Original Article

Simultaneous analysis of *ALK*, *RET*, and *ROS1* gene fusions by NanoString in Brazilian lung adenocarcinoma patients

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Background: Gene fusions have been successfully employed as therapeutic targets for lung adenocarcinoma. However, tissue availability for molecular testing of multiples alterations is frequently unfeasible. We aimed to detect the presence of *ALK*, *RET*, and *ROS1* rearrangements by a RNA-based single assay in Brazilian lung adenocarcinomas and to associate with clinicopathological features and genetic ancestry.

Methods: From a FFPE series of 444 molecularly characterized lung adenocarcinomas, 253 *EGFR/KRAS* wild-type cases were eligible for gene rearrangement analysis. Following RNA isolation, *ALK*, *RET*, and *ROS1* rearrangements were simultaneously analyzed employing the ElementsXT Custom panel (NanoString Technologies). Rearrangements were further associated with clinicopathological features and genetic ancestry of the patients.

Results: The NanoString platform was performed in subset of 142 cases. Gene fusion results were conclusive for 94.4% (n=134) cases (failure rate =5.6%). *ALK* rearrangements were observed in 21 out of 134 cases, and associated with younger, never smokers, metastatic disease, and metastases in the central nervous system. *RET* and *ROS1* fusions were detected in two and one out of 134 cases, respectively. Genetic ancestry was not associated with gene fusions. Overall, considering all cases for which a molecular analysis was conclusive (*EGFR/KRAS/ALK/RET/ROS1*), *ALK* fusions frequency was observed in 6.5% (21/325), *RET* in 0.6% (2/325), and ROS1 in 0.3% (1/325).

Conclusions: This study successfully used a RNA-based single assay for the simultaneous analysis of *ALK*, *RET*, and *ROS1* fusions employing routine biopsies from Brazilian patients lung adenocarcinoma allowing an extensive molecular testing for actionable rearrangements contributing to guide clinical strategies.

Keywords: Non-small cell lung cancer (NSCLC); rearrangements; precision medicine; multiplexed analysis

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Introduction

Lung cancer is the leading cause of cancer deaths in the world (1). Non-small cell lung cancer (NSCLC) accounts for the majority of lung cancer cases (85%), with adenocarcinoma being the most common histologic subtype (1,2). Most of the NSCLC cases are diagnosed in late stages when treatment has no curative intent. The development of oncogene-driven therapies has revolutionized the treatment of NSCLC dramatically, increasing the overall survival of advanced NSCLC patients (3-6). *EGFR* mutations are the most frequent actionable alterations in patients with lung adenocarcinomas (2,7,8). Other driver genomic alterations are *ALK*, *RET*, and *ROS1* rearrangements, which can also be targeted for tyrosine kinase inhibitors (TKIs) (2,9). Although these genetic rearrangements are actionable, and the frequency of them is broadly investigated worldwide, the frequency of *RET* and *ROS1* rearrangements is unknown in Brazilian patients (10-12). Moreover, Brazilian patients have a high admixture background, with contribution from European, African, Native American (Amerindian), and, more recently, Asian ethnicities (8,13,14). Therefore, the impact of actionable alterations on clinicopathological characteristics of NSCLC patients should be deeply explored (8,10,11,15,16).

Although significant efforts have been made on molecular techniques for the detection of actionable alterations in NSCLC, the scarcity of tumor cells in tumor biopsies due to the sampling procedures remains a challenge for molecular analysis. Targeted panels and multiplexed assays have been employed for optimizing molecular analysis of actionable genes in NSCLC, including a sequential approach for analysis' feasibility on small tissue samples (17-22). We have previously reported the frequency of *EGFR* in 444 Brazilian lung adenocarcinomas and the association with Asian ancestry as well as the rate of *KRAS* mutations and its association with an unfavorable prognosis (8). In the present study, the *ALK*, *RET*, and *ROS1* rearrangements were analyzed employing a single multiplex assay, and their associations with clinicopathological features and genetic ancestry were investigated. The detection of *ALK*, *RET*, and *ROS1* rearrangements has a direct impact on clinical management of lung adenocarcinoma patients accruing in a shorter turnaround time through the employment of a single multiplexed assay enabling the tailored treatment as early as possible.

We present the following article in accordance with the STROBE reporting checklist (available at http://dx.doi.org/10.21037/tlcr-20-740).

Methods

Study population and design

This retrospective study was conducted at the Molecular Oncology Research Center, the Department of Pathology, and the Department of Molecular Diagnosis, from patients diagnosed with lung adenocarcinoma (n=444) at Barretos Cancer Hospital from 2011 to 2014. All sociodemographics and clinicopathological data were collected retrospectively from medical records. Patients' outcomes were collected from SISOnco (institutional software) and an active search was conducted when outcomes were not available from medical records (last updated: October 2019). Data on the main clinicopathological features and *EGFR/KRAS* mutation status and genetic ancestry of these cases were recently reported (8). Since the main molecular alterations in lung adenocarcinoma are well known to be mutually exclusive with *EGFR* and *KRAS* mutations, only *EGFR* / *KRAS* wild-type lung adenocarcinoma cases (n=253) were enrolled in the present study (*Figure 1*). However, due to a lack of available tumor tissue (n=91) or low RNA quantity (lower than 100 ng, n=20), gene fusions were evaluated in 142 *EGFR/KRAS* wild-type cases (*Figure 1*).

This study was approved by the local ethics committee (Barretos Cancer Hospital IRB/Project No. 630/2012), with the exemption of informed consent. The methodology was performed following the Declaration of Helsinki (as revised in 2013).

RNA isolation

RNA isolation was performed from formalin-fixed paraffin-embedded (FFPE) tumor samples, sectioned on slides with a thickness of 10μm as previously reported (23). One slide was stained with hematoxylin and eosin (H&E) and evaluated by an experienced pathologist for identification, sample adequacy assessment, and selection of the tumor tissue area (minimum of 70% tumor area). RNA was isolated using a commercial kit (RNeasy FFPE Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Simultaneous detection of ALK, RET and ROS1 rearrangements by NanoString custom panel

Detection of *ALK*, *RET*, and *ROS1* rearrangements was

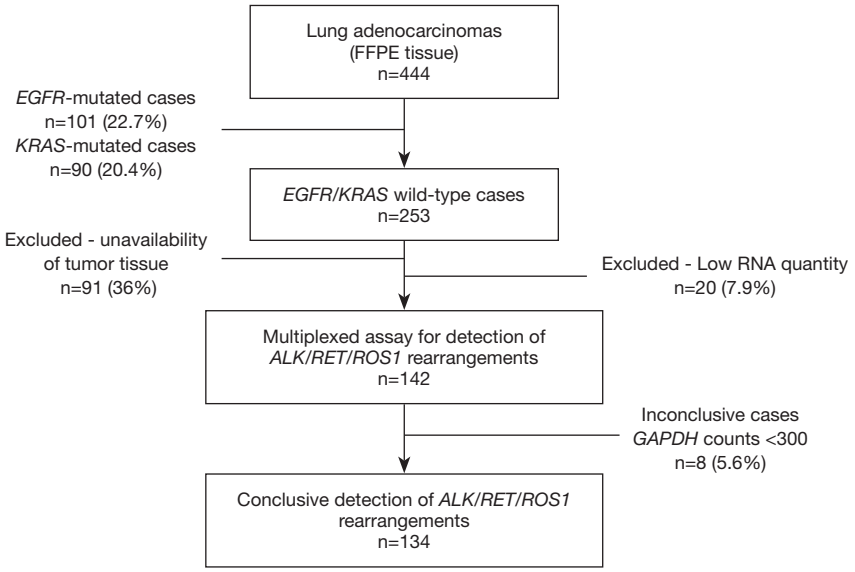

Figure 1 Sampling workflow for Brazilian lung adenocarcinoma series. FFPE, formalin-fixed paraffin-embedded tissue.

performed in 142 out of the 253 *EGFR/KRAS* wild-type cases since, in 91 cases, the tissue was unavailable and in 20 cases in which the RNA quantity was insufficient (*Figure 1*). The nCounter® Elements XT (NanoString Technologies, Seattle, WA, USA) custom panel was designed following previously described techniques for the evaluation of transcripts using specific probes. It contained 24 probes for the 5' and 3' regions of the *ALK*, *RET* and *ROS1* genes along with 12 specific probes for the rearrangement partners (*EML4-ALK*, *KIF5B-ALK*, *TGF-ALK; CCDC6-RET*, *KIF5B-RET*; *CD74-ROS1*, *EZR-ROS1*, *GOPC-ROS1*, *LRIG3-ROS1*, *SLC34A2-ROS1*, *TPM3-ROS1*, *SDC4-ROS1*) (20). Briefly, from 100 to 300 ng of total RNA samples were hybridized with specific probes for 21 hours at 67 °C. The hybridized complexes were purified in the PrepStation (NanoString Technologies) and immobilized in the cartridge. The cartridge was scanned by the Digital Analyzer (NanoString Technologies) for counting the transcripts (24). The positive controls used were an *ALK*-positive cell line (H2228 cell line) and a commercial control harboring *ALK*, *RET*, and *ROS1* rearrangements (Horizon Discovery, Cat. No.: HD784).

The transcripts counts were normalized by the nSolver Analysis® Software v4.0 (NanoString Technologies), using the ratio of geometric mean for each sample and arithmetic mean of all samples for positive assay controls and reference genes (housekeepings). Inconclusive results were considered when counts lower than 300 counts were obtained for *GAPDH*. The calculation of the imbalance probes was defined by the ratio between geometric mean of 3' probes and the average of 5' probes, considering thresholds for *ALK* rearrangement positivity equal to 2, for *RET* equal to 5 and for *ROS1* equal to 3, as previously reported (20,22). Imbalance analysis, detection of fusion partners (count more of 50), and graphical construction was performed in R environment v3.4.1 with scripts implemented in the local Galaxy server (25).

Detection of ALK, RET and ROS1 rearrangements by NanoString Lung Fusion Panel (nCounter Vantage 3D™)

Validation of the presence of *ALK*, *RET*, and *ROS1* rearrangements was performed using the Lung Fusion assay (NanoString Technologies). This assay was designed for the evaluation of transcripts using specific probes for the 5' and 3' regions from *ALK*, *RET*, and *ROS1* genes and specific probes for the rearrangement partners from *ALK*, *RET*, *ROS1* and *NTRK1* (no *NTRK* imbalance probes are provided in this assay). The transcription counts were normalized by the nSolver Analysis® Software v4.0 (NanoString Technologies). The calculation of the imbalance between the 3' and 5' probes was performed by a *t*-test comparing the log-scale data from probes. A significant P value provided the final positive result about fusion presence from the *t*-test plus the detection of fusion partners. These analyses were conducted using the Advanced Analysis v2.0

package (NanoString Technologies).

Fluorescence in situ hybridization (FISH)

Detection of *RET* and *ROS1* rearrangements was performed using commercial probes (ZytoLight SPEC *RET* Dual Color Break Apart, ZytoLight SPEC *ROS1* Dual Color Break Apart). Breast adenocarcinoma tissue was used as a negative control. For considering the sample suitable for evaluation, more than 15% of positive cells in at least 100 cells should be present. Hybridization reactions were repeated twice. FISHView 7.0 software (Applied Spectral Imaging) was employed for the analysis.

Ancestry analysis

The genetic ancestry background was previously assessed in the tumor DNA by a panel of 46-ancestry informative markers that allow estimating the ancestral proportions of African (AFR), European (EUR), Asian (ASN), and Native American (AME) populations (8).

Statistical analysis

Univariate (*t*-test/ Fisher's exact test/ chi-square test) and multivariate (Linear regression model) analyses were used to determine if gene rearrangements had a significant effect on the investigated parameters. Kaplan-Meier method and Log-rank test were used for univariate survival analysis, and Cox, the proportional hazards model, was used for multivariate survival analysis. For survival analysis, death was considered as an event, and live patients or patients who lost follow up were considered as censored. The survival analysis was conducted only for patients diagnosed at stage IV to decrease bias regarding the clinical outcome. Statistical analysis performed by IBM SPSS® Statistics Base software (IBM, Armonk, NY) with a 95% significance limit.

Results

Clinicopathological and sociodemographic features

The NanoString results were conclusive in 94.4% of the cases (n=134), resulting in a failure rate of 5.6% (8 out of 142 presented inconclusive results) (*Figure 1*). Overall, the majority of the patients was male (63%; n=85), with an average age of 60 years old, self-reported as white (77%; n=103), current or former smokers (75%; n=62 and n=38, respectively), diagnosed at stage IV (70%; n=94) and presented with metastasis in multiple sites at diagnosis (43%; n=58) (*Table 1*).

ALK rearrangements and clinicopathological associations

ALK rearrangements were detected in 15.7% of the *EGFR/KRAS* wild-type cases (21 out of 134) (*Figure 2*) (Table S1). The most frequently observed rearrangement partner was *EML4-ALK* (33.3%; 7 out of 21) (*Figure 2*). As expected, both commercial control and H2228 cell line were positive for *ALK* rearrangement, validating the assay. When considering all the 325 molecularly analyzed cases (134 plus 101 *EGFR*-mutated and 90 *KRAS*-mutated cases), we observed a frequency of 6,5% (21/325) of *ALK* gene fusions.

The presence of *ALK* rearrangements was associated with younger age at diagnosis (P=0.049), never smokers (P<0.0001), disease stage IV at diagnosis (P=0.019), metastases in multiple sites (P=0.003) and presence of metastases in central nervous system (CNS) (P=0.023; *Table 1*). Gender, self-reported color, loss of weight, genetic ancestry, ECOG PS were not associated with *ALK* rearrangements. The presence of *ALK* rearrangements was not associated with clinical outcome (P=0.486; Figure S1).

In multivariate analysis, the presence of *ALK* rearrangements was associated with never smokers (OR =12.432; P<0.0001; *Table 2*) and presence of metastasis in central nervous system (CNS) (OR =13.224; P=0.029; *Table 2*).

RET and ROS1 rearrangements

RET and *ROS1* rearrangements were detected in two and one out of 134 cases, respectively (*Figures 3*,*4*) (Table S1). No rearrangement partner was identified in the *RET* and *ROS1* positive cases. No statistical associations with the clinicopathological characteristics could be performed due to the low sample size. Overall, *RET* positive cases were male, never smokers, diagnosed at stage IV, and presented with metastases in the CNS. *ROS1* positive case was female, never smoker, diagnosed at stage IV, and presented with lymph node metastases.

When considering all the 325 molecularly for which a molecular analysis was conclusive (134 plus 101 *EGFR*-mutated and 90 *KRAS*-mutated cases), we observed a frequency of 0.6% (2/325), and 0.3% (1/325), for *RET* and *ROS1*, respectively.

Table 1 Association between *ALK* rearrangements and clinicopathological features and ancestry background of Brazilian lung adenocarcinoma patients (n=134)

Variables	Parameters	*ALK* rearrangement			
		n	Negative (%)	Positive (%)	P value
Age[1]	≤60 years	64	76.6	23.4	0.18
	>60 years	70	91.4	8.6	
Gender	Male	85	88.2	11.8	0.101
	Female	49	77.6	22.4	
Self-reported color[4]	White	103	85.4	14.6	0.209
	Brown	20	80	20	
	Black	7	85.7	14.3	
	Yellow	1	0	100	
	Missing	3			
Smoking Status	Never smoker	30	60	40	<0.0001
	Current	62	95.2	4.8	
	Former	38	86.8	13.2	
	Missing	4			
Disease staging at diagnosis	I e II	18	100	0	0.019
	III	22	95.5	4.5	
	IV	94	78.7	21.3	
Metastasis at diagnosis	No	39	97.4	2.6	0.003
	One site	37	89.2	10.8	
	Multiple sites	58	72.4	27.6	
Sites of Metastasis at diagnosis	No	39	97.4	2.6	
	CNS	35	77.1	22.9	0.023
	Others sites	60	80	20	
PS ECOG	0	10	90	10	0.417
	1	68	82.4	17.6	
	2	25	88	12	
	3	22	90.9	9.1	
	4	8	62.5	37.5	
	Missing	1			
Loss of weight[2]	No	60	85	15	0.907
	<10%	45	82.2	17.8	
	>10%	21	85.7	14.3	
	Missing	8			

Table 1 (*continued*)

Table 1 (*continued*)

Variables	Parameters	*ALK* rearrangement			
		n	Negative (%)	Positive (%)	P value
ASN ancestry[3]	Low	44	93.2	6.8	0.126
	Intermediate	43	81.4	18.6	
	High	46	8.3	21.7	
	Missing	1			
AFR ancestry[3]	Low	44	86.4	13.6	0.635
	Intermediate	44	84.6	13.6	
	High	45	80	20	
	Missing	1			
EUR ancestry[3]	Low	44	79.5	20.5	0.482
	Intermediate	44	84.1	15.9	
	High	45	88.9	11.1	
	Missing	1			
AME ancestry[3]	Low	44	88.6	11.4	0.410
	Intermediate	42	85.7	14.3	
	High	47	78.7	21.3	
	Missing	1			

n, number of patients; PS ECOG, performance status ECOG (Eastern Cooperative Oncology Group); CNS, central nervous system. [1]Age at diagnosis was dichotomized according to the average age of the series. [2]Loss of weight <10% and >10% of total body weight. [3]Cut off values were determined according to tercile categorization; [4]Self-reported race according to Brazilian Institute of Geography and Statistics (IBGE). ASN, Asian ancestry; AFR, African ancestry; EUR, European ancestry; AME, Amerindian ancestry.

Validation of the ALK, RET and ROS1 rearrangements

ALK rearrangements detection by NanoString using the same gene panel was previously validated by immunohistochemistry by our group (23). Concerning *RET* and *ROS1*, rearrangements were further analyzed by FISH; however the experiments were considered inconclusive due to the insufficient number of signals observed, probably due to pre-analytical issues of the tissue (Figure S2).

Due to unsuccessful attempts for confirming *RET* and *ROS1* rearrangements by FISH, we further validated the results for the cases exhibiting RET (n=2) and *ROS1* rearrangements (n=1) with the commercial Lung Fusion assay (Figure S3). Both *RET*-positive samples were confirmed as positive by the Lung Fusion assay (Figure S3A and S3C). In one of then, the partner was identified-*KIF5B* (Figure S3F). Although the known *KIF5B-RET* fusion partner was included in our custom panel, the variant (variant 12) that was detected is not included in our custom panel.

The *ROS1*-positive sample was also confirmed as positive by the Lung Fusion assay (Figure S3B), and no fusion partner was identified (Figure S3E).

Ancestry analysis

The mean of ancestry proportions observed among the 134 NSCLC patients was 74.7% for the EUR, 13.1% for the AFR, 5.8% for the AME, and 6.4% for the ASN (Figure S4). The mean of ancestry proportions observed among the 444 NSCLC patients was 73.1% for the EUR, 13.1% for the AFR, 6.5% for the AME, and 7.3% for the ASN (Figure S5 and Table S2) as previously reported (8). In accordance with a high percentage of EUR, most patients were self-declared white (*Table 1*). The presence of *ALK* rearrangements was not correlated with genetic ancestry (*Table 1*).

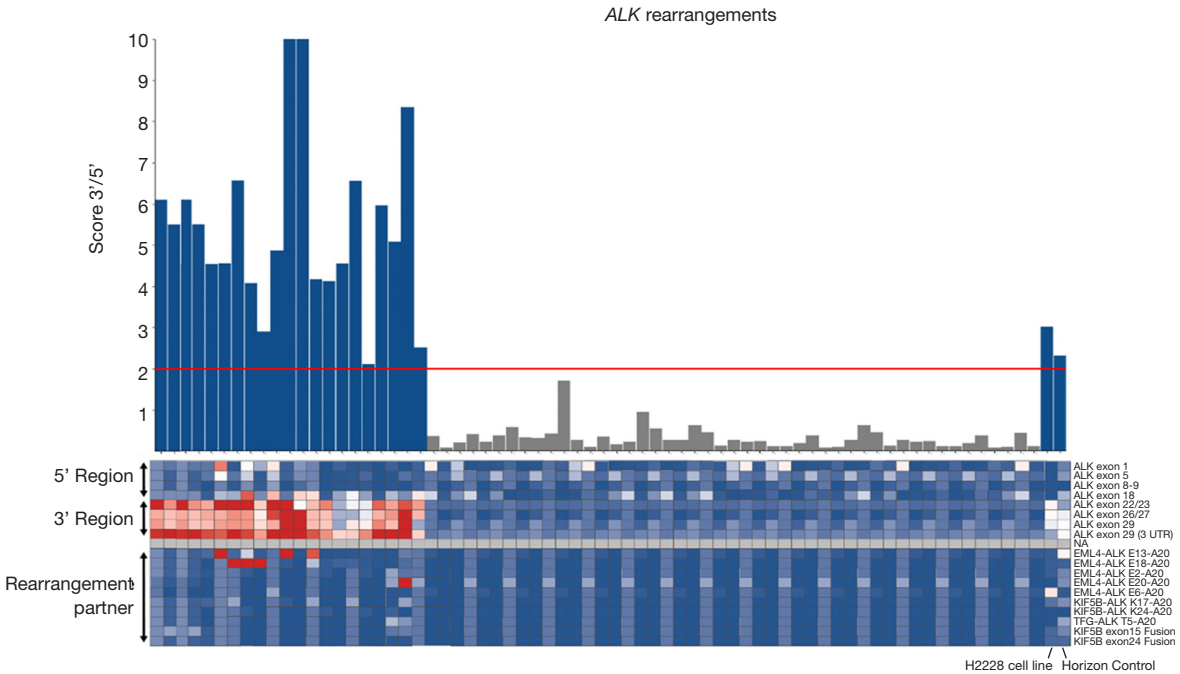

Figure 2 Representative graph of *ALK* rearrangements obtained from the analyzed samples (cut-off =2 for *ALK*) (20). The y-axis represents the packing ratio between the 3' and 5' regions for the *ALK* gene. The x-axis represents the RNA samples analyzed in the study.

Table 2 Multivariate analysis of the association between clinicopathological features and the presence of *ALK* rearrangements

Variables	Parameters	HR	95% CI	P value
Age	>60 years	Ref.	Ref.	Ref.
	≤60 years	0.338	0.103–1.077	0.067
Smoking Status	Current smoker	Ref.	Ref.	Ref.
	Former smoker	4.207	0.866–20.452	0.075
	Never Smoker	12.432	2.950–52.390	0.001*
Sites of Metastasis at Diagnosis	No	Ref.	Ref.	Ref.
	Yes, others sites	9.11	1.018–81.544	0.048*
	Yes, CNS	13.224	1.303–134.168	0.029*

Ref., reference variable; HR, hazard risk; CI, confidence interval; CNS, central nervous system; P value: significance of *t*-test. *Significant.

Although Barretos Cancer Hospital is located in the upstate of Sao Paulo, it is a reference center that assists patients from all over the country. Although not all Brazilian states are currently represented in our series and the Southeast of Brazil is the most represented region, all ancestry proportions are represented in the current series (Figure S6).

Discussion

The identification of actionable molecular alterations has conferred therapeutic relevance for advanced NSCLC patients. Although several studies have reported the frequency of these molecular alterations, they were performed mostly in European and Asian populations. Data on admixture populations remain lacking. In this

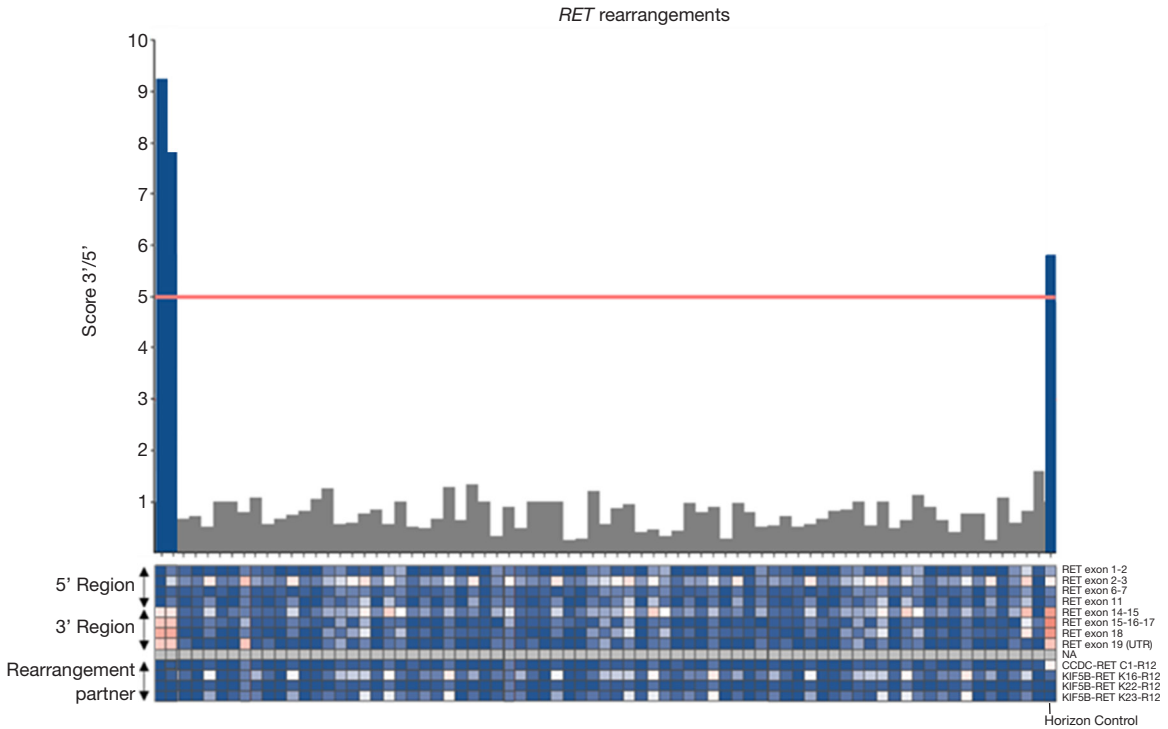

Figure 3 Representative graph of *RET* rearrangements obtained from the analyzed samples (cut-off =5 for *RET*) (20). The y-axis represents the packing ratio between the 3' and 5' regions for the *RET* gene. The x-axis represents the RNA samples analyzed in the study.

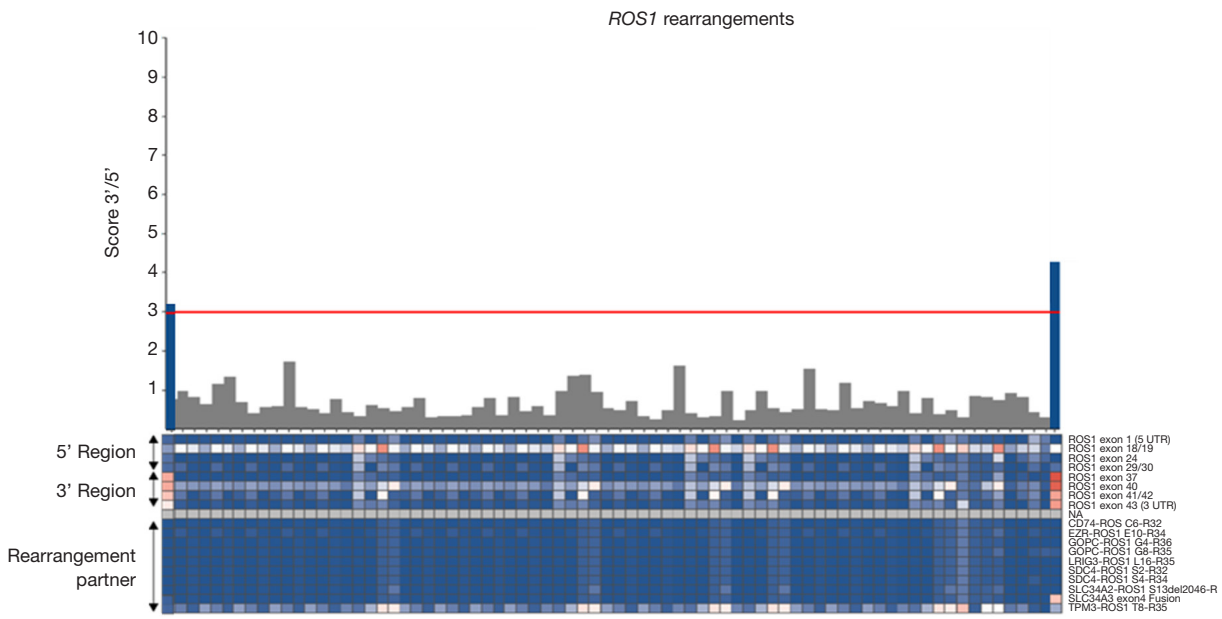

Figure 4 Representative graph of *ROS1* rearrangements obtained from the analyzed samples (cut-off =3 for *ROS1*) (20). The y-axis represents the packing ratio between the 3' and 5' regions for the *ROS1* gene. The x-axis represents the RNA samples analyzed in the study.

study, we reported the frequency of *ALK*, *RET*, *ROS1* rearrangements in a Brazilian series of lung adenocarcinoma using a single multiplexed assay, and the association of these rearrangements with clinicopathological features and ancestry.

In the present series, we have previously reported the frequency of *EGFR* (22.7%) and *KRAS* mutations (20.4%) (8). Regarding *ALK*, *RET*, and *ROS1* rearrangements, it is well known that they are all mutually exclusive and mutually exclusive with other driver mutations, so only *EGFR* and *KRAS* wild-type cases were evaluated (26-30).

We identified *ALK* rearrangements in 6.5% (21/325) of lung adenocarcinomas, which is in line with the reported worldwide. The frequency of *ALK* rearrangements in the literature varies from 3% to 10.8% (31-33). In Latin America, a study enrolling 5,130 NSCLC patients from 10 countries (excepting Brazil), the frequencies of *ALK* rearrangements ranged from 4.1% to 10.8% (Colombia 4.1%; Panama 4.4%; Uruguay 5.4%; Argentina 6.0%; Mexico 7.6%; Chile 8.6%; Venezuela 8.9%; Costa Rica 9.5%; Peru 10.8%) (34). In Brazil, two recent studies employing immunohistochemistry, one from Northeast and another from South, reported frequencies of *ALK* rearrangements of 10.4% (n=173 patients) and 4% (n=275 patients), respectively (10,12). The frequency of *ALK* rearrangements in the present work is in between other Brazilian studies. Such variation could be explained by the admixture background of the Brazilian population (35), since ancestry background is a factor that may influence the frequency of actionable alterations (36). Although the Southeast of Brazil is the most represented region in the current series, all ancestry proportions are indeed represented. Yet, our ancestry analysis did not identify any significant association.

We observed that the presence of the *ALK* rearrangements was associated with younger age, never smokers, and the presence of metastases in CNS, in line with some studies (37,38). Considering that *ALK*-positive patients are recurrently younger and associated with advanced disease at diagnosis, these cases may be more aggressive even from the beginning of the disease. For this reason, the identification of driver alterations becomes even more important to guide treatment with targeted therapies. *ALK*-positive patients are eligible for treatment with crizotinib (5). In this present study, only one *ALK*-positive patient enrolled in a clinical trial (phase III-2013) was treated with crizotinib. Although treatment with *ALK* inhibitors is approved by the Brazilian regulatory agency (ANVISA), patients do not have access to treatment via the public health system (39).

Regarding *RET* and *ROS1* rearrangements, the frequency observed in our overall series was 0.6% (2/325) and 0.3% (1/325), respectively. The frequency of *RET* rearrangements in lung adenocarcinoma is lower than 2% worldwide, ranging from 0.2-1.9% in Asian patients and 1.3% in patients from USA (27,32,40-43). The frequency of *ROS1* rearrangements in lung adenocarcinoma is variable, ranging from 1.2% in Europeans patients and reaching 3.4% in Chinese patients (40,42,44,45). There are no reports on the presence of *RET* and *ROS1* rearrangements in the Brazilian or any other admixture population. Due to the small number of *RET* and *ROS1*-positive cases, no statistical analysis could be performed. Anyhow, similarly to *ALK*-positive cases, the presence of *RET* and *ROS1* rearrangements were observed in younger patients, and never smokers (36,40,42,43,46,47). Despite the shallow frequency of *RET* and *ROS1* rearrangements, they constitute important therapeutic actionable alterations. The *ALK*-inhibitors, have also demonstrated success in the treatment of *ROS1*-positive patients, and it was approved by USA-FDA, and Brazilian ANVISA for these patients (4,48). Concerning *RET* rearrangements, the TKi selpercatinib (LOXO-292) has recently been approved by the FDA for the treatment of lung and thyroid tumors, due to the highly promising results obtained by clinical trials (ClinicalTrials.gov: NCT04268550 and ClinicalTrials.gov: NCT03157128) (49-51).

Due to a large number of driver alterations and the scarcity of tumor tissue usually available for molecular testing of NSCLC patients, the use of multiplexed assay platforms for FFPE can be a powerful tool. The NanoString technology is very robust, sensitive, easy to execute, with multiplex capabilities, and more cost-effective when custom panels are employed, surpassing FISH, IHC, and NGS techniques (22,23). We have previously shown that the NanoString platform managed to identify the presence of *ALK* rearrangements in FFPE samples, highlighting its capacity to detect RNA transcripts in highly degraded samples employing low RNA input (23). Likewise, in the extended study, the detection of rearrangements in FFPE samples was feasible, even in samples that lead to inconclusive FISH results. Moreover, due to the multiplex capability of the NanoString platform, it was possible to detect both gene rearrangements and known partners (20,22,23). Our panel includes the most observed rearrangement partners for each gene investigated as well as variants of these partners. Thus, it was possible

to identify the rearrangement partners for *ALK* and *RET*-positive cases in our study. Importantly, the possibility of custom panels, allowed to add novel gene rearrangements and new variants from fusion partners, such as with *NTRK* rearrangements (18,52). This maximizes the number of patients for oncogene-driven therapy for NSCLC with no additional tissue sample required and minimally increased in the test cost. In addition, the employment of a single multiplexed assay accrues in a shorter turnaround time for report release enabling the tailored treatment as early as possible.

One limitation of the present study is that the number of patients in the present study does not portrait the entire Brazilian lung cancer population, and further studies analyzing a higher number of cases and patients from all regions of Brazil are warranted.

Conclusions

Our study successfully used a single assay for the detection of *ALK*, *RET*, and *ROS1* fusions in FFPE biopsies of lung adenocarcinoma. The NanoString methodology allows an extensive molecular investigation of the significant actionable gene rearrangements potentially to be employed in the diagnostic routine for contributing to better guide clinical treatment strategies for Brazilian patients with lung adenocarcinoma.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the local ethics committee (Barretos Cancer Hospital IRB/ Project No. 630/2012), with the exemption of informed consent. The methodology was performed following the Declaration of Helsinki (as revised in 2013).

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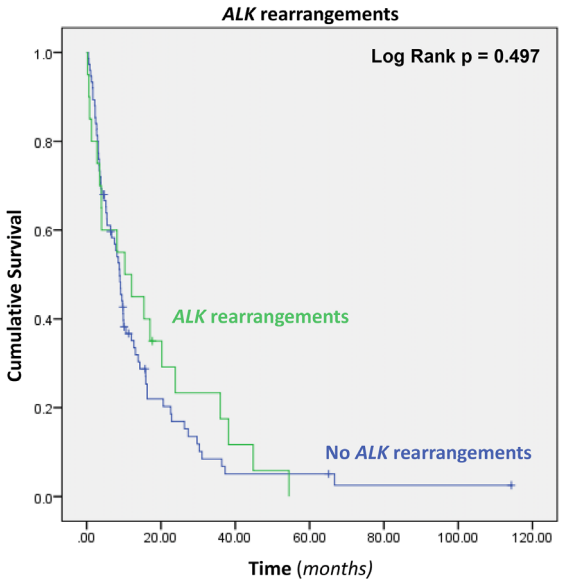

Figure S1 Kaplan-Meier curves for overall survival (OS) of NSCLC patients according to *ALK* positivity (mean OS: *ALK* Rearrangements negative = 14.81 months; Mean OS *ALK* Rearrangements positive = 16.65 months). Survival time is presented in months; p values are related to Log-rank test results.

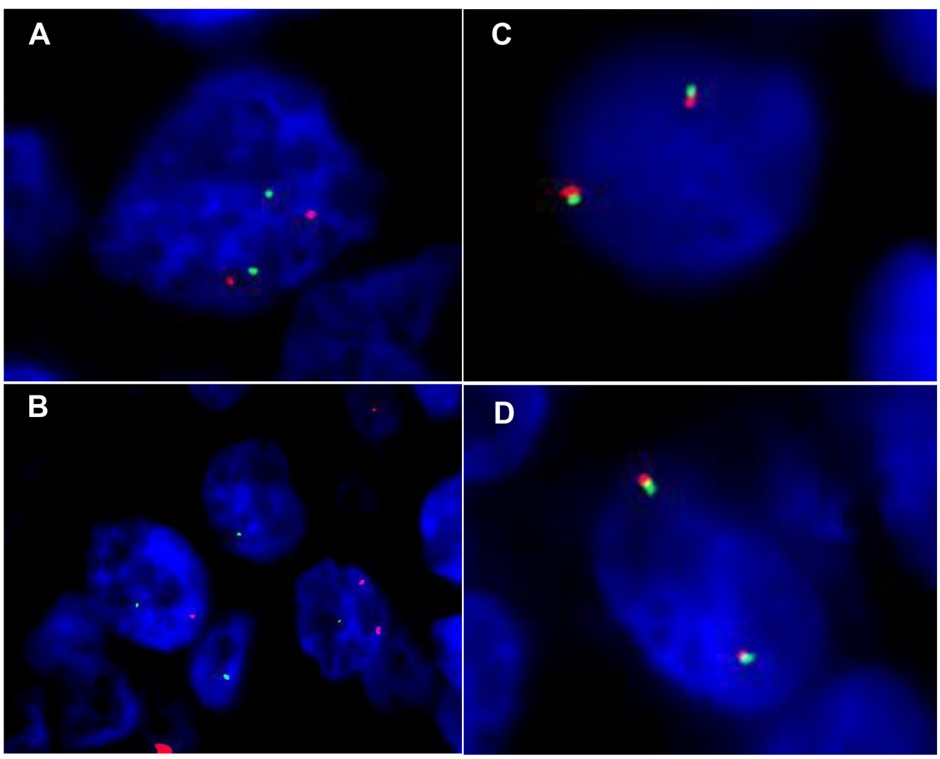

Figure S2 Representation of the fluorescence *in situ* hybridization (FISH) for (A) *RET* (n = 1) and (B) *ROS1* (n = 1) rearrangements (results obtained by the NanoString platform). These results obtained by FISH were considered "inconclusive" (low visualization of hybridization signal). Negative controls (breast tissue) for (C) *RET* and (D) *ROS1* rearrangements were also represented. All experiments were repeated twice.

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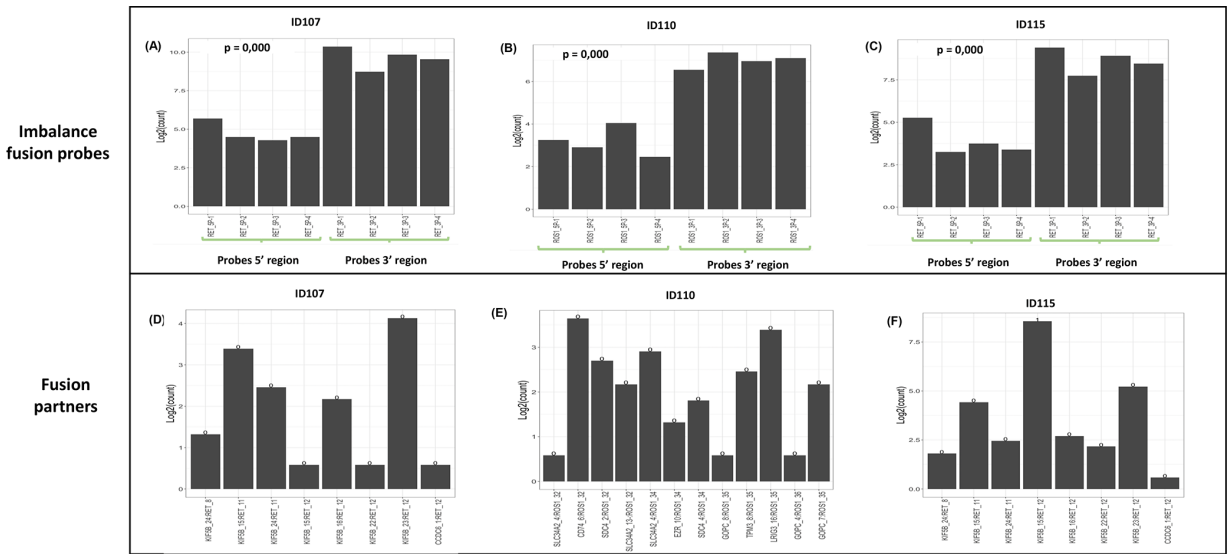

Figure S3 Representative graphs of the rearrangements obtained from the analyzed samples. The y-axis represents the significant count for imbalance probes between the 3' and 5' regions (A, B, C) and rearrangement partners (D, E, F) for the *RET* and *ROS1* genes. The x-axis represents the specific probes for 3' and 5' regions (A, B, C) and the *RET* and *ROS1* fusion partners (D, E, F). For the *RET* and *ROS1* fusion partners (D, E, F), "0" represents the absence of the specific fusion partner, and "1" represents the presence of the specific fusion partner.

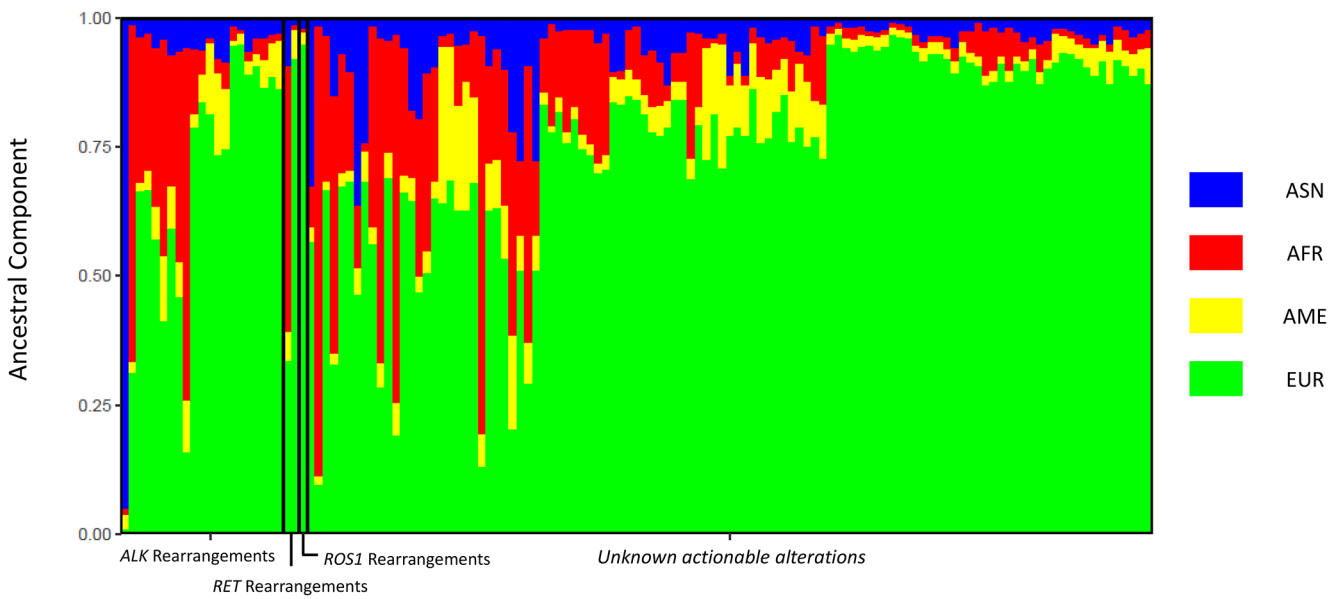

Figure S4 Genetic ancestry profiling of the Brazilian lung adenocarcinoma. Ancestry proportion of Brazilian patients (n=134) according to *ALK/RET/ROS1* positivity. The ASN (blue), AFR (red), EUR (green), AME (yellow) groups were used as reference populations. ASN, Asian ancestry; AFR, African ancestry; EUR, European ancestry; AME, Native American ancestry.

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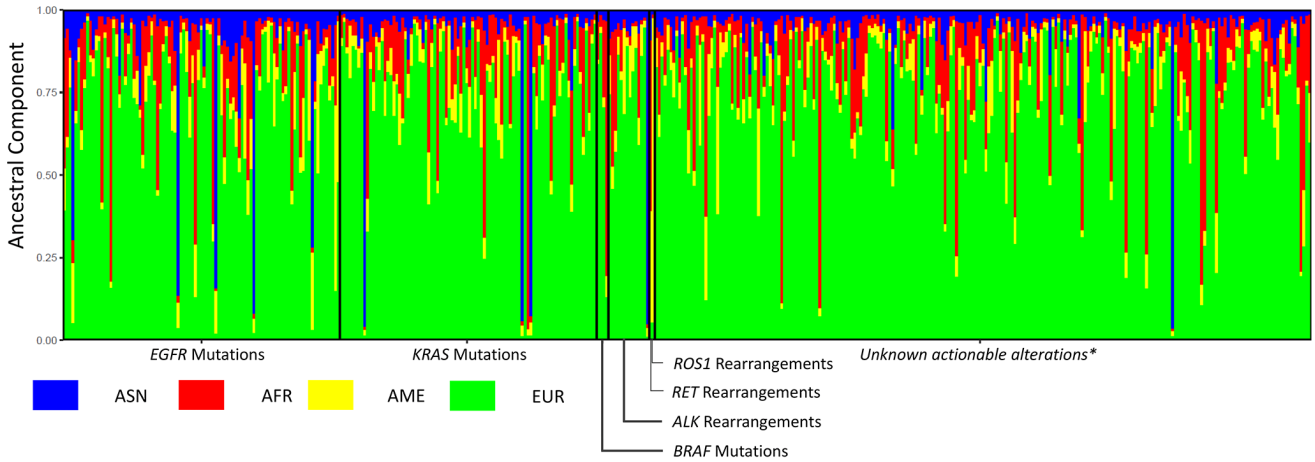

Figure S5 Genetic ancestry profiling of the Brazilian lung adenocarcinoma. Ancestry proportion of Brazilian patients (n=444) according to driver alterations. The ASN (blue), AFR (red), EUR (green), AME (yellow) groups were used as reference populations. ASN, Asian ancestry; AFR, African ancestry; EUR, European ancestry; AME, Native American ancestry.

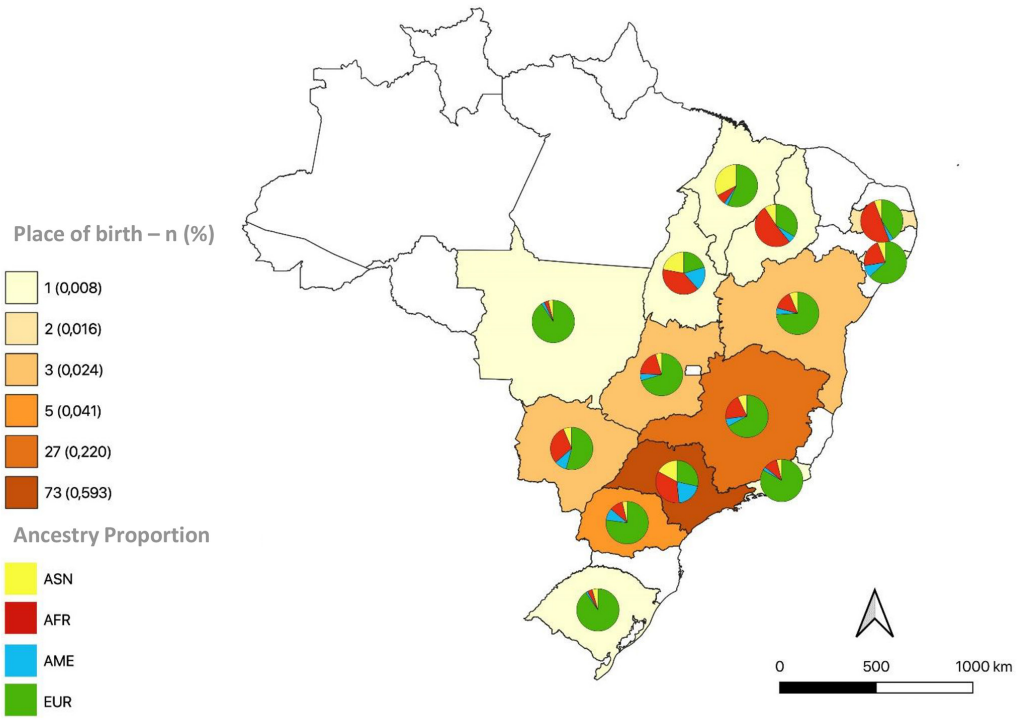

Figure S6 Geographical origin and ancestry proportions. Place of birth along with ancestry proportions of each state for *ALK*-, *RET*, *ROS1*-positive patients and patients with unknown actionable alterations included in the present study (n=143). The ASN (yellow), AFR (red), EUR (green), AME (blue) groups were used as reference populations. ASN, Asian ancestry; AFR, African ancestry; EUR, European ancestry; AME, Native American ancestry.

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Table S1 Report from Galaxy server of ratios of imbalance of ALK, RET and ROS1 in samples analyzed

	ALK ratio	RET ratio	ROS1 ratio
Horizon Commercial Control	2.59	6.19	4.76
H2228 cell line	3.54	0.33	0.50
Sample 1	6.10	0.67	0.90
Sample 2	6.66	0.70	1.02
Sample 3	5.49	0.19	0.50
Sample 4	4.56	1.06	0.14
Sample 5	6.56	0.33	0.06
Sample 6	4.07	0.76	0.48
Sample 7	2.90	0.54	0.10
Sample 8	4.87	0.60	1.88
Sample 9	1.58	0.57	1.30
Sample 10	1.92	0.18	0.72
Sample 11	8.17	0.49	1.27
Sample 12	8.55	0.13	0.83
Sample 13	2.43	0.36	1.10
Sample 14	4.30	0.24	1.25
Sample 15	6.71	1.08	1.19
Sample 16	3.07	1.19	1.10
Sample 17	3.49	0.29	1.07
Sample 18	5.96	0.17	0.51
Sample 19	5.08	0.20	1.36
Sample 20	8.12	0.49	1.27
Sample 21	2.43	0.36	1.10
Sample 22	0.39	9.85	0.13
Sample 23	0.20	7.96	0.36
Sample 24	0.52	0.64	3.02
Sample 25	0.49	1.11	0.08
Sample 26	0.88	1.00	1.08
Sample 27	1.57	1.00	0.97
Sample 28	0.28	0.27	0.75
Sample 29	0.45	0.33	0.64
Sample 30	0.33	0.85	0.33
Sample 31	0.33	0.19	0.08
Sample 32	0.24	0.70	0.20
Sample 33	0.24	0.49	0.41

Table S1 (*continued*)

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Table S1 (*continued*)

	ALK ratio	RET ratio	ROS1 ratio
Sample 34	1.70	0.58	1.05
Sample 35	0.20	0.56	0.36
Sample 36	0.05	0.12	0.05
Sample 37	0.19	0.39	0.46
Sample 38	0.23	1.39	0.35
Sample 39	0.17	0.21	0.77
Sample 40	0.09	0.36	0.05
Sample 41	0.13	0.84	0.18
Sample 42	0.53	0.30	1.62
Sample 43	0.61	0.80	0.66
Sample 44	0.32	0.23	0.16
Sample 45	0.44	0.29	0.20
Sample 46	0.41	0.44	1.18
Sample 47	0.47	1.17	0.27
Sample 48	0.52	0.54	0.21
Sample 49	0.80	0.75	0.68
Sample 50	0.11	0.27	0.08
Sample 51	0.12	0.16	0.23
Sample 52	0.56	0.58	1.78
Sample 53	0.15	0.13	0.10
Sample 54	0.20	0.21	0.11
Sample 55	0.79	1.10	0.88
Sample 56	0.37	0.56	0.60
Sample 57	0.24	1.23	1.13
Sample 58	0.34	0.87	0.03
Sample 59	0.33	0.20	0.45
Sample 60	0.33	0.28	0.10
Sample 61	0.31	0.18	0.04
Sample 62	1.78	0.57	0.12
Sample 63	0.35	0.17	1.09
Sample 64	0.46	0.28	0.35
Sample 65	0.51	1.14	1.06
Sample 66	0.41	0.28	0.20
Sample 67	0.33	0.21	0.93
Sample 68	0.35	0.49	0.76

Table S1 (*continued*)

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Table S1 (*continued*)

	ALK ratio	RET ratio	ROS1 ratio
Sample 69	0.37	0.31	0.89
Sample 70	0.48	0.25	0.23
Sample 71	0.40	0.12	0.06
Sample 72	0.43	0.78	0.04
Sample 73	0.71	0.67	0.19
Sample 74	1.76	0.51	1.01
Sample 75	0.70	0.67	0.13
Sample 76	0.43	0.36	0.49
Sample 77	0.79	0.52	0.50
Sample 78	0.20	0.10	1.07
Sample 79	0.20	0.42	0.32
Sample 80	0.26	0.20	1.03
Sample 81	0.55	0.70	0.09
Sample 82	0.68	0.31	0.10
Sample 83	0.19	1.03	0.06
Sample 84	0.31	0.04	0.07
Sample 85	0.19	0.82	0.09
Sample 86	0.71	0.12	0.77
Sample 87	0.97	0.90	0.86
Sample 88	0.76	0.09	2.05
Sample 89	0.82	0.89	0.95
Sample 90	0.20	1.05	0.77
Sample 91	0.80	1.00	1.05
Sample 92	1.00	1.00	1.00
Sample 93	1.08	0.93	0.50
Sample 94	1.00	1.00	1.34
Sample 95	0.50	0.62	0.54
Sample 96	0.33	0.60	0.71
Sample 97	0.94	0.58	1.13
Sample 98	1.00	1.03	0.67
Sample 99	0.46	0.79	1.49
Sample 100	0.98	0.94	0.65
Sample 101	1.56	1.03	0.71
Sample 102	0.40	0.57	0.79
Sample 103	0.50	2.29	0.81

Table S1 (*continued*)

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Table S1 (*continued*)

	ALK ratio	RET ratio	ROS1 ratio
Sample 104	0.28	0.50	0.65
Sample 105	0.15	0.55	0.35
Sample 106	0.51	0.55	0.70
Sample 107	0.91	0.95	0.95
Sample 108	0.68	1.02	0.91
Sample 109	0.49	0.98	0.67
Sample 110	0.62	0.72	0.87
Sample 111	0.36	0.76	0.65
Sample 112	0.46	0.97	1.46
Sample 113	0.33	0.65	0.67
Sample 114	1.19	0.94	0.86
Sample 115	0.67	1.02	1.21
Sample 116	0.27	0.74	0.96
Sample 117	0.37	0.76	0.91
Sample 118	0.53	0.63	0.84
Sample 119	0.42	0.68	0.91
Sample 120	1.90	0.87	0.89
Sample 121	0.56	0.82	0.54
Sample 122	0.49	0.71	0.82
Sample 123	0.45	1.14	0.40
Sample 124	0.46	1.14	0.79
Sample 125	0.60	1.07	0.93
Sample 126	0.60	0.82	1.45
Sample 127	0.14	0.76	1.29
Sample 128	0.67	0.83	0.54
Sample 129	1.89	0.65	0.77
Sample 130	0.53	0.85	0.61
Sample 131	0.71	1.20	0.57
Sample 132	0.75	0.63	0.54
Sample 133	0.44	0.57	0.92
Sample 134	0.20	1.02	0.86

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Table S2 Ancestry background categorization of Brazilian lung adenocarcinoma patients (n=444), according to tercile based on the percentage proportions for ethnic groups

Genetic Ancestry	Low	Intermediate	High
ASN	<0.028	0.028 - 0.055	>0.055
AFR	<0.027	0.027 - 0.125	>0.125
AME	<0.029	0.029 - 0.058	>0.058
EUR	<0.698	0.698 - 0.865	>0.865

Category boundaries were defined according to tercile categorization; ASN, Asian ancestry; AFR, African ancestry; EUR, European ancestry; AME, Amerindian ancestry.

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