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Diverse *EGFR* Exon 20 Insertions and Co-Occurring Molecular Alterations Identified by Comprehensive Genomic Profiling of Non-Small Cell Lung Cancer

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Abstract

Introduction—*EGFR* exon 20 insertions (*EGFR*ex20ins) comprise an uncommon subset of *EGFR* activating alterations relatively insensitive to first and second-generation EGFR tyrosine kinase inhibitors (TKIs). However, recent early clinical data suggests these patients may benefit from newer generation EGFR-TKIs. Comprehensive genomic profiling (CGP) identifies a broad spectrum of *EGFR*ex20ins and associated co-occurring genomic alterations (GA) present in non-small cell lung cancer (NSCLC)

Methods—Hybrid capture-based CGP was performed prospectively on 14,483 clinically annotated consecutive NSCLC specimens to a mean coverage depth of >650X for 236 or 315 cancer-related genes.

Results—Of 14,483 NSCLC cases, CGP identified 263 (1.8%) cases with *EGFR*ex20ins, representing 12% (263/2,251) of cases with *EGFR* mutations. 64 unique *EGFR*ex20ins were identified, most commonly D770_N771>ASVDN (21%) and N771_P772>SVDNP (20%). *EGFR* amplification occurred in 22% (57/263). The most common co-occurring GA effected *TP53* (56%), *CDKN2A* (22%), *CDKN2B* (16%), *NKX2-1* (14%) and *RB1* (11%); co-occurring GA in other known lung cancer drivers were rare (5%). Average tumor mutational burden (TMB) was

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low (mean 4.3, range 0-40.3 mutations/Mb). Clinical outcomes to first- and second-generation EGFR TKIs were obtained for 5 patients and none responded.

Conclusion—In the largest series of EGFRex20ins NSCLC, diverse *EGFR*ex20ins were detected in 12% of *EGFR*-mutant NSCLC, a higher frequency than previously reported in smaller single-institution studies. Clinical outcomes demonstrated lack of response to EGFR TKIs. TMB was low, consistent with non-smoking associated NSCLC. Comprehensive sequencing revealed increased proportion and wide variety of EGFRex20ins, representing a population of patients significant enough for focused efforts on effective interventions.

Keywords

EGFR Exon 20 Insertion; Genomics; Tumor Mutational Burden

Introduction

EGFR exon 19 deletions and *EGFR* exon 21 L858R represent the vast majority of EGFR activating mutations, and are exquisitely sensitive to approved EGFR TKIs such as erlotinib, gefitinib, and afatinib [1–3]. Less common *EGFR* mutations have variable sensitivity to EGFR inhibitors, though many still demonstrate clinical sensitivity to EGFR TKIs (e.g. EGFR G719X, L861Q, S768I)[4]. *EGFR* exon 20 insertions (*EGFR*ex20ins) are a collection of *EGFR* driver mutations characterized by in-frame insertions that typically serve to constitutively upregulate EGFR kinase activity similar to sensitizing mutations (but are insensitive to 1st and 2nd gen EGFR-TKIs). These alterations have previously been reported to comprise approximately 4-10% of all *EGFR* mutant lung cancers [5–8]. The diverse array of exon 20 insertions and the challenges associated with identifying them may lead to underestimation of their true frequency. Although *EGFR*ex20ins usually have the same transforming ability as more common EGFR activating mutations and are thus considered *driver* mutations, they are typically unresponsive to first and second-generation EGFR TKIs due to the modified structures of their kinase domains [5–8]. Very few of these EGFRex20ins such as A763_Y764insFQEA have demonstrated sensitivity to 1st and 2nd generation EGFR TKIs [9].

Several next-generation EGFR TKIs (some with pan-HER activity) have demonstrated pre-clinical activity against *EGFR*ex20ins and are in clinical development (EGF816, AP32788, osimertinib, poziotinib)[5, 10, 11]. In particular, a strong signal of clinical activity was demonstrated with poziotinib with an overall response rate of 64% in the first 11 patients in an early phase clinical trial[12]. However, no EGFR-TKIs are currently approved for *EGFR*ex20ins and their diversity of structures suggests that different insertion events may have divergent responsiveness to various EGFR TKIs. Thus, identifying the full array and scope of *EGFR*ex20ins is of paramount importance.

Studies examining *EGFR*ex20ins have generally been limited to cases from single institutions. Certain next-generation sequencing approaches such as comprehensive genomic profiling (CGP) permit sequencing of the entire *EGFR* gene to broadly assess for diverse *EGFR*ex20ins and co-existing genomic alterations that may be relevant to the pathogenesis of *EGFR*ex20ins-positive NSCLC. In addition to characterizing the clinical and pathologic

characteristics of *EGFR*ex20ins-positive NSCLC from a large dataset with molecular testing performed in the course of clinical care from multiple institutions, the purpose of this study was to assess the frequency and diversity of *EGFR*ex20ins in NSCLC by their pattern of sequence alterations and co-occurring genomic alterations, which may impact the development of targeted treatments for these patients.

Methods

DNA was extracted from 40-um formalin-fixed paraffin-embedded sections. *EGFR*ex20ins and co-occurring genomic alterations were identified by hybrid capture-based comprehensive genomic profiling performed during the course of clinical care on 14,483 consecutive NSCLC specimens to a mean coverage depth of >650X for 236 (version 1, July 2012 to August 2014) or 315 (version 2, August 2014 to June 2016) cancer related genes plus selected introns from 19 or 28 genes frequently rearranged in cancer. *EGFR* amplification was defined as estimated copy number greater than 6 copies[13]. Clinical data such as age, gender, stage, and histologic subtype were abstracted from the accompanying pathology report submitted by the ordering physician. Treatment outcomes from these patients were included where available. Testing was performed in a Clinical Laboratory Improvement Amendments–certified, College of American Pathologists–accredited reference laboratory (Foundation Medicine, Inc., Cambridge, MA). Patient samples were evaluated for genomic alterations (GAs), including base pair substitutions, insertions/deletions (indels), copy number alterations, and rearrangements, as described previously. Tumor mutational burden (TMB) was characterized as the number of somatic base substitution or indel alterations per megabase (MB) per previously described methods[14]. Approval for this study, including a waiver of informed consent and a Health Insurance Portability and Accountability Act waiver of authorization, was obtained from the Western Institutional Review Board (Protocol No. 20152817).

Results

Comprehensive genomic profiling (CGP) performed on 14,483 NSCLC cases in the course of clinical care identified 2,251 cases with *EGFR* mutations, 263 of these cases were *EGFR*ex20ins, representing 12% of all *EGFR*-mutant NSCLC and 1.8% of all NSCLC cases tested. *EGFR*ex20ins were the third most common type of *EGFR* mutation detected following *EGFR* exon 19 deletions (47%) and EGFR L858R (32%). Other less common *EGFR* mutations detected in this large case series include G719X (4%), L861Q (2%), S768I (1%), and cases with compound *EGFR*-activating mutations (2%) (Figure 1).

Clinical and pathologic characteristics were available for all of the 263 patients with *EGFR*ex20ins as summarized in Table 1. The large majority of cases *EGFR*ex20ins were identified in lung adenocarcinoma (90%, 237/263), followed by NSCLC *not otherwise specified* (9.1%, 24/263), and sarcomatoid histology (0.7%, 2/263). The median age for *EGFR*ex20-positive cases was 63 years, and 62% of patients were female. Of the 263 patients with *EGFR*ex20ins-positive NSCLC, treatment outcomes were available for 11 patients. Five of those patients received either first or second-generation EGFR TKIs, with none of the five patients demonstrating a response to treatment and median time to

progression of only 3.5 months (Supplementary Table 1). The remaining 6 patients did not receive EGFR targeted therapy.

Sixty-four unique *EGFR*ex20ins were identified, most commonly D770_N771>ASVDN (21%), N771_P772>SVDNP (20%) and N771_H773dupNPH (8%); 6% (15/263) harbored EGFR A763_Y764insFQEA, an *EGFR*ex20ins with preclinical and clinical evidence demonstrating sensitivity to first- and second-generation EGFR-TKI (Figures 2 and 3). Putative co-occurring driver alterations in genes including *EGFR* (ex19del and L858R), *HER2, MET* and *KRAS* mutations tended to be mutually exclusive from *EGFR*ex20ins, occurring only in 5% (12/263) of cases, and no co-occurring *ALK, ROS1,* or *RET* fusions or *BRAF* mutations were identified. Among *EGFR*ex20ins cases, *EGFR* amplification occurred in 22% (57/263). Three cases with *EGFR*ex20ins harbored co-occurring T790M, including 2 cases with A763_Y764insFQEA, which is sensitive to 1st and 2nd generation EGFR TKIs. The most common co-occurring alterations affected *TP53* (56%), *CDKN2A* (22%), *CDKN2B* (16%), *NKX2-1* (14%) and *RB1* (11%) (Figure 4). Co-occurring alterations did not substantially differ between *EGFR*ex20ins and canonical *EGFR* mutations (*EGFR* exon 19 deletion and EGFR L858R) that were negative for the most common acquired EGFR TKI resistance mutation, EGFR T790M (Table 2, Figure 4). Average tumor mutational burden (TMB) was low (mean 4.3, median 3.6, range 0-40.3 mutations/Mb) in *EGFR*ex20ins cases - comparable with TMB in *EGFR* exon 19del and EGFR L858R and lower than EGFR-WT NSCLC (Table 2).

Discussion

Herein we present the largest known dataset of *EGFR*ex20ins NSCLC, where 64 unique *EGFR*ex20ins sequence alterations are identified, reflecting the diversity of these EGFR driver mutations, which comprise 12% of *EGFR*-mutant NSCLC and 1.8% of all NSCLC in our series. With approximately 222,000 newly diagnosed lung cancer cases annually in the United States alone, this corresponds to about 3,000 newly diagnosed *EGFR*ex20ins cases per year in the US [16].

*EGFR*ex20ins were detected in 12% of *EGFR*-mut NSCLC cases, which is higher than previously reported in smaller single institution series (~4-10% frequency). Prior record of molecular testing was not available for the majority of cases. Several reasons may explain the higher frequency of *EGFR*ex20ins detected. This may reflect referral bias with enrichment for lung cancers from never smoking patients. However, for other common *EGFR* alterations, frequencies detected were comparable to those reported in the literature (15.5% of all NSCLC cases). Thus, the higher frequency of *EGFR*ex20ins is likely rather due to advancement of sequencing technology and improved detection of these alterations at lower mutant allele frequency by next-generation sequencing. Mutation specific assays in some previous studies also likely underestimate the number of *EGFR*ex20ins given the diversity of *EGFR*ex20ins we detected by next-generation sequencing.

A previous study of 27 cases at a single institution described 13 unique *EGFR*ex20ins variants[6]. This dataset herein reports 263 cases including 64 unique *EGFR*ex20ins variants with concomitant molecular alterations and tumor mutational burden (TMB), with a

comparison of these co-occurring alterations to a large dataset of *EGFR*-wild-type and canonical *EGFR*-mutant NSCLC.

Sensitivity of the EGFR L858R and exon 19 deletions to EGFR TKIs is mediated by enhanced intrinsic affinity for EGFR TKI versus substrate ATP. Structural modeling and binding affinity studies of representative *EGFR* exon 20 insertions showed that 1st generation EGFR TKI binding affinity is similar to that of wild-type EGFR[9]. Identifying the spectrum of *EGFR*ex20ins where next-generation EGFR TKI can fit into a sterically hindered *EGFR* exon 20 binding pocket is critical to demonstrating anti-tumor activity and improving clinical outcomes in patients NSCLC harboring diverse *EGFR*ex20ins[9, 15, 16]

The majority of *EGFR*ex20ins were detected in lung adenocarcinoma, though they were also found in a limited number of lung cancers with sarcomatoid histology. In this retrospective series, these insertions were detected at a higher frequency than reported in smaller series suggesting that *EGFR*ex20ins are more prevalent than previously reported. Though this dataset represents patients that were selected specifically by their physician to be tested for analysis, which may not represent a random cross-section of NSCLC, the higher frequency of *EGFR*ex20ins reported could be due in part to comprehensive sequencing of *EGFR* compared to limitations of non-comprehensive assays performed in prior studies. Nevertheless, *EGFR*ex20ins were the third most common EGFR activating mutation following exon19del and L858R, and occur at approximately the same frequency as *ROS1* gene fusions (~1% in our dataset; data not shown) and BRAF V600E mutations in NSCLC (~2% in our dataset; data not shown) where targeted therapies are approved with high rates of response and lengthy progression-free survival [17, 18]. Available treatment outcomes demonstrated lack of response to first- and second-generation EGFR TKIs such as erlotinib, gefitinib and afatinib, consistent with other series, and highlighting the need for new strategies to target *EGFR*ex20ins [19]. The EGFR A763_Y764insFQEA insertion, for which sensitivity to first- and second-generation EGFR TKIs has been described, only comprised 6% of all *EGFR*ex20ins in our series (thus representing less than 1% of all EGFR mutations). Although we detected over 60 *EGFR*ex20ins, the most frequently occurring, D770_N771>ASVDN, N771_P772>SVDNP, and N771_H773dupNPH, represent about 50% of all detected *EGFR*ex20ins in this series (Figure 3). Studies of newer generation EGFR TKIs demonstrating pre-clinical and clinical efficacy against these particular *EGFR*ex20ins would thus impact the greatest number of patients. More recently, poziotinib has demonstrated robust pre-clinical and clinical activity; a confirmed response rate of 64% in *EGFR*ex20ins NSCLC was noted in a phase 2 trial, albeit in a limited number of patients[12]. Poziotinib, due to it's small size, can circumvent steric changes resulting from the exon 20 insertion to more effectively bind to mutant EGFR. Further understanding the spectrum of *EGFR*ex20ins is important, as it is likely that among the 64 unique insertions we detected, there will be differential sensitivity to poziotinib and similar drugs due to the size and configuration of the drug binding pocket based on the specific *EGFR*ex20ins variant.

The most common genomic alterations co-occurring with *EGFR*ex20ins were tumor suppressor (e.g. *TP53* and *RB1*) and cell cycle alterations (e.g. *CDKN2A/2B*). These occurred at comparable frequency in NSCLCs samples with *EGFR* exon 19del and EGFR

L585R without EGFR T790M (Table 2, Figure 4). Since EGFR T790M is the most common acquired resistance mutation to first and second generation EGFR TKIs, these were excluded for this comparison to control as best as possible for additional genomic aberrations resulting from acquired EGFR TKI resistance. As is typical of other oncogenic drivers, the vast majority of *EGFR*ex20ins were mutually exclusive of other putative oncogenic drivers in NSCLC such as canonical *EGFR, KRAS, BRAF, MET,* and *HER2* mutations, *MET* amplification, and *ALK, ROS1* and *RET* fusions.

EGFR amplification was detected in 22% of *EGFR*ex20ins-positive NSCLCs, which is comparable to other *EGFR* mutations in our dataset when cases with co-occurring T790M are excluded (*EGFR* ex19del 23%, L858R 25%) and higher than EGFR-WT NSCLC (Figure 4, Table 2), as noted in other studies in *EGFR*-mutant NSCLC[20]. Treatment with EGFR monoclonal antibodies based on *EGFR* amplification and overexpression has modestly improved clinical outcomes of squamous NSCLC, and cetuximab added to afatinib appears to increase response rates compared to afatinib alone in patients with *EGFR* exon 19 deletion or L858R[21, 22]. Small series have described activity of the addition of cetuximab to EGFR TKIs in *EGFR*ex20ins NSCLC[23, 24].

Another important aspect of study is analysis of tumor mutational burden (TMB), which has not been previously reported for *EGFR*ex20ins. Immune checkpoint inhibition with PD-1 and PD-L1 antibodies has revolutionized the treatment of NSCLC and improved survival outcomes for many lung cancer patients[25]. However, patients with *EGFR*-mutant NSCLC appear to derive less clinical benefit from PD-1/PD-L1 blockade and in a recent meta-analysis do not have an overall survival benefit compared to chemotherapy[26]. In addition to PD-L1 expression, other features such as TMB appear to also be associated with clinical benefit to PD-1 / PD-L1 antibodies, potentially relating to an increase in neoantigen specific T-cell activity [27, 28]. TMB measured by comprehensive genomic profiling is comparable to measurements by whole exome sequencing and is typically higher in smoking associated lung cancer due to tobacco carcinogenesis[14, 29]. Our study shows comparable TMB in *EGFR*ex20ins cases to classic (exon 19 del or L858R) *EGFR*-mutant NSCLC that is lower than EGFR-WT NSCLC, likely reflecting non-tobacco associated carcinogenesis (Table 2). Less than 4% (10/263) of *EGFR*ex20ins-positive cases analyzed had intermediate-high (10-20 mutations/Mb) TMB, and only 0.7% (2/263) cases had high (>20 mutations/Mb) TMB (Table 2). Overall, low TMB suggests comparable lack of benefit to single agent PD-1 / PD-L1 antibodies in *EGFR*ex20ins NSCLC as in NSCLC harboring more common *EGFR* mutations.

This study represents the largest series of *EGFR*ex20ins with over 60 unique *EGFR* exon 20 insertions detected. With next-generation EGFR TKIs demonstrating potential activity in *EGFR*ex20ins-positive NSCLC identifying patients with these molecular aberrations is paramount [5, 8, 11, 30, 31]. In light of newly available combination trials and clinical development of fourth-generation EGFR TKIs targeting *EGFR*ex20ins, comprehensive genomic profiling to detect diverse *EGFR*ex20ins in NSCLC is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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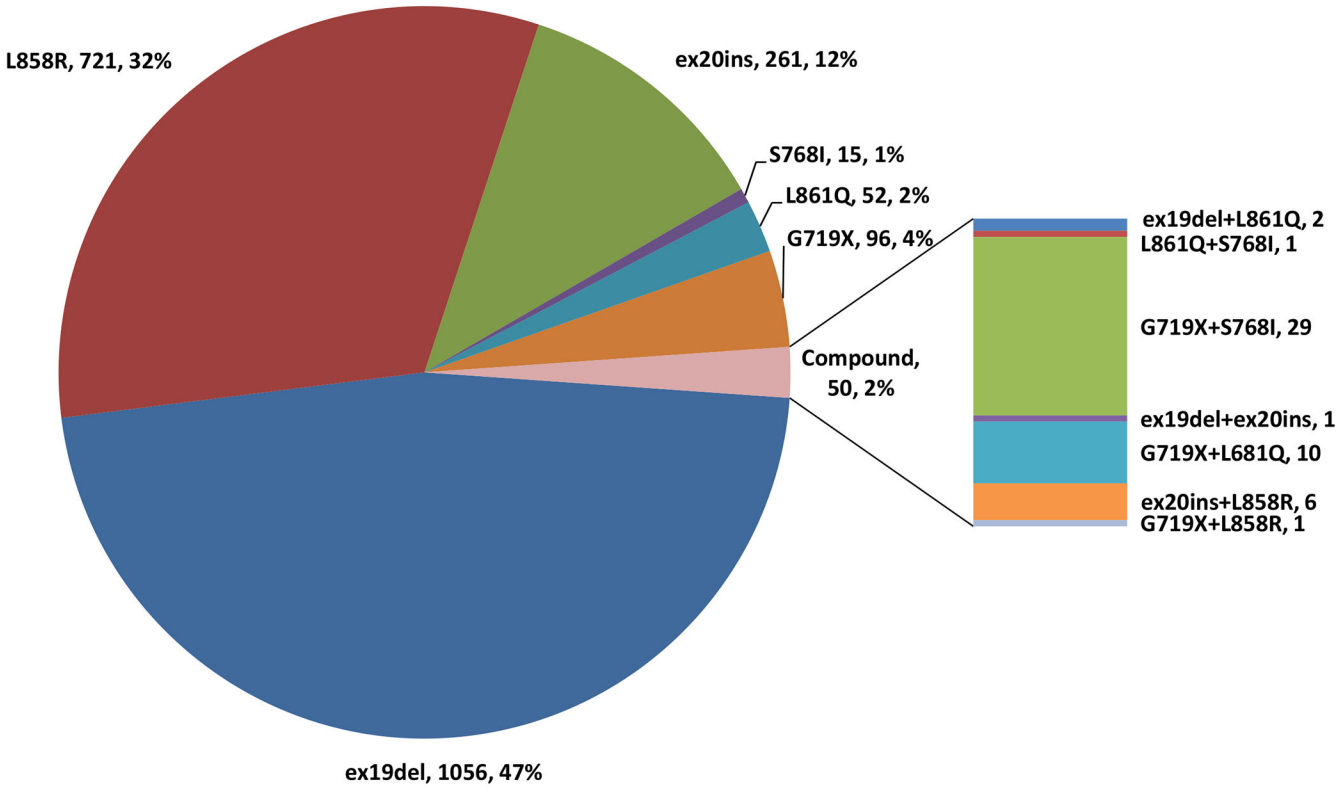

Figure 1:

Frequency and Distribution of 2,251 *EGFR* mutations in NSCLC Detected by Comprehensive Genomic Profiling. Each alteration is shown as: mutation, number of cases, frequency.

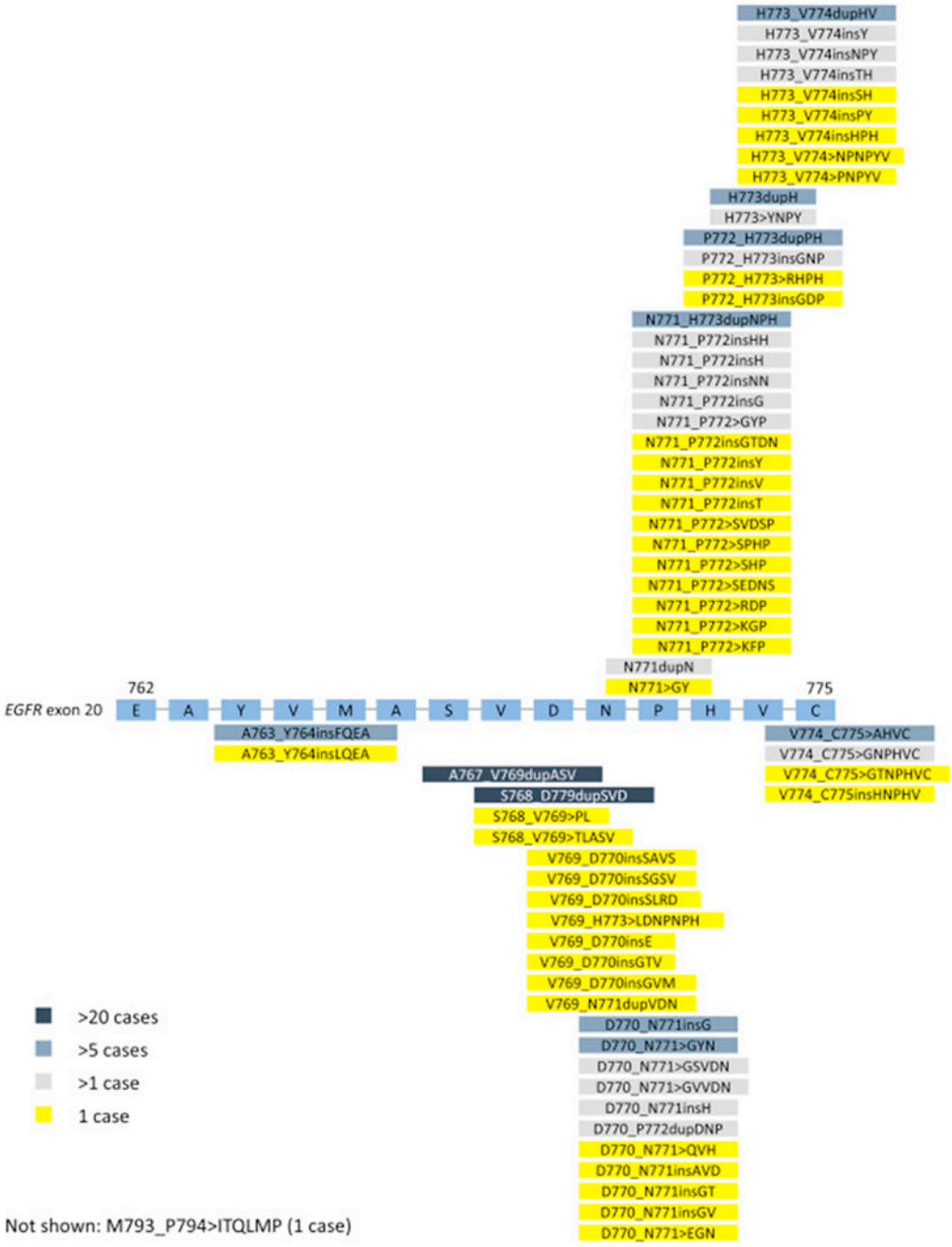

Figure 2:

Schematic of genomic positions of *EGFR* Exon 20 Insertions Detected by Comprehensive Genomic Profiling. EGFR amino acids positions are indicated.

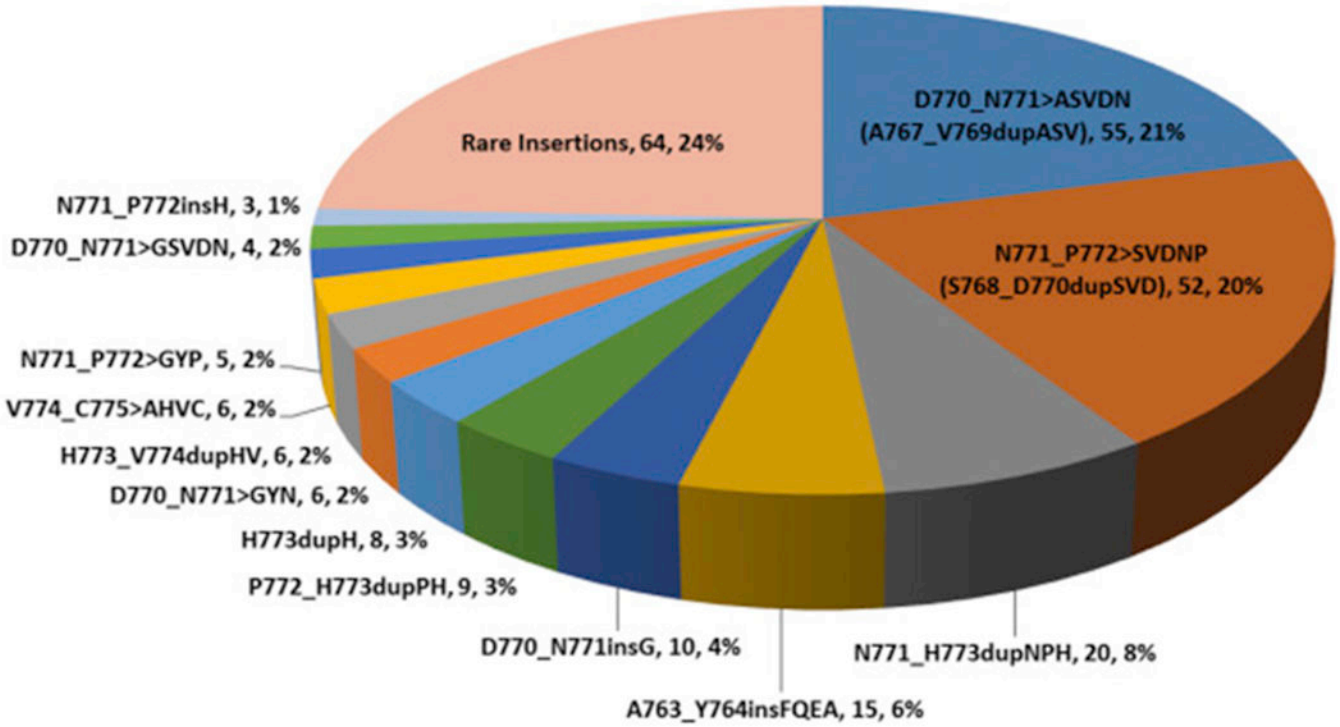

Figure 3:

Frequency of Unique *EGFR* Exon 20 Insertions Detected by Comprehensive Genomic Profiling. Each alteration is shown as: Insertion (Alternative nomenclature), number of cases, frequency.

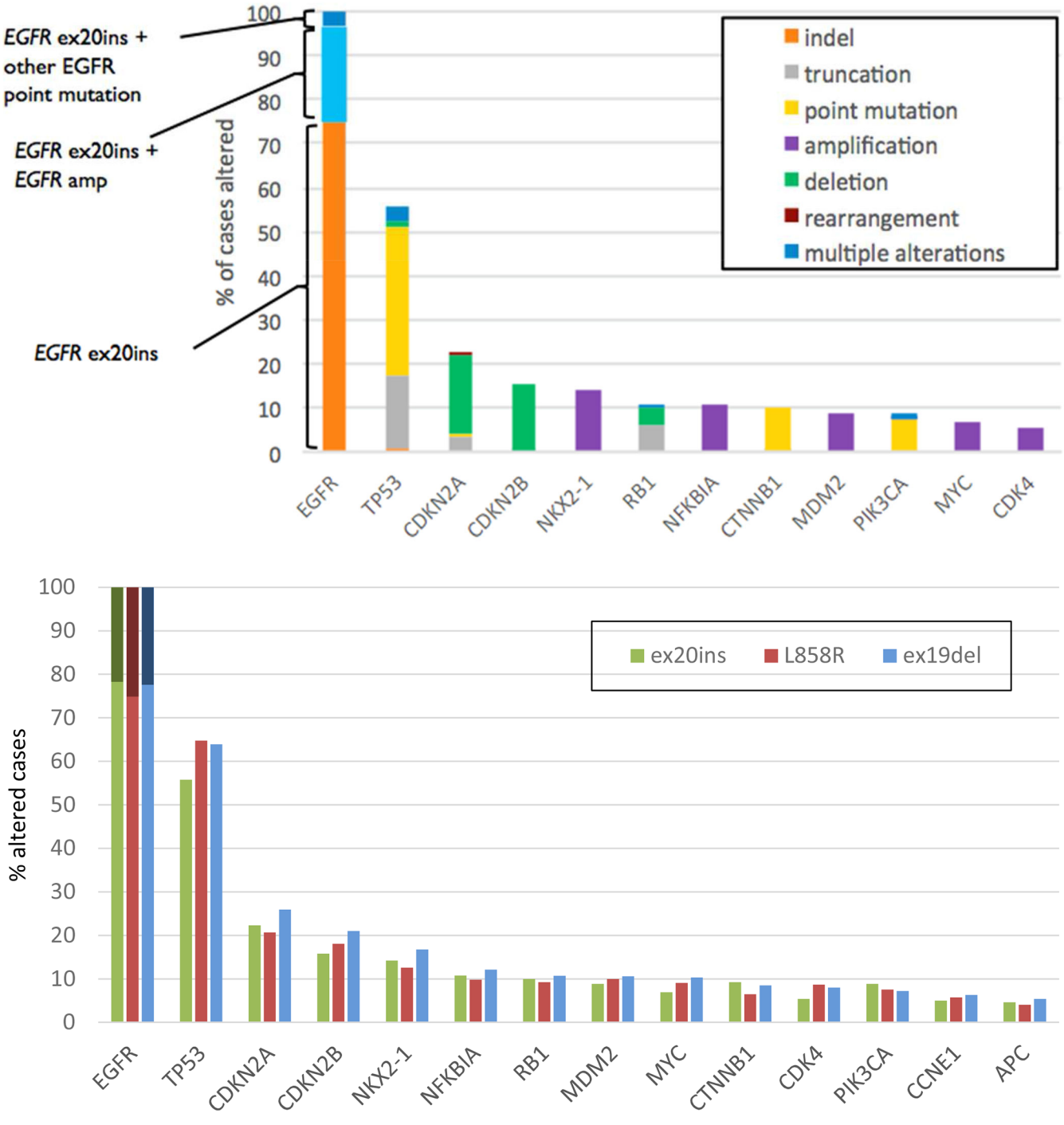

Figure 4.

A: Co-Occurring Genomic Alterations With a Frequency > 5% in NSCLC Harboring *EGFR* Exon 20 Insertions.

B: Longtail of frequently co-altered genes in NSCLC with *EGFR*ex20ins L858R, ex19del. Only cases without co-occurring T790M mutation are shown: *EGFR* ex20ins (n=260),

L858R (n=542), ex19del (n=776). Dark shading on the *EGFR* bars indicate co-occurring *EGFR* amplification.

Table 1:

Histologic and Clinical Characteristics of Non-Small Cell Lung Cancer Patients with Tumors Harboring *EGFR* Exon 20 Insertions

Histologic Subtype	**All**	**Adenocarcinoma**	**NSCLC NOS**	**Squamous/ Adenosquamous**	**Sarcomatoid**
Total Cases	14483	10272 (71%)	2197 (15%)	1942 (13%)	122 (0.8%)
N of patients with *EGFR* exon 20 Insertions	263	237 (90.1%)	24 (9.2%)	0	2 (0.7%)
Median Age (range)	63 (14-90)	63 (14-90)	71 (44-87)		50.5 (45-56)
Sex					
M	100	89	10		1
F	163	148	14		1

Table 2:

Comparison of Molecular, Pathologic and Clinical Characteristics of EGFR-WT, EGFR-mutant (E19del/ L858R positive / T790M negative) and EGFR ex20ins NSCLC. Mann Whitney test for age. Chi-square tests (2-sided, alpha <0.01) used for sex, mutation data, histology (Adenocarcinoma vs all others). TMB (tumor mutational burden) by category: 2-way ANOVA, Bonferroni posttest for significance.

	EGFR-WT NSCLC	**EGFR-mutant NSCLC (E19del and L858R – T790M neg)**	**EGFR ex20ins NSCLC**	**p-value (EGFR-WT vs. EGFR ex20ins)**	**p-value (EGFR-mutant E19del and L858R vs. ex20ins)**	**p-value (EGFR-WT vs. EGFR-mutant E19del and L858R – T790M neg)**
Total Cases (N)	**12,551**	**1,318**	**260**			
Median Age, years (range)	65 (6-99)	65 (25-95)	63 (14-90)	**0.0007**	0.02	0.07
Sex, F/M (%F)	6,246/6,304 (50%)	888/430 (67%)	161/99 (62%)	**<0.0001**	0.089	**<0.0001**
Histologic Subtype						
Adenocarcinoma	8,572 (68%)	1,149 (87%)	235 (90%)	**<0.0001**	0.15	**<0.0001**
Squamous/Adeno squamous	1,835 (15%)	50 (3.8%)	0			
NSCLC-NOS	2,027 (16%)	115 (8.7%)	23 (8.8%)			
Sarcomatoid	117 (0.9%)	4 (0.3%)	2 (0.8%)			
Frequency of Co-Occurring Genomic Alterations						
Concurrent *EGFR* Copy Number Gain	355 (2.8%)	311 (24%)	57 (22%)	**<0.0001**	0.63	**<0.0001**
Concurrent *TP53* alteration	7,748 (62%)	844 (64%)	146 (56%)	0.0773	0.0163	0.107
Concurrent *RB1* alteration	771 (6.1%)	133 (10%)	28 (11%)	**0.0035**	0.7413	**<0.0001**
Concurrent *CDKN2A/2B* alteration	3,222 (26%)	317 (24%)	57 (22%)	0.1939	0.5232	0.2113
TMB (mutations per MB) Median	**8.1**	**3.6**	**3.6**	**<0.0001**	0.31	**<0.0001**
TMB Low (<5)	3,785 (30%)	832 (63%)	179 (69%)	**<0.01**	NS	**<0.01**
TMB Intermediate Low (5-10)	3,470 (28%)	400 (30%)	69 (27%)	**<0.01**	NS	**<0.01**
TMB Intermediate High (10-20)	3,325 (26%)	82 (6.2%)	10 (3.8%)	**<0.01**	NS	**<0.01**
TMB High (>20)	1,971 (16%)	4 (0.3%)	2 (0.8%)	NS	NS	NS