

## Genomic alterations of ERBB receptors in cancer: clinical implications

Rosalin Mishra<sup>1</sup>, Ariella B. Hanker<sup>2</sup> and Joan T. Garrett<sup>1</sup>

<sup>1</sup>Division of Pharmaceutical Sciences, James L. Winkle College of Pharmacy, University of Cincinnati, Cincinnati, Ohio, U.S.A

<sup>2</sup>Department of Medicine, Breast Cancer Program, Vanderbilt-Ingram Cancer Center, Vanderbilt University Medical Center, Nashville, Tennessee, U.S.A

**Correspondence to:** Joan T. Garrett, **email:** joan.garrett@uc.edu

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### ABSTRACT

The ERBB family of receptor tyrosine kinases has been implicated in carcinogenesis for over three decades with rigorous attention to EGFR and HER2. ERBB receptors, consisting of EGFR, HER2, HER3, and HER4 are part of a complicated signaling network that activates downstream signaling pathways including PI3K/AKT, Ras/Raf/MAPK, JAK/STAT and PKC. It is well established that EGFR is amplified and/or mutated in gliomas and non-small-cell lung carcinoma while HER2 is amplified and/or over-expressed in breast, gastric, ovarian, non-small cell lung carcinoma, and several other tumor types. With the advent of next generation sequencing and large scale efforts to explore the entire spectrum of genomic alterations involved in human cancer progression, it is now appreciated that somatic ERBB receptor mutations occur at relatively low frequencies across multiple tumor types. Some of these mutations may represent oncogenic driver events; clinical studies are underway to determine whether tumors harboring these alterations respond to small molecule EGFR/HER2 inhibitors. Recent evidence suggests that some somatic ERBB receptor mutations render resistance to FDA-approved EGFR and HER2 inhibitors. In this review, we focus on the landscape of genomic alterations of EGFR, HER2, HER3 and HER4 in cancer and the clinical implications for patients harboring these alterations.

### INTRODUCTION

The ERBB family of receptor tyrosine kinases (RTKs), consisting of EGFR (also known as ERBB1, HER1), HER2 (ERBB2, neu), HER3 (ERBB3) and HER4 (ERBB4), were first implicated in cancer in the beginning of the 1980s when it was discovered that EGFR had close sequence homology to avian erythroblastosis tumor virus (AEV) [1, 2]. HER2/neu was first identified in rat carcinogen-induced tumors with a transmembrane domain mutation, V664E, that made its tyrosine kinase constitutively active [3]. The V664E mutation in HER2 supports receptor dimerization and greater tyrosine kinase activity [4]. The HER2<sup>V664E</sup> mutation has not yet been found in human tumors. HER3 and HER4 were subsequently identified due to their sequence homologies to EGFR [5-7]. Each member of the ERBB family is composed of an extracellular ligand-binding domain,

a single transmembrane domain and an intracellular domain which includes the tyrosine kinase (TK) domain. Signaling in the EGFR family is typically initiated when ligands bind the ectodomain, causing conformational changes that allows for homo- or heterodimerization with other ERBB family members. Dimerization activates cytoplasmic catalytic activity resulting in trans- and autophosphorylation of tyrosine residues in the cytoplasmic tails. These tyrosine residues serve as docking sites for several adaptor proteins which initiate multiple signaling cascades, ultimately resulting in deregulated cell proliferation, cell survival, angiogenesis, and metastasis.

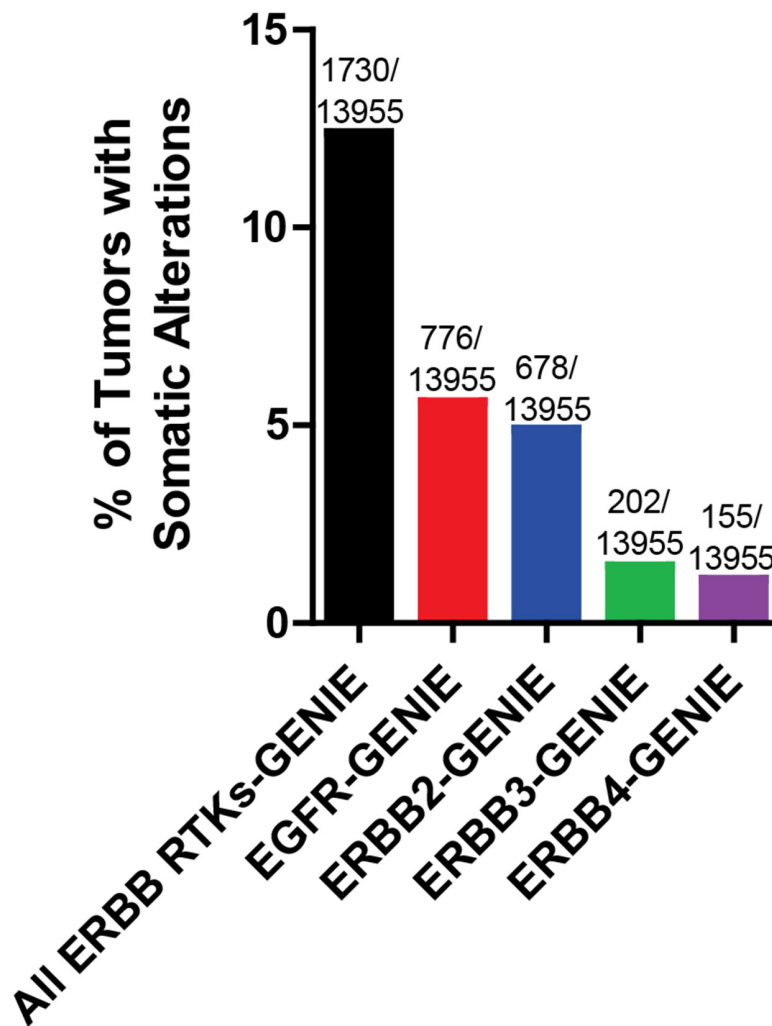
The advent of next generation sequencing technology has allowed for many large scale projects exploring whole genome or exome analysis of tumors including TCGA (The Cancer Genome Atlas) and ICGC (International Cancer Genome Consortium) [8]. Figure 1 indicates the frequency of ERBB receptor copy number

amplification and putative driver mutations across all cancer types from the GENIE data set [9]. A putative driver mutations is defined as frequency >5 in cBioPortal or COSMIC databases, or a HotSpot or OncoKB driver annotation in cBioPortal. In accordance with an abundance of literature indicating the oncogenic role of *EGFR* and *ERBB2*, these two family members each have a greater than three-fold higher incidence of somatic alterations compared to *ERBB3* or *ERBB4*. Nevertheless, the frequency rate of somatic alterations of EGFR account for only 5.6% of all cancer types, with other ERBB family members having a lower rate of somatic alterations within the GENIE dataset [9]. However, we note that this may be an underestimate, as many of the sequencing assays used in project GENIE fail to detect gene rearrangements and large deletions, such as the EGFR type III variant, frequently found in glioblastoma. Collectively, greater than 12% of all cancers examined in the GENIE data

set harbor somatic alterations in one or more members of the ERBB family. Further efforts are underway to distinguish between ERBB receptor mutations that drive cancer progression versus passenger mutations. Passenger mutations are not thought to contribute to cancer growth; rather, they simply accrue during the course of tumor development as a result of genomic instability. In this review, we discuss recent advances in our understanding of genomic alterations of the ERBB family members in cancer and efforts to target these alterations.

## EGFR MUTATIONS IN NON-SMALL CELL LUNG CANCER

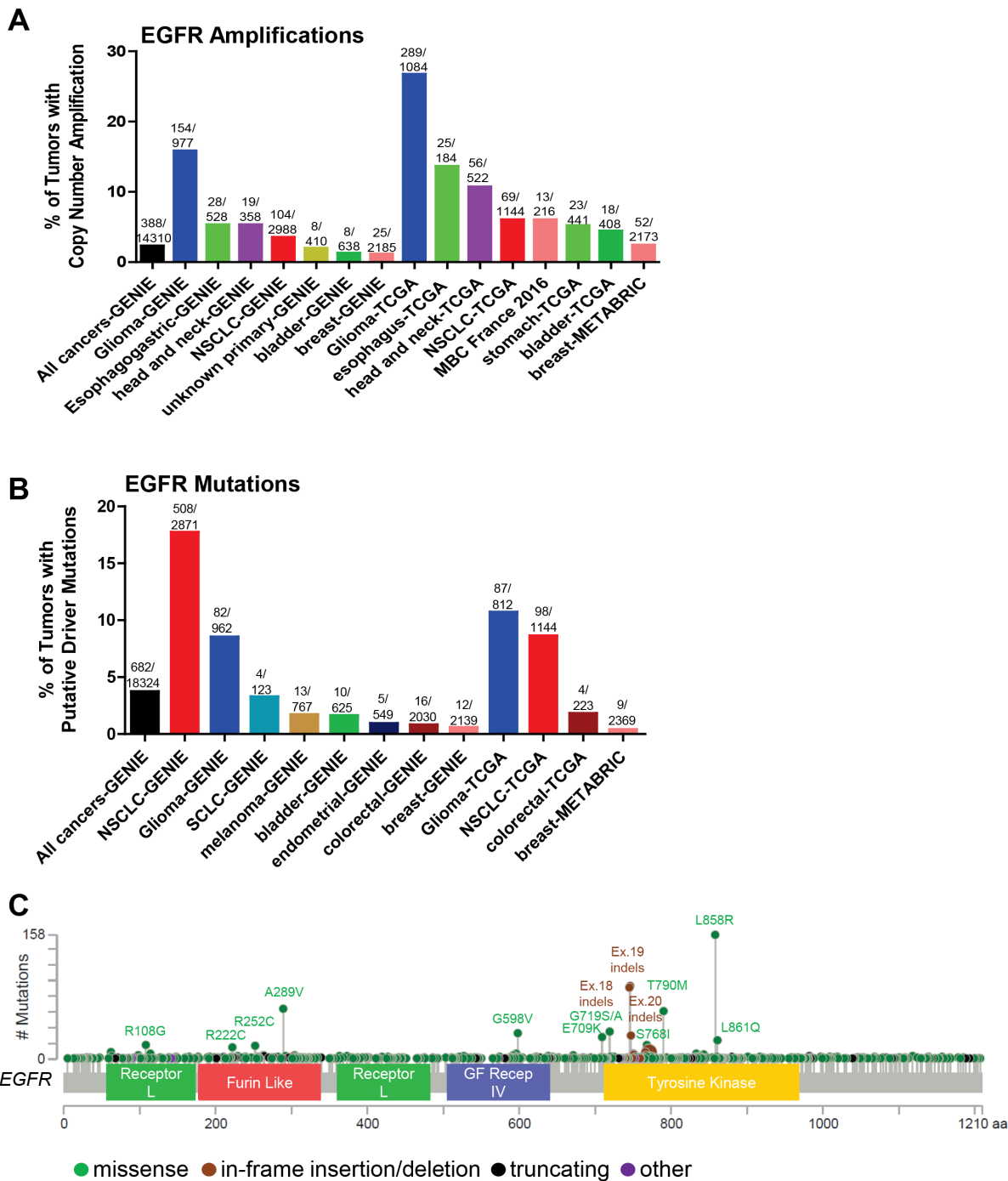
The selective response of non-small cell lung cancer (NSCLC) patients to EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib allowed for the



**Figure 1: Frequency of somatic alterations of ERBB receptors in cancer.** The Project GENIE dataset was analyzed for frequency of *EGFR*, *ERBB2*, *ERBB3*, and *ERBB4* copy number amplification and putative driver mutations across all cancer types (N=13955 tumors with copy number and mutation data for all 4 genes). Some tumors harbored multiple alterations. Putative driver mutations are defined as: cancer hotspot or OncoKB driver annotation (defined by cBioPortal.org) or number >5 in cBioPortal or COSMIC datasets.

identification of oncogenic EGFR mutations [10–13]. Many EGFR activating mutations are found in the catalytic kinase domain (exons 18-24) including small in-frame deletions found at amino acids 747-750 of exon 19 and the L858R mutation in exon 21, the most frequent EGFR mutation (Figure 2C). These activating mutations are clustered around the ATP-binding pocket of

the enzyme [14] and display up to a 50-fold acceleration in catalysis by disrupting autoinhibitory interactions [15]. Increased kinase activity of EGFR results in pro-survival and anti-apoptotic signals via activation of downstream targets including PI3K-AKT, ERK and STAT. Thus, these mutations represent classic cases of oncogene addiction [16]. As such, the efficacy of the first-line EGFR inhibitors



**Figure 2: Somatic alterations of EGFR in cancer. (A, B)** Frequency of EGFR copy number amplifications (A) or putative driver mutations (B) in selected cBioPortal and GENIE datasets. (C) Distribution of somatic variants within EGFR across its domain-annotated protein structure in all cBioPortal studies. NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; GF Recept IV, Growth Factor Receptor IV domain.

gefitinib and erlotinib over cytotoxic chemotherapy in patients with EGFR-mutant NSCLC has been well-established [17].

Despite the successes of EGFR targeting agents in patients with activating catalytic kinase domain EGFR mutations compared to chemotherapy, patients invariably progress within several years of treatment. The first identified mechanism of acquired resistance to EGFR TKIs was the EGFR T790M mutation [18, 19]. The T790M mutation structurally corresponds to the mutated gatekeeper residue T315I in BCR-ABL, T670I in c-KIT and T674I in PDGFR $\alpha$  [20]. The EGFR T790M mutation has increased affinity to ATP, resulting in decreased sensitivity to ATP-competitive reversible inhibitors [21]. Notably, the T790M mutation is one of the most frequently found mutations in EGFR (Figure 2C), with the caveat that 57 of 63 of these T790M EGFR mutant tumors come from the MSK-IMPACT cohort [22]. While the T790M mutation is very rare in primary untreated tumors [23], it has a much higher frequency in the project GENIE dataset [9], which includes tumors that have relapsed following treatment with EGFR TKIs. However, there is evidence showing that the T790M mutation can be found in primary, untreated tumors, and also in germline cells in families with inherited lung cancer [24, 25]. Thus, the detection of the EGFR T790M mutation in tumors could indicate a dependence on EGFR due to increased ATP-binding to EGFR. Afatinib is a second-generation irreversible, covalently-bound inhibitor of EGFR that has more recently been approved to treat NSCLCs harboring EGFR activating mutations [26] but still may have decreased sensitivity to tumors harboring T790M mutations [27]. Other classes of drugs targeting EGFR-mutant tumors are in various stages of development, including mutant-selective irreversible inhibitors, such as osimertinib (AZD9291), based on a pyrimidine scaffold that forms a covalent bond with Cys797 at the edge of the ATP binding pocket [28]. In 2015, osimertinib received accelerated FDA approval for NSCLC patients carrying the T790M mutation, and had recently shown promising efficacy as a first-line treatment for EGFR-mutant NSCLC [29]. However, as with first- and second-generation EGFR TKIs, resistance to osimertinib ultimately develops. Potential mechanisms of resistance include loss of the T790M mutation [30], mutations in the RAS pathway [31], amplification of *ERBB2* or *MET* [29, 30], and the EGFR C797S mutation, altering the amino acid that binds osimertinib [29, 32, 33]. The latter mutation was found in 40% of a small cohort of NSCLC patients following acquired resistance to osimertinib [33]. EA1045 is an inhibitor that binds the allosteric pocket of EGFR rather than the ATP binding pocket, and is selective for drug-resistant EGFR mutants [34]. The combination of EA1045 and cetuximab blocked the growth of mouse models of lung cancer harboring EGFR T790M and C797S.

Other recurrent EGFR mutations in NSCLC include the exon 18 mutations E709K and G719A/C/D/S, the exon 20 S768I missense mutation, and exon 20 insertions (Table 1 and Figure 2C). While there is evidence that the missense mutations respond to second-generation EGFR inhibitors such as afatinib, the Exon 20 insertions are thought to be less sensitive [35]. Mutant-specific EGFR inhibitors with preclinical activity against the exon 20 insertion mutations, such as AP32788 and EGF816, are now in clinical development [36, 37].

## THERAPEUTIC CHALLENGES IN GLIOMA WITH EGFR GENETIC ALTERATIONS

Grade IV glioblastoma multiform (GBM) is the most common and aggressive cancer originating in the central nervous system (CNS), exhibiting a high frequency of recurrence and dismal prognosis due to the invasive nature of the tumor. The standard of care for GBM patients is surgical resection followed by radiation plus the chemotherapeutic temozolomide, with a median overall survival of 15 months from diagnosis [38]. GBM is frequently associated with molecular changes in EGFR (Figure 2). The most common and best-studied EGFR alteration in glioblastomas is the EGFR type III variant (EGFRvIII), a constitutively active genomic deletion variant lacking exons 2 to 7 of the EGFR gene, usually occurring in EGFR-amplified tumors [39–41]. EGFRvIII lacks domains I and II of the extracellular region of wild-type (WT) EGFR. Lacking the domain II loop, EGFRvIII is thought to avoid formation of the tethered, inactive conformation, causing a shift in the equilibrium to the open, active conformation [42]. Large-scale genomic studies reported that EGFR is a key driver of GBM, defining a subtype of GBM [43]. Genetic alterations including mutations, rearrangements, alternative splicing and focal amplifications occurred in 57% of primary GBMs [44]. Thus, EGFR represents a prime therapeutic target for glioblastoma. Unfortunately, the use of the EGFR tyrosine kinase inhibitors erlotinib [45, 46], gefitinib [47, 48], afatinib [49], or lapatinib [50], either alone or in combination with other agents, has resulted in disappointing results in the clinic. In addition to drug delivery concerns in glioblastoma, there are several important caveats to consider. The disappointing exploration of EGFR as a target in glioblastoma includes well-documented intra-tumoral heterogeneity of EGFR and amplification of other RTKs that could bypass EGFR inhibition [51–54]. One mechanism for *de novo* resistance in glioblastoma to EGFR inhibitors is the ability of these cancers to reversibly up-regulate or suppress mutant EGFR expression, resulting in distinct cellular phenotypes to reach an optimal equilibrium for growth [55]. Although one of the most characteristic features of glioblastoma is alterations in EGFR, therapeutically targeting EGFR

**Table 1: Selected ERBB family mutations found in patients**

<b>ERBB mutation</b>	<b>Principle tumor type (cBioPortal)</b>	<b>Drug sensitivity</b>	<b>Drug insensitivity</b>	<b>Acquired resistance</b>
<b>EGFR R108G/K</b>	Glioma	preclinical: erlotinib [143]		
<b>EGFR A289D/I/N/T/V</b>	Glioma	preclinical: erlotinib [143]		
<b>EGFR E709A/K/Q</b>	NSCLC	preclinical: afatinib, neratinib [144]		
<b>EGFR exon 18 insertions/deletions</b>	NSCLC	preclinical, clinical: afatinib, neratinib [144]		
<b>EGFR G719A/C/D/S</b>	NSCLC	clinical: erlotinib, gefitinib, afatinib [145], [35]		
<b>EGFR exon 19 insertions/deletions</b>	NSCLC	clinical: erlotinib, gefitinib, afatinib [146]		
<b>EGFR exon 20 insertions/deletions</b>	NSCLC	preclinical: EGF816, AP32788 [36, 37]	clinical: afatinib, gefitinib, erlotinib [147],[148],[35]	
<b>EGFR S768G/I/T</b>	NSCLC	clinical: afatinib, gefitinib, erlotinib [149], [35]		
<b>EGFR T790M</b>	NSCLC	clinical: osimertinib [28]; preclinical: EA1045 and cetuximab [34]	clinical: afatinib [26, 27]	clinical: gefitinib, erlotinib, afatinib [18, 19]
<b>EGFR C797S/Y</b>	NSCLC	preclinical: EA1045 and cetuximab [34]		clinical: osimertinib [32, 33]
<b>EGFR L858R</b>	NSCLC	clinical: gefitinib, erlotinib, afatinib [10],[11]		
<b>EGFR L861Q/R</b>	NSCLC, lung squamous	clinical: gefitinib, erlotinib, afatinib [145], [35]		
<b>ERBB2 D277G/H/V/Y</b>	bladder	preclinical: lapatinib, afatinib [80]		
<b>ERBB2 S310F/Y</b>	bladder, breast, esophagogastric, colorectal, lung, cervical	preclinical: trastuzumab, lapatinib, neratinib, afatinib [77, 79, 80, 90]; clinical: neratinib [103, 105]	preclinical: cetuximab, panitumumab [79]	
<b>ERBB2 R678Q</b>	esophagogastric, colorectal, bladder	preclinical: lapatinib, afatinib, neratinib [77, 80]		
<b>ERBB2 L755S</b>	breast, bladder, colorectal	preclinical: neratinib, afatinib [77, 112]; clinical: neratinib [98, 103, 105]	preclinical: trastuzumab, lapatinib, cetuximab, panitumumab [77, 79, 85, 112]	preclinical: lapatinib [112]

*(Continued)*



ERBB mutation	Principle tumor type (cBioPortal)	Drug sensitivity	Drug insensitivity	Acquired resistance
<b>ERBB2 D769H/Y</b>	breast, bladder, esophagogastric, colorectal	preclinical: trastuzumab, lapatinib, neratinib [77]; clinical: neratinib [103]		
<b>ERBB2 exon 20 insertions/deletions</b>	NSCLC	preclinical: lapatinib, afatinib, neratinib, AP32788 [36, 73, 77, 82, 84, 93]; clinical: trastuzumab, afatinib, neratinib, dacomitinib [96, 100-103, 105] [150], [97]	preclinical: erlotinib, gefitinib [73, 82, 93]	clinical: osimertinib [29]
<b>ERBB2 V777L</b>	breast, colorectal, esophagogastric	preclinical: trastuzumab, lapatinib, neratinib [77, 79, 85] clinical: neratinib [91]	preclinical: cetuximab, panitumumab [79]	
<b>ERBB2 T798I/M</b>	breast	preclinical: afatinib [91]	preclinical: trastuzumab, lapatinib, neratinib [85, 91, 111]	clinical: neratinib [91]
<b>ERBB2 V842I</b>	colorectal, breast, esophagogastric, endometrial	preclinical: trastuzumab, lapatinib, neratinib [77, 79]; clinical: neratinib [103]	preclinical: cetuximab, panitumumab [79]	
<b>ERBB2 L869R</b>	breast	preclinical: neratinib, afatinib [91]; clinical: neratinib [92, 103, 105]	preclinical: lapatinib [91]	
<b>ERBB3 V855A</b>	NSCLC	preclinical: pertuzumab and afatinib [128]		
<b>ERBB3 mutations</b>	multiple tumor types		clinical: neratinib [105]	
<b>ERBB4 KD mutations</b>	melanoma, esophagogastric, colorectal	preclinical: lapatinib [132]		

is currently not efficacious likely due to heterogeneity of EGFR signaling networks and redundant alternative signaling pathway activation [56].

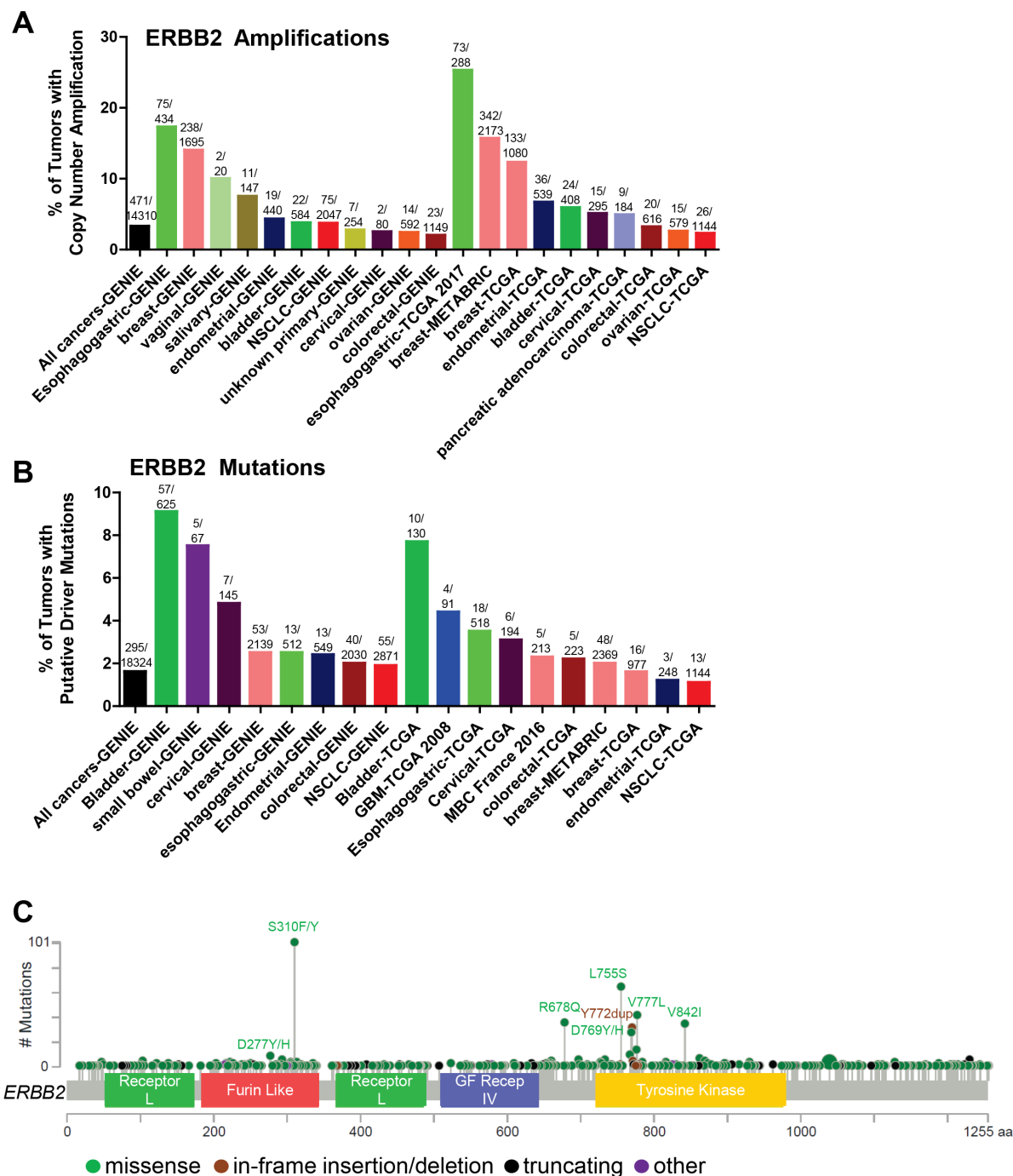
Novel strategies to target EGFR in GBM are currently being explored. One area of interest for patients harboring EGFR amplifications are antibody-drug conjugates (ADCs) [57]. ADCs enable therapeutic delivery of cytotoxic agents specifically to tumor cells by linkage to an antibody targeting a protein that is expressed more highly on tumor cells than normal cells. The ADC ABT-414 is comprised of an EGFR antibody linked to monomethyl auristatin F (MMAF), an inhibitor of tubulin assembly. ABT-806, the parental antibody of ABT-414, was found to accumulate specifically in the tumor in both mouse models and in patients with glioma [58, 59] and showed impressive antitumor activity in GBM xenografts harboring EGFR amplification or EGFRvIII [60]. Phase

I trials found that ABT-414 demonstrated an acceptable safety profile, although ocular toxicity was very common [61], and several partial responses were observed. ABT-414 is currently in phase II studies as monotherapy or in combination with temozolomide. Since EGFRvIII is specific to tumor cells, it has also been an attractive target for immunotherapeutic approaches [62]. These include rindopepimut (CDX-110-KLH), a 14 amino acid peptide vaccine corresponding to the fusion junction of EGFRvIII [63]. This peptide was then used as the basis for a dendritic cell (DC) vaccine. In preclinical studies, DCs pulsed with CDX-110-KLH caused prolonged immunity and a significant elongation of survival in mouse models [64]. However, this vaccine failed to prolong overall survival in a phase III trial [65]. Due to the substantial intracellular heterogeneity in GBM, combination therapies may be needed to block the growth of all tumor cells.

## HER2 ALTERATIONS IN CANCER

HER2 has long been known to be amplified and overexpressed in breast [66], gastric [67], and bladder cancers [68] and large scale copy number analysis confirms that HER2 is amplified most frequently in gastric followed by breast cancer (Figure 3A). As such, several decades of drug discovery efforts have resulted

in five HER2 inhibitors that are currently FDA-approved to treat HER2-amplified/overexpressing breast cancers. These include the monoclonal antibodies trastuzumab and pertuzumab, the ADC trastuzumab emtansine, and the EGFR/HER2 TKIs lapatinib and neratinib. These drugs, in combination with chemotherapy, have significantly improved outcomes for HER2-amplified breast cancer patients, particularly in the adjuvant setting. However,



**Figure 3: Somatic alterations of *ERBB2* in cancer.** (A, B) Frequency of *ERBB2* copy number amplifications (A) or putative driver mutations (B) in selected cBioPortal and GENIE datasets. (C) Distribution of somatic variants within *ERBB2* across its domain-annotated protein structure in all cBioPortal studies. NSCLC, non-small cell lung cancer; GBM, glioblastoma multiform; MBC, metastatic breast cancer; GF Recep IV, Growth Factor Receptor IV domain.

resistance to HER2 inhibitors in metastatic cancers remains a problem. Potential mechanisms of resistance include activation of downstream signaling pathways, such as the PI3K/AKT pathway, HER2 truncations (p95 HER2) and splice variants, upregulation of ERBB ligands, and activation of other RTKs [69].

Unlike breast cancer, only trastuzumab is approved to treat HER2-amplified gastric cancer. Trastuzumab emtansine and lapatinib failed to prolong survival over standard treatment. The combination of pertuzumab, trastuzumab, and chemotherapy is currently being evaluated in the phase III JACOB trial for HER2-positive gastric cancers [70]. Trastuzumab and lapatinib and trastuzumab emtansine are currently being investigated in the clinic for other types of cancer with HER2 amplifications, including lung and colorectal cancers.

Somatic mutations in the kinase domain (KD) of the *ERBB2* gene (primarily exon 20 insertions) were first reported in a low frequency of lung cancers [71, 72] and were subsequently shown to increase HER2 protein activation [73]. More recently, HER2 mutations have been found in a variety of cancer types [74, 75] (Figure 3B), including breast [76–78], colorectal [79], and bladder cancers [80]. HER2 somatic mutations correlate with poor survival in HER2-negative (non-amplified) breast cancer [81]. Nearly 2% of all cancers harbor hotspot or putative activating mutations in *ERBB2* (Figure 3B), suggesting that these cancers may be sensitive to HER2-targeted therapies. The most frequent HER2 mutations are found in the extracellular domain ECD (primarily S310F/Y) and the KD (exon 20 insertions/deletions, L755S, V777L, V842I; Figure 3C). The S310F/Y mutation is most frequently found in bladder cancer, whereas the KD mutations L755S and V777L occur frequently in breast cancer, and V842I is most commonly found in colorectal cancer. The majority of HER2 mutations in lung cancer are the exon 20 insertions/deletions, the most prevalent being the Y772\_A775 duplication (also known as the A775\_G776 YVMA insertion). The exon 20 insertions were the first HER2 mutations to be extensively characterized [73, 82]. The exon 20 insertions are similar to those found in EGFR [71] and are thought to induce a conformational change of the autoinhibitory  $\alpha$ C- $\beta$ 4 loop in the kinase domain, narrowing the ATP-binding cleft and leading to enhanced kinase activity [83]. HER2<sup>YVMA</sup> was further shown to increase phosphorylation of HER2, EGFR, and downstream signal transducers including AKT and ERK, transform bronchial and mammary epithelial cells, and promote tumor formation in nude mice [73]. Inducible expression of HER2<sup>YVMA</sup> in the mouse lung epithelium promoted development of adenosquamous lung tumors that were sensitive to HER2 kinase inhibition [84].

Bose et al. characterized the HER2 activating mutations G309A, D769H/Y, V777L, P780ins, and V842I. These mutations promoted the HER2 kinase activity, phosphorylation of HER2, EGFR, HER3, and ERK, and

the transformation of mammary epithelial cells [77]. Similarly, the L755S/P, V777L, and T862A mutations promoted colony formation in NMuMg mouse mammary epithelial cells [85], and the L755S, V777L, V842I, and S310F mutations were shown to transform colonic epithelial cells [79]. Of note, the V777L mutation is homologous to EGFR V769L, a rare mutation associated with NSCLCs (Table 2), and ALK F1174L, a known activating mutation in neuroblastoma [77, 86–89]. The V777 residue abuts the conserved DFG motif involved in tyrosine kinase activity [77].

Greulich et al. reported that the HER2 ECD mutations G309E, S310F, and S310Y, in addition to several rare ECD mutants identified in glioblastoma, increased colony formation in NIH 3T3 cells [90]. While the rare G309E mutation promoted covalent homodimerization mediated by intermolecular disulfide bond formation, HER2<sup>S310F</sup> functioned more similarly to the kinase domain mutations, and increased c-terminal tail phosphorylation. Several studies reported that many non-“hotspot” HER2 variants of unknown significance (VUS) do not appear to activate HER2 kinase activity or signaling [77, 80, 90]. However, we recently reported that the relatively rare HER2<sup>L869R</sup> mutation increased HER2-mediated signaling and growth of MCF10A mammary epithelial cells; in addition, a breast cancer patient with this mutation showed an excellent response to neratinib [91]. Therefore, more studies are needed to characterize the large number of HER2 VUS appearing in databases such as cBioPortal and GENIE in order to determine if they are gain-of-function driver mutations or neutral passenger mutations.

Recent evidence suggests that HER2 mutants, when expressed at endogenous levels, demonstrate weak oncogenic properties [92] and require additional cooperating mutations to transform cancer cells. Such cooperating alterations may include co-occurring mutations in *PIK3CA* [92] and *ERBB3* [91]. In addition, low-level copy number gain of *ERBB2* is frequently observed in HER2-mutant tumors ([www.cbioportal.org](http://www.cbioportal.org)); therefore, elevated expression of the HER2 mutants may contribute to their oncogenic function.

## THERAPEUTIC TARGETING OF MUTANT HER2

There has been considerable interest in determining whether the growing number of anti-HER2 therapies initially developed to treat HER2-amplified breast cancer could also block cancers harboring HER2 mutations. A number of studies have examined whether cells engineered to express various HER2 mutants are sensitive to HER2 TKIs and monoclonal antibodies. Moderate sensitivity to trastuzumab was observed in MCF10A cells expressing various HER2 mutants [73, 77]. Some mutants, including S310F/Y and V777L, were sensitive to lapatinib [77, 85,



**Table 2: Sequence homology of missense mutations found in ERBB family members**

EGFR	ERBB2	ERBB3	ERBB4
R108G/K (glioma)	R103Q (bladder)	ND	R106C/H (multiple)
ND	S310F/Y (multiple)	ND	S303F/Y (multiple)
S768I (NSCLC)	G776V/S (multiple)	ND	ND
V769L (NSCLC)	V777A/L/M (multiple)	ND	ND
T790M (NSCLC)	T798I (breast)	ND	ND
L858R (NSCLC)	ND	V855A (NSCLC)	ND
L861R/Q (NSCLC; lung squamous)	L869R/Q (breast)	ND	ND
ND	V842I (multiple)	ND	V840I (multiple)

ND (no data): mutation not reported/not found in cBioPortal or GENIE.

90], whereas others, such as L755S, L869R, and exon 20 insertions/deletions, displayed lapatinib resistance *in vitro* [77, 85, 91]. Most mutants tested were sensitive to irreversible EGFR/HER2 inhibitors such as afatinib and neratinib [73, 77, 90, 93]. Perera et al. found that transgenic mouse tumors driven by the HER2 YVMA insertion were somewhat sensitive to afatinib, but the combination of afatinib with the mTOR inhibitor rapamycin was more efficacious [84].

Several studies have examined the sensitivity of cells lines harboring naturally occurring HER2 mutations to HER2 inhibitors. Early studies indicated that the H1781 lung cancer cell line, harboring the HER2 G776\_VC insertion, is sensitive to combined treatment of lapatinib and trastuzumab, the pan-HER inhibitor CI-1033, and neratinib [73, 82]. Urinary bladder cancer (UBC) cell lines harboring the HER2 mutations S653C, R678Q, and S310F overall were more sensitive to lapatinib than HER2 WT cell lines [80]. Bose and colleagues examined whether colorectal cancer patient-derived xenografts (PDXs) harboring HER2 mutations responded effectively to HER2 inhibitors [79]. PDXs harboring HER2<sup>S310Y</sup> or HER2<sup>L866M</sup> were resistant to the EGFR monoclonal antibodies cetuximab and panitumumab, but were sensitive to neratinib. The HER2<sup>S310Y</sup>-expressing PDX was partially sensitive to trastuzumab or lapatinib as single agents. In both xenografts, the combination of neratinib and trastuzumab led to more complete inhibition of tumor growth, suggesting that this combination should be explored further.

In addition to preclinical studies, there are several case studies reporting that individuals with HER2-mutant metastatic lung or breast cancer respond to trastuzumab, trastuzumab + pertuzumab, or neratinib [94–97]. Due to the robust preclinical data, there is significant interest in using the irreversible EGFR/HER2 inhibitors neratinib and afatinib to treat HER2-mutant cancers. Responses to these agents have been documented in individuals with HER2-mutant lung and breast cancers [91, 96, 98–101].

Early results of clinical trials testing HER2 TKIs in HER2-mutant cancers are promising. In 30 patients with NSCLC harboring HER2 exon 20 mutations (primarily insertions) treated with the irreversible EGFR/HER2/HER4 inhibitor dacomitinib, 3 partial responses were seen [102]. The MutHER and SUMMIT trials tested the efficacy of neratinib in HER2-non-amplified, HER2-mutant metastatic breast cancer. Clinical benefit rates of 31% and 41.7% to neratinib monotherapy were observed in the MutHER and SUMMIT trials, respectively [103, 104]. The clinical benefit rate increased to 58.3% in patients treated with neratinib in combination with the ER antagonist fulvestrant in the SUMMIT trial. Complete responses were observed in patients with V777L, L755S, and S310F missense mutations and the GSP and YVMA insertions. In the MutHER trial, no responses were seen in the uncharacterized VUS S609F and P802S [103]. The SUMMIT trial also tested the efficacy of neratinib monotherapy in other cancer types with HER2 mutations (n=125 patients representing 21 cancer types and 30 HER2 mutations) [105]. The greatest clinical activity was seen in breast, cervix, and biliary cancers, and with tumors harboring kinase domain missense mutations. These results strongly suggest that some HER2 mutations are true “driver” mutations in these cancers. No clinical benefit was observed in HER2-mutant colorectal cancers, regardless of mutation type, suggesting that overall response rates may be influenced by both tissue type and mutation type.

As with most targeted therapies in advanced cancers, acquired resistance to neratinib monotherapy is expected. An acquired HER2<sup>T798I</sup> “gatekeeper” mutation, homologous to the EGFR<sup>T790M</sup> mutation in EGFR inhibitor-resistant lung cancer (Table 2), was recently identified in a HER2-mutant breast cancer patient following progression on neratinib [91]. Structural modeling of the HER2<sup>T798I</sup> mutant suggested that the increased bulk of the isoleucine in place of the threonine sterically blocked neratinib binding. Afatinib, but not neratinib, blocked the growth

of HER2<sup>T798I</sup>-expressing cells, perhaps because afatinib is smaller than neratinib and may not be occluded from the binding pocket. Interestingly, the same mutation was also found in another HER2-mutant breast cancer patient following progression on neratinib [103]. Several other acquired HER2 mutations were also found in the circulating tumor DNA (ctDNA) of patients following progression on neratinib, including R678Q, V697L, T862A, and I767M; more than one acquired HER2 mutation was found in the same patient. These studies strongly suggest that these tumors were “addicted” to the initial driver alteration(s) in HER2, and required re-activation of HER2 for continued growth. Recently, the HER2<sup>C805S</sup> mutation, homologous to the drug-resistant EGFR<sup>C797S</sup> mutation, was found to promote resistance to irreversible HER2 TKIs in HER2-mutant cells *in vitro* [106], but this mutation has not yet been found in patients. Other potential mechanisms of acquired resistance to neratinib include *PIK3CA* mutations and amplification of the mutant *ERBB2* allele [96, 107].

Although activating HER2 mutations are found in ~2-6% of HER2-amplified breast cancers (www.cbioportal.org; [78, 108]) whether they promote resistance to HER2-targeted therapy in HER2-amplified breast cancer has not yet been clearly established. Boulbes *et al.* sequenced the kinase domains of *EGFR*, *ERBB2*, and *ERBB4* in 76 primary HER2+ breast cancers and found 6 mutations in *EGFR*, 3 in *ERBB2*, and 3 in *ERBB4*. None of the patients with ERBB mutations responded to trastuzumab, whereas 32% of patients with ERBB-WT cancers achieved partial responses. They further showed that the novel HER2<sup>L726F</sup> mutation detected in a patient reduced lapatinib efficacy in HER2-amplified BT474 breast cancer cells [109]. Larger studies are needed to confirm whether ERBB family mutations are associated with *de novo* resistance to HER2 inhibitors. In another study, acquired HER2 mutations were identified in 5/16 metastatic breast cancer samples treated with adjuvant trastuzumab. The mutations were not detected in matched primary samples. Three of these were the known lapatinib-resistant L755S mutation, whereas two were the novel K753E mutation. The authors further showed that HER2<sup>K753E</sup> expression promoted resistance to lapatinib and trastuzumab [110]. Similarly, recent preclinical studies have identified HER2 L755S and T798M as mutations that promote resistance to lapatinib or trastuzumab [111, 112]. In addition, HER2<sup>T798M</sup> was shown to be insensitive to neratinib in BT474 cells [91]. Afatinib retained its ability to block HER2 in these cells, while cells expressing HER2<sup>L755S</sup> retained sensitivity to afatinib and neratinib [112]. Therefore, HER2-positive patients progressing on anti-HER2 therapy should be profiled for acquired drug-resistant HER2 alterations.

Amplification of HER2 is a well-established mechanism of acquired resistance to EGFR TKIs in NSCLC [113, 114]. However, whether HER2 mutations

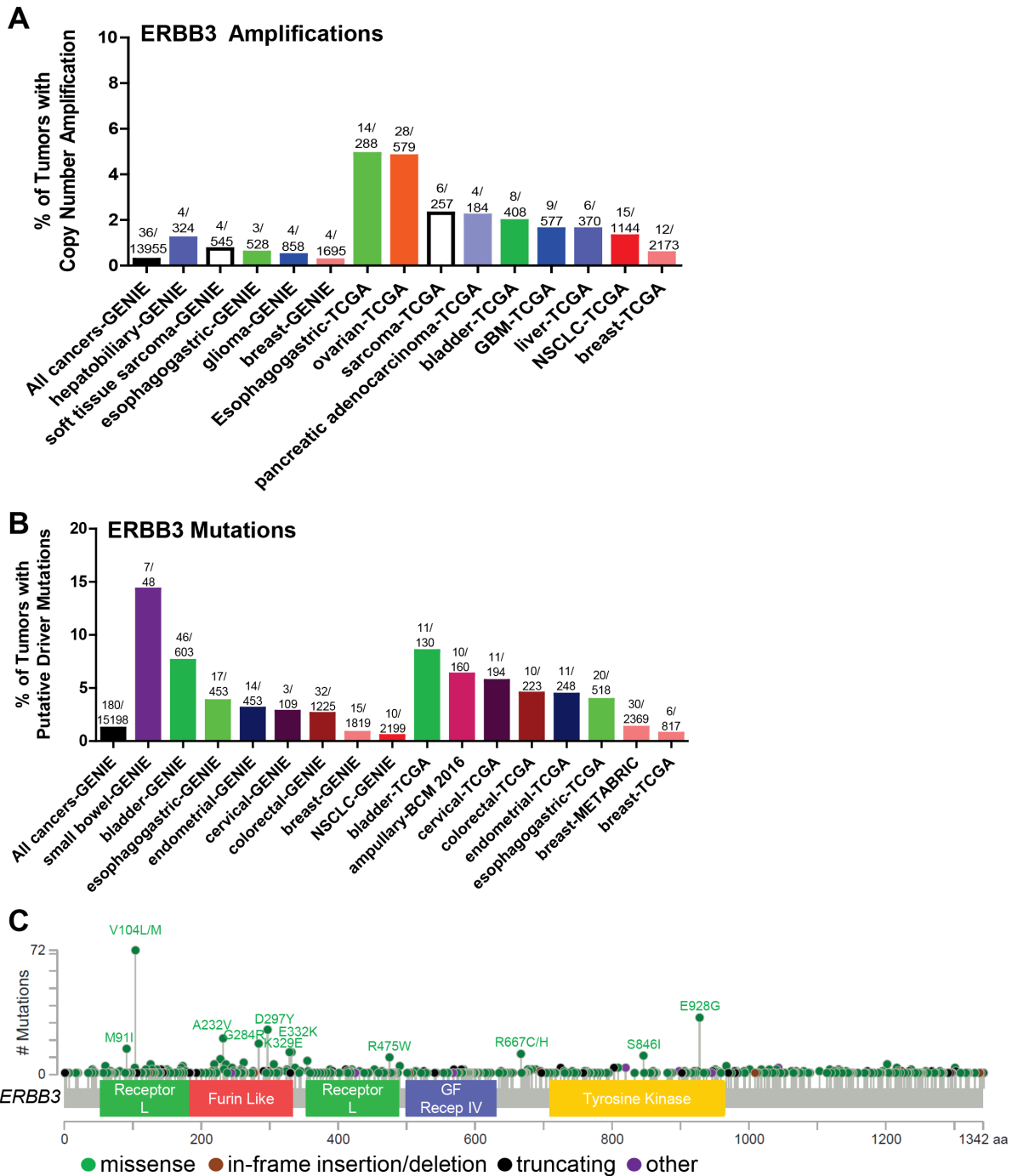
also promote acquired resistance to EGFR TKIs is not yet known. Interestingly, in primary lung tumors, HER2 and EGFR activating mutations are mutually exclusive [115], suggesting that they have overlapping roles in oncogenesis. A HER2 exon 20 insertion was recently found in an EGFR-mutant lung tumor with acquired resistance to osimertinib; no additional cancer-associated mutations were found in this tumor [29]. Future studies should investigate whether HER2 mutations directly promote resistance to osimertinib and other EGFR TKIs.

## HER3 MUTATIONS AND AMPLIFICATIONS

Although somatic mutations and alterations associated with EGFR and HER2 have been studied more rigorously due to their well-established roles as oncogenes, HER3 (*ERBB3*) mutations have been in the limelight recently as HER3 is an irrefutable partner in HER2-HER3 heterodimer signaling, and HER3 mediates resistance to EGFR- and HER2-targeted therapies [116]. Most *ERBB3* mutations have been identified in the ECD and few in the intracellular KD (Figure 4C). *ERBB3* missense mutations were first reported in 2006, when Jeong *et al.* found that 1 of 100 colon cancer samples tested had a missense mutation at S846I [117]. However, the authors failed to detect any mutations in 48 lung carcinomas. Ding *et al.* also identified 3 *ERBB3* somatic mutations (2 missense and 1 non-sense) [118]. Several other studies identified various somatic *ERBB3* mutations in 4% of breast cancer [119], 10% of gastric [120], 1% of ovarian [121, 122], 1% of colon cancer [117], 1% of glioblastoma [123]; 0.5% of squamous carcinomas, and 1% of head and neck cancer [124]. A whole exome sequencing analysis of 72 primary colon tumor specimens by Seshagiri *et al.* identified *ERBB3* somatic alterations at a rate of 8% (6 out of 72) [125]. Jaiswal *et al.* performed *ERBB3* exon sequencing of 507 primary tumors [126] and reported *ERBB3* alterations in 1% NSCLC (1/67 squamous; 1/71 adenocarcinoma;), 12% of gastric (11/92), 11% of colon cancers (11/100). Using *in vitro* transformed colonic and breast epithelial model systems, HER3 mutants (V104, A232A, P262H, G284R, T389K, Q809R, S846I and E928G) promoted anchorage-independent growth as compared to WT control in the presence but not in the absence of kinase-active HER2 in a ligand-independent manner [126]. This indicates that mutant HER3 may not be able to induce oncogenic transformation alone but requires HER2 expression to enhance tumor growth, consistent with HER3 being highly kinase-impaired [127] (in the absence of mutations that alter its kinase activity). However, recent studies in our lab indicate that a patient-derived HER3 ECD mutation (T355I) is transforming *in vitro* in the absence of HER2 overexpression. ER+ breast cancer cells (T47D and MCF-7 cells) overexpressing HER3<sup>T355I</sup> show enhanced colony formation in 3D-Matrigel and enhanced cell proliferation

as compared to WT control (manuscript in preparation). The HER3 ECD mutants (V104, A232, P262, G284, D297, G325, and T355I) are common hot-spot mutations across multiple cancers [126] (Figure 4C). Recurrent HER3 KD mutations include S846I and E928G [76]. Umelo et al. identified a novel somatic HER3 kinase mutant (V855A) homologous to EGFR-L858R activating mutation to be a

primary driver in lung pathogenesis [128] (Table 2). Using a murine hematopoietic system or transformed human embryonic kidney cells, in the presence of WT ERBB2, ERBB3-V855A demonstrated enhanced transformation of cells upon stimulation with ERBB3's ligand, NRG1. Figure 4 illustrates the somatic *ERBB3* copy number amplifications (A) and putative activating mutations



**Figure 4: Somatic alterations of *ERBB3* in cancer.** (A, B) Frequency of *ERBB3* copy number amplifications (A) or putative driver mutations (B) in selected cBioPortal and GENIE datasets. (C) Distribution of somatic variants within *ERBB3* across its domain-annotated protein structure in all cBioPortal studies. NSCLC, non-small cell lung cancer; GBM, glioblastoma multiforme; GF Recep IV, Growth Factor Receptor IV domain.

sequenced from 15198 patients in cBioPortal and GENIE datasets (Figure 4B).

## TARGETED THERAPEUTICS AGAINST HER3 MUTANTS

Multiple agents known to target ERBB receptors directly or indirectly are effective against various ERBB3 mutants. Use of small-molecule inhibitors or antibodies targeting ERBB family members, particularly lapatinib, trastuzumab, and anti-ERBB3 antibodies, were effective in inhibiting mutant ERBB3-mediated oncogenic activity both *in vitro* and *in vivo* [126]. Pertuzumab and afatinib were effective in inhibiting the transforming potential of the HER3<sup>V855A</sup> mutant [128]. As a part of the SUMMIT trial, neratinib was tested against 16 patients harboring *ERBB3* gene mutations. No clinical activity was observed in *ERBB3* mutant cohort in response to neratinib [105]. One explanation for the lack of clinical activity of neratinib in ERBB3 mutant tumors is the possibility that tumors are not “addicted” to the *ERBB3* mutations. If this is the case, inhibition of HER3 would not block tumor growth. Another prospect is that neratinib, a TKI of EGFR and HER2, may not be effective in *ERBB3*-mutant tumors, where HER3 functions independently of HER2 and an inhibitor specifically targeting HER3 may be necessary. In support of this, shRNA knockdown of *ERBB3* *in vivo* in cells with endogenous *ERBB3* mutations moderately but statistically significantly delayed tumor growth [126]. Another potential strategy to treat HER3-mutant tumors may be with antibodies targeting HER3 and other ERBB receptors. For example, Jacobsen et al. generated a mixture of six antibodies (Pan-HER) for synergistic targeting of EGFR, HER2 and HER3, with the goal of preventing compensatory activation of ERBB receptors when only one receptor is inhibited. The authors analyzed the efficacy of Pan-HER2 antibody mixture against 100 different cancer cell lines, including cell lines harboring EGFR or HER2 mutations and/or amplifications, and found that Pan-HER significantly suppressed cancer cell proliferation and outperformed the reference antibodies (cetuximab, trastuzumab and MM-121). Whether Pan-HER blocks the growth of HER3-mutant cancer cells is not known [129].

## HER4 ALTERATIONS

Among the members of the ERBB family, *ERBB4* activating mutations and amplifications are the least frequent (Figure 1) and have not been extensively explored. Stephens et al. analyzed 25 breast cancer samples and reported 1 *ERBB4* mutation (4%) outside the KD [130]. Soung et al. screened 595 samples from various cancers including gastric, lung, colon and breast and identified 12 (2%) that contained *ERBB4* KD mutations. Mutations were detected in 1 of 94 breast carcinomas

(1.1%), 3 of 104 colorectal carcinomas(2.9%), 5 of 217 non-small cell lung cancers (2.3%) and 3 of 180 gastric carcinomas (1.7%). The authors also analyzed the somatic mutations of *EGFR*, *ERBB2*, *PIK3CA*, *KRAS*, and *BRAF* genes in these 12 samples harboring *ERBB4* mutations and detected a *KRAS* mutation in 1 gastric cancer sample [131]. Prickett et al. screened 79 melanoma patients and identified 24 somatic *ERBB4* mutations in 19% of melanoma patients. Most of these mutations (14) spanned across the extracellular domain (ECD), while three *ERBB4* mutations were identified in the KD. Additionally, there were several mutations which were not associated with any functional domain (P700S, P1033S and R1174Q) and 1 (S1246N) found in His-Me endonuclease domain. The authors also identified that several mutations were multi-mutational hot spots for other oncogenes including NRAS and BRAF. The tumors harboring the *ERBB4* ECD mutants (L39F, R393W, E452K, R491K and R544W) and KD mutant E836K also harbored BRAF mutations. NRAS mutations co-occurred with several *ERBB4* mutants (M313I, E317K, E452K, E542K, E563K, P700S R1174Q). The HER4 mutants E317K, E452K, E542K, R544W, E563K, E836K, and E872K induced autophosphorylation as compared to WT control in HEK293T and melanoma cells. HEK293T cells transiently transfected with these HER4 mutants also showed increased *in vitro* kinase activity. Melanoma cells harboring endogenous HER4 mutations showed activation of AKT signaling compared to WT. However, the MAPK pathway was not activated in these cell lines. Knocking down endogenous HER4 using shRNA significantly reduced proliferation of melanoma cells harboring endogenous HER4 mutations as compared to control [132]. Lau et al. described the sequencing techniques to analyze the hot-spot and non-hotspot *ERBB4* gene mutations dispersed across its multiple exons. This technique has been applied within a clinical trial to select patients with *ERBB4*-mutant melanoma for lapatinib treatment [133]. Kurppa et al. functionally characterized 9 HER4 somatic mutations (N181S, T244R, Y285C, R306S, V348L, D595V, H618P, D931Y and K935I) in NSCLC. Out of these, 2 were located in the ECD (Y285C, D595V) and 2 in KD (D931Y and K935I); these mutants were oncogenic and enhanced both basal and NRG1-induced HER4 phosphorylation. All of these HER4 variants also increased activation of endogenous HER2 in the presence of NRG1. The HER4 ECD mutants Y285C and D595V efficiently formed HER4 homodimers when stimulated with NRG1 in NIH 3T3 cells. The above mutants increased phosphorylation and heterodimerization of HER2 in the presence of NRG1 in NIH3T3 cells. The HER4 mutants Y285C, D595C, and K935I promoted prolonged cell survival in NIH 3T3 cells in the absence of serum. These mutants increased HER4 cleavage, resulting in functionally active HER4 soluble intracellular domains (ICDs) as compared to WT, both basally and upon serum starvation in NIH 3T3 cells. This indicates

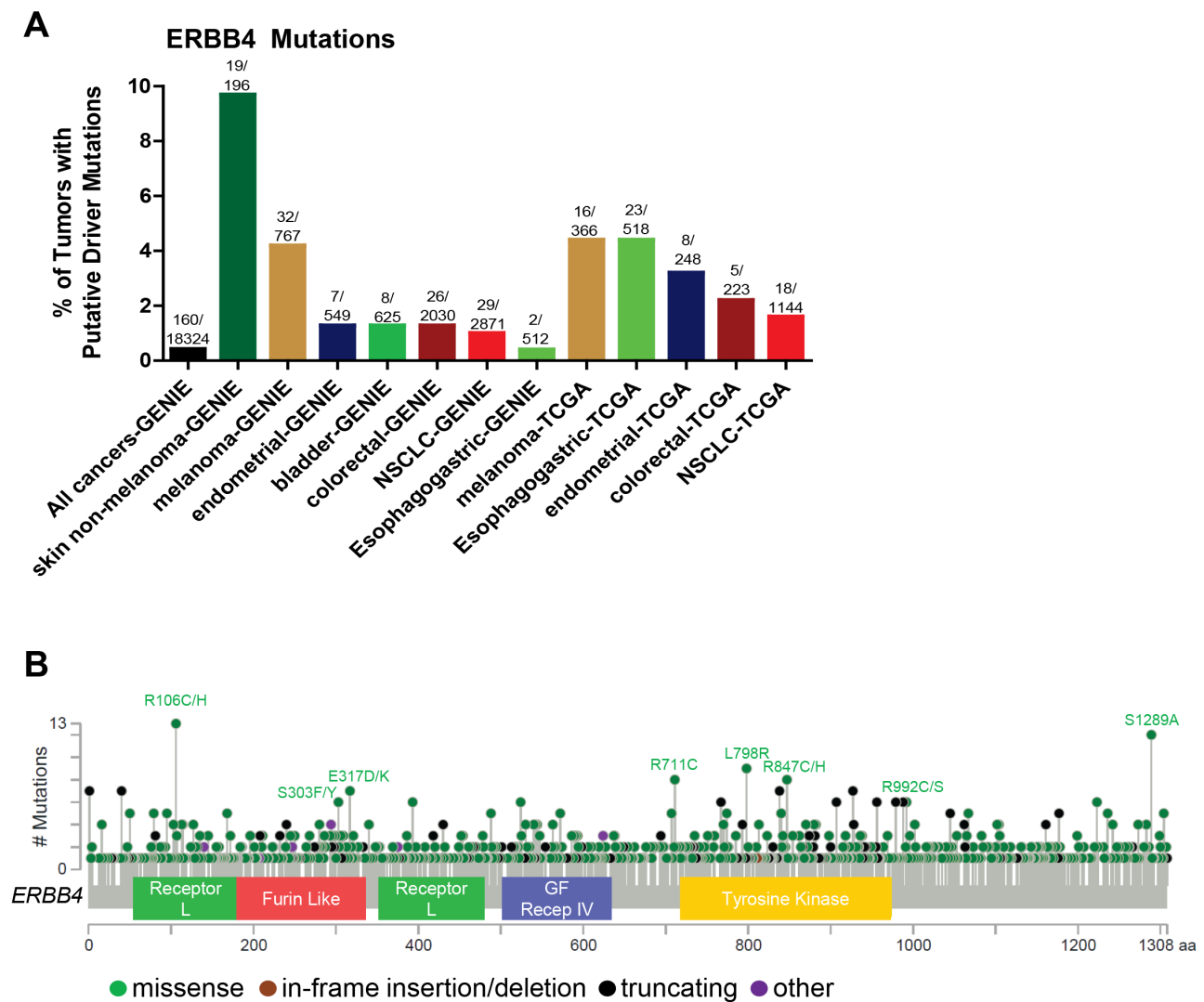


that oncogenic HER4 signaling is transduced through the regulated intra-membrane proteolysis (RIP) pathway rather than canonical RAS-MAPK signaling [134]. This recent identification of HER4 mutations presents a need for a better mechanistic understanding of how HER4 mutations are oncogenic [135]. 160/18324 (0.87%) of all cancers have putative *ERBB4* driver mutations (defined as frequency >5 in cBioPortal or COSMIC databases, or a HotSpot or OncoKB driver annotation in cBioPortal) as per the cBioPortal and GENIE databases (Figure 5). *ERBB4* copy number amplification is very rare. The GENIE database indicates that *ERBB4* driver mutations are most common in skin non-melanomas, but are also found in melanomas, endometrial cancers, bladder cancers, colorectal cancers, NSCLC, and esophagogastric cancers. According to the TCGA, the percentage of tumors

with putative *ERBB4* driver mutations are highest in melanoma and esophagogastric, followed by endometrial cancer, colorectal cancer, and NSCLC (Figure 5A). Figure 5B summarizes the distribution of the most common *ERBB4* mutations.

## SENSITIVITY OF HER4 MUTANTS TO DRUGS

The majority of HER4 mutations have been studied in malignant melanoma, where the mutants display a gain-of-function phenotype and were targeted using common EGFR/HER2 tyrosine kinase inhibitors such as lapatinib. Prickett et al. screened several HER4 mutants and targeted the transforming HER4 mutants using the EGFR/HER2 TKI lapatinib. Lapatinib treatment resulted in 10-250



**Figure 5: Somatic alterations of *ERBB4* in cancer. (A)** Frequency of *ERBB4* putative driver mutations in selected cBioPortal and GENIE datasets. *ERBB4* copy number amplification is very rare. **(B)** Distribution of somatic variants within *ERBB4* across its domain-annotated protein structure in all cBioPortal studies. NSCLC, non-small cell lung cancer; GBM, GF Recep IV, Growth Factor Receptor IV domain.



fold inhibition of cell proliferation in cells harboring endogenous HER4 mutants as compared to WT control. Lapatinib inhibited receptor autophosphorylation in a dose-dependent manner. There was also specific inhibition of HER4-induced AKT signaling and markedly enhanced apoptosis in melanoma cells harboring exogenous mutant HER4 as compared to cells harboring WT HER4 [132]. Similarly, Lau et al. highlighted the sensitivity of HER4 mutants to lapatinib. The authors stated that melanoma patients having multiple HER4 mutations showed a wide range of sensitivity to lapatinib and also emphasized the need of comprehensive sequencing strategies for patients harboring two or more HER4 alterations [131]. Unfortunately, no clinical responses to lapatinib were observed in a Phase II trial in HER4-mutant patients [136]. Either a better compound may be needed and/or confirmation of the oncogenic activity of these mutants in additional models is required. Lapatinib is a much less potent inhibitor of HER4 than of EGFR and HER2 [137], and the concentrations needed to inhibit HER4 may not be clinically achievable. Instead, neratinib or afatinib, which are more potent inhibitors of HER4, may be more promising inhibitors of mutant HER4; clinical investigation of these agents in HER4-mutant tumors is warranted.

## CONCLUSION

While inhibitors of EGFR and HER2 have transformed the clinical care of patients harboring alterations in these genes, resistance to EGFR and HER2 inhibitors remains a major problem. Tumor cell plasticity and tumor heterogeneity are major hurdles to prevent acquired resistance to ERBB inhibitors in advanced cancers. The favorable toxicity profile of third-generation EGFR inhibitors such as osimertinib may more easily enable combinations with other agents. Osimertinib is currently in clinical trials in combination with agents including MET inhibitors and immune checkpoint inhibitors, such as PD-L1 inhibitors [138]. Similarly, immune checkpoint blockade is being investigated in combination with HER2 monoclonal antibodies in HER2-amplified breast cancer [139].

Another approach to preventing/delaying drug resistance is to stay one step ahead of the tumor. We are nearing an age in which we can detect new mutations and target them as soon as they are detectable, before tumors have progressed too far. Molecular profiling of drug-resistant tumor tissue or ctDNA following progression on HER family TKIs should become routine in clinical trials in order to identify additional mechanisms of drug resistance. As with anti-HER2 therapies in HER2-amplified breast cancer, moving targeted HER family therapies earlier, to the adjuvant setting, when tumor burden is low and the pool of dividing tumor cells that

can acquire new mutations is small, may well result in improved outcomes [69].

More work is needed to systematically characterize the drug sensitivity of each individual ERBB family member mutation. A more detailed understanding of which particular mutations are blocked by which therapies will further enable the promise of precision oncology. In addition, more specific EGFR or HER2 TKIs that spare the considerable toxicity associated with WT EGFR/HER2 inhibition [140, 141] are needed. The HER2-specific TKI tucatinib (ONT-380) does not block EGFR and thus may have more favorable side effects [142]; whether this drug blocks mutant HER2 has not yet been tested. The small molecule EGFR/HER2 TKI AP32788 is more specific for HER2 and EGFR with exon 20 insertions relative to WT receptors [36] and is currently being tested in NSCLC tumors harboring these mutations. Whether AP32788 blocks all known activating HER2 mutants, including non-kinase domain mutants, is not known. Therefore, future drug discovery efforts should focus on agents that selectively block ERBB family member mutants but spare the WT receptors. We are optimistic that the combination of potent, selective inhibitors of ERBB mutants, together with inhibitors of other pathways involved in drug resistance or with immunotherapy, will ultimately lead to durable responses and perhaps cures for the hundreds of thousands of patients with ERBB-driven cancers.

## CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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