The future of lung cancer therapy: Striding beyond conventional *EGFR* and *ALK* treatments

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Received February 20, 2018; Accepted October 16, 2018

DOI: 10.3892/mco.2019.1811

Abstract. Lung cancer, one of the most frequently diagnosed cancers worldwide has long relied on testing for the molecular biomarkers *EGFR*/*ALK*. However, achieving superior clinical outcomes for patients with lung cancer requires developing comprehensive techniques beyond contemporary *EGFR*/*ALK* testing. Current technologies are on par with molecular testing for *EGFR*/*ALK* in terms of efficacy, most of them failing to offer improvements perhaps primarily due to skepticism among clinicians, despite being recommended in the NCCN guidelines. The present study endeavored to minimize chemotherapy-dependence in *EGFR*/*ALK*-negative patient cohorts, and use evidence-based methods to identify ways to improve clinical outcomes. In total, 137 lung cancer cases obtained from 'PositiveSelect NGS data', comprising 91 males and 46 females, were investigated. *EGFR*- and *ALK*-positivity was used for data dichotomization to understand the therapeutic utility of rare gene alterations beyond just *EGFR*/*ALK*. Statistics obtained from PositiveSelect were collated with data from international studies to construct a meta-analysis intended to achieve better clinical outcomes. Upon dichotomization, 23% of cases harbored *EGFR* variants indicating that treating with *EGFR* TKIs would be beneficial; the remaining 77% exhibited no *EGFR* variants that would indicate favorable results using specific currently available chemotherapy practices. Similarly, 28% of cases had *EGFR+ALK* variants favoring *EGFR*/*ALK*-based targeted therapeutics; the remaining 72% harbored no *EGFR*/*ALK* variants with known beneficial chemotherapy routes. The present study aimed to overcome current inadequacies of targeted therapies in patients with a conventional *EGFR*/*ALK*-positive diagnosis and those in *EGFR+ALK*-negative cohorts. Upon analysis of the negative cohorts, significant and clinically relevant single nucleotide variants were identified in *KRAS*, *ERBB2*, *MET* and *RET*, with frequencies of 7, 1, 2 and 3% in patients who were *EGFR*-negative and 6, 1, 1, and 3% in patients who were *EGFR* and *ALK*-negative, respectively, enabling the use of targeted therapeutics aside from *EGFR*/*ALK* TKIs. From the results of the current study only 35% of the two negative arms (*EGFR* negative and *EGFR+ALK* negative) would be recommended NCCN or off-label chemotherapy; prior to the current study, the entire cohorts would have been recommended this treatment. The present study emphasizes the potential of comprehensive genomics in identifying hallmarks of lung cancer beyond *EGFR*/*ALK*, using broad-spectrum genetic testing and data-sharing among medical professionals to circumvent ineffective chemotherapy.

Introduction

Epidermal growth factor receptor (*EGFR*), present on the cell surface, facilitates intercellular communication. *EGFR* selectively targets growth signals and permits the transfer of information (1,2). Cancer, being a progressive disease, employs *EGFR* as a target, which, when impaired, can successfully deregulate the downstream cascade and favor tumorigenesis. As per mycancergenome (https://www.mycancergenome.org/content/disease/lung-cancer/), ~35% of patients with non-small cell lung cancer (NSCLC) in East Asia have tumors associated with positive *EGFR* expression.

Clinical diagnostics have evolved through multiple facets, allowing difficult medical decisions to be accomplished easily. Cancer is one field where opinions are widespread and diverse, requiring extensive research and development. Cancer diagnostics are complex and always changing, involving techniques such as tissue-based immunohistochemistry (IHC), chromosome based fluorescence *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH), DNA-based gold-standard sequencing techniques such as Sanger sequencing, and next generation sequencing (NGS). These

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Key words: epidermal growth factor receptor, NCCN, targeted therapy, chemotherapy, next generation sequencing

techniques not only support the diagnosis of cancer, but also help to predict treatment success and repercussions. NGS encompasses DNA-based genetic modifications, such as single nucleotide variations (insertions, deletions and rearrangements), in its therapeutic prediction, using massive parallel sequencing operandi. Single nucleotide variations constitute the substitution of one nucleotide base for another, thus impairing the formation of hydrogen bonds between the strands. These variations can be synonymous or non-synonymous; when they occur at conserved domains they are able to impair normal function of the translated protein. Insertions and deletions refer to the introduction or removal of one or more nucleotides from the DNA strand. Rearrangement variations occur because of chromosomal breakage causing broken segments to adhere elsewhere on the chromosome. These can include small or large segments of DNA, with effects depending on the size and domain. NGS has been gradually introduced into many clinical fields, especially oncology, for which genetic modifications are a major factor (3). Lung cancer, one of the most common types of cancer, has *EGFR* as a major therapeutic predictor. The present study highlights the significance of other genomic alterations in the therapeutic prediction of lung cancer, most of which are frequently neglected.

Notable associations have been documented between somatic mutations (deletions and single nucleotide variants) in *EGFR* exons 19 and 21 and the corresponding tyrosine kinase inhibitors (TKIs). Small molecule TKIs and monoclonal antibodies are major treatment regimens pursued for cases of lung cancer (4). *EGFR* TKIs have also been approved by the Food and Drug Administration (FDA) as a first line regimen for the treatment of NSCLC. Of all *EGFR* TK domain mutations, 50% were exon 19 in-frame deletions, 42% were exon 21 missense mutations, 7% were exon 18 mutations, and 3% were in exon 20 (5,6). Noronha *et al* (6) showed that, the overall frequency of *EGFR* mutations, including single nucleotide alterations, deletions and insertions, in cases of Indian patients with adenocarcinoma is 26%, compared with 3.8% in squamous cell carcinoma. TKIs and monoclonal antibodies are useful in the presence of somatic *EGFR* mutations, the absence of which creates agitation in treating oncologists, as patients are unable to have TKI treatment (7). Thus, new methods and techniques for managing lung cancer, beyond *EGFR*, are required.

Technology improved clinical diagnostic and therapeutic methods at an enormous rate over recent years. Single gene testing is used for the effective determination of mutations in established genes and regions (8,9). Routine diagnostic testing of functionally significant genes is recommended by NCCN guidelines as it is beneficial in the treatment of lung cancer, generally using formalin-fixed paraffin-embedded (FFPE) tissue extracted from tumor samples. Despite its clinical significance, the method also presents uncertainty with 'tumor heterogeneity' (10-12). To overcome the limitations of hotspot testing, comprehensive genomics-based testing panels have been developed to provide a comprehensive picture of the genome, exome or targeted regions to study various other rare or barely studied oncogenic drivers (13-15). It is a technique with a great capacity to identify alterations beyond *EGFR*, along with other benefits such as early treatment response and resistance evaluation, the assessment of molecular heterogeneity, early detection of disease, and, most importantly, the identification of genetic determinants for targeted therapy (16-18). Herein, we present our data analysis and characterization of pathogenic and targetable variants beyond *EGFR* for the effective prediction of clinical outcomes in lung cancer.

Materials and methods

Reports were investigated pertaining to 137 patients with lung cancer who had previously undergone commercially available comprehensive hybrid capture NGS (PositiveSelect). This included patients with different stages of the disease and a wide therapeutic spectrum. All cases were predominantly diagnosed at stage 4 with progressive disease condition and metastasis, as presented in Table I. Few had previously undergone molecular testing for NCCN recommended genes like *EGFR*, *ALK* or *ROS1*. Molecular diagnostics mostly comprised *EGFR*, with occasional testing for *ALK* and other NCCN recommended genes. A few cases were previously treated with targeted and chemotherapeutics based on hotspot molecular diagnostics reports and NCCN guideline recommendations, respectively. The PositiveSelect assay protocol for NGS (sequencing and analysis) employed in the study is not yet been published. The parameters utilized for NGS are briefly described below. Hybrid capture libraries of 350 genes and 35 selected introns frequently rearranged in cancer were sequenced in parallel with a high, uniform coverage (x1,000). The resultant raw data were demultiplexed using NextSeq reporter software (CASAVA 2.2; Illumina, Inc., San Diego, CA, USA) for acquisition of individual samples. The acquired data are often subjected to trimming to eliminate primer artifacts, followed by alignment with a human reference genome, variant calling and prioritization. Strict cut-off parameters were subjected to variant calling algorithms that segregate variants of allelic fractions greater than 1 as clonal and those greater than 0.1, but less than 1 were regarded as subclonal variations for further analysis. The detection of subclonal alterations was accomplished effectively with the utilization of liquid biopsy-based samples. The algorithm further followed annotation according to databases with notable functional evidence of genomic alterations, which includes dbsnp, ClinVar, COSMIC, TCGA and ExAC, among others. Interpretation of the results holds significance for the determination of clinically relevant classes of alterations, such as base-pair substitutions, insertions/deletions, copy number alterations and rearrangements. Clinically relevant genomic alterations were thus defined as those associated with responses to therapies currently available or in target-driven clinical trials. In the present study the clinically relevant alterations were dichotomized based on *EGFR* and *ALK* positivity to understand the therapeutic utility of rare and frequently occurring alterations beyond *EGFR* and *ALK*. The analytical perspectives from the clinically relevant classes of alterations characterized by PositiveSelect were collated and assessed with international studies, constructing a pooled meta-analysis executed from cBioPortal and proving clinical concordance of our study with the international study spectrum (http://www.cbioportal.org/).

Results

Our analysis revealed a notable percentage of genomic alterations beyond *EGFR* and *ALK* recommended by NCCN

Table I. Cohort characteristics included in our analysis.

	Total number of cases (n=137)			
Age (years)	Total (n)	Males (n=91)	Females (n=46)	Disease stage
30-40	7	5	2	4
40-50	20	13	7	2, 3B and 4
50-60	47	29	18	1C, 2A, 3B and 4
60-70	37	26	11	2, 3B and 4
70-80	26	18	8	3 and 4

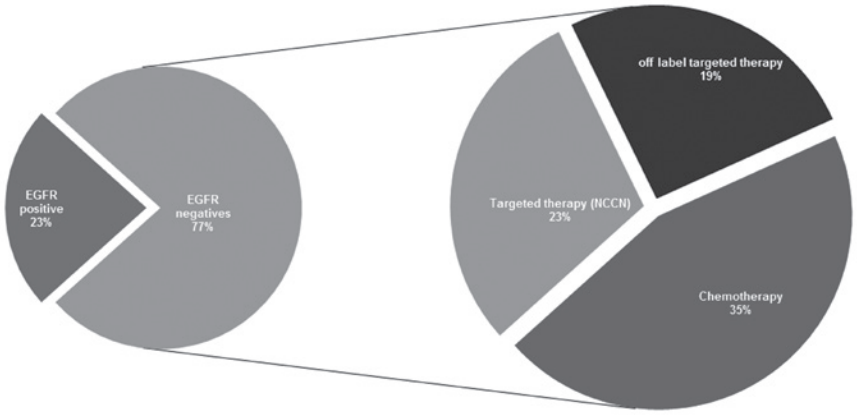

Figure 1. Comprehensive genome analysis aimed to extend the options of targeted therapeutics in EGFR-negative cases. In depth analysis of the EGFR-negative cohort revealed that 42% were eligible for NCCN or off-label targeted therapy. EGFR, epidermal growth factor receptor; NCCN, National Comprehensive Cancer Network.

guidelines in the study population, which are as follows: Single nucleotide variations in *KRAS*, *RET* and *BRAF* were detected at 6, 2 and 2%, respectively; single nucleotide variation and amplification detected in *MET* and *ERBB2* at 3 and 2%, respectively. Though the percentages do not appear appreciable, these become significant when considered in light of the clinical implications. This verifies the study objective of moving beyond *EGFR* and *ALK* to achieve better clinical outcomes.

EGFR positivity and beyond. Of the 137 lung cancer cases in our cohort, 23% (n=31) were identified to be *EGFR*-positive, indicating benefit from the use of *EGFR*-TKI. However, the 77% (n=106) of patients were *EGFR*-negative cases, which still pose a therapeutic challenge. The present study provides new hope for the treatment of patients who fall under the criteria of *EGFR*-negative status. It was identified that 23% of patients, who were *EGFR*-negative, may benefit from NCCN-recommended targeted therapeutics as they possess non-*EGFR* genomic alterations, while 19% of patients, who were *EGFR*-negative, were identified to have other gene alterations indicating that they may benefit from the use of off-label targeted therapeutics. The remaining 35% of patients, who were *EGFR*-negative, become eligible for NCCN-recommended, and/or off-label chemotherapy, which would have otherwise been the entire 77% as per current clinical practice. This finding has also been summarized in Fig. 1. According to our analysis, of the *EGFR*-negative population that may benefit from NCCN guideline recommended targeted

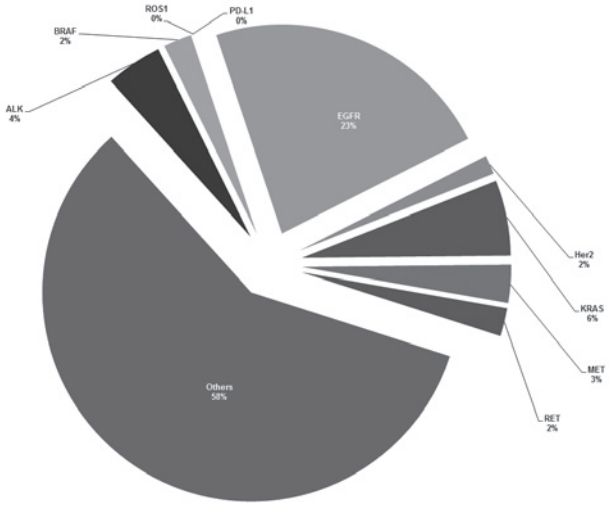

Figure 2. Frequency distributions as detected by broad-spectrum genome analysis with distinctive emphasis on NCCN-recommended genes for lung cancer. Fig. 2 shows the representation of gene frequency dispersal, with 42% constituting NCCN guideline-recommended genes for lung cancer. The figure also highlights other significant gene variants, which comprises 58% of the cohort, which may predict future therapeutic efficacy. NCCN, National Comprehensive Cancer Network.

therapeutics, we identified 7% (n=7) to bear a *KRAS* pathogenic single nucleotide mutation, which allows the utilization of downstream *MEK*, *PIK3CA* and *mTOR* inhibitors, such as

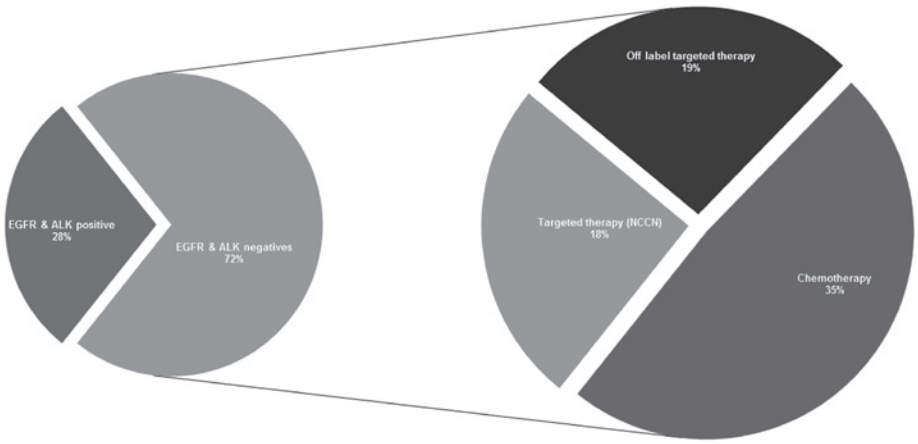

Figure 3. Targeted therapy distributions in *EGFR+ALK* negative cases. Of the cohort proportion identified as *EGFR+ALK* negative, 37% were eligible for NCCN or off-labeled targeted therapy. *EGFR*, epidermal growth factor receptor; NCCN, National Comprehensive Cancer Network.

Trametinib, Buparlisib and Everolimus, to target the downstream *KRAS*/*MEK*/*ERK* and *KRAS*/*PIK3CA*/AKT/*mTOR* signaling pathways, respectively. Apart from *KRAS*, other pathogenic alterations detected include *ALK* rearrangements in 3% (n=3), and *ALK* resistance mutations detected in 4% (n=4), *BRAF* in 2% (n=2), *ERBB2* amplifications and mutations detected in 1% (n=1) each, *MET* in 2% (n=2) and *RET* in 3% (n=3). A summary of the overall variant frequencies of NCCN recommended genes in the 137 lung cancer cases is presented in Fig. 2.

EGFR and ALK dichotomy. Based on *EGFR* and *ALK* dichotomization, our study indicated that only 28% of the total cohort was eligible for targeted therapy based on *EGFR* and *ALK* positivity, taken as the two most frequently tested genes in lung cancer therapeutics. The remaining major cohort (72% of patients), who were negative for *EGFR* and *ALK*, received conventional chemotherapy treatment rather than being subjected to further molecular tests. Contrary to typical approaches, our comprehensive NGS analysis identified other favorable treatment options for the *ALK*- and *EGFR*-negative cohort, of whom 18% harbored genomic alterations which made them eligible for NCCN guideline recommended targeted therapy. A further 19% of the cohort was identified to benefit from off-label targeted therapy due to alterations detected in *PTEN* and *TSC1/2*. Specifically, we detected *KRAS* pathogenic single nucleotide variations in 6% (n=6) and amplifications in 2% (n=2) of the negative population. Furthermore, 35% of the patients who were *ALK* negative and *EGFR* negative, with no additional genomic alterations, have been as potentially receiving benefiting from chemotherapy, as no additional genomic alterations were identified as highlighted in Fig. 3.

Meta-analysis and a pooled assessment with International Lung Cancer studies. During a meta-analysis of three international studies on the variant distribution frequency of the nine NCCN recommended lung cancer genes, we observed concordance between our data and the global published literature (Table II) (19-21). Of the 137 lung cancer cases analyzed in this study, 23% (n=31) were identified with targetable *EGFR* variants, marginally concordant with the 19% reported in a relevant international lung cancer study in 2012 by Imielinski *et al* (19) (Table II). Additionally, *ALK* (4%), and *RET* (2%) correlated with the statistics unveiled by the Broad Institute of Harvard and MIT (Cambridge, MA, USA) in their study, which reported frequencies at 4 and 2% for *ALK* and *RET*, respectively (19). Additional similarities were observed between our frequencies and those reported by international studies, validating the detection of additional genomic alterations in *EGFR* and *ALK* mutation-negative cohorts (20,21). The results of the aforementioned study demonstrated that 20% of the mutations harbored by the *EGFR*-negative cohort occurred in other significant genes, including *PIK3CA*, *CDK4/6* or *CCND*, *KDR*, *TSC1/2*, *mTOR*, *TP53* and DNA repair genes recommended as drug targets by the NCCN. This enhances the clinical benefits of the patients harboring variants in these genes. It is evident that this 20% population from the *EGFR*-negative cohort in the current study may also benefit from the aforementioned gene-targeted therapy.

However, 44% of the total cohort harbored ≥1 gene mutation from the NCCN recommended panel for lung cancer. Ideally, patients can be recommended agents with an improved therapeutic benefit based on if they possess targetable and clinically actionable gene mutations aside from the NCCN recommended genes that may distinguish them from the conventional chemotherapy group. A compilation of identified mutations, including single nucleotide variations, insertions/deletions and copy number alterations, in *EGFR*- and *ALK+EGFR*-negative cohorts, and their corresponding recommended therapeutics, is listed in Table III.

Discussion

In lung cancer, *EGFR* is the most commonly targeted gene and is regarded as a potential candidate for small molecule therapeutics. The prevalence of *EGFR* mutations across various countries was highlighted by a study conducted at Tata Memorial Hospital, and has been tabulated in Table S1. Targeting *EGFR* has been a hot research topic in the scientific community, and has modeled current therapeutic interventions for lung cancer (22,23). The present analysis is based on the

Table II. Comparison of frequencies with published international literature.

	Gene variant distribution % (n) in all lung cancer cases					Gene variant distribution % (n) in *EGFR* negative lung cancer cases				
	1. Broad (19)	2. Nature (20)	3. MSKCC (21)	Meta analysis (1, 2 and 3)	Positive Select	1. Broad (19)	2. Nature (20)	3. MSKCC (21)	Meta analysis (1, 2 and 3)	Positive Select
EGFR	19 (34)	3 (4)	6 (2)	12.3 (40)	23 (31)	81 (149)	96 (106)	94 (32)	88 (287)	77 (106)
ALK	4 (10)	5 (7)	0	4 (17)	4 (6)	5 (8)	7 (7)	0	5 (15)	3 (3)
BRAF	6 (14)	2 (2)	3 (1)	4 (17)	2 (3)	8 (12)	2 (2)	3 (1)	5 (15)	2 (2)
ERBB2	2 (3)	1 (1)	0	2 (4)	2 (2)	2 (3)	1 (1)	0	1 (4)	1 (1)
KRAS	19 (48)	0	23 (8)	14 (56)	8 (6)	32 (47)	0	25 (8)	19 (55)	6 (7)
MET	4 (8)	2 (2)	6 (2)	4 (12)	3 (4)	4 (8)	2 (2)	6 (2)	4 (12)	2 (2)
RET	2 (6)	5 (6)	0	3 (12)	2 (3)	3 (5)	5 (5)	0	4 (10)	3 (3)

EGFR, epidermal growth factor receptor.

Table III. Treatment approaches recommended by our analysis in *EGFR*- and (*EGFR*+*ALK*)-negative cases.

NCCN recommended panel of genes	Frequency % (n) *EGFR* negative	Frequency % (n) (*EGFR*+*ALK*) negative	Potential therapeutic recommendations
ALK	3 (3)	0 (0)	*ALK* inhibitors (Crizotinib, Ceritinib)
BRAF	2 (2)	0 (0)	*BRAF* inhibitors (Dabrafenib, Vemurafenib)
ERBB2	1 (1)	1 (2)	*ERBB2* inhibitors (Neratinib, Lapatinib)
KRAS	7 (7)	6 (8)	*MEK* inhibitors (Trametinib), *PI3K* inhibitors (Buparlisib)
MET	2 (2)	1 (1)	*MET* inhibitors (Crizotinib)
RET	3 (3)	3 (3)	*RET* inhibitors (Cabozantinib, Lenvatinib, Sorafenib)

EGFR, epidermal growth factor receptor.

essential functionality of every gene in a cell, not just their statistics. Using this strategy, distinguishing driver mutations beyond from *EGFR* becomes straightforward. Thus, the aim was to determine the maximum number of patients, who are currently over-prescribed chemotherapy, that could potentially benefit from targeted therapeutics beyond *EGFR*.

In current oncology, NCCN guidelines are a repository of treatment protocols for patients with various cancer types. As per the recently updated NCCN guidelines for NSCLC, testing for 9 genes (*EGFR*, *ALK*, *ROS1*, *PDL-1*, *RET*, *KRAS*/*NRAS*, *BRAF*, *MET*, *ERBB2*) is recommended as routine. Fig. 2 shows the distribution of alterations of 9 NCCN recommended genes in the study population. Of these, routine hotspot molecular tests using conventional methods generally include *EGFR*, *ALK* and *ROS*, due to the availability of approved targeted therapeutics (24). However, this type of limited testing leaves the majority of patients diagnosed as *EGFR*- and *ALK*-negative with no alternatives to chemotherapy. The *EGFR*-negative population was identified to be 77% of the total cohort, and accounts for the majority of patients with lung cancer, who are then either subjected to genetic testing to detect *ALK* status or recommended chemotherapy in current practice. In our study, 42% of this negative population (23% NCCN recommended targeted therapy + 19% off-label targeted therapy) was identified to present other mutations in genes including *KRAS*, *BRAF*, *ERBB2*, *MET*, *RET*, *PTEN* and *TSC1/2*, among others. Similarly, 72% of the population did not present with pathogenic mutations in *EGFR* and *ALK*, of which 37% (18% NCCN recommended targeted therapy + 19% off-label targeted therapy) exhibited mutations in other genes such as *KRAS*, *MET*, *RET*, *PTEN* and *TSC1/2*. Only 35% of both the cohorts were classified to benefit from chemotherapy. Though these therapeutic decisions are challenging, the data could be beneficial to improving clinical outcomes in the future.

The mutation frequencies listed in Table II assert that these genetic alterations do have a significant impact on lung

carcinogenesis. It is understood that these alterations could develop as onco-drivers under certain microenvironmental pressures. In this way, patients would not have to deal with the genotoxic and cytotoxic effects on the normal healthy cellular population caused by chemotherapy. Though the current study approach may not boost therapeutic practice, it will certainly enhance medical oncology clinical outcomes in the near future.

There are a few targeted therapy options based on genomic alterations beyond *EGFR* and *ALK* detected in the present study population. In *ALK* and *MET* pathogenic cases (*ALK*-rearrangements, *MET*-amplifications and exon-skipping mutations), TKIs such as crizotinib and ceritinib would deliver better treatment responses. *BRAF* inhibitors such as dabrafenib and vemurafenib could be effective against *BRAF* single nucleotide variant pathogenicity (25). *RET* single nucleotide variant pathogenicity could be efficiently targeted by inhibitors such as cabozantinib, lenvatinib and sorafenib. A few inhibitors, such as neratinib and lapatinib, have been recommended for *ERBB2* mutations including, amplification and single nucleotide variations.

Future cancer therapeutics can achieve better clinical outcomes with drugs targeting additional cancer drivers such as *PTEN*, *PIK3CA* and *TSC1/2*, among others, which contribute to cancer growth and progression. The present study also unveiled novel avenues for genomic biomarker discovery to assist cancer diagnosis and therapy.

In summary, lung cancer is a rapidly progressive class of cancer. The current state of oncology is such that the treatment regimen is determined based on the mutation status of specific guideline-recommended genes, tested for using routine diagnostic procedures. The majority of routine diagnostic testing focuses on established regional variants of *EGFR* and *ALK*. Treatment strategies are straightforward if testing indicates positivity for *EGFR* and *ALK*, or any of the nine genes recommended by the guidelines. Unfortunately, the only treatment option available for negative results is chemotherapy. Additional parameters beyond *EGFR* and *ALK* were examined to better deal with the toxicity by bringing in more targeted therapeutic options in place of chemotherapy alone. Additional genes beyond *EGFR* and *ALK* were examined to increase the utilization of gene-specific-targeted drugs in place of just chemotherapy. Thus, patients administered chemotherapy due to their non-responsiveness to *EGFR*/*ALK*-based targeted therapy could be minimized. The results of the present study will allow reforms to the current cancer diagnostics framework, and facilitate the development of better therapeutics.

Acknowledgements

Not applicable.

Funding

No funding received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GS designed the study concept and reviewed the manuscript. SI performed study design, analyzed and interpreted the data and contributed to manuscript preparation. SK contributed to data acquisition, algorithms and performed statistical analysis and figure generation. RP contributed to data acquisition, algorithms and performed statistical analysis and figure generation. AR contributed to data acquisition, statistical analysis, figure generation and manuscript preparation. MB performed statistical analysis and figure generation and contributed to manuscript review. ST contributed to manuscript preparation and review. RV contributed to data interpretation and analysis. RN contributed to data interpretation. PK performed data analysis and interpretation. AB contributed to manuscript preparation. VM contributed to manuscript preparation and review. VM contributed to manuscript review. SCT contributed to data interpretation and manuscript review. MS contributed to manuscript preparation and review. AR contributed to manuscript preparation and review.

Ethical approval and consent to participate

All the experiments were carried out according to the appropriate guidelines and are approved by the PositiveATGC's (ACADEMY for TRAINING in GENOMICS and CLINICAL APPLICATION) scientific review committee. Written informed consent was acquired from all subjects as per the protocol of the review committee for the utilization of clinical samples in this study.

Patient consent for publication

The patient, or parent, guardian or next of kin provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare no competing interests.

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