erbB in NSCLC as a molecular target: current evidences and future directions

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ABSTRACT

A number of treatments have been developed for HER1, 2 and 3-driven non-small cell lung cancer (NSCLC), of which the most successful have been the epidermal growth factor receptor-tyrosine kinase inhibitors in HER1-mutant tumours resulting in highly improved progression-free survival. Human epidermal growth factor (HER)2 and 3-driven tumours represent the minority of NSCLC, and effective therapies in these patients still represent an unmet medical need. The encouraging results seen with anti-HER2 and anti-HER3 monoclonal antibodies need to be validated in larger studies, even if the greatest obstacle is represented by the exiguous number of patients bearing deregulated HER2/3 system and abnormalities of signal transduction pathway. Considering NSCLC tumour heterogeneity, which affects response and resistance to treatment, combined multiparametric approaches, such as liquid biopsy together with radiomics, may provide a better understanding of the tumour dynamics and clonal selection during the treatments.

THE BIOLOGICAL ROLE OF THE ERBB FAMILY OF PROTEINS IN NON-SMALL CELL LUNG CANCER
ErbB1/HER1-driven non-small cell lung cancer

The epidermal growth factor receptor 1 (HER1, EGFR) belongs to the HER/ErbB family of membrane-bound proteins (ErbB1-4) and plays a pivotal role in cell proliferation and survival. These receptors are characterised by four different domains with diverse functions: the extracellular domain is involved in ligand biding, the transmembrane domain serves for receptor anchorage, the cytoplasmic domain contains the tyrosine kinase activity and the carboxy terminal and is involved in signal transduction.[1] HER1 is associated with a complex signalling network, activated both in a ligand-dependent or independent manner.[2] Ligand binding induces receptor homodimerisation (HER1/HER1) or heterodimerisation (HER1/HER2) that, in turn, promotes autophosphorylation at the tyrosine kinase domain. HER1 recognises different ligands, including EGF and transforming growth factor-α. EGF family ligands are present as preproteins in a membrane-docked form and membrane-anchorage metalloproteases were found to be involved in their cleavage. The release of ligands and the consequent EGFR dimerisation can trigger multiple signalling transduction pathways, including Rat Sarcoma (RAS), Rapidly Accelerated Fibrosarcoma (RAF), Mitogen Activated Protein Kinases (MAPKs), phosphatidylinositol-3 kinase (PI3K/Akt), signal transducer and activator of transcription (STAT) and mammalian target of rapamycin (mTOR).[3]

Ligand-independent (constitutive) activation of ErbB1/HER1 due to receptor aberration was found in multiple cancer types, particularly in lung cancer.[2] For example, EGFRvIII is generated by an in-frame deletion of the extracellular domain and this mutant receptor neither requires a ligand to be activated nor forms dimers. Furthermore, EGFRvIII was only found in cancer cells and its constitutive state was associated with an invasive phenotype.[4] Somatic HER1 mutations at the tyrosine-kinase domain make non-small cell lung cancer (NSCLC) sensitive to selective EGFR tyrosine kinase inhibitors (TKIs).[5] Class I mutations are in-frame deletions encoded by exon 19; class II are single-nucleotide polymorphisms in exons 18–21, and class III mutations are in-frame duplications and/or insertions in exon 20.

EGFR activation is also involved in the immune system regulation. Stimulation of EGFR or its constitutive activation can trigger the MAPK cascade and upregulate Programmed Death-Ligand 1 (PD-L1), a checkpoint protein that plays a key role as a negative regulator of the antitumor immune response.[6] Furthermore, constitutive EGFR activation also plays a role in tumour angiogenesis through the upregulation of hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF).[7]

ErbB2/HER2-driven NSCLC
HER2 is a member of the HER/erbB family and it is well characterised in breast cancer, in which HER2 overexpression is associated

with sensitivity to anti-HER2 drugs.[8] HER2 does not have a specific ligand and can transmit signals downstream through the formation of homodimers and heterodimers with HER1 and HER3. Furthermore, HER2 is less incline to internalisation and degradation than other EGFR members and can remain activated for a long time on the cell membrane.[9] It is worth noting that increased signalling of HER2/HER1 heterodimer compared with HER1/HER1 homodimer may account for the high sensitivity of EGFR-activating NSCLC with HER2 overexpression to selective EGFR inhibitors.[10] Somatic ErbB2 mutations were found in 1%–4% of patients with lung adenocarcinoma, particularly in exon 18–21, and were associated with worse prognosis.[11 12] HER2 YVMA consists in a 12 bp duplication/insertion of the amino acid sequence YVMA in exon 20 at codon 776 that change receptor conformation leading to an increased tyrosine kinase activity, compared with the wild-type.[10 13] NSCLCs harbouring this type of mutation were found to be less sensitive to osimertinib than those having HER1 amplification.[14]

ErbB3/HER3-driven NSCLC

HER3 is characterised by a reduced kinase activity, compared with other EGFR members. HER3 binding to heregulin led to the formation of HER2 heterodimers able to activate signalling pathways (ie, PI3K/AKT) involved in tumour progression[15] and resistance to fist-generation EGFR TKIs in NSCLC.[16] Somatic HER3 mutations were found in multiple cancer types, including NSCLC; among them, HER3 V855A is a novel mutation homologous to EGFR L858R found in a 14-year-old patient with NSCLC.[17] HER3 V855A can enhance heregulin-induced transactivation of HER2, compared with HER3 wild type, thus increasing the transforming potential of cancer cells. Co-transfection experiments demonstrated that HER3 V855A were sensitive to afatinib and pertuzumab,[18] suggesting a possible targeting strategy in patients harbouring this type of mutation. Notably, hepatocyte growth factor receptor (HGFR or MET) amplification was found to induce resistance to TKIs in NSCLC by driving HER3-dependent activation of PI3K pathway.[19]

erbB receptor activation as a target

Anti-HER1 TKIs

EGFR is mutated in 10%–20% of lung adenocarcinomas in Caucasian patients, being more common in never smokers, younger, female and Asian patients; whereas EGFR mutations are uncommon in other lung cancer histotypes.[20 21] The most common EGFR mutations are inframe deletions of exon 19 (ex19del; 45%) and the missense mutation L858R of exon 21 (40%–45%).[5] These mutations are predictive factors of response to treatment with EGFR TKIs. Indeed, gefitinib and erlotinib demonstrated only a mild activity in unselected NSCLC populations.[22 23] On the contrary, gefitinib and erlotinib were effective treatments in EGFR mutant NSCLC.[24–27] Different randomised clinical trials evaluated the role of gefitinib and erlotinib comparing with standard chemotherapy for the first line of metastatic NSCLC, harbouring common EGFR mutations. Gefitinib and erlotinib demonstrated a better progression-free survival (PFS) compared with chemotherapy,[24 27] however, none of these trials showed an improvement in overall survival (OS), due to the high rate of crossover between treatments. Since TKIs were less toxic than chemotherapy, erlotinib and gefitinib become the standard of care for the first line treatment of advanced NSCLC with EGFR mutations.[28–30]

Second-generation TKIs include afatinib and dacomitinib, and are irreversible EGFR inhibitors. Afatinib prolonged PFS compared with chemotherapy in LuxLung3 and LuxLung6, and in the LuxLung7 demonstrated no significant differences compared with gefitinib/erlotinib.[31–34] However, any difference in OS was not observed in both trials. The combined data of these trials suggested a better OS in patients with ex19del mutation treated with afatinib compared with chemotherapy; whereas, differences in OS were not significant in patients with L858R mutation. Dacomitinib was compared with gefitinib obtaining a longer PFS and OS. Second generation TKIs, afatinib and dacomitinib, were more toxic with higher grade of diarrhoea, paronychia, acneiform dermatitis and stomatitis.[35]

Osimertinib is a third-generation TKI, specifically designed to interact with mutated EGFR and to spare wild type receptors, and is also effective in patients harbouring the T790M EGFR mutations. In patients who progress to first line TKIs that developed T790M mutation as mechanism of acquired resistance, osimertinib obtained a better overall response rate (ORR) compared with chemotherapy (71% vs 21%) and prolonged PFS (median 10.1 vs 4.4 months).[36] Osimertinib was compared with first generation TKIs,[37] obtaining improvement in ORR (80% vs 76%), a better PFS (18.9 vs 10.2 months) and a better OS (38.6 vs 34.5 months),[37] becoming the new standard of treatment for these patients.

To improve efficacy, EGFR TKIs have been combined with anti-angiogenic agents and chemotherapy. The combination of bevacizumab plus erlotinib improved PFS compared with erlotinib in a preplanned interim analysis.[38] Similarly, the combination of ramucirumab with erlotinib improved PFS compared with erlotinib.[39] In both reports, data were still not mature for OS analysis.

Several attempts were aimed at overcame the emergence of resistance by combining chemotherapy with an EGFR TKI. Based on that strategy, in the NEJ009 phase III trial, the combination of carboplatin and pemetrexed with gefitinib prolonged PFS and OS compared with gefitinib monotherapy, in EGFR mutant NSCLC patients.[40] Noronha et al performed a phase III randomised trial adding pemetrexed and carboplatin chemotherapy to geftinib as first-line treatment, demonstrating that the association significantly prolonged PFS (16 vs 8 months, combination vs gefitinib alone, respectively; p<0.001) and OS (not reached vs 17 months, combination vs gefitinib alone, respectively; p<0.001), despite an increased

toxicity (51% vs 25%, combination vs gefitinib alone, respectively; p<0.001).[41] Anti PD-1 and PD-L1 antibodies (Abs) have a mild activity in EGFR mutated tumours with 4% ORR compared with 23% in the wild type population.[42] However, there is a biological rationale for the combination of TKIs with immunotherapy and, in the IMPower150 trial, the combination of atezolizumab with carboplatin, paclitaxel and bevacizumab seemed to improve PFS and OS in the small subgroup of patients with EGFR mutations. The combination of osimertinib or gefitinib with durvalumab was too toxic for clinical development because of a high rate of pneumonitis, whereas preliminary data suggest that the combination of erlotinib with atezolizumab or pembrolizumab could be feasible.[43] A single-centre experience on the KEYNOTE-001 trial suggested that TKI naïve patients had superior outcome when treated with pembrolizumab. Therefore, a phase II trial evaluated pembrolizumab efficacy in TKI naive patients with EGFR mutant, PD-L1-positive (≥1%), advanced NSCLC. The study was interrupted due to lack of efficacy after 11 of 25 planned patients were treated (ORR 0%; 46% of treatment adverse events).[44]

Beside ex19del and L858R mutations, less common EGFR mutations can be diagnosed. Only some of them are predictive factors of response to EGFR-TKIs. Objective responses have been obtained using afatinib in tumours with G719X, L861Q and S768I EGFR mutations.[45] Considering the remaining EGFR mutations two possibilities can be presumed: (1) the mutation does not transform the proto-oncogene EGFR in an oncogene; (2) TKIs are ineffective to inhibit tyrosine phosphorylation in that specific mutation. This second option seems to be the case of exon 20 insertion. Preliminary data suggest that specific inhibitors, such as poziotinib and TAK788, can be effective in this subgroup of patients. Although poziotinib obtained 58% ORR in the first treated patients, it failed to meet its primary endpoint in the phase II ZENITH20 trial with only 15% ORR. TAK788 obtained an interesting 50% ORR in the first 14 treated patients.[46] A selection of the most important trials in EGFR mutant tumors is reported in table 1.

Anti-HER2 TKI and moAbs

Trastuzumab emtansine (T-DM1), an Ab drug conjugate of trastuzumab and a mytansinoid potent inhibitor of cellular microtubules, has been evaluated in NSCLC patients.

A study evaluated 18 patients with advanced, previously treated HER2-mutant NSCLC. The response rate was remarkably high, 44%, and met the primary endpoint

Table 1 Summary of EGFR TKI trials for the first line treatment of NSCLC patients harbouring EGFR mutations

Trial	Arms	Patients	ORR	mPFS (months)	HR (95% CI)	mOS (months)	HR (95% CI)	Reference
EURTAC	Erlotinib	86	58%	9.7	0.37 (0.25 to 0.54)	19.3	1.04 (0.65 to 1.68)	28
	Chemotherapy	87	15%	5.2		19.5		
NEJ002	Gefitinib	115	74%	10.8	0.31 (0.24 to 0.44)	27.7	0.89 (0.63 to 1.24)	27 29
	CBDCA+TXL	115	31%	5.4		26.6		
IPASS†	Gefitinib	132	71%	9.5	0.48 (0.26 to 0.64)	21.6	1 (0.76 to 1.33)	30
	CBDCA+TXL	129	47%	6.3		21.9		
LuxLung3	Afatinib	230	56%	11.1	0.58 (0.43 to 0.78)	28.2	0.88 (0.66 to 1.17)	32 33
	CDDP+Pem	115	23%	6.9		28.2		
LuxLung6	Afatinib	242	70%	11	0.28 (0.2 to 0.34)	23.1	0.93 (0.72 to 1.22)	33 34
	CDDP+Gem	122	23%	5.6		23.5		
LuxLung7	Afatinib	160	70%	11.1	0.73 (0.57 to 0.95)	27.9	0.88 (0.66 to 1.12)	31
	Gefitinib	159	56%	10.9		24.5		
Archer1050	Dacomitinib	227	75%	14.7	0.59 (0.47 to 0.74)	34.1	0.76 (0.58 to 0.99)	35
	Gefitinib	225	70%	9.2		26.8		
FLAURA	Osimertinib	279	80%	18.9	0.46 (0.37 to 0.57)	38.6	0.8 (0.64 to 1.00)	37
	Gefitinib	277	76%	10.2		34.5		
NEJ026	Erlotinib+Bev	114	72%	16.9	0.61 (0.42 to 0.88)	Not mature		38
	Erlotinib	114	66%	13.3				
RELAY	Erlotinib+Ram	224	76%	19.4	0.59 (0.46 to 0.76)	Not mature		39
	Erlotinib	225	75%	12.4				
NEJ009	Gef +CBDCA+Pem	172	84%	20.9	0.49 (0.39 to 0.62)	50.9	0.72 (0.55 to 0.95)	40
	Gefitinib	173	67%	11.9		38.8		

EGFR, epidermal growth factor receptor; mPFS, median progression free survival; NSCLC, non-small cell lung cancer; ORR, overall response rate; OS, median overall survival; TKIs, tyrosine kinase inhibitors.
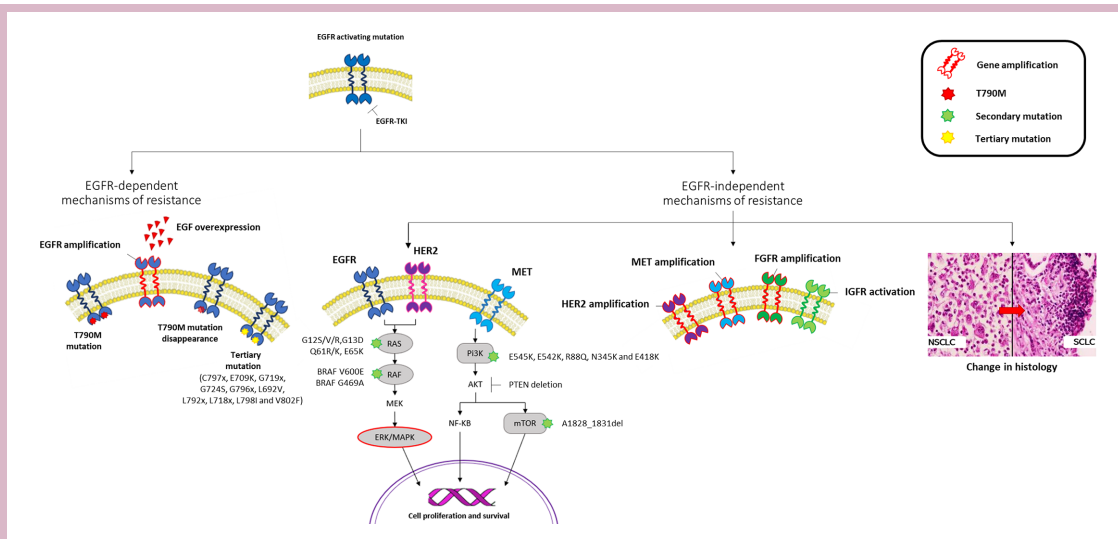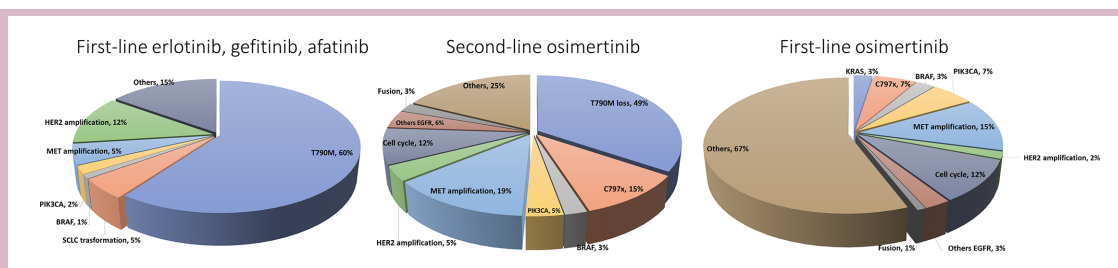
Figure 1 EGFR dependent and independent mechanisms of resistance to EGFR TKIs. EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor 2; IGFR, insulin-like growth factor receptor; mTOR, mammalian target of rapamycin; NF-KB, nuclear factor kappa-light-chain-enhancer of activated B cells; NSCLC, non-small cell lung cancer; PI3T, phosphatidylinositol-3 kinase; TKIs, tyrosine kinase inhibitors.

of the study. Subjects with mutations of the HER2 exon 20, consisting of insertions and point mutations in the different domains of HER2, including the kinase, transmembrane and extracellular, responded to the treatment. The selection of patients based on the immunohistochemical evaluation of HER2 expression did not predict response, since responders also had low HER2 score. Median PFS was 5 months and adverse events consisted of infusion reactions (grade 1 or 2), thrombocytopenia, and elevated AST/ALT. No patient discontinued treatment due to toxicity and no toxic deaths were recorded.[47] This study demonstrated that T-DM1 is an active treatment in patients with NSCLC harbouring mutations of HER2.[47]

The EUHER2 retrospective study was conducted in patients with advanced NSCLC harbouring HER2 exon 20 insertion and administered chemotherapy with or without anti-HER2 drugs.[48] The largest group of patients received trastuzumab or T-DM1 (n=57/1), while the remaining were treated with the EGFR TKIs neratinib (n=14), afatinib (n=11), or lapatinib (n=5). The ORR was 50.9% and PFS was 4.8 months with trastuzumab or T-DM1 while the group receiving neratinib, lapatinib, and afatinib showed an ORR of 7.4% and PFS of 3.4

months.[48] Overall, the study demonstrated good clinical activity of trastuzumab/T-DM1, while TKIs showed only modest effect.

A phase II study evaluated the activity of single agent T-DM1 in patients with advanced, multitreated NSCLC with HER2 overexpression or mutation.[49 50] The HER2 status assessed by immunohistochemistry was 3+in 33% subjects; 2+ combined with positive fluorescence in situ hybridisation (20%), while 47% of patients had exon 20 mutation. Only one subject achieved partial response (ORR 6.7%) and PFS was 2 months; thrombocytopenia (40%) and liver toxicity (20%) were the most serious adverse events.[50] In conclusion, T-DM1 had a limited efficacy for HER2-positive NSCLC. A clinical trial on HER2-overexpressing NSCLC patients evaluated the clinical effect of T-DM1 in subjects whose tumours scored 2+/3+ at immunohistochemistry.[51] No responses were detected in the HER2 2+ cohort, while four patients with HER2 3+ NSCLC showed partial response (ORR, 20%). Response duration ranged from 2.9 to 10.8 months and no differences were observed in PFS between 2+ and 3+ NSCLC.[51] The study demonstrated limited activity of T-DM1 in HER2 overexpressing tumours and concluded that HER2

Figure 2 Incidence of mechanisms of resistance to EGFR TKIs based on first, second and third-generation TKIs. EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor 2; TKIs, tyrosine kinase inhibitors.

overexpression as a single marker had unsatisfactory predictivity.

The DESTINY-Lung01 study enrolled two cohorts of patients (HER2-overexpressing (IHC3 +or IHC2+) and HER activating mutations) to be treated with trastuzumab–deruxtecan. Trastuzumab deruxtecan is an Ab drug conjugate composed of a humanised anti-HER2 IgG1 moAb with the same aminoacid sequence of trastuzumab, a topoisomerase I inhibitor payload, and a tetrapeptide-based cleavable linker. Trastuzumab deruxtecan has a higher drug-to-Ab ratio, retaining a favourable pharmacokinetic profile. The tetrapeptide-based linker is stable in plasma and selectively cleaved by upregulated cathepsines in tumour cells; the payload easily crosses the cell membrane and has a short half-life, allowing higher cytotoxic effect with minimally systemic exposure. The interim analysis on the HER2 mutant cohort showed an ORR of 61.9%, disease control rate (DCR) 90.5% and an estimated mPFS of 14 months. The safety profile was generally consistent with previously reported studies. Based on the clinical activity demonstrated in the interim analysis and the safety profile, trastuzumab–deruxtecan represent a new treatment option for patients with HER2-mutated NSCLC.[52]

The administration of pertuzumab to 43 patients with advanced, pretreated NSCLC unselected for HER2 expression was associated with lack of responses and only 20.9% patients had stable disease at 12 weeks.[53] The PFS was 6.1 weeks and four patients (9.3%) reported grade 3/4 adverse events but no cardiac toxicity.[53] Overall, the administration of pertuzumab yielded modest clinical effect in an unselected population.

Preclinical evidence on neratinib shoedw that the drug has remarkable antitumour activity in mouse xenografts using NSCLC cells overexpressing wild-type HER2 or bearing HER2 mutations.[54] The results of the present study strongly suggest that neratinib has potential as promising therapeutic option for the treatment of HER2-altered NSCLC. However, no robust clinical data on HER2-altered NSCLC patients are available.

Anti-HER3 agents in NSCLC
To exploit the concept that HER3 dimerises with other HER family members and is involved in the development of resistance to HER-targeting drugs, the human anti-HER3 monoclonal Ab patritumab was evaluated in combination with erlotinib in 24 patients with previously treated advanced NSCLC.[55] PFS was 44 days in nine NSCLC patients with wild-type EGFR and 107.0 days in 13 subjects bearing EGFR-activating mutation. No relationship was found between efficacy and HER3 expression in NSCLC tissues. The most frequent toxicities were gastrointestinal or dermatological, all characterised by a manageable profile.[55] The preliminary results of the U3-1402, a HER3-Ab conjugated with the topoisomerase I inhibitor DX-8951 in patients with advanced or metastatic EGFR TKI-resistant, EGFR-mutant NSCLC demonstrated six partial responses in 26 patients evaluable for

efficacy; HER3 overexpression was demonstrated in all tumour tissues.[56] The dual HER1/HER3 inhibitor duligotuzumab was evaluated in a phase I trial and demonstrated encouraging activity in patients affected by squamous cell carcinomas of head and neck displaying high tumour expression heregulin, the HER3 ligand; three patients with NSCLC had stable disease lasting ≥8 weeks.[57] Finally, the humanised anti-HER3 monoclonal Ab lumretuzumab was evaluated in combination with carboplatin and paclitaxel in first-line treatment of 12 patients with squamous NSCLC.[58] Mild and manageable adverse events were gastrointestinal, haematological and neurological (central); partial responses were observed in three subjects with high heregulin mRNA tumour levels and lasted from 81 to 207 days.[58] Overall, lumretuzumab in combination with carboplatin and paclitaxel showed promising activity in tumours characterised by high HER3 mRNA levels.

Mechanisms of resistance to anti-HER1 agents
The mechanism underlying the resistance to an EGFR-TKI reflects the TKI potency against the target and its pharmacological characteristics. First-generation TKIs are characterised by reversible binding to both the wild type and the mutant EGFR, while second-generation TKIs are irreversible EGFR inhibitors covalently binding to HER1, HER2 and HER4. Third-generation TKIs are irreversible mutant EGFR inhibitors.[59] Since TKIs activity against the EGFR is not equal, it is reasonable that also the relative mechanisms of resistance are not equal as well. In detail, the highest is the potency of the TKI, the highest is the possibility that the acquired resistance may occur through an EGFR independent mechanism. Therefore, mechanisms of resistance to EGFR-TKIs may be divided as EGFR dependent and independent. The EGFR dependent mechanisms include the appearance of EGFR secondary/third mutations and EGFR overexpression; while the EGFR independent mechanisms include HER-2 and MET amplification, appearance of mutations in alternative pathways, and the small cell histologic transformation (figure 1).[60]

In patients progressed under first/second-generation TKIs, the molecular aberrations include the T790M mutation (~50%),[61] MET (5%–15%)[62] and HER2 (12%) amplification,[63] PIK3CA mutations (5%),[64] BRAF (1%)[65] and transformation into small-cell histology (3%–14%).[64] Moreover, the epithelial to mesenchymal transition has been related to acquired resistance (figure 2).[64]

The T790M mutation plays a major role in resistance to first/second-generation EGFR TKIs (~50%).[66] In the case of gefitinib and erlotinib, EGFR reversible binders, it is expected that the main mechanism of resistance is EGFR dependent. The T790M is a gatekeeper mutation affecting the ATP binding pocket of the EGFR kinase domain, able to confer resistance by increasing the affinity for the ATP, so that the inhibitors are outcompeted. In addition, being the methionine larger than threonine, it directly blocks the inhibitor binding to the active site.[67]
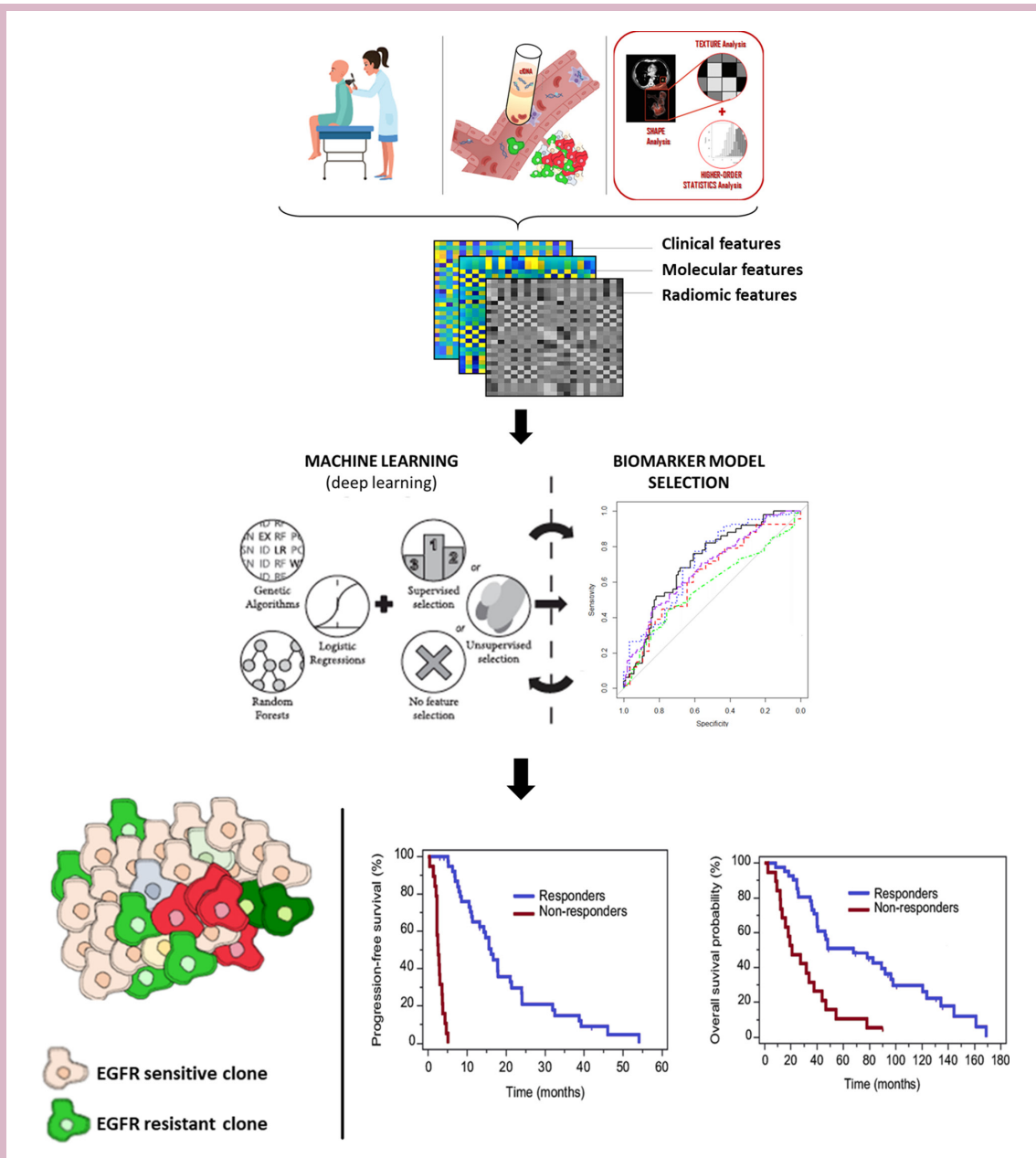
Figure 3 Multiomics approach as predictive biomarker in NSCLC. EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

A preclinical study demonstrated that afatinib is the most potent inhibitor of EGFR ex19del or L858R mutant, followed by gefitinib, erlotinib and osimertinib.[68] Being afatinib an irreversible EGFR binder, the appearence of the T790M as a mechanism of resistance may be reduced in patients treated with afatinib.[69] However, while the mechanisms of resistance to gefitinib/erlotinib are well known and described, few data are available for afatinib.

MET gene amplification is also reported as acquired mechanism of resistance, causing ERBB3 phosphorylation, which activates the PI3K/Akt signal downstream. Therefore, even with the TKI inhibiting ERBB3 phosphorylation by EGFR, the proliferation signal is not inhibited because of the maintenance of the ERBB3

phosphorylation by MET.[19] MET amplification has been reported in 5% of patients treated with gefitinib, erlotinib, afatinib as first line. Other mechanisms of resistance, that is, PI3K, BRAF mutations are reported as lower than 3% (figure 2).

The landscape of mechanisms of resistance dramatically changes considering osimertinib. Multiple coexisting EGFR-dependent or independent mechanisms frequently occur when osimertinib is administered as first/second line. The appearance of the EGFR C797S mutation accounts for 6%–10% after osimertinib as first line and 10%–26% as second line.[36] C797S acquisition has potential implications for treatment: when C797S and T790M occur on the same allele (*cis*), no response

to EGFR TKIs alone or in combination can be expected, while the C797S in trans with the T790M mutation confers sensitivity to a combination of first/third-generation drugs.[70–72] A number of rare point mutations have been identified in circulating tumour DNA (ctDNA). A study on 93 osimertinib-resistant NSCLC patients showed the coexistence of C797S with novel tertiary EGFR C797G in 24% of cases[73]; and besides C797X, other mutations such as those in the G796, L792, L718, G719 and G724 residue have been showed to sterically interfere with the osimertinib-EGFR interaction.[74–78] T790M loss is another common mechanism demonstrated in about 50%–60% of patients at osimertinib progression.[79–81] Acquired EGFR mutations (C797S, 14%) was observed in 21% of cases while 49% of patients showed the T790M loss at progression in ctDNA. An association has been demonstrated between the T790M loss and a shorter time to treatment discontinuation (6.1 vs 15.2 months).[82] Lastly, other EGFR-dependent mechanisms of resistance to osimertinib include exon 20 insertion (1%), EGFR S768I (<1%),[83] and EGFR amplification (4%–35%).[79 84]

TKIs resistance is also mediated by activation of alternative pathways or histological transformation, together with the afore-mentioned mutations. The ErbB2 overexpression, coexisting with EGFR G796S+MET amplification (1%) and PIK3CA amplifications (1%), was identified in 5% of patients who acquired secondary resistance to osimertinib.[85 86] BRAF mutation has been identified as responsible for osimertinib resistance in ~5%–7% of cases[65 87] both as first and second line. V600E and G469A mutations seem to coexist with EGFR T790M.[65] KRAS and NRAS mutations have also been reported after osimertinib failure.[86] MET amplification was observed in nearly 19% of the samples at disease progression, opening-up the possibility for future combinations to overcome resistance.[88] Concluding, oncogenic fusions (ie, FGFR3–TACC3, RET–ERC1, NTRK1–TPM3, ESYT2–BRAF) and histological and phenotypic transformation have also been confirmed after progression on second-line osimertinib.[89]

Multiparametric approach to treatment monitoring
Liquid biopsy
The analysis of circulating nucleic acids through liquid biopsy may be sufficiently sensitive and comprehensive to understand the clonal evolution driving resistance mechanisms in EGFR NSCLC and to identify new genomic targets. Liquid biopsy was first introduced in NSCLC for the research of the T790M in patients with metastatic NSCLC progressing on a first/second-generation TKI. The analysis of ctDNA quickly replaced the molecular analysis of tumour tissue, allowing real-time sampling of multifocal clonal evolution, catching tumour heterogeneity.[90 91] Several studies demonstrated that liquid biopsy has good sensitivity and specificity compared with tissue, able to detect single nucleotide polymorphisms, insertions/deletions, amplifications and rearrangements; therefore, its potential in clinical practice increased,

becoming an useful tool for the appearance of new mutations, and to monitor tumour dynamics and clonal evolution.[92 93] Liquid biopsy is usually performed on plasma samples, however, data on ctDNA testing in other body fluids such cerebrospinal fluid and urine are also available.[94] From a technical point of view, there is still a lack of standardisation across platforms. The complexity of Next Generation Sequencing (NGS) workflow, data analysis and costs can be challenging, and on the other side PCR-based tests are more accessible and cheaper and have a shorter turnaround time, and allow a limited number of target to analyse. However, mutual agreement has been demonstrated between NGS and digital PCR in ctDNA on specific mutations with comparable results.[95] The chance to find a mutation in liquid biopsy is strictly dependent both on the analytical method and the clinical characteristics of patients. It is well known that intrathoracic lesions, CNS and bone metastasis yield lower amounts of ctDNA.[96] Moreover, a proportion of tumours appear not to shed ctDNA into the peripheral blood and seem to have a better prognosis, being correlated with tumour burden.[92]

Furthermore, ctDNA is a possible tool for detection of minimal residual disease among treated early-stage NSCLC patients,[97] and it is possible to follow the amount of mutant DNA during anti-EGFR treatments. The amount of EGFR mutation in plasma decreases during the treatment, and its disappearance is correlated with tumour responses according to RECIST criteria and radiological evaluation.[98 99]

However, despite several advantages of ctDNA analysis, mechanisms of resistance involving histological transformation (ie, SCLC) can be captured by tissue biopsy only. Recent data showed that ctDNA dynamics in global copy number may predict the histological transformation into SCLC, however, its clinical validation is still needed.[12] The major issue for liquid biopsy remains the risk of false negative results. Moreover, being tumour heterogeneity a complex entity to consider to personalised treatment, a multiomics approach may be the best solution for a good predictive biomarker.

Radiogenomics and artificial intelligence
Image processing and machine learning methods can be proposed in support to understand the molecular profile and cancer dynamics during treatment. In this regard, radiomics is emerging as a novel tool for the extraction of qualitive and quantitative features from images, to develop potential non-invasive biomarkers for detection and characterisation of disease.[100] Radiomic features provide information about the grey-scale patterns, interpixel relationships, shape and spectral properties within regions of interest on radiological images,[101] which are able to reflect the patho-physiological processes and the heterogeneity of tumours, including transcriptomics, metabolomics, proteomics and genomics.[100] Radiomics deals with development of deep artificial neural networks, inspired by biological neural networks in our

brain to perform accurate segmentations and recognise patterns,[102] succeeding an appropriate auto-training of the underlying functions, minimising the difference between ground truth and prediction (ie, deep learning).[103] Several data showed that NSCLC texture analysis can classify tumour with EGFR mutations.[104] However, the lack of standardised statistical processes for features selection and the heterogeneity of the analysed cohorts allowed several selected radiomic features among different research groups. Deep learning approaches may overcome these limitations by computing the most distinguishable radiomic features through *n*-fold bootstrap training sets, incorporating them into a model for EGFR status prediction. Most frequently, top ranking features were incorporated in a signature *via* multinomial logistic regression,[104] naive bayesian classifier, K-nearest neighbour, random forest, support vector machine and decision tree.[105] Performance models are measured by using area under the curve of receiver operating characteristic curve analysis in the validation cohort, evidencing good capability with acceptable representativeness for predicting EGFR status.

Since radiomic analysis may be performed using routinely diagnostic scans, some limitation may come from the heterogeneous scanning protocols: images are acquired using scanners manufactured by different companies, with a range of image reconstruction algorithms, different slice thicknesses, with and without contrast, using different dosages; despite normalisation, these factors potentially adding noise to the data.[106]

Radiomics together with liquid biopsy may have great potential, since are both minimally invasive, easy to perform, and can be repeated over time, enabling the extraction of the overall tumour load. This approach, may help decode tumour information regarding type, aggressiveness, progression and response to treatment.[107] This approach may provide new diagnostic support, being able to suggest a change in treatment strategy earlier than with conventional methods (figure 3).

CONCLUSIONS

A wide array of treatments have been developed for NSCLC driven by HER1, 2 and 3, the most successful being EGFR-TKI in HER1-mutant tumours. HER2 and 3-driven tumours represent the minority of NSCLC and in these patients effective therapies still represent an unmet medical need. The encouraging results seen with anti-HER2 and with anti-HER3 monoclonal Abs need to be validated in larger studies even if the greatest obstacle is represented by the scarce number of patients bearing deregulated HER2/3 expression/activation and corresponding abnormalities of the signal transduction pathway.

Considering NSCLC tumour heterogeneity, which affects response and resistance to treatment, combined multiparametric approaches, such as liquid biopsy together with radiomics, may provide a better understanding of the tumour dynamics during treatments.

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