

BRIEF REPORT

ERBB2 VARIANTS DISTRIBUTION IN NON-SMALL CELL LUNG CANCER: AN ITALIAN REAL-WORLD EXPERIENCE

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ABSTRACT: Lung cancer still remains the leading cause of cancer-related mortality worldwide. Among the emerging biomarkers predictive for target therapy, Erythroblastic oncogene B (ERBB2) is recommended by the main international guidelines to be investigated, according to several novel therapeutic approaches. Aim of this retrospective study was to analyze the distribution of ERBB2 mutations and their location along the gene in a set of 331 consecutive lung cancer tissues analyzed by next generation sequencing (NGS) technology in the Structure of Oncological Molecular Pathology, Azienda USL Toscana Centro, Italy. ERBB2 variants were detected in 95 out of 331 samples (28.7%) and statistical analysis showed a 3% distribution of oncogenic variants, in line with literature data. Moreover, NGS technology allowed to identify a substantial amount of transmembrane domain mutations, not detectable by single hot spot assay: they should represent future clinical target in lung cancer, since nowadays many trials are investigating their clinical benefit.

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Key words: NSCLC; ERBB2; NGS.

Impact statement: This report examined the distribution of entire ERBB2 variants along the protein structure and the frequency of oncogenic ones in a set of lung cancer samples.

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INTRODUCTION

As of today, lung cancer still remains the leading cause of cancer-related mortality for both men and women worldwide (1). Lung cancer is subdivided into two major histological types: small cell lung carcinoma (SCLC) and non-small-cell lung cancer (NSCLC). The latter has a much higher incidence, comprising approximately 80-85% of all lung cancer cases. The highly complex and heterogeneous molecular nature of NSCLC, as well as late diagnosis (2) is a major factor responsible

for the statistically low survival rates of these patients. Ever since the implementation of multiplex molecular panels in clinical practice and the consequent identification of new actionable driver mutations, the treatment scenario for these patients has gradually evolved from chemotherapy-based approaches to molecularly targeted therapies. Currently, the National Comprehensive Cancer Network (NCCN), the American Society of Clinical Oncology (ASCO), and the European Society of Medical Oncology (ESMO) guidelines recom-

mend that all NSCLC tumors containing an adenocarcinoma component be tested for the following actionable biomarkers: Epidermal Growth Factor Receptor (EGFR), V-Raf Murine Sarcoma Viral Oncogene Homolog B (BRAF), Mesenchymal Epithelial Transition exon14 skipping (METex14), Anaplastic Lymphoma Kinase (ALK), ROS proto-oncogene 1 receptor tyrosine kinase (ROS1), REarranged during Transfection (RET), and Neurotrophic Tyrosine Receptor Kinase (NTRK) 1, 2, 3 (3-5). In addition, they also recommended that many other genes, classified as “emerging biomarkers”, be tested not as routine stand-alone assays but as part of a larger testing panels. Unsurprisingly, the ESMO has recently recommended the use of Next Generation Sequencing (NGS), especially in the presence of scant starting material (3). Among these newly emerging biomarkers is Erythroblastic oncogene B (ERBB2), also known as Human Epidermal growth factor Receptor 2 (HER2) or Cluster of Differentiation 340 (CD340).

The ERBB2 gene is 28,515 base pairs in length: its transcript encodes the plasma membrane-bound receptor tyrosine kinase ERBB2 protein comprising a ligand-bound extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular domain (ICD). This latter subunit is divided by a juxtamembrane domain (JMD), a tyrosine kinase domain (TKD), and a carboxy terminal tail domain (CTD) (6).

In NSCLC, ERBB2 alterations are commonly amplified (2-23%) and/or overexpressed (11-32%), and only seldom mutated (1.6-4%) (1, 7). Mutations in ERBB2 lead to constitutive activation of the receptor and are considered poor prognostic predictors: fortunately, these mutations are sensitive to small-molecule tyrosine kinase inhibitors (TKIs). Although previous studies have identified the majority mutations in the TKD, more recent research using NGS has described oncogenic mutations even in TMD, ICD and ECD. Preclinical studies indicate these mutations constitute promising candidates for targeted anti-ERBB2 therapies: small molecule inhibitors and anti-ERBB2 antibodies have also been shown to be highly effective against non-TKD oncogenic mutations such as exon 17 mutations p.V659E and p.G660D in the TMD and exon 8 mutation p.S310F in the ECD (8).

The rationale of this study was to provide clinicians with grounded information regarding the plentitude of mutations potentially occurring outside

cancer-type specific domains in the hope of offering patients' better NSCLC treatments once new targeted drugs become available.

Thus, the purpose of this retrospective study was to analyze the distribution of ERBB2 mutations in a set of 331 consecutive NSCLC tissues by using NGS. We first verified whether the frequency of oncogenic activating mutations was in line with literature data. Then, we examined the frequency of mutations occurring outside the activating domains and their distribution along the protein structure.

MATERIALS AND METHODS

A total of 398 NSCLC tissue samples underwent NGS analysis from June 2019 to October 2021 were retrieved from the electronic archive of the Structure of Oncological Molecular Pathology, S. Stefano Hospital, Oncological Department, Azienda USL Toscana Centro, Italy.

In brief, NGS was performed on genomic DNA isolated from formalin-fixed paraffin-embedded (FFPE) NSCLC tumor tissues. Prior to molecular analysis, an experienced pathologist selected representative tumor tissue areas (>20% neoplastic cells) from Hematoxylin-Eosin stained slide. DNA extraction was performed with the QIAamp FFPE Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and resuspended it in 20 µL of ATE buffer (Qiagen). DNA quantification was assessed by Qubit dsDNA HS assay (Life Technologies, Carlsbad, CA, USA) on Qubit™ Fluorometer (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA).

Samples with adequate DNA content were then analyzed by NGS performed on the GeneReader platform (QIAGEN), according to the manufacturer's protocol. Briefly, 40 ng of DNA was used to generate libraries with the GeneReadQIAact Actionable Insight Tumor Panel (QIAGEN). This panel covers 773 unique variant positions in 12 genes (KRAS, NRAS, KIT, BRAF, PDGFRA, ALK, EGFR, ERBB2, PIK3CA, ERBB3, ESR1 and RAF1). Data was analyzed by the QIAGEN Clinical Insight (QCI™) Analyze software (QIAGEN). A 200X minimum read coverage cutoff was used for variant calls at any position in the panel, whereas a cutoff of 5% sensitivity criteria was chosen for variant calls at the clinical level. All variants were detected with >99% confidence based on allele-frequency and amplicon coverage.

RESULTS

Out of 398 samples, 331 (83%) had adequate DNA content for subsequent NGS analysis. Within the 331 adequate NSCLC tissues, 95 (28.7%) ERBB2 mutations were detected in 89 samples, histologically divided in adenocarcinoma (73, 82%), squamous (4, 4.5%) and not otherwise specified (12, 13.5%) subtypes, respectively. The distribution denotes previous works reporting that ERBB2 mutations predominantly occur in adenocarcinoma compared with other histological subtypes (9). Most of ERBB2 mutations (85, 89.5%) were located in the TMD and comprised germinal like mutations (p.I654V and p.I655V, exon 17). The remaining 10 (10.5%) were located in the ECD and in the TKD (exon 8 and 20, respectively) and classified as activating mutations. Of the 10 non-TMD mutations, 7 (70.0%) were exon 20 insertions located in the TKD, whereas 3 (30.0%) were p.S310F exon 8 point mutations located in the ECD (**Table 1**).

Regarding the distribution of the mutations in the TMD, 91.8% (78/85) were p.I655V and 8.2% (7/85) were p.I654V. Six samples harbored a double TMD mutation, namely, p.I654V and p.I655V. Within the ERBB2 variants, 7.4% (7/95) and 92.6% (88/95) constituted classical activating mutations and rare non-TKD mutations, respectively (**Figure 1**). As for the recurrent variants, exon 17 p.I655V was the most frequent one (82.1%, 78/95), followed by exon 17 p.I654V (7.4%, 7/95), exon 20 p.Y772_A775dup (5.3%, 5/95), exon 8 p.S310F (3.2%, 3/95), exon 20 p.G778_P780dup (1%, 1/95), and exon 20 p.A775_G776insV (1%, 1/95). Clinically, ERBB2 mutations are classified as oncogenic, benign, and uncertain significance. Oncogenic function was significantly stronger in TKD mutations than in non-TKD mutations. Intriguingly,

though up to 37.5% of ERBB2 oncogenic mutations were within the non-TKD. Based on the OncoKB classification, our results displayed 10/95 (10.5%) oncogenic variants (exon 20 insertion and exon 8 point mutation p.S310F) and 85/95 (89.5%) benign/uncertain significant variants (exon 17 p.I654V and p.I655V).

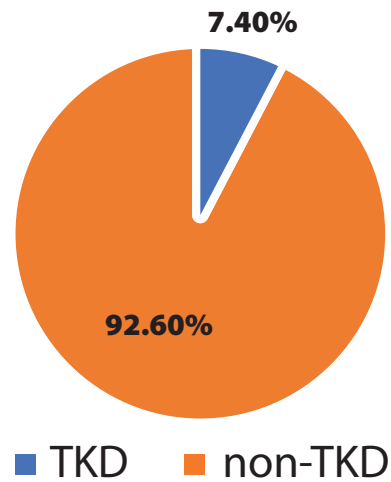


Figure 1. TKD and non-TKD ERBB2 variants distribution detected in 331 consecutive NSCLC tissues.

DISCUSSION

As the ERBB2 gene has recently emerged as a promising biomarker in NSCLC patients, the main international guidelines strongly recommend the routine use of NGS (10) in clinical practice to identify the highest possible number of aberrations present in ERBB2 mutations. The reason for this major endeavor is to find potentially actionable mutations and thus improve targeted treatments for NSCLC patients. Indeed, as

Table 1. Oncogenic ERBB2 variants detected in 331 consecutive NSCLC tissues.

SAMPLE	MUTATION	EXON	DOMAIN
1	G778_P780dup	20	TKD
2	S310F	8	ECD
3	Y772_A775dup	20	TKD
4	A775_G776insV	20	TKD
5	S310F	8	ECD
6	Y772_A775dup	20	TKD
7	Y772_A775dup	20	TKD
8	Y772_A775dup	20	TKD
9	Y772_A775dup	20	TKD
10	S310F	8	ECD

of today, traditional chemotherapy for ERBB2 in NSCLC is far from satisfactory. However, in recent years, several lines of investigation have demonstrated the clinical benefits of several therapeutic approaches in ERBB2-positive NSCLC patients (11).

These therapies, which are based on small molecule, second generation irreversible TKIs, include, for instance, afatinib, neratinib, tucatinib, and pyrotinib.

Clinical benefits of afatinib in ERBB2 ex20ins patients have been demonstrated by De Grève and colleagues. The Authors observed that patients harboring G778_P780dup and G776delinsVC mutations derived favorable outcomes from this treatment (12).

In addition, an *in vivo* preclinical study assessing the activity of various drugs against ERBB2 mutation variants found that neratinib, as well as afatinib, was more effective than other inhibitors in patients harboring the ERBB2^{YVMA} mutation subtype (13). An equally interesting molecule is pyrotinib, *i.e.*, an oral, irreversible pan-HER TKI. A phase II clinical study has shown the high efficacy of this drug in patients with advanced ERBB2 mutant lung cancer. Noteworthy, the use of NGS to detect ERBB2 alterations enabled the research team to identify mutations outside exon 20. Another clinical trial demonstrated that ERBB2-positive NSCLC patients harboring non-exon 20 aberrations achieved an ORR similar to that of patients with exon 20 mutations (14).

Finally, the most recently developed oral TKI is tucatinib: in the human lung cancer cell line NCI-H1781 inhibits ERBB2 phosphorylation. Lastly, an ongoing basket trial is also evaluating the clinical activity of tucatinib in combination with trastuzumab (NCT04579380) in patients with solid tumors harboring ERBB2 alterations, including a cohort for NSCLC (15).

Based on the above-mentioned druggable scenario ERBB2 testing showed a great potential in NSCLC clinical administration. Peters *et al.* identified a positive Objective Response Rate (ORR 20%, ranging from 5.7%-43.7%, CI 95%) in a series of NSCLC patients exhibiting 3+ score by testing ERBB2 with immunohistochemistry (IHC) under Trastuzumab Emtansine (T-DM1) administration. Moreover, an improving in response duration was also observed in same experimental arm (10.8 months). IHC is sometime afflicted by some technical and interpretation limitations, so we opted to analyze ERBB2 alterations by NGS (16).

The principal aim of this report was to investigate whether the distribution of ERBB2 oncogenic mutations in NSCLC samples in our real-world experience

was consistent with the literature data. To this aim, we used the GeneReadQIAact Actionable Insight Tumor Panel (QIAGEN) for our NGS analysis. Such system investigated 489 missense mutations along exons 2-6, 8, 10-26, 28 of the ERBB2 gene and encompassed all three domains, namely, ECD, TMD, and TKD.

Interestingly, in our cohort of 331 samples, the prevalence of ERBB2 was higher than that reported in previous studies (28.7% vs. 2-4%) (17). We reasoned that this discrepancy was most likely due to the fact that the previous studies detected only TKD mutations, particularly exon 20 mutations. In fact, ERBB2 mutations are clinically categorized as oncogenic, benign, and uncertain significance. Studies have shown that whereas oncogenic function is significantly stronger in TKD mutations than in non-TKD mutation, up to 37.5% of ERBB2 oncogenic mutations are present within the non-TKD (11). Based on the OncoKB classification, ERBB2 variants located at exons 20 and 8, are considered oncogenic. Indeed, in our samples, we found seven mutations at exon 20 (represented by Y772_A775dup, A775_G776insV, G778_P780dup), and three at exon 8 (represented by S310F), as shown in **Table 1**. In our experience, oncogenic variants made up 3% (10/331) of the total samples analyzed, which is consistent with the literature data.

The secondary aim of our study was to investigate the presence and distribution of activating mutations along the ERBB2 gene. We found a significant fraction of TMD mutation: 89.5% (85/95) germinal-like mutations at exon 17, precisely at aminoacidic positions 654 and 655. However, since they are considered non-oncogenic germline alterations, their association with increased risk of developing cancer is still controversial (18). Previous data indicate that p.L655V is more frequent than p.L654V: consistently, in our experience distribution was 91.8% (78/85) vs. 8.2% (7/85), respectively. Although today these mutations do not represent clinical targets in lung cancer, many recent clinical trials and case reports have instead highlighted the clinical benefits of targeted treatments for NSCLC patients harboring ERBB2 TMD mutations (19). Furthermore, we also found mutations both in the TKD (exon 20) and in the ECD (exon 18), with frequencies up to 7.4% (7/95) and 3.1% (3/95), respectively. Variants located in the TKD and in the ECD are classified as oncogenic: in our experience 10.5% of the total mutations were oncogenic and consistent with the literature data.

CONCLUSIONS

ERBB2 gene has recently emerged as a promising biomarker in NSCLC patients, regarding both oncogenic variants located at TKD or ECD, and germinal-like mutations at TMD. For the oncogenic targets, drugs are nowadays available, so the importance to fast identify them. Even though many more studies are warranted to establish the best therapeutic option for patients with non-TKD ERBB2 mutations, identifying and monitoring these alterations are crucial steps toward developing more efficient therapeutic strategies for NSCLC patients.

The identification of TKD and non-TKD ERBB2 mutations in a real-world cohort of NSCLC patients would not have been possible without the implementation of NGS. This amazing technology provided several advantages. First, it allowed us to analyze multiple druggable targets simultaneously, thereby reducing the time needed to obtain a complete diagnostic molecular classification of our patients' tumors. Second, it enabled us to examine multiple biomarkers in scant specimens. Finally, it allowed us to analyze non-canonical alterations, which are generally undetectable by single hot spot technologies. The technical efficiency of our approach was indeed confirmed by the fact that the distribution of the oncogenic alterations we detected in our patients was consistent with that of previous literature. Finally, we hope that our present findings, together with the burgeoning literature in the field, may help clinicians improve the overall clinical and therapeutic framework of their patients by relying on NGS technologies to detect NSCLC targetable mutations.

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COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of interests

Umberto Malapelle has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientifics, Eli Lilly, Diaceutics, GSK, Merck and AstraZeneca, Janssen, Diatech, Novartis and Hedera unrelated to the current work. Pasquale Pisapia has received personal fees as speaker bureau from Novartis, for work performed outside of the current study. The other Authors have nothing to disclose.

Availability of data and materials

N/A.

Authors' contributions

SB, UM, MB: conceptualization; SB, MO, PP, FP: formal analysis; MO: software analysis; SB: writing-original draft preparation; UM, PP, FP: writing-review and editing; MB, GT: supervision.

Ethical approval

Human studies and subjects

The study was conducted in accordance with the ethical standards established in Declaration of Helsinki; informed consent was obtained from all subjects involved.

Animal study

N/A.

Publication ethics

Plagiarism

The contents of the article are original and any overlaps with other articles are by the Authors themselves and appropriately cited.

Data falsification and fabrication

All the data correspond to the real.

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