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Review Article

EGFR SIGNALING AND INHIBITION BY EGFR INHIBITORS IN NSCLC

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Abstract

Lung cancer is the third most cancer among the population. The American society's estimation for lung cancer in the United States for 2014 states that about 2,24,210 people are suffering from the lung cancer and 1,59,260 deaths are occur from lung cancer. Among all the types of lung cancer, NSCLC (Non-Small cell Lung Cancer) represents 85% of the lung cancer. The estimated spread of NSCLC is 2, 26,160 and 1, 60, 340 cases are of death in 2012**.** One of the risk factor for NSCLC is over expression of epidermal growth factor receptor (EGFR) and its intracellular signaling pathways. EGFR is over expressed in 40 -80 % cases of NSCLC. EGFR belongs to the ErbB family of receptor tyrosine kinases (RTK) having molecular weight 170 to 185 kDa. Epidermal Growth Factor (EGF) binds to the EGFR at its extracellular domain and this binding leads to the homo or hetero dimerization and autophosphorylation of EGFR which initiates the several intracellular pathways. Several mutations in EGFR or in any of the proteins of the pathway leads to the growth and survival of the tumor cells.so in order to inhibit the growth of tumor cell, several EGFR inhibitors and targeted therapies are found to target the particular mutations.

Key Words: NSCLC; EGFR Signaling; EGFR inhibitors; Drug Resistance.

Introduction

Lung cancer is the uncontrolled growth of abnormal cells that start from the one or both lungs; usually from the epithelia of the trachea, bronchi or lungs. The abnormal cells do not develop into healthy lung tissue. They divide rapidly and form tumors. As tumors become larger and more numerous, they undermine the lung ability to provide the oxygen.

Lung cancer is a main cause of cancer death in the United States and World Wide (Doebele *et al.*, 2010; Gaughan *et al.*, 2012) . It is the third most cancer among the population. Second most cancer in males after prostate cancer and in females second most cancer after breast cancer. Approximately, 1, 62, 000 Americans and1.4 million people were dead around the world in 2008 (Doebele, Oton *et al.* 2010). The American society's estimation for lung cancer in the United States for 2014 states that, about 2,24,210 (Siegel *et al.*, 2014) (1,16,000 in men and 1,08,210 in women) are suffering from the lung cancer and 1,59,260 deaths are occur from lung cancer(86,930 in men and 72,330 in women) as shown in Fig.1, it is about 27% of all cancer deaths. It is more common among men than women and has a peak onset at age 75–84. 85% of cases diagnosed after the age of 60 (Blackhall and Chaplin 2009). On average 28% of cancer death in males and in females, lung cancer accounts for 10% of death. Cigarette smoking has been clearly established as a major cause of this malignancy (Blackhall and Chaplin 2009). Other risk factors include environmental exposure to radon gas, industrial carcinogens, air pollution and a positive family history (Blackhall and Chaplin 2009). 10-20% of lung cancer is developed in never smokers. The advance research established the understanding of certain molecular mechanisms involved in tumor genesis. Specifically mutation in EGFR gene and translocations involving the anaplastic lymphoma kinase (ALK) gene are the main pathways involve in pathogenesis of lung cancer (Köhler and Schuler 2013) According to the World Health Organization (WHO), there are four types of lung cancer: adenocarcinoma, squamous carcinoma, large-cell carcinoma, and small-cell carcinoma (Blackhall and Chaplin 2009) as shown in Fig. 2. First three types have the same treatment and staging paradigms. So, they are grouped together as non-small cell lung cancer (NSCLC). It represents about 85% of all lung cancer types (Doebele *et al.*, 2010).

Most patients are diagnosed with advance-stage disease (stage IIIB and IV) requiring systemic therapy. The prognosis for such patients is poor so, survival rate of stage IIIB and IV diseases is 8% and 2% respectively (Doebele, Oton *et al.* 2010). A family history of lung cancer is also related with the increased risk of non-small lung cancer (NSCLC), for both smokers and never smokers (Gaughan, Cryer *et al.* 2012).Thus non-small-cell lung cancer (NSCLC) remains a therapeutic challenge. Although there is some progress, it remains a leading cause of cancer related deaths in the United States. The estimated spread of

NSCLC is 2,26,160 and 160340 cases are of death in 2012(Bar and Onn, 2012)

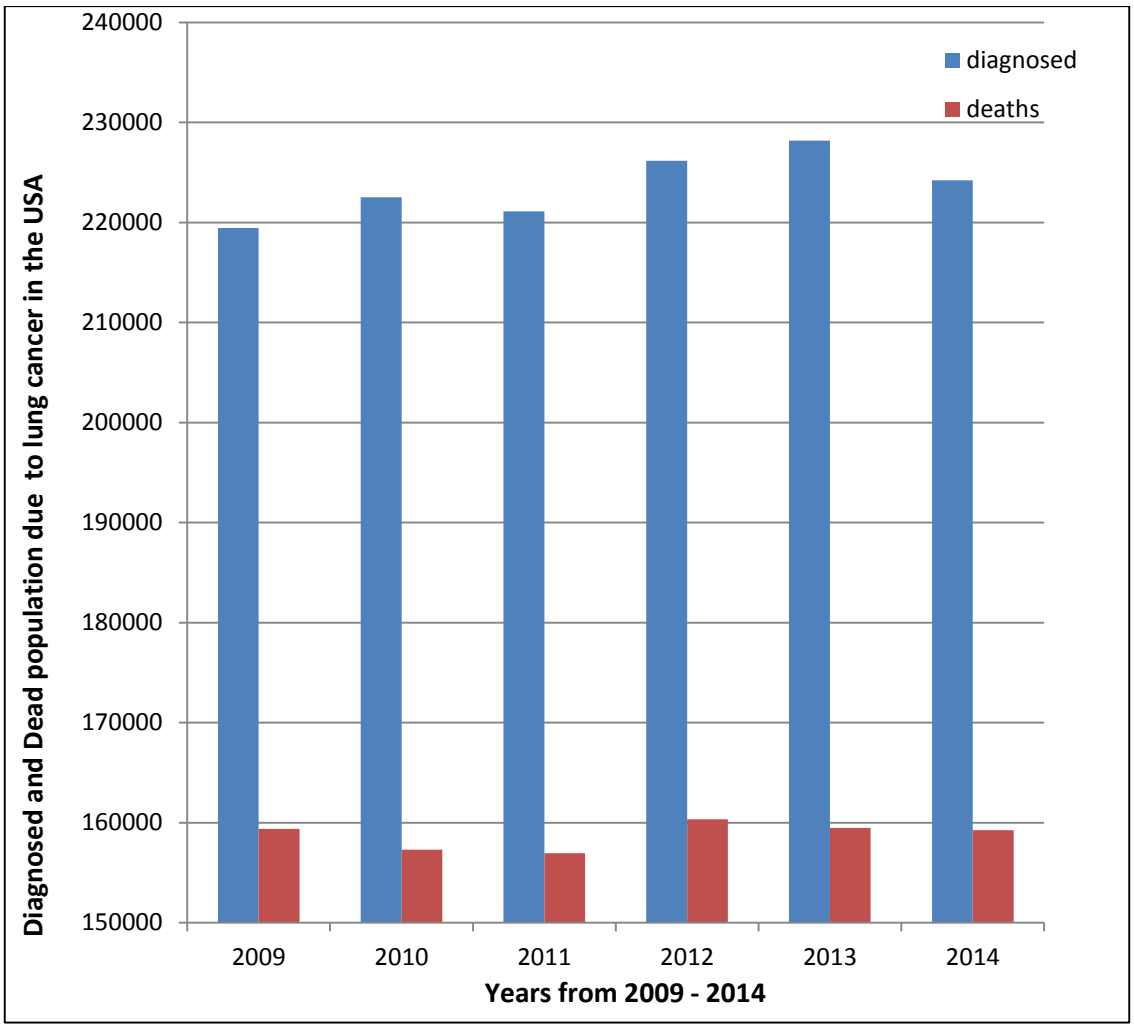

Fig 1: Lung cancer spread in the USA and World Wide

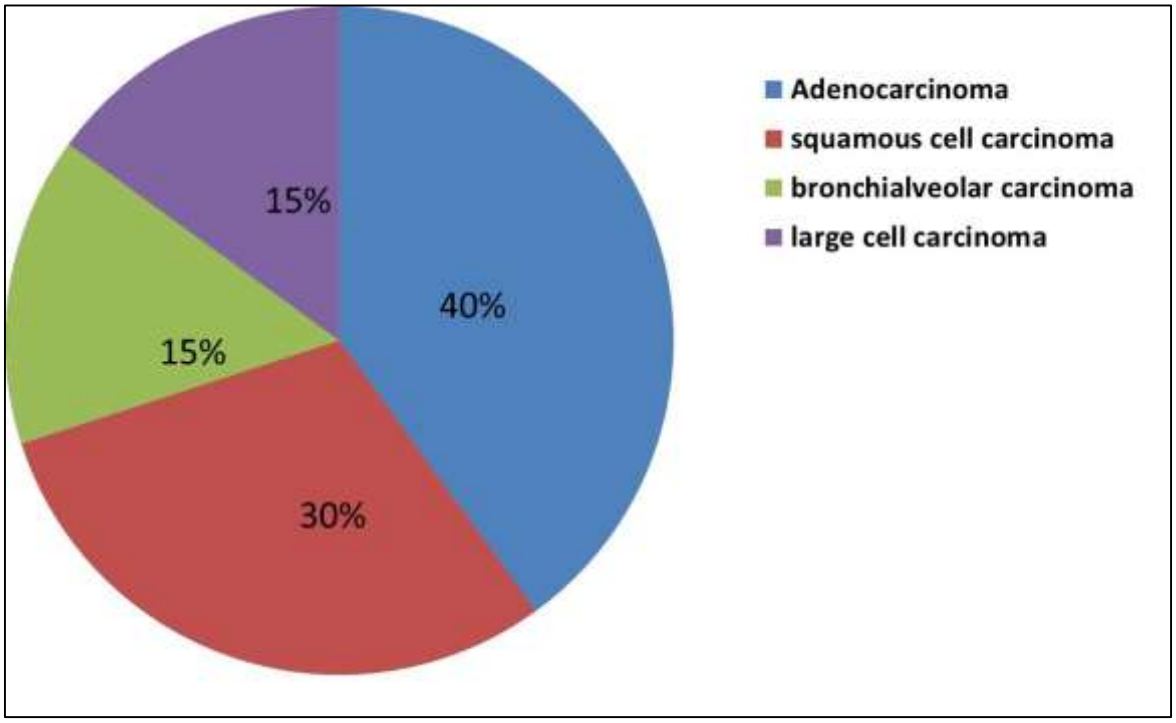

Fig 2: Different types of NSCLC

EGFR Biology and Signaling

In NSCLC and other epithelial malignancies, the epidermal growth factor receptor (EGFR) – first isolated by Stanley Cohen in 1962 – as a cell surface glycoprotein receptor belonging to a family of receptor tyrosine kinase (TKIs) (Wheeler, Dunn *et al.* 2010) . EGFR is overexpressed in 40-80% of NSCLC cases(Lynch, Bell *et al.* 2004). Other members of this family are HER2/ ErbB2, HER3/ErbB3 and HER4/ErbB4. (Giaccone and Pinedo 1996, Chinnaiyan, Huang *et al.* 2005, Normanno, De Luca *et al.* 2006). This Her/ErbB receptor family has been identified as such a critical oncogene. These are transmembrane glycoproteins having molecular weight of 170 to 185 kDa (Hong, Kim *et al.* 2010, Seshacharyulu, Ponnusamy *et al.* 2012). EGFR and its family contain three domains – an extracellular binding domain, A transmembrane domain and an intracellular domain (Doebele, Oton *et al.* 2010). EGFR mediates several cell functions and cell proliferation, cell migration and survival. Over expression of EGFR leads to the several intracellular pathways which contribute to the development of lung cancer (Gaughan *et al.*, 2012). Primary pathways activated EGFR include RAS/RAF/MEK/ERK (Martin, Kelly *et al.* 2006, Vincenzi, Schiavon *et al.* 2008) and PIK3/AKT/mTOR. Moreover, Src tyrosine kinases, PLCγ, PKC and STAT pathway is also activated by EGFR (Wheeler, Dunn *et al.* 2010, Köhler and Schuler 2013). Binding of EGF (Epidermal Growth Factor) to this receptor and results in to homo or hetro dimerization (Martin, Kelly *et al.* 2006) and autophosphorylation of the cytoplasmic receptor domain and then subsequently activates these downstream pathways (Lynch, Bell *et al.* 2004). These activated pathways may ultimately counteract apoptosis and enhance cellular metabolism, proliferation and survival of a tumor cell. Whereas non-malignant cells are regulatory programs for receptor tyrosine kinase (RTK) functions, the EGFR activity in cancer cells can be dysregulated by oncogenic processes like increased *EGFR* gene copy number, EGFR overexpression, and activating gene mutations. Thus, the deregulated EGFR is a promising cellular target for the treatment for lung cancer (Köhler and Schuler 2013).

EGFR inhibitors

In order to inhibit downstream pathways it is essential to target EGFR at its intracellular and extracellular domain. So, according to that mainly two kinds of EGFR inhibitors are derived (Dassonville, Bozec *et al.* 2007). First is TKIs - reversible (erlotinib and gefitinib) and irreversible (afatinib, neratinib, vandetanib, lapatinib etc.). These inhibitors compete with ATP-binding site reversibly or irreversibly at intracellular domain. Second class is monoclonal antibody (cetuximab, panitumumab and trasuzumab etc.). These inhibitors bind to the receptor at tyrosine kinase's extratracellular domain. Thus, inhibitors of these both the classes bind to the EGFR receptor and stop binding of EGF (Epidermal Growth Factor) to the receptor, overexpression of EGFR, homo or hetro dimerization and autophosphorylation of EGFR and Thus, prevent all the molecular pathways and ultimately leads to the apoptosis of the tumor cells.

(1) Tyrosine kinase inhibitors

EGFR- TKIs are low molecular weight containing small molecules administered orally(Arora and Scholar 2005, Wheeler, Dunn *et al.* 2010). They have a favorable safety profile and they can easily combine with other therapies like chemotherapy or radiation therapy. TKIs have very effective antitumor activity (Arora and Scholar 2005). They bind to the intracellular domain (Brugger and Thomas 2012) of tyrosine kinase by compete with ATP to bind to the (ATP)-binding site of domain and inhibit the auto phosphorylation and tyrosine kinase activity and ultimately prevent the downstream signal transduction and thus it leads to the cell apoptosis. According to the binding of TKIs to the specific binding site of the EGFR intracellular domain, there are two types of them. (1) Reversible TKIs. This type includes gefitinib and Erlotinib, and another type is (2) Irreversible, TKIs. This type includes afatinib, lapatinib, vandatinib (Arora and Scholar 2005, Wheeler, Dunn *et al.* 2010), semaximab (SU5416), canertinib (CI- 1033) etc. (Wheeler, Dunn *et al.* 2010).

a) Reversible Tyrosine kinase Inhibitors

Erlotinib

Erlotinib hydrochloride (trade name Tarceva, Genentech/OSIP, originally coded as OSI-774) drug is approved by food and drug administration in November2004. It is used to treat non-small cell lung cancer, pancreatic cancer and several other types of cancer. It is orally available, potent and reversible drug (Hata, Katakami *et al.* 2011). Erlotinib specifically inhibits the epidermal growth factor receptor (EGFR) tyrosine kinase at nano-molar concentration and blocks the cell cycle progression in the G1 phase (Moyer *et al.*1997). It reversibly binds to the to the adenosine triphosphate (ATP) binding site of the receptor. Erlotinib has recently been shown to be a potent inhibitor of JAK2V617F activity. JAK2V617F is a mutant of tyrosine kinase JAK2, is found in most patients with polycythemia vera (PV) and a substantial proportion of patients with idiopathic myelofibrosis or essential thrombocythemia. The study suggests that erlotinib may be used for treatment of JAK2V617F-positive PV and other myeloproliferative disorders (Wheeler, Dunn *et al.* 2010).

Gefitinib

Gefitinib (ZD1839/Iressa) is an anilinoquinazoline derived EGFR tryrosine kinase inhibitor and was first characterized in the year 1996. It is approved by US Food and Drug Administration as a second and third line treatment of NSCLC. It is an orally active low-molecular-weight EGFR inhibitor with selective tyrosine kinase activity but does not

inhibit serine-threonine kinase activity. The FDA approved gefitinib through a new accelerated process at May-15, 2003(Comis 2005) as a mono therapy for the treatment of patients with locally advanced or metastatic NSCLC after failure of both platinum-based and docetaxel chemotherapies (Chang, Chang *et al.* 2013). As a condition of accelerated approval, the FDA required demonstration of a survival benefit in a subsequent clinical trial. After three prospective studies (INTACT 1, INTACT 2 and ISEL) showed no improvement in overall survival, so, the original FDA approval was modified in 2005, limiting the indication to cancer patients who, in the opinion of their treating physician, are currently benefiting or have previously benefited from gefitinib treatment (Wheeler, Dunn *et al.* 2010). Studies with gefitinib have revealed that estimated measurement of dosages is 250 or 500 mg daily in the IDEAL (Iressa Dose Evaluation in Advanced Lung cancer) 1 trial and also in the IDEAL-2 trial (Jiang 2009, Seshacharyulu, Ponnusamy *et al.* 2012). Half-life of the gefitinib is 28 hours (eshacharyulu, Ponnusamy *et al.* 2012). Gefitinib has a 200-fold greater affinity for EGFR relative to the other ErbB family members (Dubois and Cohen 2009). It has also been observed that gefitinib inhibits other tyrosine kinase receptors including HER2 at concentrations more than 100-fold(Seshacharyulu, Ponnusamy *et al.* 2012). Gefitinib prevents autophosphorylation of EGFR in various tumor cell lines and xenografts (Arteaga and Johnson, 2001) by binding to its intracellular domain of tyrosine kinase and prevent the further downstream signal transduction. It is speculated that it inhibits the cyclin-dependent kinase activity by upregulation of p27 via EGFR kinase inhibition and arrest in the G1 cell cycle phase (Arteaga and Johnson, 2001). It also inhibits tumor neo angiogenesis(Arora and Scholar 2005).

b) Irreversible Tyrosine Kinase Inhibitors

In recent years, newer class or a second generation of small-molecule HER family inhibitors, called irreversible TKIs is derived(Doebele, Oton *et al.* 2010, Bar and Onn 2012). This class contain a novel compounds which active against EGFR mutations which are resistant to reversible TKIs like gefitinib and earlotinib (Doebele, Oton *et al.* 2010, Ohashi, Maruvka *et al.* 2013) for example T790M mutation (Doebele, Oton *et al.* 2010, Chang, Chang *et al.* 2013) at lower concentration. They are more potent than gefitinib and erlotinib (Ohashi, Maruvka *et al.* 2013). Most of these agents are active against HER2 and EGFR protein and other members of EBBR (ERBB2,ERBB4) family of kinase (Gazdar 2009) and some inhibit HER4 also(Doebele, Oton *et al.* 2010). In addition, preclinical studies have shown that compounds of this class develop less resistance compared to reversible TKIs (Doebele, Oton *et al.* 2010). Moreover, irreversible inhibitors have the advantage of prolonged clinical effects and a decreased need for frequent dosing, although it may compromise specificity and tolerability (Seshacharyulu, Ponnusamy *et al.* 2012).Second-generation EGFR TKIs include canertinib (CI-1033), neratinib (HKI-272), afatinib(BIBW 2992), and dacomitinib (Nguyen, Kobayashi *et al.* 2009, Chen 2013) These irreversible agents are ATP-competitive and make irreversibly covalent bonds with a nucleophilic cysteine residue at position 797 in EGFR unlike reversible TKIs (Martinelli, De Palma *et al.* 2009, Seshacharyulu, Ponnusamy *et al.* 2012).

Afatinib (BIBW 2992)

This irreversible EGFR/HER2 inhibitor(Bar and Onn 2012) act against wild type and mutant forms of EGFR, including T790M. This is more potent than erlotinib and gefitinib including cell death of NSCLC cell lines, including those harboring wild type EGFR, the activating L858R mutation and erlotinib resistant T790M mutation (Doebele, Oton *et al.* 2010).

Neratinib

It is an irreversible inhibitor of EGFR and HER2. In preclinical studies, HKI 272 was more potent than gefitinib in suppressing EGFR autophosphorylation and downstream signaling and growth inhibiting NCI-H1975 BAC cells harboring L858R and T790M mutation in EGFR(Doebele, Oton *et al.* 2010, Dienstmann, De Dosso *et al.* 2012).

Dacomitinib (PF0299804)

PF029904 is irreversible inhibitor of the EGFR/HER1, HER2, and HER4 tyrosine kinase. It is given orally. Preclinical data showed the activity of PF0299804 against EGFR mutations and T790M (Bayraktar and Rocha-Lima 2013).

Drug resistance to TKIs

Despite of many clinical benefits achieved with EGFR TKIs in selected patient populations, resistance to those inhibitors has proven to be the major clinical issue. Certain molecular mechanisms have been implicated in resistance to EGFR TKIs treatment (Doebele, Oton *et al.* 2010). Resistance to inhibitors is generally occurred due to mutations in the kinase domains of a receptor which decrease the affinity of the TKIs to binding domain. Some mutations may occur around the binding site, which make extensive conformational changes, there by impeding TKIs approach through steric hindrance. Moreover, some mutations may render the predominance of ATP to competitive binding to the kinase. Up to now, more than 100 mutations have been described affecting more than 70 amino acids causing resistance by heterogeneous molecular mechanisms (Engelman and Jänne 2008) . Among, all patients with EGFR activating mutations, 70% patients respond to TKI treatment, while remaining 30% show intrinsic resistance to these inhibitors(Siegelin and Borczuk 2013). Among 90% of the EGFR mutations are clustered in exon 19-24 (Gridelli, Bareschino *et al.* 2007, Messersmith and Hidalgo 2007, Wheeler, Dunn *et al.* 2010). These mutations include L747S and D761Y both on exon 19 (Wu, Yang *et al.* 2010)

around codons 746-750 (45% -50% of all EGFR mutations)(Gridelli, Bareschino *et al.* 2007, Seshacharyulu, Ponnusamy *et al.* 2012) in exon 21 around codon 858 (T854)(Wu, Yang *et al.* 2010) accounts for another 40% of mutations(Gridelli, Bareschino *et al.* 2007) . These both are most frequent mutations(Chinnaiyan, Huang *et al.* 2005, Seshacharyulu, Ponnusamy *et al.* 2012). While remaining 10-20% of mutations are in exon18 and 20 (Seshacharyulu, Ponnusamy *et al.* 2012). These all mutations are sensitive to TKIs with compare to exon 20 (T790M) mutation, T790M germ line mutations have been identified in a European family with genetic susceptibility to EGFR signaling in lung cancer(Wangari-Talbot and Hopper-Borge 2013). This is a secondary acquired mutation(Chen and Fu 2011) it means the patients carrying this mutation is initially responsive to TKI therapy and then become resistant to TKI therapy(Wangari-Talbot and Hopper-Borge 2013). This is a gate keeper residue (Bean, Brennan *et al.* 2007, Nguyen, Kobayashi *et al.* 2009) and initially conserves the threonine residue located in the hinge region of the kinase domain at the back of the ATP-binding pocket but after mutation amino acid substitution at position 790 from threonine to methionine. This point mutation of EGFR leads to steric hindrance during binding of TKIs due to bulky methionine placed in EGFR instead of threonine. The most common resistance pathway involves simplification of the MET oncogene, which occurs in 40% of erlotinib and gefitinib–treated patients(Bean, Brennan *et al.* 2007, Chen and Fu 2011, Panagiotou, Tsiambas *et al.* 2012).The MET gene encodes a transmembrane tyrosine kinase receptor (Panagiotou, Tsiambas *et al.* 2012). Downstream activation of RAS/RAF/MEK/ERK and PI3K/AKT/mTOR via alternative pathways is an important mechanism of resistance to EGFR TKIs. This signaling has been described with mesenchymale epithelial transition factors (MET) amplification. There is preclinical evidence showing that coupling of the MET receptor tyrosine kinase to HER3 leads to the activation of the PI3K/AKT/mTOR pathway, via epidermal growth factor receptor 3 (ERBB3) driven mechanisms(Goffin and Zbuk 2013).

MET amplification represents the strongest evidence that cells treated with TKIs tend to acquire genetic alterations to tolerate the inhibition (Brugger and Thomas 2012). Moreover, Crosstalk of EGFR with the Insulin like Growth Factor Receptor 1 (IGFR-1) can also induce intrinsic resistance to EGFR targeted therapies. In a study of surgically resected patients, high co-expression of EGFR with IGFR-1 was reported to have a poor prognosis and was associated with decreased survival. The activation of IGFR-1 by binding of IGF-I and IGF-II to its extracellular domain results in the activation of both MAPK and PI3K/AKT pathways. It is through the activation of PI3/AKT pathway that IGFR-1 can promote resistance to EGFR targeted therapies (Wangari-Talbot and Hopper-Borge 2013). Furthermore, intrinsic resistance to EGFR inhibitors is associated with *PTEN* loss (Panagiotou, Tsiambas *et al.* 2012, Wangari-Talbot and Hopper-Borge 2013) or inactivation(Soria, Lee *et al.* 2002). PTEN antagonizes the (PI3K)/AKT pathway, so loss of PTEN induce (PI3K)/AKT pathway excessively(Soria, Lee *et al.* 2002). Along with the deletion and mutation of PTEN, promoter methylation and translational modification can also lead to the PTEN inactivation(Panagiotou, Tsiambas *et al.* 2012). Mutations in the PI3KCA(Chen and Fu 2011) and P110α subunits of PI3K can accounts for resistance to EGFR TKIs through constitutive activation of AKT(Chen and Fu 2011, Wangari-Talbot and Hopper-Borge 2013). The 15-30% mutations observed in KRAS are point mutations on codons 12, 13 and 61 due to lack of response to erlotinib and gefitinib in NSCLC(Pao, Miller *et al.* 2005, Bayraktar and Rocha-Lima 2013, Wangari-Talbot and Hopper-Borge 2013). 37% of the erlotinib-resistant tumors showed more than 2-fold increase in HER2 expression compared with normal lung cells(Ohashi, Maruvka *et al.* 2013). In one of the in vitro study, it is confirmed that ectopic expression of BRAFV600E or BRAF G469A showed resistance to erlotinib in PC9(Nguyen, Kobayashi *et al.* 2009).

Resistance to reversible TKIs is generally results due to certain mutations or changes during the TKIs treatment such as T790M germ line mutations, MET amplification, KRAS and Insulin like Growth Factor Receptor 1 (IGFR-1) mutations, HER2 mutation, Downstream activation of RAS/RAF/MEK/ERK and PI3K/AKT/mTOR via alternative pathway, then increased signaling through (PI3K)/AKT pathway as a consequences of the PTEN loss, Mutations in the PI3KCA etc. as mention in Fig.3.

Overcome the Drug resistance

There are several strategies to overcome resistance to reversible TKIs. One of them is development of second and third generation of irreversible TKIs. Second generation of irreversible TKIs includes afatinib, neratinib, decomitinib, etc. These inhibitors irreversibly bind to the ATP binding site while third generation TKIs are under the study. This class includes AP-26113, CO-1686and WZ- 4002. They are structurally altered, and have greater efficacy to inhibit mutated forms of EGFR, includingT790M(Yewale, Baradia *et al.* 2013). WZ4002 have been initiated and a phase I/II clinical trial for CO-1686 started in January 2012 (NCT01526928). Another new EGFR inhibitor is AP26113. This drug was originally characterized as an ALK inhibitor but study of preclinical model proved that this inhibitor is also used as an *EGFR* T790 inhibitor. A phase I/II clinical trial for this agent began in September 2011(Ohashi, Maruvka *et al.* 2013) [29]. Second strategy is the use of Salvage chemotherapy or chemotherapy plus original EGFR-TKI. Among these two, only salvage chemotherapy is considered as better choice(Chen 2013). Increasing evidence has suggested that solid tumors with multiple salvage pathways and resistance pathways allow them to

circumvent inhibition of a single signaling pathway(Bayraktar and Rocha-Lima 2013). The combinatorial therapy of different drugs has been also demonstrated the significant advantage over EGFR TKIs resistance. The study of 22 patients with stage IIIB or stage IV NSCLC in phase I evaluated the combination of erlotinib with celecoxib (cyclooxygenase-2 inhibitor). This study also determined the optimal dose of celecoxib in combination with fixed dose of erlotinib 150 mg/day (Bar and Onn 2012). On other side the combination of erlotinib with sorafenib was also proved beneficial targeted therapy in its phase I study against advanced NSCLC(Dienstmann, De Dosso *et al.* 2012).

Moreover the combination of anti-EGFR antibody with EGFR TKIs has been also studied to overcome resistance to EGFR TKIs. Erlotinib with cetuximab did not how any effect in patients having EGFR TKIs resistance, while the combination of afatinib and cetuximab showed a positive effect against resistance (Ohashi, Maruvka *et al.* 2013).

The novel combinations are the combination of low doses (eg.gefitinib) and high doses (eg.afatinib) of TKIs, and also suggest the significant advantage to overcome the EGFR TKIs resistance rather than the use of high-doses alone (Ohashi, Maruvka *et al.* 2013). Preclinical studies suggested that vascular endothelial growth factor (VEGF) pathway activation may also be involved in resistance to first-generation EGFR TKIs. So, many clinical trials have evaluated combine inhibitors of EGFR and VEGF pathways to inhibit both the pathways(Dienstmann, De Dosso *et al.* 2012). Sunitinib and sorafinib are inhibitors of VEGFR-1,-2 and -3 and PDGFR, KIT and RET activities. Axitinib is also one of the potent and selective inhibitor of VEGFR-1,-2 and -3 compare to other VEGFR-TKIs. Another inhibitors are Vatalanib, Motesanib against VEGFR-1,-2 and -3, PDGFR, KIT and RET are recently studied(Méndez, Custodio *et al.* 2011).

Another strategy is to use MET inhibitors to inhibit the MET amplification resistance as it is one of the cause of drug resistance to TKIs. It is under investigation. The combination of an irreversible TKIs and MET inhibitor is also a good approach to use against resistance of TKIs(Bar and Onn 2012) . Tivantinib (ARQ-197),(Méndez, Custodio *et al.* 2011, Bar and Onn 2012) is one of the MET inhibitor was tested in trial Phase IIIa in combination with erlotinib but this study was terminated early because of lack of its usefulness and other data which shows that the major mechanism of this agent is may be microtubule stabilization rather than MET inhibition(Bean, Brennan *et al.* 2007). Study of XL184 with erlotinib showed some responses against MET amplification and T790M mutation in phaseIb clinical trial. Crizotinib(Bean, Brennan *et al.* 2007, Méndez, Custodio *et al.* 2011), which is extremely active against lung cancers with the echinoderm microtubule-associated protein- like 4 gene – anaplastic lymphoma kinase(EML4-ALK)translocation also act as MET inhibitor and is being studied in combination with for etinibapan-ERB inhibitor(Bean, Brennan *et al.* 2007, Méndez, Custodio *et al.* 2011).

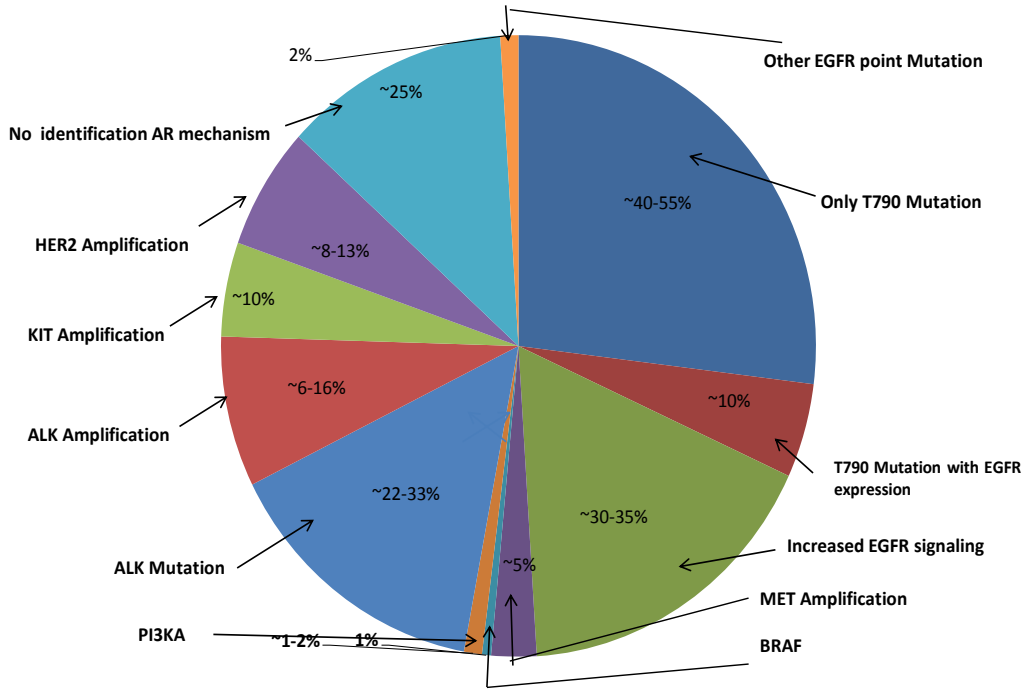

Fig 3 : Mutations responsible for the drug resistance

Recently, the use of Met mAb with earlotinib is also evaluated in a phase II clinical trial (Bar and Onn 2012). As for example, onartuzumab which acts as a monovalent antibody for the MET receptor(Bean, Brennan *et al.* 2007), has been also studied in PhaseIII of clinical trial in a combination with erlotinib in a populations in whom NSCLC is detected positive [34].Moreover, another agent is ficlatuzumab the HGF ligand - neutralizing antibody is also in study in phase I/II. Phase I study suggested the activity of ficlatuzumab in combination with gefitinib in partially treated population(Bean, Brennan *et al.* 2007).

Next strategy is to use the combination of TKIs with monoclonal antibodies against the TKIs resistance(Soria, Lee *et al.* 2002, Yewale, Baradia *et al.* 2013). The combination of EGFR antibodies with first generation of TKIs has not shown any activity, whereas the combination of second generation of TKIs with cetuximab shows some activity against TKIs resistance(Soria, Lee *et al.* 2002). The large scale FLEX (fracture Intrvention Trial Long-Term Extension) trial made a combination of cetuximab with cisplastin and vinorelbine and got success with a modest improvement. On other side, the combination of cetuximab with carboplatin and paclitaxel and also with pemetrexed or docetaxel are failed to show improvement(Yewale, Baradia *et al.* 2013). In xeno graft models of lung cancer, the combination of cetuximab with erlotinib or gefitinib achieved a greater tumor regression and regrowth delay compare to either of the drug used alone. Next strategy to overcome TKIs resistance is the use of mTOR/PI3K Inhibitors as the activation of PI3K/Akt signaling pathways by either MET amplification(Messersmith and Hidalgo 2007) or by PI3KC mutation is also a major cause of EGFR TKIs resistance. So, mTOR, PI3K and both mTOR/PI3K are either use alone or use with combination of erlotinib or gefitinib. So, PI3K specific agents BKM-120888 and XL-147(Bao and Chan 2011) have shown effective activity in phase I and then used with combination with erlotinib and gefitinib . Moreover, the combination of MET inhibitors and PI3K inhibitors is also used to treat the KRAS mutation bin NSCLC. After the early signs of antitumor activity observed in ongoing phase I clinical trial suggested the combinations of the MEK inhibitor GDC-0973 and PI3K inhibitor GDC-0941 and the combination of MEK inhibitor AZD6244 and AKT inhibitor MK-2206 as shown in Fig. 4 (Dienstmann, De Dosso *et al.* 2012). Another strategy is to use Anti-IGF1-R (Chang, Chang *et al.* 2013) and Anti-HER-2 agents(Stahel, Peters *et al.* 2013). The combination of an IGF1-R and EGFR inhibitor is an effective treatment to overcome IGF1-R mediated resistance.

Furthermore phase III study showed that the combination of figitumumab (CP-735,871) with erlotinib and also with carboplatin and paclitaxel was rejected because of serious side effects like dehydration, hyperglycemia, and hemoptysis. The ongoing phase II clinical trial evaluated the combination of erlotinib with cexutumumab (IMC-A12) to target both EGFR and IGFR-1 in advance NSCLC patients. Moreover the use ofOSI-906 (IGF1-R TKI)(Bayraktar and Rocha-Lima 2013) in combination with erlotinib is ongoing to treat the advance NSCLC patients(Bar and Onn 2012). MK-0646 (dalotuzumab) is another IGF1-R antibody which is studied in NSCLC patients(Lynch, Bell *et al.* 2004). The combination of erlotinib and R1507 (a recombinant monoclonal antibody) is failed as it did not provide any significant advantage over the use of erlotinib alone (Lynch, Bell *et al.* 2004) . The use of anti HER3 mAbs in advance NSCLC is an interesting approach but still it is an early stage of development (Dienstmann, De Dosso *et al.* 2012).

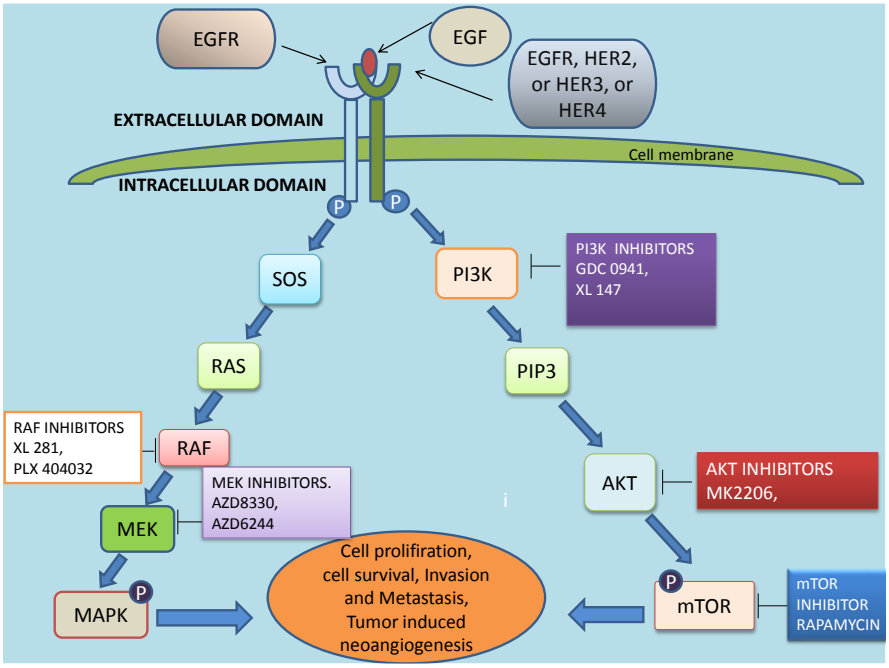

Fig 4: Different inhibitors to target different mutations. Different mutations in the EGFR pathway are targeted by their respective inhibitors.

The use of combination of HDAC (histone acetylases and histone deacetylases) inhibitor and EGFR TKIs is reported in NSCLC cell lines. The combination of vorinostat, an HDAC inhibitor and gefitinib has been studied in Phase I/II clinical trial. Moreover the combination of another HDAC inhibitor entinostat and erlotinib is recently reported in phase II clinical trial CI-994 is an orally available inhibitor of HDAC (Stahel, Peters *et al.* 2013)

(2) Anti-EGFR monoclonal antibodies

EGFR is the first molecular target against which mAbs have been developed for cancer therapy. Anti-EGFR mAbs are highly selective to receptor because they recognize EGFR exclusively(Yewale, Baradia *et al.* 2013) . Anti-EGFR mAbs act against the extracellular region of EGFR and prevent the receptor dimerization, auto-phosphorylation and downstream EGFR signaling. Apart from these monoclonal antibodies will induce receptor internalization, ubiqutinization, degradation and prolonged down regulation(Seshacharyulu, Ponnusamy *et al.* 2012). Moreover, there are two other proposed mechanisms of action, where the binding of the monoclonal antibody may lead to the induction of antibody dependent cell mediated cytotoxicity resulting in the induction of endocytosis and to a lesser extent, complement mediated cytotoxicity(Seshacharyulu, Ponnusamy *et al.* 2012) .

Antibody approaches have several potential advantages, including a prolonged half-life, allowing a decreased frequency of drug administration; receptor down regulation (permanent or temporary disappearance of the receptor from the cancer cell surface); and a favorable toxicity profile(Ray, Jablons *et al.* 2010).

A guideline of the American Medical Association for monoclonal antibody nomenclature proposes that all monoclonal antibody names should end with the suffix ‘mab’, indicating ‘monoclonal antibody’.

The name of an antibody whose source is mouse should add the letter ‘o’ to the suffix to become ‘omab’. For a chimeric antibody the name should add the letters ‘xi’ to the suffix to become ‘ximab’.The name for a humanized antibody should add the letters‘zu’ to the suffix to become ‘zumab(Ray, Jablons *et al.* 2010). So, the types of monoclonal antibodies include cetuximab, trastuzumab, or bevicizumab, matuzumab, and panitumumab (Martinelli, De Palma *et al.* 2009, Ray, Jablons *et al.* 2010). These mAbs are mainly successful treatments in head and neck cancer and colorectal cancer(Dassonville, Bozec *et al.* 2007) but these have also established a role in a NSCLC treatment as EGFR inhibitors(Egri and Takats 2006).

mAbs act against EGFR by the following mechanism of action: (1) extracellular binding (2) internalization of receptor-antibody complexes (3) inhibition of EGFR signal transduction (4) stimulation of immunological response(Pirker and Filipits 2011).

Cetuximab

Cetuximab (marketed as Erbitux®; Dako, Copenhagen, Denmark) is a 152 kDa chimeric monoclonal antibody belongs to the IgG1 subclass(Pirker and Filipits 2011, Bayraktar and Rocha-Lima 2013). It consists of two identical heavy chains made up of 449 amino acids each and two light chains of 214 amino acids each. Cetuximab has a 5-to 10-fold higher affinity for EGFR than the native ligand, resulting in inhibition of the receptor function(Martinelli, De Palma *et al.* 2009). It is also able to mediate antibody-dependent, cell-mediated cytotoxicity and receptor down regulation(Pirker and Filipits 2011). Cetuximab functions by blocking ligand binding to the extracellular domain of EGFR thus preventing ligand mediated EGFR signaling (Egri and Takats 2006). Apart from EGFR inhibition, cetuximab also down regulated VEGF, IL-2 and bFGF(Owonikoko, Sun *et al.* 2009, Brand, Iida *et al.* 2011, Yonesaka, Zejnullahu *et al.* 2011). Cetuximab, is an effective treatment alone or in combination with chemotherapy for patients with colorectal cancer (CRC), squamous cell cancer of the head and neck (HNSCC) and non-small cell lung cancer(NSCLC) particularly for those with *KRAS* and *BRAF* wild type cancers(Brand, Iida *et al.* 2011, Yonesaka, Zejnullahu *et al.* 2011). KRAS is a small GTPase responsible for coupling EGFR to the RAF/MEK/ERK pathway. KRAS binding to GTP leads to conformational changes in RAF and activation of the downstream-signaling pathway(Yonesaka, Zejnullahu *et al.* 2011, Seshacharyulu, Ponnusamy *et al.* 2012). Lievre *et al.* reported that KRAS gene with mutations at codon 12 or 13 is one of the most notable predictive biomarkers of response to cetuximab (Owonikoko, Sun *et al.* 2009, Seshacharyulu, Ponnusamy *et al.* 2012). cetuximab resistance is partly due to the ability of cells to re-activate pro-angiogenic factors via alternate pathways. In 2001, Viloria-Petit *et al.* demonstrated that tumors resistant to EGFR blocking antibodies have increased VEGF production. Raben *et al* investigated the antitumor activity of cetuximab on four non-small cell lung cancer (NSCLC) cell lines, with H157 (high), A549, H226 (moderate) and H322 (low) EGFR expression, in monotherapy, and in combination therapy with ionizing radiation where cetuximab reduced the rate of cellular proliferation in vitro(Seshacharyulu, Ponnusamy *et al.* 2012). Cetuximab in phase I/II studies has shown encouraging results in combination with different drugs like paclitaxel/carboplatin, with gemcitabine/carboplatin and with gemcitabine/platinum. Cetuximab is currently undergoing phase III trials in advanced NSCLC. The recent FLEX study demonstrated that the combination of cetuximab with cisplastin/venorelbine resulted in a small but significant improvement in patients with advanced NSCLC(Bayraktar and Rocha-Lima 2013).

Panitumumab

Panitumumab is the first FDA (2006) approved fully human IgG2 kappa monoclonal antibody used for the treatment of

EGFR-expressing metastatic colorectal cancer (Messersmith and Hidalgo 2007, Dubois and Cohen 2009, Seshacharyulu, Ponnusamy *et al.* 2012). It is developed by immunizing transgenic mice or Xeno-Mouse transgenic Technology(Messersmith and Hidalgo 2007, Martinelli, De Palma *et al.* 2009). This methodology is based on inactivation of mouse immunoglobulin genes which are replaced by mega base gene containing light and heavy chains. This technology generated fully human antibodies which do not contain murine portions of the IgG molecule. This avoids the formation of human anti mouse antibodies, which may be resulted in to more frequent hypersensitivity(Engelman and Jänne 2008). Panitumumab is used for the treatment of metastatic colorectal cancer after failure of other cytotoxic drugs(Dubois and Cohen 2009). It targets the EGFR by binding to it and prevents the binding of EGF to it and induces internalization of EGFR. Thus, panitumumab prevent the intracellular processes like dimerization, autophosphorylation and signal transduction and ultimately leads to the increased apoptosis, reduced growth and development of tumor cells and reduced angiogenesis but as like other EGFR-targeting agents, preclinical studies of panitumumab show synergistic effects when combined with chemotherapy and radiation therapy(Messersmith and Hidalgo 2007, Dubois and Cohen 2009).

Drug resistance to monoclonal antibodies

Dysregulation of EGFR receptor internalization and degradation causes upregulation of EGFR, which promotes the dimerization and transactivation of HER2 and HER3, confers acquired resistance to NSCLC. Gene amplification and strong activation of hepatocyte growth factor receptors have also been implicated in development of resistance to cetuximab in NSCLC(Bean, Brennan *et al.* 2007, Wheeler, Dunn *et al.* 2010). Although no point mutations are known to be associated with resistance to cetuximab or panitumumab, preclinical models analyzing the EGFR variant III (EGFRvIII), which lacks the ligand - binding domain has role in resistance to cetuximab therapy. EGFR ubiquitination has been also identified as a mechanism of acquired resistance to cetuximab. Epithelial-mesenchymal transition has also been implicated in the resistance to cetuximab (Wheeler, Dunn *et al.* 2010). Cetuximab resistance is partly due to the ability of cells to re-activate pro-angiogenic factors via alternate pathways. In 2001, Viloria-Petit *et al.* demonstrated that tumors resistant to EGFR blocking antibodies have increased VEGF production. Thus mechanisms of resistance to cetuximab, are dysregulation of EGFR internalization and degradation, subcellular localization of EGFR, constitutive activation of EGFR efffector molecules. ERBB2 causes cetuximab resistance by activating ERK 1/2 signaling (Yonesaka, Zejnullahu *et al.* 2011).

Overcome the resistance to monoclonal antibodies

As like resistance to TKIs, several changes or the over expression of a receptor during the treatment with monoclonal antibodies also leads to the resistance to the mAbs. So to overcome these problems there are certain agents and therapies (Table 1).

The first approach to overcome resistance to mAbs was to use the combination of mAbs and VEGF inhibitors. Next strategy is to develop such mAbs which have more efficiency to bind with EGFR to overcome the resistance to the mAbs. For example Sym004, is a combination of two chimeric mAbs and target the non-overlapping epitopes of domain III of the EGFR and stop the growth and proliferation of the cells which are responsible for the resistance to mAbs. Both chimeric antibodies bind to the extracellular domain of EGFR simultaneously, and lead to the highly efficient internalization and degradation of the receptor on cancer cells(Owonikoko, Sun *et al.* 2009, Wu, Yang *et al.* 2010). Second strategy is to use figitumumab (CP-751871). It is fully human IgG2 mAb act against the IGF1-R. It binds to the IGF1-R and prevent the binding of other ligand to it and thus stop the over expression of IGF1-R. There are also other anti-IGF-1R mAbs agents which are used as a first or second line treatment for NSCLC like R1507, AMG-479 and MK-0646(Owonikoko, Sun *et al.* 2009).

Another strategy to overcome resistance is to enhance the efficiency of anti EGFR mAbs by increasing immune-mediated cytotoxicity. RG 7160 is humanized glycol engineered anti-EGFR IgG1 mAb having increased binding affinity for all FcgRIIIa variants expressed on immune effector cells. This property improves the number of dead cells by ADCC assay(Dienstmann, De Dosso *et al.* 2012).

A recent study demonstrate the resistance to the cetuximab can be overcome by dual targeting of EGFR and erbB3 as erbB3 is also one of the cause of resistance to mAbs. U3-1287/AMG-888 is fully humanized anti-erbB3 monoclonal Ab. It inhibits the proximal and distal erbB3 signaling and enhances the internalization of erbB3. AMG-888 also inhibits the growth of cell lines of lung cancer and which shows resistance to erbB3 inhibitors and its inhibitory effect is also observed in xenograft models(Brand, Iida *et al.* 2011). Another anri-erbB3 mAbs are MM-121 and MM-111 (Ma, Lyu *et al.* 2014). Thus ErbB3 may be considered as a valuable biomarker to target the EGFR/erbB2 in NSCLC treatment (Siegelin and Borczuk 2013).

Recently, MEHD7945A is found as a dual target antibody against EGFR and HER3. It shows its activity by inhibiting the ligand dependent EGFR and HER3 mediated downstream signaling(Huang, Li *et al.* 2013). MK-0646 (dalotuzumab) is a humanized IgG1 mAb against IGF-1R, which is under development in advanced NSCLC(Méndez, Custodio *et al.* 2011). Now a days, the combination of

cetuximab and radiation therapy is ongoing treatment to overcome drug resistance to mAbs in NSCLC(Bar and Onn 2012).

Mechanism of action of EGFR inhibitors

Epidermal growth factor receptor is expressed on the cell surface of normal cells as well as cancer cells(Wheeler, Dunn *et al.* 2010). Epidermal growth factor (EGF) binds to this receptor, the tyrosine residues which are associated with this receptor are phosphorylated and leads to the different downstream signaling pathways which results in to the growth and proliferation of cells. In a cancer cells, the overexpression of this EGFR receptor or mutation of any of the factors of the signaling pathway enhance the survival and development of cancer cells. So, in order to stop the development of cancer cells, it is necessary to stop the binding of EGF to EGFR and different downstream signaling pathways. So, there are mainly two strategies to inhibit the growth of tumor cells - one is the tyrosine kinase inhibitors which are either reversible or irreversible and second is the monoclonal antibodies(Chen 2013).

First therapeutic approach is tyrosine kinase inhibitors (TKIs). Generally most of these TKIs are divided in to four groups (1) ATP-competitive inhibitors which bind to the binding site of a kinase when it is in an active form. (2) Inhibitors which recognize and bind to the site when it is in an inactive form. (3) Allosteric inhibitors which bind outside of the binding site and alter the structure of receptor and disrupt the interaction between ATP and kinase pocket. (4) Covalent inhibitors which covalently bind to the binding site of a receptor(Dienstmann, De Dosso *et al.* 2012).

TKIs compete with adenosine triphosphate (ATP) to the ATP binding site on the intracellular catalytic domain of receptor tyrosine kinase (Martinelli, De Palma *et al.* 2009, Seshacharyulu, Ponnusamy *et al.* 2012) reversibly or irreversibly and inhibit the EGFR autophosphorylation and further downstream signaling (Wheeler, Dunn *et al.* 2010) and ultimately stop the development and prolifiration of cancer cells (Seshacharyulu, Ponnusamy *et al.*, 2012). Reversible inhibitors non covalently bind to the receptor, so when mutation is shifted to the other member of the pathway then these inhibitors can easily detached from its binding site and attach to the binding site of the other member where mutation has taken place. While irreversible inhibitors covalently bind to the binding site of a receptor by specifically reacting with nucleophilic cysteine 773 residues (Harari 2004, Seshacharyulu, Ponnusamy *et al.* 2012) and change the confirmation of the receptor, so the growth factor will be unable to bind to the receptor. Irreversible inhibitors have an advantage of prolonged clinical effects and reduce the need of frequent dosing (Seshacharyulu, Ponnusamy *et al.* 2012). Along with the EGFR, these TKIs also inhibit other member of the EGFR family like vascular endothelial growth factor receptor (Martinelli, De Palma *et al.* 2009).There are several drugs which belong to TKIs class such as gefitinib, erlotinib, They are specific to target EGFR, while other drugs like lapatinib, vandetanib, AEE788 target and inhibit other receptors also along with EGFR such as HER2 and VEGFR2 (Wheeler, Dunn *et al.* 2010). In some cases these drugs are used in a combination with other drugs to target the specific receptor. In short, the mechanism of action of TKIs include following steps (1) intracellular binding (2) prevention of tyrosine kinase activation and (3)inhibition of EGFR signaling pathways and (4) potential stimulation of an immunological response (Harari 2004).

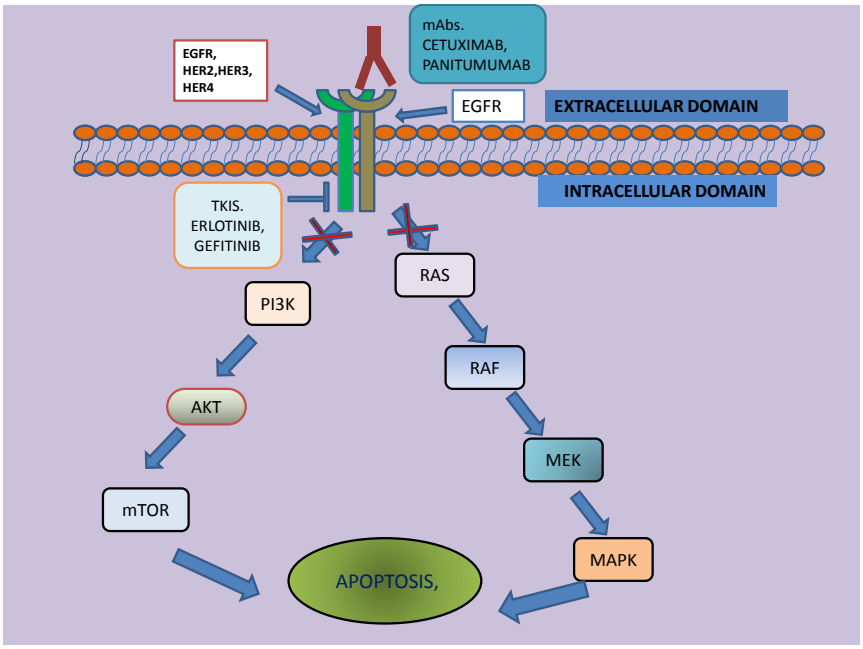

Fig 5: Mechanism of action of EGFR inhibitors. TKIs and mAbs bind to the intracellular and extracellular domain of EGFR and inhibit the further signal transduction which ultimately prevents the cell proliferation and survival or leads to the apoptosis.

Table 1: Comparison between Tyrosine Kinase Inhibitors and Monoclonal antibodies.

Characteristics	**Anti EGFR- mAbs**	**Anti EGFR- TKIs**
Nature of molecule	Recombinant immunoglobulin either of type IgG (cetuximab) or IgG2 type (panitumumab)	These are synthetic chemicals
Size Size	Large in size having 150 kDa	Small molecules having 500D
FDA approval	one mAb is approved to target EGFR	Two TKIs are approved to target EGFR
Specificity and selectivity	Highly specific and selective for the binding site	Less specific compare to mAbs. Either selective to specific nucleotide binding site, or multi selective.
Half life	3.1-7.8 days	Erlotinib: approximately 36 hrs. gefitinib: approximately 48 hrs
Mode of drug administration and dosage	Given intravenously and weekly	Given orally and daily
Toxicity	Less toxic Less toxic	Mild or highly toxic
Interaction with the Receptor	They interact with extracellular domain of tyrosine kinase due to inability to pass through the cell membrane.	They have ability to enter in to the cytoplasm so they interact with both intracellular and extracellular domain of tyrosine kinase.
Mode of action	Interaction of mAbs with the receptor, results in to the partial occlusion of ligand binding region (L2) and steric hindrance thus preventing receptor to adopt the extended confirmation which is required for the dimerization and thus inhibit downstream signaling	TKIs (gefitinib and erlotinib) specifically inhibit EGFR auto phosphorylation and dimerization and thus inhibit downstream signaling. TKIs (gefitinib and erlotinib) specifically inhibit EGFR auto phosphorylation and dimerization and thus inhibit downstream signaling.
Concentration level for Inhibition	Inhibition is achieved at lower concentration	Higher concentration is required to achieve Inhibition
Degradation system	Less compare to TKIs.	It might very more in individuals compare to mAbs.
Immune response to the Therapy	Immune-antibody response, which will render the antibody therapy ineffective.	No such reactions are Found
Response to therapy	Less or ineffective against EGFRvIII. They fail to recognize extracellular ligand binding domain.	EGFR independent constituently activated KRAS signaling. This will impair the inhibitory response to the therapy.
Cost	Expensive	Inexpensive
Success rate in clinical trials	Higher(18% chimeric and 24% humanized)	Lesser as compared to mAbs (5%).
Advantage	Highly specific and Selective	Specific for tyrosine kinase Domain
Adverse effects	Body aches or pain, tightness in the chest, rashes, tingling of the hands or feet, trouble with breathing on exertion, trouble with swallowing, wheezing, difficult and painful urination	Bloating of stomach, blood in urine, bloody nose, skin rashes, chills, darkened urine, fast or irregular breathing, pain in stomach, side, or abdomen, possibly radiating to the back, severe stinging of the eyesore, throat sore, ulcer, white spots in mouth or on lips

Another approach is to use monoclonal antibodies against EGFR. mAbs class include cetuximab, panitumumab, metuzumab, bevicuzumab etc. mAbs bind to the extracellular domain of EGFR(Seshacharyulu, Ponnusamy *et al.* 2012, Yewale, Baradia *et al.* 2013) due to having inability to cross the cellular membrane. They compete with ligand to bind with the receptor with high affinity(Seshacharyulu, Ponnusamy *et al.* 2012) compare to endogenous ligands, EGF and TGF-α(Harari 2004) and prevent the receptor dimerization and auto-phosphorylation and downstream signaling as shown in Fig 5(Seshacharyulu, Ponnusamy *et al.* 2012). Along with that, mAbs also induce the internalization of receptor, ubiqutinization, degradation and downregulation. Cetuximab which is chimeric IgG1 monoclonal antibody bind to the second L2 domain of EGFR and induce the internalization of receptor(Seshacharyulu, Ponnusamy *et al.* 2012, Huang, Li *et al.* 2013).

Recent studies have identified that X-ray crystal structure of the antigen binding (Fab) fragment from cetuximab (Erbitux), an inhibitory anti-EGFR antibody, in complex with the soluble extracellular region of EGFR (sEGFR). Cetuximab interacts with domain III of sEGFR, partially occluding the ligand binding region on this domain and prevent the receptor from adopting the extended

conformation required for dimerization. These both effects contribute to potent inhibition of EGFR activation(Li, Schmitz *et al.* 2005). The binding of monoclonal antibody may lead to the induction of antibody dependent cell mediated cytotoxicity which results in to the induction of endocytosis and also to the complement mediated cytotoxicity(Vincenzi, Schiavon *et al.* 2008). In short, the mechanism of action of mAbs includes following steps (1) the inhibition of ligand binding and receptor dimerization, with the consequent inhibition of downstream signaling, (2) internalization of the EGFR and its degradation without phosphorylation and activation of receptor, (3) immune effects which expert their activities by antibody dependent cell mediated cell cytotoxicity (ADCC) (Dienstmann, De Dosso *et al.* 2012).

Summery

The findings described in this review article strongly support that the overexpression of EGFR is involved in tumor pathogenesis and progression. In normal cells, growth factor like EGF binds to the EGFR and homo or hetrodimerization and autophosphoryaltion of EGFR occurs that leads to the several signaling pathways which ultimately results into the cell growth and angiogenesis. While in case of tumor cell, several mutations in EGFR or any member of its signaling pathway results into the overexpression of EGFR which starts the several intracellular pathways and ultimately lead to the growth and survival of tumor cell. So, in order to inhibit the growth of tumor cell, different EGFR inhibitors are found which act on either intracellular domain of EGFR (TKIs) or at extracellular domain of EGFR (mAbs) but overexpression of EGFR is also responsible for the drug resistance and due to drug resistance, tumor cells do not respond tothe drugs like reversible TKIs (erlotinib and gefitinib). So, in order to overcome resistance to reversible TKIs, second class of TKIs is developed (irreversible inhibitors). mAbs which act on extracellular domain of EGFR are also used in order to overcome drug resistance to reversible TKIs. In some cases, tumor cells do not respond to any single agent or mAbs. So, the drugs are used in combination as a treatment to prevent the over expression of EGFR. Targeted therapies are also developed to target the particular mutations in EGFR signaling pathway by their respective inhibitors in order to inhibit the proliferation and survival of tumor cells.

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