *EGFR* mutations (Pan *et al.*, 2005; Tapia *et al.*, 2009) was found in only one case (1.1%) in the present study. This patient has

features more common to NSCLC patients with the L585R mutation in exon 21, i.e. female, non-smoker with adenocarcinoma histology (Kondo *et al.*, 2005; Shigematsu *et al.*, 2005; Riely *et al.*, 2006). One plausible explanation for the disparities between the rates of the *EGFR* mutations in the present study and other studies is the type of tumours and patients analysed. The cases analysed in the present study are mostly men, smokers, with a high African ancestry. Other studies showed that the prevalence of *EGFR* mutations among African Americans have yielded conflicting results (Cote *et al.*, 2011; Reinersman *et al.*, 2011). Indeed, in a previous study focusing on Brazilian NSCLC cases, Amoedo *et al.* (2009) studied only exon 19 of *EGFR* and reported one 746_750del in 64 paraffin-embedded tissue samples analysed (~1.5%). These data combined with the data presented herein support the notion of a low prevalence of somatic *EGFR* mutations in Brazilian NSCLC cases.

Somatic activating *KRAS* mutations in NSCLC can usually be detected in codon 12 (Gly12Asp), less frequently in codon 13 (Gly13Asp) and rarely at codon 61 (Gln61His) (Riely *et al.*, 2009). The frequency and type of *KRAS* activating mutations in NSCLC are in part determined by the specific tumour histology, patients' ethnicity and smoking history (Riely *et al.*, 2008; Amoedo *et al.*, 2009); these mutations are more commonly encountered in Caucasians, smokers with adenocarcinoma histology (Suda *et al.*, 2010). In the present study, the mutation in codon 12 was noted in male patients diagnosed with adenocarcinoma. Unlike the reported mutational spectrum in the *KRAS* gene in other world populations (Dearden *et al.*, 2013), the sequence alteration described in codon 61 was detected in two female smokers, diagnosed with SCC. Although environmental factors could be involved, the aetiology of this alteration remains to be established.

The rate of somatic *KRAS* mutations in the present study was low (7.9%), similar to the low rates (11/173; 6.3%) reported by Lee *et al.* (2010) who analysed 173 Korean cases. These rates are well below the rates reported for Japanese (12.8%) (Lee *et al.*, 2010), Latin American (16.6%) (Arrieta *et al.*, 2011) and Caucasian populations (41.9%) (Borràs *et al.*, 2011). These apparent differences in somatic mutation rates in the *KRAS* gene may also be attributed to different ethnic background of the studied populations and may indicate that the malignant transformation process in Brazilian NSCLC cases may have different pathways than those in some other, ethnically diverse, populations.

Although several studies have shown the relationship of *BRAF* and *PTEN* with the tumorigenesis of lung cancer, several studies confirm the low frequency of mutations of these genes in NSCLC (Forgacs *et al.*, 1998; Yokomizo *et al.*, 1998; Sasaki

et al., 2006; Pratilas *et al.*, 2008; Jin *et al.*, 2010; Marchetti *et al.*, 2011). In the Brazilian population analysed here, we have not found any mutations in *BRAF* and *EGFR* genes thus confirming the rarity of this alteration in NSCLC. To our knowledge, our work represents the first data of a *PTEN* and *BRAF*^{V600E} mutation search in Brazilian patients diagnosed with NSCLC.

Approximately 80% of adenocarcinomas express TTF-1, and this marker has been used to differentiate the histological subtypes of NSCLC (adenocarcinoma and SCC) (Maeshima *et al.*, 2008). Our results showed that 40.7% of SCCs analysed were TTF-1 (+) demonstrating that a subset of SCCs should be considered NSCLC-favour adenocarcinoma (Travis *et al.*, 2013). According to Ordóñez (2012), the introduction of target therapies can result in dramatically different outcomes based on histological subtype. Thus, the use of markers such as TTF-1 can help to discriminate between adenocarcinoma and SCC subtypes, what is indispensable to define a personalized treatment.

The Brazilian population has a major Caucasian contribution (Pena *et al.*, 2009) but our study found a greater African component in patients with NSCLC than in the control group. African-American patients with NSCLC are significantly less likely to harbour activating mutations in *EGFR* genes and their signalling pathway when compared with Caucasians (Riely *et al.*, 2006). This notion is further supported by Reinersman *et al.* (2011), who reported that African Americans are less likely to have *KRAS* mutations in NSCLC when compared with Caucasians, a finding corroborated in our study.

As demonstrated, our data show a low frequency of mutations in *EGFR*, *KRAS*, *BRAF* and *PTEN*. This may be related to the high African component found in our patients, which has been discussed in previous studies (Maxwell *et al.*, 2000; Kumar *et al.*, 2009; Leidner *et al.*, 2009; Pena *et al.*, 2009; Reinersman *et al.*, 2011) but had not yet been analysed in the Brazilian population. Thus, our data add to a growing body of evidence that highlight the genetic heterogeneity of the *EGFR* pathway in NSCLC among different populations, which highlights the need to incorporate these differences in designing clinical therapies and agents for the inhibition of this pathway.

The limitations of the present study should be pointed out: this is a relatively small study from a single medical centre that serves a population that might not reflect the ethnic diversity of the entire Brazilian population. In addition, it must be said that patients are referred to treatment when surgical procedures are no longer acceptable and further investigations inappropriate. Hence it is important to stress that these results should be expanded and validated in larger studies focusing on Brazilian patients.

In conclusion, Brazilian lung cancer patients display a low frequency of somatic mutations in *EGFR*, *KRAS*, *BRAF* and *PTEN*. This may be related to the high African ancestry component found in our patients, but an expansion and validation of these preliminary data is needed.

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Statement of Interest

None.

References

- Amoedo, N. D., Castelo-Branco, M. T., Paschoal, M. E., Pezzuto, P., Esperança, A. B., Rumjanek, V. M. & Rumjanek, F. D. (2009). 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. *International Journal of Molecular Medicine* **23**, 609–614.
- Arrieta, O., Cardona, A. F., Bramuglia, G. F., Gallo, A., Campos-Parra, A. D., Serrano, S., Castro, M., Avilés, A., Amorin, E., Kirchuk, R., Cuello, M., Borbolla, J., Riemersma, O., Becerra, H. & Rosell, R. (2011). Genotyping non-small cell lung cancer (NSCLC) in Latin America. *Journal of Thoracic Oncology* **6**, 1955–1959.
- Bastos-Rodrigues, L., Pimenta, J. R. & Pena, S. D. (2006). The genetic structure of human populations studied through short insertion-deletion polymorphisms. *Annals of Human Genetics* **70**, 658–665.
- Berghmans, T., Mascauxa, C., Hallerb, A., Meerta, A. P., Van Houttec, P. & Sculier, J. P. (2008). EGFR, TTF-1 and Mdm2 expression in stage III non-small cell lung cancer: a positive association. *Lung Cancer* **62**, 35–44.
- Borràs, E., Jurado, I., Hernan, I., Gamundi, M. J., Dias, M., Martí, I., Mañé, B., Arcusa, A., Agúndez, J. A., Blanca, M. & Carballo, M. (2011). Clinical pharmacogenomic testing of KRAS, BRAF and EGFR mutations by high resolution melting analysis and ultra-deep pyrosequencing. *BMC Cancer* **11**, 406.
- Brevet, M., Johnson, M. L., Azzoli, C. G. & Ladanyi, M. (2011). Detection of EGFR mutations in plasma DNA from lung cancer patients by mass spectrometry genotyping is predictive of tumor EGFR status and response to EGFR inhibitors. *Lung Cancer* **73**, 96–102.
- Cantley, L. C. & Neel, B. G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proceedings of the National Academy of Sciences USA* **96**, 4240–4245.
- Cantwell-Dorris, E. R. (2011). BRAFV600E: Implications for carcinogenesis and molecular therapy. *Molecular Cancer Therapeutics* **10**, 385–394.
- Cote, M. L., Kardia, S. L., Wenzlaff, A. S., Ruckdeschel, J. C. & Schwartz, A. G. (2005). Risk of lung cancer among white and black relatives of individuals with early-onset lung cancer. *JAMA* **293**, 3036–3042.
- Cote, M. L., Haddad, R. & Edwards, D. J. (2011). Frequency and type of epidermal growth factor receptor mutations in African Americans with non-small cell lung cancer. *Journal of Thoracic Oncology* **6**, 627–630.
- Dearden, S., Stevens, J., Wu, Y. L. & Blowers, D. (2013). Mutation incidence and coincidence in non small-cell lung cancer: meta-analyses by ethnicity and histology (mutMap). *Annals of Oncology* **24**, 2371–2376.
- Dhomen, N. & Marais, R. (2009). BRAF signaling and targeted therapies in melanoma. *Hematology/Oncology Clinics of North America* **23**, 529–545.
- Endersby, R. & Baker, S. J. (2008). PTEN signaling in brain: neuropathology and tumorigenesis. *Oncogene* **27**, 5416–5430.
- Forgacs, E., Biesterveld, E. J., Sekido, Y., Fong, K., Muneer, S., Wistuba, I. I., Milchgrub, S., Brezinschek, R., Virmani, A., Gazdar, A. F. & Minna, J. D. (1998). Mutation analysis of the PTEN/MMAC1 gene in lung cancer. *Oncogene* **17**, 1557–1565.
- Gaughan, E. M. & Costa, D. B. (2011). Genotype-driven therapies for non-small cell lung cancer: focus on EGFR, KRAS and ALK gene abnormalities. *Therapeutic Advances in Medical Oncology* **3**, 113–125.
- Girard, N. (2013). Other signalization targets. *Target Oncology* **8**, 69–77.
- Haiman, C. A., Stram, D. O., Wilkens, L. R., Pike, M. C., Kolonel, L. N., Henderson, B. E. & Le Marchand, L. (2006). Ethnic and racial differences in the smoking-related risk of lung cancer. *New England Journal of Medicine* **354**, 333–342.
- Han, S. W., Kim, T. Y., Jeon, Y. K., Hwang, P. G., Im, S. A., Lee, K. H., Kim, J. H., Kim, D. W., Heo, D. S., Kim, N. K., Chung, D. H. & Bang, Y. J. (2006). Optimization of patient selection for gefitinib in non-small cell lung cancer by combined analysis of epidermal growth factor receptor mutation, K-ras mutation, and Akt phosphorylation. *Clinical Cancer Research* **12**, 2538–2544.
- Heigener, D. F. & Reck, M. (2011). Mutations in the epidermal growth factor receptor gene in non-small cell lung cancer: impact on treatment beyond gefitinib and erlotinib. *Advances in Therapy* **28**, 126–133.
- Hunt, J. D., Strimas, A., Eyer, M., Haddican, M., Lockett, B. G., Ruiz, B., Axelrad, T. W., Backes, W. L. & Fontham, E. T. (2002). Differences in KRAS mutation spectrum in lung cancer cases between African Americans and Caucasians after occupational or environmental exposure to known carcinogens. *Cancer Epidemiology, Biomarkers and Prevention* **11**, 1405–1412.
- Irmer, D., Funk, J. O. & Blaukat, A. (2007). EGFR kinase domain mutations – functional impact and relevance for lung cancer therapy. *Oncogene* **26**, 5693–5701.
- Jin, G., Kim, M. J., Jeon, H. S., Choi, J. E., Kim, D. S., Lee, E. B., Cha, S. I., Yoon, G. S., Kim, C. H., Jung, T. H. & Park, J. Y. (2010). PTEN mutations and relationship to EGFR, ERBB2, KRAS, and TP53 mutations in non-small cell lung cancers. *Lung Cancer* **69**, 279–283.
- Kadara, H., Kabbout, M. & Wistuba, I. (2011). Pulmonary adenocarcinoma: a renewed entity in 2011. *Respirology* **17**, 50–65.
- Kawaguchi, T., Matsumura, A., Fukai, S., Tamura, A., Saito, R., Zell, J. A., Maruyama, Y., Ziogas, A., Kawahara, M. & Ignatius, S. H. (2010). Japanese ethnicity compared with Caucasian ethnicity and

- never-smoking status are independent favorable prognostic factors for overall survival in non-small cell lung cancer: a collaborative epidemiologic study of the National Collaborative Organization Study Group for Lung Cancer (NHSGLC) in Japan and a Southern California Regional Cancer Registry databases. *Journal of Thoracic Oncology* **5**, 1001–1010.
- Keedy, V. L., Temin, S., Somerfield, M. R., Beasley, M. B., Johnson, D. H., McShane, L. M., Milton, D. T., Strawn, J. R., Wakelee, H. A. & Giaccone, G. (2011). American Society of Clinical Oncology provisional clinical opinion: epidermal growth factor receptor (EGFR) Mutation testing for patients with advanced non-small-cell lung cancer considering first-line EGFR tyrosine kinase inhibitor therapy. *Journal of Clinical Oncology* **29**, 2121–2127.
- Kondo, M., Yokoyama, T., Fukui, T., Yoshioka, H., Yokoi, K., Nagasaka, T., Imaizumi, K., Kume, H., Hasegawa, Y., Shimokata, K. & Sekido, Y. (2005). Mutations of epidermal growth factor receptor of non-small cell lung cancer were associated with sensitivity to gefitinib in recurrence after surgery. *Lung Cancer* **50**, 385–391.
- Kumar, K., Brim, H., Giardiello, F., Smoot, D. T., Nourai, M., Lee, E. L. & Ashktorab, H. (2009). Distinct BRAF (V600E) and KRAS mutations in high microsatellite instability sporadic colorectal cancer in African Americans. *Clinical Cancer Research* **15**, 1155–1161.
- Lahiri, D. K. & Nurnberger, J. I. (1991). A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Research* **11**, 5444.
- Lee, S. Y., Kim, M. J., Jin, G., Yoo, S. S., Park, J. Y., Choi, J. E., Jeon, H. S., Cho, S., Lee, E. B., Cha, S. I., Park, T. I., Kim, C. H., Jung, T. H. & Park, J. Y. (2010). Somatic mutations in EGFR signaling pathway genes. *Journal of Thoracic Oncology* **5**, 1734–1740.
- Leidner, R. S., Fu, P., Clifford, B., Hamdan, A., Jin, C., Eisenberg, R., Boggan, T. J., Skokan, M., Franklin, W. A., Cappuzzo, F., Hirsch, F. R., Varella-Garcia, M. & Halmos, B. (2009). Genetic abnormalities of the EGFR pathway in African American patients with non-small-cell lung cancer. *Journal of Clinical Oncology* **27**, 5620–5626.
- Li, G., Zhao, J., Peng, X., Liang, J., Deng, X. & Chen, Y. (2012). The mechanism involved in the loss of PTEN expression in NSCLC tumor cells. *Biochemical and Biophysical Research Communications* **418**, 547–552.
- Li, T., Kung, H. J., Mack, P. C. & Gandara, D. R. (2013). Genotyping and genomic profiling of non-small-cell lung cancer: implications for current and future therapies. *Journal of Clinical Oncology* **31**, 1039–1049.
- Maeshima, A. M., Omatsu, M., Tsuta, K., Asamura, H. & Matsuno, Y. (2008). Immunohistochemical expression of TTF-1 in various cytological subtypes of primary lung adenocarcinoma, with special reference to intratumoral heterogeneity. *Pathology International* **58**, 31–37.
- Marchetti, A., Felicioni, L., Malatesta, S., Grazia Sciarrotta, M., Guetti, L., Chella, A., Viola, P., Pullara, C., Mucilli, F. & Buttitta, F. (2011). Clinical features and outcome of patients with non-small-cell lung cancer harboring BRAF mutations. *Journal of Clinical Oncology* **29**, 3574–35479.
- Matsuo, K., Ito, H., Yatabe, Y., Hiraki, A., Hirose, K., Wakai, K., Kosaka, T., Suzuki, T., Tajima, K. & Mitsudomi, T. (2007). Risk factors differ for non-small-cell lung cancers with and without EGFR mutation: assessment of smoking and sex by a case-control study in Japanese. *Cancer Science* **98**, 96–101.
- Maxwell, G. L., Risinger, J. I., Hayes, K. A., Alvarez, A. A., Dodge, R. K., Barrett, J. C. & Berchuck, A. (2000). Racial disparity in the frequency of PTEN mutations, but not microsatellite instability, in advanced endometrial cancers. *Clinical Cancer Research* **6**, 2999–3005.
- Miller, S. A., Dykes, D. D. & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* **16**, 1215.
- Mok, T. S., Wu, Y. L., Thongprasert, S., Yang, C. H., Chu, D. T., Saij, N., Sunpaweravong, P., Han, B., Margono, B., Ichinose, Y., Nishiwaki, Y., Ohe, Y., Yang, J., Chewaskulyong, B., Jiang, H., Duffield, E. L., Watkins, C. L., Armour, A. A. & Fukuoka, M. (2009). Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *New England Journal of Medicine* **361**, 947–957.
- Murray, S., Timotheadou, E., Linardou, H., Vrettou, A. V., Kostopoulos, I., Skrickova, J., Papakostantinou, C., Christodoulou, C., Pectasides, D., Samantas, E., Papakostas, P., Skarlos, D. V., Kosmidis, P. & Fountzilas, G. (2006). Mutations of the epidermal growth factor receptor tyrosine kinase domain and associations with clinicopathological features in non-small cell lung cancer patients. *Lung Cancer* **52**, 225–233.
- Ordóñez, N. G. (2012). Thyroid transcription factor-1 is not expressed in squamous cell carcinomas of the lung: an immunohistochemical study with review of the literature. *Applied Immunohistochemistry and Molecular Morphology* **20**, 525–530.
- Oxnard, G. R., Binder, A., & Jänne, P. A. (2013). New targetable oncogenes in non-small-cell lung cancer. *Journal of Clinical Oncology* **31**, 1097–1104.
- Paez, J. G., Jänne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M. J., Sellers, W. R., Johnson, B. E. & Meyerson, M. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**, 1497–1500.
- Pan, Q., Pao, W., Ladanyi, M. (2005). Rapid polymerase chain reaction-based detection of epidermal growth factor receptor gene mutations in lung adenocarcinomas. *The Journal of Molecular Diagnostics* **7**, 396–403.
- Pandolfi, P. P. (2008). P-TEN exciting years: from the cytosol to the nucleus and back to keep cancer at bay. *Oncogene* **27**, 5386.
- Pao, W., Wang, T. Y., Riely, G. J., Miller, V. A., Pan, Q., Ladanyi, M., Zakowski, M. F., Heelan, R. T., Kris, M. G. & Varmus, H. E. (2005). KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Medicine* **2**, e17.
- Pena, S. D., Bastos-Rodrigues, L., Pimenta, J. R. & Bydlowski, S. P. (2009). DNA tests probe the genomic ancestry of Brazilians. *Brazilian Journal of Medical and Biological Research* **42**, 870–876.
- Pratilas, C. A., Hanrahan, A. J., Halilovic, E., Persaud, Y., Soh, J., Chitale, D., Shigematsu, H., Yamamoto, H., Sawai, A., Janakiraman, M., Taylor, B. S., Pao, W., Toyooka, S., Ladanyi, M., Gazdar, A., Rosen, N. & Solit, D. B. (2008). Genetic predictors of MEK dependence in non-small cell lung cancer. *Cancer Research* **68**, 9375–9383.
- Reinersman, J. M., Johnson, M. L., Riely, G. J., Chitale, D. A., Nicastrì, A. D., Soff, G. A., Schwartz, A. G., Sima, C. S., Ayalew, G., Lau, C.,

- Zakowski, M. F., Rusch, V. W., Ladanyi, M. & Kris, M. G. (2011). Frequency of EGFR and KRAS mutations in lung adenocarcinomas in African Americans. *Journal of Thoracic Oncology* **6**, 28–31.
- Reungwetwattana, T., Weroha, S. J. & Molina, J. R. (2012). Oncogenic pathways, molecularly targeted therapies, and highlighted clinical trials in non-small-cell lung cancer (NSCLC). *Clinical Lung Cancer* **13**, 252–266.
- Riely, G. J., Politi, K. A., Miller, V. A. & Pao, W. (2006). Update on epidermal growth factor receptor mutations in non-small cell lung cancer. *Clinical Cancer Research* **12**, 7232–7241.
- Riely, G. J., Kris, M. G., Rosenbaum, D., Marks, J., Li, A., Chitale, D. A., Nafa, K., Riedel, E. R., Hsu, M., Pao, W., Miller, V. A. & Ladanyi, M. (2008). Frequency and distinctive spectrum of KRAS mutations in never smokers with lung adenocarcinoma. *Clinical Cancer Research* **14**, 5731–5734.
- Riely, G. J., Marks, J. & Pao, W. (2009). KRAS mutations in non-small cell lung cancer. *Proceedings of the American Thoracic Society* **6**, 201–205.
- Rosell, R., Moran, C., Queralt, C., Porta, R., Cardenal, F., Camps, C., Majem, M., Lopez-Vivanco, G., Isla, D., Provencio, M., Insa, A., Massuti, B., Gonzalez-Larriba, J. L., Paz-Ares, L., Bover, I., Garcia-Campelo, R., Moreno, M. A., Catot, S., Rolfo, C., Reguart, N., Palmero, R., Sánchez, J. M., Bastus, R., Mayo, C., Bertran-Alamillo, J., Molina, M. A., Sanchez, J. J. & Taron, M. (2009). Screening for epidermal growth factor receptor mutations in lung cancer. *New England Journal of Medicine* **361**, 958–967.
- Sasaki, H., Kawano, O., Endo, K., Suzuki, E., Haneda, H., Yukiue, H., Kobayashi, Y., Yano, M. & Fujii, Y. (2006). Uncommon V599E BRAF mutations in Japanese patients with lung cancer. *Journal of Surgical Research* **133**, 203–206.
- Shigematsu, H., Lin, L., Takahashi, T., Nomura, M., Suzuki, M., Wistuba, I. I., Fong, K. M., Lee, H., Toyooka, S., Shimizu, N., Fujisawa, T., Feng, Z., Roth, J. A., Herz, J., Minna, J. D. & Gazdar, A. F. (2005). Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *Journal of the National Cancer Institute* **97**, 339–346.
- Simpson, L. & Parsons, R. (2001). PTEN: life as a tumor suppressor. *Experimental Cell Research* **264**, 29–41.
- Su, Z., Dias-Santagata, D., Duke, M., Hutchinson, K., Lin, Y. L., Borger, D. R., Chung, C. H., Massion, P. P., Vnencak-Jones, C. L., Iafrate, A. J. & Pao, W. (2011). A platform for rapid detection of multiple oncogenic mutations with relevance to targeted therapy in non-small-cell lung cancer. *Journal of Molecular Diagnostics* **13**, 74–84.
- Suda, K., Tomizawa, K. & Mitsudomi, T. (2010). Biological and clinical significance of KRAS mutations in lung cancer: an oncogenic driver that contrasts with EGFR mutation. *Cancer Metastasis Review* **29**, 49–60.
- Tang, J. M., He, Q. Y., Guo, R. X. & Chang, X. J. (2006). Phosphorylated Akt overexpression and loss of PTEN expression in non-small cell lung cancer confers poor prognosis. *Lung Cancer* **51**, 181–191.
- Tang, M., Iijima, M., Kamimura, Y., Chen, L., Long, Y. & Devreotes, P. (2011). Disruption of PKB signaling restores polarity to cells lacking tumor suppressor PTEN. *Molecular Biology of the Cell* **22**, 437–447.
- Tapia, C., Savic, S., Bihl, M., Ruffe, A., Zlobec, I., Terracciano, L. & Bubendorf, L. (2009). EGFR mutation analysis in non-small-cell lung cancer: experience from routine diagnostics. *Der Pathologe* **30**, 384–392. [In German]
- Travis, W. D., Brambilla, E. & Riely, G. J. (2013). New pathologic classification of lung cancer: relevance for clinical practice and clinical trials. *Journal of Clinical Oncology* **31**, 992–1001.
- Vlastos, F., Zinzner, J., Hussenet, T., du Manoir, S., Vordonis, L., Nikolakopoulou, S., Hardavella, G., Lacomme, S., Vignaud, J. M. & Martinet, N. (2010). Mapping EGFR1 mutations in patients with lung adenocarcinoma. *Diagnostic Molecular Pathology* **19**, 209–217.
- Wislez, M., Antoine, M., Baudrin, L., Poulot, V., Neuville, A., Pradere, M., Longchamp, E., Isaac-Sibille, S., Lebitasy, M. P. & Cadranet, J. (2010). Non-mucinous and mucinous subtypes of adenocarcinoma with bronchioloalveolar carcinoma features differ by biomarker expression and in the response to gefitinib. *Lung Cancer* **68**, 185–191.
- Xing, M. (2005). BRAF mutation in thyroid cancer. *Endocrine Related Cancer* **12**, 245–262.
- Yatabe, Y., Kosaka, T., Takahashi, T. & Mitsudomi, T. (2005). EGFR mutation is specific for terminal respiratory unit type adenocarcinoma. *American Journal of Surgical Pathology* **29**, 633–639.
- Yokomizo, A., Tindall, D. J., Drabkin, H., Gemmill, R., Franklin, W., Yang, P., Sugio, K., Smith, D. I. & Liu, W. (1998). PTEN/MMAC1 mutations identified in small cell, but not in non-small cell lung cancers. *Oncogene* **17**, 475–479.
- Zuo, Z., Chen, S. S., Chandra, P. K., Galbincea, J. M., Soape, M., Doan, S., Barkoh, B. A., Koeppen, H., Medeiros, L. J. & Luthra, R. (2009). Application of COLD-PCR for improved detection of KRAS mutations in clinical samples. *Modern Pathology* **22**, 1023–1031.