REVIEW OF LITERATURE
1. POTENTIAL DRUG TARGETS IN CANCER:

The excerption of appropriate target is of paramount importance in process of drug discovery. To attain this expeditiously, there is need to explore potential candidates from the available extensive therapeutic targets so far reported. Unremitting attempts to search the targets of extremely successful drugs, and accelerated advancement related to molecular mechanisms of disorders uncovered numerous unique targets. Previous data revealed that there were in total 500 targets reported out of which 120 were exclusive targets of commercialized drugs. Currently, there are 1494 protein subtypes, 41 nucleic acids and 997 distinct proteins, are reported as targets in the Therapeutic Target Database inclusive of 268 successful and 1267 research targets. The considerable raise in the figure of successful and research targets is perhaps due to growing knowledge of protein particular to disease (of existing targets) and recent data about formerly unidentified targets of presently available pharmaceutical compounds and investigational drugs. Numerical survey of disease associated genes and proteins proposed that there are 600 to1500 overall anticipated potential targets in the human genome (Andrew L. et al., 2002). Below figure represents total count of research and successful targets in each class of disease.

Of the total count of research and successful targets, correspondingly. While the second largest group of targets is represented by factors and regulators as compared with the rest of target population.

![Target distribution in different disease class](image)

**Fig 10: Distribution of targets in different disease classes.**

The gray, black, and white bars represent successful, research, and all targets respectively.
Apart from congenital abnormality, noteworthy increase is appearing at the level of discovery of targets in all disease classes as indicated by the presence of huge number of research targets than successful targets, which gives an idea about rigorous attempts undertaken in order to find effectual therapeutic alternative for every diseases. Maximum numbers of targets are observed in disease classes of neoplasm with presence of 78 successful and 468 research targets.

With the accessibility of the statistical data regarding appreciably more number of targets than that used in the latest investigations, it is of prime importance to re-examine bio-chemical classification of therapeutic targets. Allocation of targets to their respective biochemical classes is depicted in the figure. Biochemical classes include enzymes, transcription factors and regulators that constitute major part while the remaining part is attributed to regulatory enzymes, structural & adaptor protein, nucleic acids, etc (Trine H. Mogensen, 2009).

**Fig 11: Biochemical Classification of various targets**
Targets unable to allocate into any of these families are temporarily clustered separately as “others”. The class with the largest number of targets explored is enzymes that predominantly consist of protein tyrosine kinases along with many other sub-classes (Jacques et al., 2014) which in summation include 551 research and 134 successful targets representing 44 and 50%

On an average, about ninety tyrosine kinases have been witnessed by decrypting the human genome (Anne Goriely and Andrew O.M. Wilkie, 2012). This class of protein is mainly concerned with signal transduction, cellular proliferation, differentiation, apoptosis & a variety of regulatory processes which makes it extremely appealing target group for curative interventions, thus has taken a centre stage as a persuasive target for cancer therapeutics(Wei ZHANG and Hui Tu LIU, 2002). With this concern, tyrosine kinases correspond to about 30% of all protein targets under survey by pharmaceutical companies. Presently, more than 20 distinct tyrosine kinases are in investigational phases of drug discovery projects against various neoplasms (Zachary A. et al., 2010). With this concern, tyrosine kinases correspond to about 30% of all protein targets under survey by pharmaceutical companies. Presently, more than 20 distinct tyrosine kinases are in investigational phases of drug discovery projects against various neoplasms (Zachary A. et al., 2010).

2. PROTEIN TYROSINE KINASES: MAJOR CLASS OF DRUG TARGET

Within human genome, enzyme protein kinases are considered as one of the biggest target family that have conserved kinase domain that catalyses phosphate transfer (Lynette A. Smyth and Ian Collins, 2009).

Fig 12: Structure and Mechanism of Tyrosine Kinase: (a) Schematic representation of the mode of action of tyrosine kinase. PK represents protein kinase and PP stands for protein phosphatase. (b) Ribbon diagram representing the main features of kinase catalytic domain. The N-lobe is shown in green, the C-lobe is shown in purple and the activation loop is colored yellow. The ATP binding site is coded in red (Manash K. Paul and Anup K. Mukhopadhyay, 2004)
They are divided into two sub-classes *Ser/Thr kinases* (phosphorylate serine and threonine residues) and *Tyr kinases* (phosphorylate tyrosine residues) with the foremost structural difference within these protein kinases in reference to substrate binding site, situated in kinase domain. Ser/Thr kinases are implicated in signaling pathways that governs variety of cellular functions, while Tyr kinases are frequently concerned with cell growth and regulation (Zhimin Lu and Tony Hunter, 2009)(figure 12).

### 2.1 CLASSIFICATION AND MECHANISM OF ACTION

Tyrosine kinases are divided into *receptor tyrosine kinases* (RTKs) e.g. EGFR, PDGFR, FGFR, etc and *non-receptor tyrosine kinases* (NRTKs) e.g. SRC, ABL, JAK2, etc (Veena S. et al., 2012) (Manash K. Paul and Anup K. Mukhopadhyay, 2004). The RTKs are transmembrane/receptors proteins present on surfaces of cells having kinase activity (Harvey et al., 2000). Their structural organization consists of an extracellular ligand binding domain, a hydrophobic transmembrane helix and a cytoplasmic segment having tyrosine kinase domain with regulatory sequences present at end of both, N and C terminal (Alexander O. Shpakov, 2011). The NRTKs are cytoplasmic/non-receptor proteins, having a kinase domain along with other protein-protein interacting domains like SH2, SH3 and PH domain. The tyrosine kinase domain extent across 300 residues and comprises of an N terminal lobe consist of one α helix and five β sheet whereas C terminal is a large cytoplasmic domain which predominantly α helical. ATP attaches within the cleft of the two lobes and the tyrosine bearing sequence of the protein substrate interacts with the residues of the C terminal lobe (Gareth Williams, 2010).

(A)

---

**Receptor Tyrosine Kinases**

---

Figure: Receptor Tyrosine Kinases

- Kinase
- Cytoplasm
- Protein
- Lobe Type
- Lobe Size
- Lobe Function
- Activity

---

Cont. (A)
When there is no ligand, RTKs remains inactive in its monomeric and unphosphorylated form (Ichiro N. Maruyama, 2014). While for some RTK enzymes are inhibited by interaction of the cytoplasmic juxtamembrane region with the kinase domain (Barbara P. et al., 2007; Edwin Li and Kalina Hristova, 2010). Ligand binding to the extracellular domain results in their activation, causing oligomerization of receptor by disrupting the autoinhibitory interactions of juxtamembrane domain and autophosphorylation of a regulatory tyrosine located in activation loop (Fig. 13)(Robert Roskoski Jr., 2013). These alterations realign crucial amino acid residues, thus responsible for enhancement of enzymes catalytic activity. After activation, auto-phosphorylation creates typical binding position for signaling molecules, leading to commencement of various downstream signaling pathways.

Whilst, NRTKs retains in their inactive state via intra-molecular auto-inhibition with help of cellular proteins/lipids (Yi-fan Chen and Li-wu Fun, 2011). Dissociation of inhibitors results in activation of NRTKs by recruiting various intracellular signals to transmembrane receptors (causing autophosphorylation & oligomerization) (Suneet Shukla et al., 2012) and through trans-phosphorylation by other kinases as described in the figure. Termination of TK signaling is undertaken by the induction of inhibitory molecules and tyrosine phosphatases that hydrolyze tyrosyl phosphates (Latanya M. et al., 2010).
REVIEW OF LITERATURE

2015

Figure 14. Activation mechanism of tyrosine kinase: 1) Receptor expression at membrane claveola 2) Ligand binding 3) Hetero/homodimerization leading to tyrosine kinase activation and tyrosine transphosphorylation 4) Signal transduction 5) Receptor internalization 6) Receptor degradation or re-expression.

2.2 ONCOGENIC ACTIVATION/DYSREGULATION OF TKs IN CANCER

There are manifold stages of regulation of tyrosine kinases and cancerous cells often possesses genetic/epigenetic modifications in genes coding TKs, to attain advantage related to growth, survival, invasion and migration over normal cells. The altered protein so formed is known as oncogenic TK (Klein et al. 2005). It is implied that mutational activation of these oncogenes can occur by numerous mechanisms (Alberto and Eugenio, 2011). But amongst them are two major mechanisms that lead to deregulation of TKs consequently resulting into tumor development as discussed below:

2.2.1 Chromosomal Translocations:

Translocation defines the type of chromosomal rearrangements that involves exchange of genomic segment between two non-homologous chromosomes (Colby Chiang, 2012) (Beverly S. and Sulagna C., 2007). the translocations are commonly divided into reciprocal (Balanced) and non-reciprocal (Unbalanced) translocations (Zhishou Ou et al., 2011). Reciprocal translocation occurs when interchange of fragment takes place between any two chromosomes at different location across the chromosomal length (A. Farre et al., 2012). Non-reciprocal translocation (also known as Robertsonian) arises when two acrocentric chromosomes combines together in
close proximity to the centromeric portion causing the loss of short arms and decrease in the chromosomal number (Beverly S. and Sulagna C., 2007).

2.2.1.1 Consequences of Translocations: Chromosomal translocations either results in (a) **Juxtaposition of promoter/enhancer** elements to oncogenes or (b) **formation of chimeric protein**.

Figure 15. Consequences of Chromosomal translocations. (a) Juxtaposition of promoter/enhancer elements to oncogenes (b) formation of chimeric protein (Felix Mitelman et al., 2007).

Translocations results in **juxtapositioning** of transcriptionally active promoter/enhancer of one gene close to the coding region of other gene leading to its over-expression. e.g includes IgH-BCL2, IgH-MYC, and BCL6 translocations in follicular, Burkitts and diffuse large B cell lymphoma respectively and TMPRSS2-ETS rearrangements in prostate cancers (Fig. 15a) (Mridula et al., 2008).

Balanced chromosomal translocation commonly leads to **fusion** of RTKs/NRTKs with a partner protein which results in constitutive activation of the TKs (Ming Huj et al., 2008). e.g fusion of ABL-BCR, NPM-ALK, ETV6-NTRK3 and EWS translocations in CML, ALCL, Breast carcinoma and Ewing's sarcoma correspondingly (Fig 15b).

2.2.1.2 Translocation deregulates two main protein classes:

DNA translocation involves and deregulates predominantly two main classes of proteins.

(a) **Transcriptional regulators** (~40%) Recurrent chromosome translocations are frequently associated with sarcomas. Either of two genes implicated in these
translocations, frequently codes transcription factors and thus the resultant oncogenic fusion proteins so formed have abnormal transcriptional activity that in turn stimulates series of signaling events linked with activation of numerous genes involved in growth and proliferation of tumor cells (Mukund et al., 2012; Mridula et al., 2008).

(b) **Tyrosine kinases (~6%)** A number of TKs that are associated with gene fusions shows typical feature of retaining their catalytic domain and eliminating the inhibitory domains. Along with this feature, it is also noticed that the breakpoint regions of TKs are often positioned one to three introns upstream of the kinase domain and are usually conserved. These fusion genes interrupt many biological cascades by varying the target genes expression, and thereby modifying cellular attributes that is responsible for tumorigenesis (Mridula et al., 2008).

Initially it was assumed that recurring chromosome translocations leading to oncogenic fusions are only the hallmarks of leukemias/lymphomas but development of Innovative and sophisticated technologies for genome sequencing ended up in identifying more than 600 Gene fusions as molecular signature in broad range of solid tumors (table 6) (Peter D. Aplan, 2006). In order to explicate the cause of these chromosomal breaks, numerous potential biological mechanisms such as DNA repair by non-homologous end joining (NHEJ), Alu arbitrated homologous recombination, illegitimate V (D) J recombination, topo II cleavage sites, DNase I hypersensitive sites, repair of DNA breaks by non homologous end joining (NHEJ), fragile sites and various others have been suggested (Neil J. and David P., 2012; Mridula et al., 2008; Mikhail et al., 2012).

With recent Biological understanding, it is apparent that a substantial proportion of genomic diseases occurred due to genomic reorganization induced by anomalous recombination that takes place at chromosomal region with definite low copy repeats, also called segmental duplications. Segmental duplications correspond to a novel group of DNA repeats that has been recognized within genome in recent times. They emerged due to duplication of large fragments of genomic material ranging from in 1 to 400 kb in length and are alienated into two classes (a) Inter-chromosomal duplications (between two chromosomes) and (b) Intra-chromosomal duplications (within same chromosome).
Table 6: Chromosomal translocations associated with (a) hematological and (b) solid malignancies

The cytogenetical variations arbitrated by segmental duplications incorporates translocations, deletions, inversions and interstitial duplications which in turn results in altered amount of gene(s) copy or, instead, might disturb the integrity of a single gene. Latest verification has implicated a lot of intrachromosomal duplications in the etiology of chromosomal translocations related to genomic defects. Former examinations explain the probable mechanism of direct participation of SDs in the event of t (11; 22). Also, there is an experimental verification towards contribution of
SDs in the origin of the t (9; 22) translocation in CML (Shaoguang Li et al., 1999) (Francesco et al., 2010) (table 7).

<table>
<thead>
<tr>
<th>Genomic disorder</th>
<th>Chromosomal rearrangement</th>
<th>Chromosomal location</th>
<th>Rearrangement size (Mb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charcot-Marie-Tooth disease type 1A (CMT1A)</td>
<td>Intersitial duplication</td>
<td>17p12</td>
<td>1.5</td>
</tr>
<tr>
<td>Hereditary neuropathy with pressure palsies (HNPP)</td>
<td>Deletion</td>
<td>17p12</td>
<td>1.5</td>
</tr>
<tr>
<td>Smith–Magenis syndrome (SMS)</td>
<td>Deletion</td>
<td>17p11.2</td>
<td>5</td>
</tr>
<tr>
<td>Duplication 17p11.2</td>
<td>Intersitial duplication</td>
<td>17p11.2</td>
<td>5</td>
</tr>
<tr>
<td>Neurofibromatosis type 1 (NF1)</td>
<td>Deletion</td>
<td>17q11.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Prader–Will syndrome (PWS)</td>
<td>Deletion</td>
<td>15q11–15q13</td>
<td>4</td>
</tr>
<tr>
<td>Angelman syndrome (AS)</td>
<td>Deletion</td>
<td>15q11–15q13</td>
<td>4</td>
</tr>
<tr>
<td>Inverted duplication 15 inv dup (15)</td>
<td>Supernumerary marker chromosome</td>
<td>15q11–15q14</td>
<td>4</td>
</tr>
<tr>
<td>Williams–Beuren syndrome (WBS)</td>
<td>Deletion</td>
<td>7q11.23</td>
<td>1.6</td>
</tr>
<tr>
<td>DiGeorge and velocardiofacial syndromes (DG/VCFs)</td>
<td>Deletion</td>
<td>22q11.2</td>
<td>3</td>
</tr>
<tr>
<td>Cat eye syndrome (CES)</td>
<td>Supernumerary marker chromosome</td>
<td>22q11.2</td>
<td>3</td>
</tr>
<tr>
<td>X-linked ichthyosis</td>
<td>Deletion</td>
<td>Xq22</td>
<td>1.9</td>
</tr>
<tr>
<td>Haemophilia A</td>
<td>Inversion</td>
<td>Xq28</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 7: Genomic disorders that involves chromosomal rearrangement mediated by segmental duplications (Beverly S. and Sulagna C., 2007).

Alu elements being most copious set of interspersed repeat that constitutes up to 5 to 10% of the genome (Francesc et al., 2006). Short while ago been it has been manifested that numerous kinds of leukemia-related chromosomal relocations often harbor deletions just next to the translocation breakpoint areas. Notably, these deletions come out to probably occur when the regions subject to reorganization hold a high density of Alu repeats. This implies that these repeats could render hot spots for homologous recombination, and could arbitrate the process of translocation and thus raises the chances of further forms of chromosomal rearrangements to happen. If imbalanced crossing over takes place among Alu sequences intrachromosomally (in-cis), result is either duplication or deletion of intervening sequences. When Alu-liaised recombination happens interchromosomally (in-trans), it often leads to more intricated rearrangements like translocations (Monica Bayes et al., 2003). Below Table 8, portrays the role of Alu mediated recombination in few or more malignancies. Antibody productions rely on breakage and reuniting of Genomic segments and this process is named as V (D) J recombination that appears during early developmental stages of B and T cells. Abnormal V (D) J recombination leads
to translocations that triggers oncogenes and are assumed in the etiology of B- and T-cell lymphoma.

Table 8: Alu/Alu mediated recombination associated with cancer.

Couple of recombination signal sequence-like sequences made up of heptamer- and nonamer like motifs set-apart by 12 or 23 bp spacers, was there in the neighborhood of the deletion breakpoints in lymphomas, companied by palindromic nucleotides at junction points. Recent researches undertaken to directly evaluate the formation of rearrangements with the oncogenic substrates carrying cryptic recombination signal sequences affirmed the occurrence of illegitimate V(D)J recombination and its function in lymphomagenesis (Deok Ryong Kim and Marjorie A. Oettinger, 1998; Xianming et al., 2000). Most common V(D)J recombination-mediated translocations includes t(14;18)-BCL-2/IgH in follicular lymphomas(Maria Sol et al., 2011), t(8;14)-c-MYC/IgH in Burkitt’s lymphoma, t(11;14)-BCL-1/IgH in mantle cell lymphoma. Along with lymphomagenesis, sporadic lymphoid cancers often harbor genomic aberrations that arise due to mis-repair of RAG-breaks (Joos S et al., 1992; Busch K et al., 2004).

For acquisition of in-depth knowledge pertaining to these molecular mechanisms, there is requisite to punctuate that whether there is direct link between the particular pattern of local genomic sequence and breakpoint regions which may provide us with the clue for the cellular processes that promote chromosome rearrangements (Eugene and Artem,2009; Abhijit et al., 2012). Interpreting such mechanisms is clinically imperative as they may endow with the basis for the detection & prognosis of malignancy and also aid in discovery of new targets for cancer therapeutics. The detection of the intracellular targets of these fusions will harbor new and important insights into molecular pathways that underlie tumor development (M. Kelly et al., 2006). Ultimately, combining these modalities with
conventional treatments may provide a powerful new approach to treat these fusion-positive tumors. The series of functional events giving rise to these translocations, however, remain unclear. Better in-depth knowledge of causal mechanism behind these rearrangements can be gained by assessing the direct correlation of chromatin architecture with region of breakages that may be dynamic location for biological processes that endorse translocations and through characterization of breakpoints regions (Hyung-Goo Kim et al., 2012). The fundamental question is that why and how in the genome do these breakpoints occur so specifically leading to these rearrangements. In order to address this question, one important aspect of particular interest is to study the nature of the DNA sequence in the neighborhood of the breakpoints which will elucidate potential role of the genomic architecture and will also delineate what those potential features may be. For this, functional annotation needs to be accompanied with physical information to understand the structure, dynamics and the common functionality of genomic DNA, which helps to correlate several genomic features with the incidence of breakpoints. So, to get a detailed insight into the molecular mechanism of carcinogenesis and a holistic idea about the behavioral patterns of these breakpoints, we contemplated to examine various parameters such as segmental duplicons (SDs), repeat elements, destabilization (SIDD) profile, Recombination signal sequences (RSS), MAR elements, physico-chemical characteristics of nucleic acid (Flexibility, Stability, Stacking energy), GC content and palindrome. Though, the enormous role of particular genomic architectures has been already established as casual mechanism of recurrent rearrangements but till date, this information is only confined to individual translocation cases (Franziska et al., 2011; Aaron R. and Ira M., 2012).

2.2.2 Point Mutations:

Human cancers typically arise by virtue of multistep process that continues over a long period of time (Douglas and Robert A. 2011). Multistep process of carcinogenesis is driven by the assemblage of genetic/epigenetic anomalies in numerous oncogenes & tumor suppressor genes which are involved in diversified functions in many cancer types (figure 16). Recently, overall mutational investigation divulged that cancer genome has been heavily reported with mutational load than previously thought (Maya Kansara et al., 2014; Michael Gundry and Jan Vijg, 2012)(table 9).
**Figure 16:** The lineage of mitotic cell divisions from the fertilized egg to a single cell within a cancer showing the timing of the somatic mutations acquired by the cancer cell and the processes that contribute to them (Mihael R. et al., 2009). Mutations may be acquired while the cell lineage is phenotypically normal, reflecting both the intrinsic mutations acquired during normal cell division and the effects of exogenous mutagens. During the development of the cancer other processes, for example DNA repair defects, may contribute to the mutational burden. Passenger mutations do not have any effect on the cancer cell, but driver mutations will cause a clonal expansion. Relapse after chemotherapy can be associated with resistance mutations that often predate the initiation of treatment.

On basis of various experimental studies, genetic modifications can be sorted as mutations rendering proliferation & survival benefits, and mutations governing self renewal & differentiation. Current studies examined that few of the genetic changes causative of tumorigenesis may be inherent, but the majority is somatically attained during the transition of a normal cell to a cancerous cell (Robert Strauss et al., 2012).

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Chromosome location</th>
<th>Tumour type</th>
<th>Genetic alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABL</td>
<td>9q34</td>
<td>CML</td>
<td>Translocation/fusion with BCR or ETV6</td>
</tr>
<tr>
<td>BRAF</td>
<td>7q34</td>
<td>Melanoma</td>
<td>Point mutation</td>
</tr>
<tr>
<td>EGFR</td>
<td>7p12</td>
<td>NSCLC</td>
<td>Point mutations and small in-frame deletions/insertions, increased copy number</td>
</tr>
<tr>
<td>FLT3</td>
<td>13q12</td>
<td>Colorectal, AML</td>
<td>Point mutation</td>
</tr>
<tr>
<td>HER2</td>
<td>17q21</td>
<td>Breast, Lung</td>
<td>Amplification, Point mutation</td>
</tr>
<tr>
<td>KIT</td>
<td>4q11</td>
<td>GIST</td>
<td>Point mutation</td>
</tr>
<tr>
<td>MET</td>
<td>7q31</td>
<td>HRRCC, HCC</td>
<td>Point mutation and small in-frame deletions, Point mutation, Translocation/fusion with multiple partners</td>
</tr>
<tr>
<td>NTRK1</td>
<td>1q21</td>
<td>PTC</td>
<td>Translocation/fusion with TEL</td>
</tr>
<tr>
<td>NTRK3</td>
<td>15q25</td>
<td>Congenital fibrosarcoma, secretory breast carcinoma</td>
<td>Translocation/fusion with multiple partners</td>
</tr>
<tr>
<td>PDGFR A</td>
<td>4q12</td>
<td>CMFD, CEL, GIST</td>
<td>Translocation/fusion with BCR or FIP1L1</td>
</tr>
<tr>
<td>PDGFRB</td>
<td>5q33</td>
<td>CMML, CMPD, AML</td>
<td>Translocation/fusion with multiple partners</td>
</tr>
<tr>
<td>RET</td>
<td>10q11</td>
<td>MEN-2A, FMTC, MEN-2B</td>
<td>Point mutation</td>
</tr>
</tbody>
</table>

**Table 9:** Genetic alterations (including translocations and point mutations) in tyrosine kinases related with cancer (Justin M. Drakea et al., 2014)
Moreover, the Next-Generation Sequencing has enlightened with knowledge of new cancer-related genes within cancer genome. In reality, sequencing over and over again for recognition of polymorphism have disclosed and discriminated ‘driver’ mutations that impart the changeover of a cells from normal to cancerous and ‘passenger’ mutations that purely develops during normal and ungoverned somatic cell reproduction and propagation. A logical perceptive of molecular and inherited causal factor of tumors has already begun to have a transformative impression on the cancer research and remedies (Dana Pe’er and Nir Hacohen, 2011), predominantly by recognition of wide array of genetic variation in protein kinase genes, which are highly linked with the disease. As kinases are salient therapeutic targets for treating tumor cells, analysing the affect of genomic changes on tumorigenesi s process is both well timed and reasonable. Efficient characterization of protein kinases in malignancies unveiled major driver mutations and their entailment in tumorigenesis (Anshuman Dixit et al., 2009; Anshuman Dixit et al., 2009). In contrast to multistep tumorigenesis, the “oncogene addiction” theory implies that few tumors originate through mutation of single gene (driver mutation) that are self sufficient for stimulation and maintenance of tumorigenesis. Targeting these drivers of oncogenic addiction has been field of extensive research and advancement in last few years (Francesca AC et al., 2013). Cancers harboring driver mutations are dependent on protein product of this gene for their cancerous trait and targeting this protein can strongly hinders its expansion giving resultant advantage to patients. In Support for this oncogene addiction model appear from the growing count of instances of the therapeutic effectiveness of drugs that target precisely driver oncogenes including EGFR, ALK, K-RAS & B-RAF on the EGFR downstream signaling cascade in lung adenocarcinoma (Ji Luo et al., 2009).

2.3 TARGETING TYROSINE KINASES IN LUNG CANCER:

Across the world, lung cancer is leading cause of cancer-related deaths with the frequency over 1.8 lakhs of new cases annually in both genders. Lung cancer alone is responsible for very high numbers deaths as compared together with colon, breast and prostate cancer (T. Tharakan et al., 2008; O T Brustugun et al., 2014)(figure 17B). More than 85% of carcinomas of lung are because of tobacco & smoking while non-smokers approximately accounts for 12-15% of cases which are often due to exposure
to chemical like asbestos (Cancer Facts & Figures 2012), radon gas or by genetic factors.

(A)

Figure 17: Lung cancer statistics and Classification (a) Survey done during 2014 depicts that total estimated cancer death in lung cancer is seen to be very high as compared with summation of other three major cancer subtypes. (b) Lung cancer classification on basis of histological morphology and the mutated genes frequently found to be associated with adenocarcinoma are shown here (Travis WD et al., 2013).

Depending upon cellular morphology, lung cancer is primarily classified as Small Cell Lung Carcinoma (SCLC) & Non-Small Cell Lung Carcinoma (NSCLC) and the later comprises of about 80%. NSCLC is further divided into adenocarcinoma (highest rate of occurrence 40%), squamous carcinoma and large-cell carcinoma due to differentiated histological subtypes (Travis WD et al., 2011; Rossi G et al., 2009; Kalemkerian GP et al., 2012)(figure 17A). The systemic treatments for lung cancer consist of classical surgery, standard chemotherapy and radiotherapy either individually or in combination (Jayshree Mishra et al., 2013). The foremost innovation in lung cancer therapeutics appeared through arrival of platinum-based chemotherapeutics (N Mollberg et al., 2011). Though, the use of these therapies along with compounds like docetaxel, vinorelbine, and pemetrexed was practiced, they have shown their limitations in terms of effectiveness beyond certain point. Regardless of these conventional therapies, the 5 year survival rate has remained disappointingly
low at only 15% for more than four decades (Maria L Rossi et al., 2014). But, examination of “oncogene addiction” brought radical change in cancer treatment (Ji Luo et al., 2009).

Figure 18: Mutations and activation of downstream pathways in lung cancer (a) A diagram of proximal and distal lung cells, indicating markers that are retained in carcinomas and putative squamous cell carcinoma (SCC) and adenocarcinoma (ADC) cells of origin. (b) The binding between EGFR and ligand triggers downstream intracellular signaling pathways including the PI3K/AKT pro-survival, STAT transcription, and RAS/RAF/MEK proliferation pathways. The anaplastic lymphoma kinase (ALK) fusion proteins mainly activate the RAS/RAF/MEK and PI3K/AKT pathways. Amplification of the EGFR and ALK signaling pathways drives cell proliferation, cell motility, and carcinogenesis (Markus D and Alain C, 2014)

Improved efforts to delineate and elucidate the molecular mechanisms in lung carcinogenesis clearly states that there is functional association between the pathways that drives lung tumorigenesis and key “addicted oncogenes” having mutations. These oncogenic driver mutations activate signaling cascades in constitutive manner leading to uncontrolled cell growth and proliferation (Suchithra Menon et al., 2008) (figure 18). As a result, novel treatment modalities which directly target the driver mutations responsible for the process of lung tumorigenesis were discovered, reducing the lethal secondary effects of conventional curatives. Imatinib was the first such novel targeted treatment developed against chronic myeloid leukemia thus, constructed a new frontier in the fight against various tumors (Ji Luo et al., 2009).

Only some of these targetable mutations have been known in lung adenocarcinoma and treatment projected against these mutations reflected positive outcomes for either single-line cure or in amalgamation with the classical chemotherapy. In this context, receptor tyrosine kinase inhibitors (TKIs), which target epidermal growth factor receptor (EGFR) along with its downstream molecules of
Ras/Raf signaling pathway and anaplastic lymphoma kinase (ALK), have shown great promise in tailoring treatments to common kinase mutations found in NSCLC (Min Luo and Li-Wu Fu, 2014; Miriam Méndez et al., 2011).

2.3.1 **EGFR: Oncogenic Mutations in NSCLC:**

EGFR is expressed by many solid malignancies, including NSCLC, gliomas, colorectal, pancreas, esophageal, gastric, bladder, kidney, prostate, etc (Henk M. and Herbert M., 2007). In NSCLC, EGFR is found to be deregulated with poor predictive implications by enhanced ligand reproduction, over-expression & phosphorylation of receptor, raised counts of gene or occurrence of genetic mutations. This deregulation impels unrestrained production of cancerous cells, imparting the capability to escape apoptosis, promoting migration and metastasis.

2.3.1.1 **EGFR Protein And Its Functional Pathways:**

Human Epidermal Growth Factor Receptor (EGFR) is member of the ErbB family located at chromosome 7p11.2. This gene contains 28 exons that codes for protein of 464 amino acids (170 kilodaltons) that consists of an extracellular ligand-binding domain, transmembrane region and an intracellular domain comprises of a juxtamembrane domain restoring TK activity & C-terminal domain and any change in this have direct control on ligand binding (Langenhan et al., 2013)

---

![Figure 1](image-url)  
*Figure 1* ligand-induced dimerization and downstream activation process of EGFR. (a) These RTKs comprise a ligand-binding extracellular domain, a transmembrane link and an intracellular catalytic domain. Binding of growth factors to the extracellular domain leads to homo- or hetero-dimerization of the respective receptor, with subsequent (b) activation of RTK activity and regulation of multiple key intracellular signaling substrates as shown in the Figure (Xiaochun Yu et al., 2002; Jessica P. et al., 2005).
Notably, phosphorylation of many tyrosine residues in the C-terminal domain persuades signal transduction that ultimately causes internalization of the dimerized protein (Ream Al-Hasani and Michael R., 2011). Receptor dimerization occurs due to binding of ligands to EGFR causing autophosphorylation of TK domain which in turn excites downstream signal events via activation of series of sequential cascades, together with RAS/RAF/MEK/MAPK pathway, PI3K/AKT pathway, and JAK/STAT pathway, which governs cellular growth, survival and programmed cell death (Fig. 19). NSCLC patients are frequently diagnosed with EGFR-TK somatic mutations and over expression of EGFR (Jeremy S. and Deborah K., 2012).

2.3.1.2 Primary EGFR mutations as molecular biomarkers:

Mutations of EGFR are often observed in East Asian women having non-smoking history associated with lung adenocarcinoma. Most of mutations have been documented in exons 18–21 and are generally alienated in three groups: missense mutations (exons 18–21), insertion mutations (exon 20) and in-frame deletions (exon 19) (Fig. 20) (Samuel A Yousem, 2012). Most frequent EGFR mutations that influence the ATP binding crevice are located in exon 19 and 21. The majority common deletions in exon 19 comprises of L747- T751insS, L747-P753insS and E746-A750.

*Figure 20: Epidermal growth factor receptor (EGFR) mutations in NSCLC. (a) Structure of the kinase domain of EGFR in complex with erlotinib and location of the most common EGFR mutations. (b) Frequency, exon location, and sensitivity to EGFR inhibitors of the most common EGFR mutations. TKIs: tyrosine kinase inhibitors (Martínez-Navarro EM, 2011; Jorge SE, 2014).

The subsequent main mutation is L858R present within exon 21 with rarer L861Q and L861R. Third category of mutations incorporates G719C/S/A, and S720F found
in exon 18 (AF Gazdar, 2009). Numerous insertions counting D770-N771insNPG/SVQ/G and point mutations such as V769L, T790M, and N771T found on exon 20 are often co-related with non-responsiveness of EGFR-TKIs. T790M is foremost mutation allied with a minute portion of NSCLC patients with primary resistance and more than 50% of patients reported with acquired resistance to EGFR TKI (Figure 20).

2.3.1.3 EGFR Tyrosine Kinase Inhibitors for NSCLC:

2.3.1.3.1 First-Generation EGFR TKIs

Suppression of EGFR autophosphorylation followed by inhibition of receptor activation & downstream signaling cascades can be achieved by EGFR TKIs small molecules. Gefitinib and Erlotinib are reversible first-generation TKIs available for oral intake. These are synthetic derivatives of aniline-quinazolines that binds with higher affinity to TK domain hindering ATP binding (Shunzhou Wan and Peter V., 2011; Michelle Arkin and Mark M. Moasser, 2008) (figure 22).

(A) Gefitinib (ZD1839; IRESSA®)

Gefitinib precisely binds within ATP binding pocket thus repressing TK functionality that results in blockage of EGFR activation and its succeeding pathways related to proliferation and survival. It also provokes signaling event that ultimately leads to apoptosis. Iressa suppresses cell growth by arresting cell cycle at G0/G1 phase through decreasing cyclin D1 expression, p15INK4b up regulation and aggregation of p21WAF1/CIP1 & p27KIP1 (Si-Kyoung Jo et al., 2012). Obstruction of EGF- stimulated cytoskeleton remodeling and in-vitro invasiveness was witnessed in keratinocytes and cutaneous squamous tumors cells, when treated with gefitinib. Significant anti-cancerous effects of gefitinib were visualized in Phase I & II trials, stimulating response in 12% and amending indications in 39% of NSCLC patients having local or metastatic disease refractory to docetaxel and platinum-based standard chemotherapeutics. Good response rate more than 70% and yearly survival of 80% were achieved in patients having EGFR TKD mutations. Gefitinib was investigated as first-line of treatment for advanced untreated NSCLC in numerous phase II clinical trials (Caio M. Rocha-Lima et al., 2009; Miriam Méndez et al., 2011). On the whole, observed response in random populations was 5 to 10% with a median survival of 2.5 to 7.5
month but for adenocarcinoma response rate from 10 to 16% having median survival of more than year is seen when treated with gefitinib. Superior results with improved response rate of 30% to 54% and median survival of 9.5 to 22 months were reported in North American patients with TKD mutations. Additionally, gefitinib induced admirable response rate of 60 to 65% with a mean survival of 18 to 20 months in noticed in Asian patients harboring EGFR TKD mutations (Woondong Jeong et al., 2013). Eventually, patients of NSCLC having non-smoking history showed excellent response of 80% and an expected 1-year survival of 73% (Julian R. Molina et al., 2008).

(B) Erlotinib (OSI-774; TARCEVA®)

Erlotinib particularly inhibits EGFR as compared with rest of ErbB family members. Experimental studies revealed that many human tumors demonstrates sensitivity to erlotinib that inhibits cancer cell growth by blocking cell cycle at G1 phase and disrupts anti-apoptotic signals (Robert Roskoski Jr, 2014). In phase I clinical trials Tarceva, at daily dosage of 150 mg exhibited anti-tumour potential against prolonged stable disease in patients with various cancers unmanageable with conventional therapies, together with including NSCLC (S.J Shuttleworth et al., 2011). Erlotinib was examined as first-line therapy for complex/previous treated NSCLC in several phase II trials (A A Farsi and P.M Ellis, 2014). Response of 9 to 30% with average survival of 5 to 14 months and one year survival of 25 to 55% were attained for erlotinib when administered alone to random group of patients. Efficient outcome visualized in non smoker adenocarcinoma females, with response of 25 to 35% with average survival of 13 to 15 months. In preferred wild type subjects, merely response of 3% and the mean survival of 9 months were remarked. Response rates of 20%, 31% and 88% have been reported for selected patients of adenocarcinoma, female gender or presence of TKD mutations, correspondingly. In conclusion, erlotinib is the exclusive EGFR TKI that has been confirmed with betterment of response rate, progression free survival and overall survival in locally advanced or metastatic NSCLC as a secondary or further line of treatment choice in experimental studies (F Kyle and J Spicera, 2008).
2.3.1.3.2 Acquired Resistance to first generation EGFR TKIs:

Elongated administration of gefitinib/erlotinib offers discriminatory stress that leads to expansion of cancerous cells (which possess growth potentials) regardless of drug inhibition (Jonathan E and Carol S, 2012; J Wangari and E H Borge, 2013). From biological point of view the causal process behind secondary resistance phenomenon are subject of substantial investigation and up till now, only few mechanisms are identified. A number of experimental studies have verified that the two major mechanisms are accountable for acquired resistance (a) Amplification of mesenchymal epidermal transition (MET) causing up-regulation of the sequential signaling pathway (Bai-Hua Luo et al., 2014) and (b) the manifestation of T790M “gatekeeper” mutation in EGFR TKD (Cai-Hong Yun et al., 2008; Monica Red Brewer et al., 2013). Other mechanisms comprises of PI3KCA mutations, switching from epithelial to mesenchymal differentiation and EGFR amplifications (figure 21).

![Figure 21: Biochemical pathways leading to resistance to small molecule EGFR drugs.](image)

(A) Simplified pathway diagram of EGFR signaling through RAS/MEK/ERK and PI3K/PDK1/AKT indicating the points of mutation/amplification in EGFR TKI resistance. The resistance mechanisms include the EGFR T790M gatekeeper mutation, amplification of EGFR T790M, MET amplification, and PI3KCA mutation. (b) Erlotinib bound to the EGFR TKD, the gatekeeper residue (T790) is highlighted in green; cysteine-797, which forms a covalent bond with 2nd and 3rd generation irreversible EGFR inhibitors, is highlighted in green (D Gonzalez de Castro et al., 2013).

Amplification of MET is correlated with acquired resistance in about 5% of cases and MET inhibition either by TKIs or MAbs, individually or in permutation with another targeted compounds are presently under investigational research (JP
Hughes et al., 2011). Exon 20 of EGFR gene harbors a typical T790M “acquired” point mutation is allied with inadequate action of first generation TKIs and is culpable for secondary resistance in more than 50% of subjects treated with erlotinib or gefitinib. T790 is “gatekeeper residue” and two major molecular mechanisms elucidate how T790M gives rise to drug acquired resistance. First, replacement of a huge Methionine residue in place of threonine at location 790 results into distorted binding of drug in ATP cleft. Next, phenomenon of the T790M mutation enhances the ATP affinity of the L858R mutant in manner similar to that of wild-type EGFR. This seal down the therapeutic window that is otherwise accessible by the lessened ATP affinity of the mutants, which are usually effortlessly repressed proportionate to EGFR wild-type (D G de Castro et al., 2013; Wei-Yun Lai et al., 2014).

2.3.1.3.3 Second-Generation EGFR TKI

Acquisition of secondary resistance against first generation EGFR TKIs has provoked the clinical ontogenesis of second-generation TKIs with the anticipation to surmount the mechanisms of resistance to first generation TKIs and have the prospective to be efficient to greater extent than gefitinib/erlotinib. The majorities of these TKIs (afatinib, dacomitinib and neratinib) forms irreversible covalent linkage with the Cys797 existing in ATP-binding pocket of EGFR mutant (Qingsong Liu et al., 2013), and possibly possess additional activity against other family members of EGFR or receptors comprising of similar structures (Kathryn M. Ferguson, 2008). Due to their inimitable attributes these irreversible TKIs appeared to be the ideal agents to evade resistance to 1st generation TKIs (table 10) (figure 22).

(A) Afatinib (BIBW2992)

Afatinib is an irreversible small molecule inhibitor that might probably prevail over the resistance of previous-generation TKIs (Irene Stasi and Federico Cappuzzo, 2014; Manolo D’Arcangelo and Fred R Hirsch, 2014). In in-vitro investigations, afatinib has confirmed activity against lung cancer harboring EGFR mutations. In LUX-Lung 2 phase II trial afatinib was assessed in advanced NSCLC patients with EGFR mutations either untreated or formerly treated with chemotherapy. Around 130 patients when given afatinib as first or second line therapy it was reported that mean PFS was more than 9 months and average OS was about 25 months. Both PFS and OS were found to be higher in figures with
first-line as compared to second-line treatment (Wen-Qian Zhang et al., 2014; Jane E. Rogers et al., 2014). Populations with L858R mutations and deletion-19 mutations showed the mean PFS of 14 months while in contrast to this, median PFS of only 3.5 months is remarked in patients having rest of mutations. Likewise, OS also demonstrated outcomes in parallel fashion (to results of PFS) with mean time of 39 months, 30 months and 16 months for exon 19 deletions, L858R mutations and other mutations respectively. Initial outcomes of biggest potential clinical trial LUX-Lung 3 investigating the effectiveness of afatinib as first line in contrast to cisplatin and pemetrexed chemo-regimen against EGFR mutation-positive lung cancer verified the notably extended PFS and enhanced Response of 55% vs. 22% with clear postponement in symptoms affiliated to lung adenocarcinoma. Ongoing LUX-Lung 6 phase III clinical studies in particular patients, estimates the efficiency of first-line afatinib compared to combination chemotherapy of gemcitabine and cisplatin (Shu Fang and Zhehai Wang, 2014). Afatinib was approved as first-line therapy for advanced NSCLC with L858R mutations and exon 19 deletions (Keating GM, 2014). Nevertheless, cancers harboring resistant mutations like exon 20 insertions and T790M seemed to be less responsive (Susumu Kobayashi et al, 2013).

(B) Dacomitinib (PF-00299804)

In contrast to 1st generation irreversible pan-inhibitor, dacomitinib showed striking pharmacokinetic features and demonstrated experimental activity with a enhanced inhibition in both erlotinib/gefitinib sensitive and EGFR-T790M resistant (Annette O. Walter et al., 2013). Phase I trials confirmed the daily safety of 45mg of dacomitinib in NSCLC population for EGFR mutations (Helena A. Yu and Gregory J. Riely, 2013), Her2 amplifications and wild type KRAS wild type patients. 62 Subjects who had received chemotherapy & erlotinib were considered for Phase II study that evaluated showed 3 definite PRs and More than 6 weeks of steady disease was observed in 35 patients. For safety measures and effectiveness, a randomized phase II Clinical trials of dacomitinib/erlotinib was undertaken in NSCLC patients after cytotoxic chemo-modalities demonstrated longer mean PFS in contrast to erlotinib. ARCHER is another randomized phase III clinical studies ongoing with same sub group of population (Barbara Melosky et al., 2014). Another set of Phase II trials on slight smokers/adenocarcinoma subset of patients
with sensitizing EGFR mutation tells about dacomitinib as first line setting and 35 out of forty-six patients responded partially with mean PFS of 17 months. This study is currently ongoing with HER2 mutated patients.

(C) Neratinib (HKI-272)

An oral irreversible inhibitor, Neratinib belongs to HER family is under in-vitro examination with cell lines having T790M along with other mutations and it was observed that neratinib effectively repressed cell proliferation compared to gefitinib. In phase I study conducted on pretreated NSCLC patients where the maximum tolerated dose (MTD) of 320 mg was observed. After that, neratinib was studied in a phase II clinical trials during which, more than half of patients developed diarrhea (grade three) at MTD and hence the daily oral dosage was reduced to 240 mg. Regrettably neratinib showed response rate of below 5% and no response in EGFR mutant patients and T790M mutated population incorporated in phase II trial, respectively. The ineffectiveness is perhaps due to required elevated neratinib concentration to inhibit T790M mutation in preclinical tests and this chief drawback scattered the inquisitiveness to further investigate neratinib in lung adenocarcinoma (Miriam Méndez at al., 2011).

![Figure 22: Selected first, second and third generation EGFR inhibitors for NSCLC.](image)
2.3.1.3.4 Third-Generation EGFR TKIs

Although, Dacomitinib/afatinib have demonstrated their potential effect in experimental studies, but inadequate activity in pharmaceutical trials against patients harboring acquired resistance with regard to earlier EGFR-TKIs and owing to development of toxic side effects, paved way towards discovery of new group of molecules to target T790M. Among these, CO-1686 and AZD9291 are third-generation EGFR-TKIs currently in an early clinical development with availability of some clinical information (figure 22).

(A) CO-1686

The 3rd generation TKI CO-1686, probably comes out with better efficacy against T790M resistant mutants. Preliminary report from current phase I/II clinical trial (NCT01526928) indicated regarding tolerable dosage and overall decrease in tumor volume in subjects who turned out to be unsuccessful with classical chemotherapeutics or above mentioned therapy (Yuxin Lin et al., 2014).

(B) AZD9291

One more oral irreversible inhibitor, AZD9291 has been developed against EGFR-TKI-sensitive and resistance mutations. This agent has demonstrated good activity at various dosages with negligible adverse effect and no severe effects of grade III/I in a phase I clinical trial, with 10/19 patients with T790M who responded partially.

<table>
<thead>
<tr>
<th>EGFR tyrosine kinase inhibitor</th>
<th>Phase</th>
<th>Trial registration</th>
<th>Targets</th>
<th>Trial design</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lapatinib</td>
<td>II</td>
<td>NCT00528281</td>
<td>EGFR, HER2</td>
<td>Single-arm study with penetratinex</td>
</tr>
<tr>
<td>Neratinib</td>
<td>II</td>
<td>NCT00266877</td>
<td>EGFR, HER2</td>
<td>Three-group study</td>
</tr>
<tr>
<td>Icotinib</td>
<td>II, III</td>
<td>NCT01609290</td>
<td>EGFR</td>
<td>Monotherapy and with chemotherapy, radiation or other targeted therapies</td>
</tr>
<tr>
<td>(BP-2009H)</td>
<td></td>
<td>NCT01707329</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT01516983</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT01719536</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afatinib</td>
<td>II, III</td>
<td>Multiple studies</td>
<td>Pan-HER family</td>
<td></td>
</tr>
<tr>
<td>Dacomitinib</td>
<td>III</td>
<td>NCT0174721</td>
<td>Pan-HER family</td>
<td>Monotherapy versus gefitinib or erlotinib</td>
</tr>
<tr>
<td>(PF00299504)</td>
<td></td>
<td>NCT01360554</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pitolinib</td>
<td>II</td>
<td>NCT01894028</td>
<td>Pan-HER and TEC family</td>
<td>First- and second-line monotherapy</td>
</tr>
<tr>
<td>(H781-36B)</td>
<td></td>
<td>NCT0178047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZD9291</td>
<td>I</td>
<td>NCT01802632</td>
<td>EGFR mutation specific</td>
<td>Monotherapy in previously treated EGFR mutant NSCLC</td>
</tr>
<tr>
<td>CO-1686</td>
<td>I, II</td>
<td>NCT01526928</td>
<td>EGFR mutation specific</td>
<td>Monotherapy in previously treated EGFR mutant NSCLC</td>
</tr>
<tr>
<td>HM61713</td>
<td>I</td>
<td>NCT01588145</td>
<td>EGFR mutation specific</td>
<td>Monotherapy in previously treated EGFR mutant NSCLC</td>
</tr>
<tr>
<td>AP26113</td>
<td>I, II</td>
<td>NCT01494961</td>
<td>Dual ALK and EGFR</td>
<td>Monotherapy in NSCLC with ALK gene rearrangement inhibitor or mutant EGFR</td>
</tr>
</tbody>
</table>

Table 10: Summary of Clinical trials of EGFR tyrosine kinase inhibitor in development in NSCLC with EGFR mutations (AF Gazdar, 2009).
Till date, therapeutic approaches for population with drug acquired resistance are still limited. Though, Second-generation TKIs proved to be effective against untreated EGFR-mutant adenocarcinoma but were unsuccessful against T790M mutation due to preclinically unattainable concentrations and dosage limiting toxicity associated with unselected Wild type EGFR inhibition. Existing information for afatinib in adenocarcinoma with acquired resistance to gefitinib/erlotinib pointed out the modest activity of compound. Hence, there is urgent pre-requisite to develop new approaches that can more efficiently targets T790M mutations.

2.3.2 ALK: Oncogenic Fusion in NSCLC

Anaplastic lymphoma kinase (ALK) was initially recognized as tyrosine kinase part of oncogenic fusion resulted from translocation, in anaplastic large-cell lymphoma. Genomic rearrangements include ALK amplification, mutation and translocations results in constitutive activation of ALK leading to various cancers including neuroblastoma, NSCLC and breast cancer. There are experimental evidences demonstrating potential role of ALK in tumorogenic progression, and thus targeting ALK results in anti cancerous efficacy (Natasha Y. Frank et al., 2010).

2.3.2.1 ALK fusions And Its Functional Pathways:

The EML4-ALK oncogenic fusion is one of the recently recognized prime targets in lung adenocarcinoma which occurs due to inversion of short arm of chromosome 2 that unites with exons 1 to 13 of EML4 to exons 20 to 29 of ALK resulting in fusion product that comprises of N terminal of EML4 and C terminal of ALK (TKD). Numerous other EML4-ALK iso-forms have been found that contains cytoplasmic portion of ALK as conserved portion and truncations of EML4 as variable part. ALK also forms fusion with TGF, TRK and KIF5B but have been less frequently reported in lung adenocarcinoma (figure 23B).

Similar to other receptor kinases ALK belongs to the super family of insulin receptor and consists of an extracellular ligand-binding domain, a transmembrane region, and an intracellular tyrosine kinase domain. Ligand binding causes homodimerization resulting into trans-phosphorylation and activation of kinase. ALK fusion partners render domain dimerization, facilitating ligand-independent kinase activation. Cytoplasmic localization of these fusion products may also cause ALK dysfunctioning (Justin M. Drake et al., 2014). The chief and immediate downstream
molecules of ALK include PI3K/Akt, Ras/Raf/Mek/Erk, and JAK/STAT3 signaling cascades (figure 23C).

**Figure 23:** Domain structure of ALK, different fusion gene variants of EML4-ALK and downstream signaling pathways. (a) The extracellular region contains a signal peptide (Sig P)—two meprin, A-5 protein, receptor protein tyrosine phosphatase mu (MAM) domains; one low-density lipoprotein (LDL) domain. The transmembrane domain (TM) connects the extracellular and intracellular regions. The intracellular region contains the juxtamembrane domain and the tyrosine kinase catalytic domain, which contain three tyrosine phosphorylation sites necessary for activation. (b) ALK fusion emerges on exon 20 of the kinase. Alternative variants depend on different EML4 cut points. INS, insertion; V, variant. (c) The binding of ligands to receptor leads to the activation of the tyrosine kinase domain, and concomitant activation of multiple signaling pathways that regulate cellular processes such proliferation and cell survival (Yongjun Li et al., 2011).

**2.3.2.2 ALK Inhibitors For NSCLC**

**2.3.2.3.1 First-Generation AKI TKIs:**

(A) **Crizotinib**

An oral, ATP competitive, selective small molecule c-MET inhibitor Crizotinib in experimental investigations exemplified good activity for c-MET and ALK mutated cell lines. Crizotinib binds within enzymes ATP pocket thereby stalling autophosphorylation, which is otherwise necessary for enzymes activation. From Phase I, dose acceleration trial, daily dose 250 mg of crizotinib is identified as monotherapy in patients with complex solid malignancies.
Surprisingly, two NSCLC subjects harboring EML4-ALK fusions showed remarkable symptomatic betterment in dosage escalation trials with crizotinib. This striking examination led to a huge prospective screening of ALK-positive NSCLC sub-groups and ended up with enrollment of population with ALK fusion, into an extended molecular cohort at MTD 250 mg twice on daily bases. The clinical efficacy of crizotinib on ALK-positive sub-population was rapidly praised after PROFILE 1001 phase trials (Brendan D Looyenga et al., 2011). Response of 55% and 30% of patients were reported with stable disease. Crizotinib showed good tolerance, except mild gastrointestinal indications. The OS rates in this cohort of 82 patients at 1 and 2 years were 74% and 54%, respectively.11 Preliminary data obtained from phase I clinical studies conducted on 150 patients described an overall response of 60% with average PFS of 9 months. Likewise, current PROFILE 1005 phase II investigations indicated response of 59% and a mean PFS of 8 months. On these bases FDA in 2011 approved crizotinib. Patients with disease progression subsequent to prior platinum-based therapy were considered for PROFILE 1007 (phase III) open trial, where crizotinib was equated with docetaxel/pemetrexed demonstrated appreciably response rate of 66% vs. 19% and lengthy mean PFS 7 vs. 3 months respectively (Míriam Méndez et al., 2011). From all clinical data, most frequent grade III/IV adverse effects of crizotinib include increased transaminase levels and pneumonitis. In this study, there was no significance difference in overall survival between 2 subsets which may be due to switching of patients from chemotherapy to crizotinib. Nevertheless, retrospective study evaluated crizotinib-treated ALK-positive subjects, depicted lengthened overall survival when compared with ALK-positive controls untreated with crizotinib. Outcomes of the PROFILE 1014 trials, demonstrating the comparison of crizotinib with chemotherapy as first line of treatment are still awaited.

2.3.2.3.2 Acquired Resistance to first generation ALK TKI:
Regardless of noteworthy preliminary response to crizotinib, most of patients decline within one year of treatment due to the drug acquired resistance. Mechanisms of acquired resistance can be divided into two common groups (figure 24).

The first group includes ALK amplification or mutation. Sequencing of samples from relapsed patient undergone crizotinib therapy uncovered two mutations
L1196M and C1156Y each was self-sufficient to confer resistance. L1196M signifies the “gatekeeper” mutation which causes steric hindrance in binding of drugs. Succeeding examination described other various mutations counting G1269A, L1152R, 1151Tins, S1206Y and G1202R. Additionally, ALK amplification is also recognized as contributing to mechanism of resistance. On the whole, there are more than half of patients showing regression to crizotinib owing to these amplification/mutations (fig. 24).

**Fig 24: Crizotinib bound to ALK domain and mechanisms leading to crizotinib resistance.**

(a) Crizotinib bound to ALK domain and with location of secondary mutation known to confer acquired resistance, highlighted in green. (b) The figure depicts crizotinib-sensitive cancer cells expressing EML4–ALK. Crizotinib binds and inactivates the ALK fusion, disrupting downstream signaling. The right panel depicts two general classes of crizotinib resistance: one mediated by genetic mutation of the target so that crizotinib can no longer bind to the active site, and the other mediated by activation of alternative pathways (or bypass tracks) that can engage downstream signaling pathways even when ALK is inhibited (Sen Zhang et al., 2011).

The second mechanism is commencement of alternative cascades that bypasses ALK (fig. 24). Activation of EGFR signaling has been reported experimentally in ALK-mutant crizotinib resistant cell lines. Unswerving with these data, approximately more than 50% of ALK mutated tumors depicts EGFR activation simultaneously along with crizotinib resistance (Justin F. Gainor et al., 2013). Another probable sidestep to circumvent the resistance is amplification of cKIT gene. Additional possible bypass mechanisms such as KRAS/EGFR mutations have also been anticipated. Remarkably, it is noticed that single subject may hold several mechanisms at a time leading to resistance, signifying the call for various therapeutic permutation to stimulate long term remittances in resistant patients. In clinical trials,
development of CNS disease resulted in 45% crizotinib treated patients. It was observed that cerebrospinal fluid showed extremely low drug level as compared with plasma; symptomatic of reduced infiltration in CNS. This investigation probably aroused pharmacological matter called insufficient drug exposure due to which high rate of CNS regression in observed in ALK-mutants. Numerous next generation inhibitors (discussed below) are being developed in part to address this potential liability of crizotinib. Whether ALK gene alterations or bypass signaling may also contribute to resistance in the CNS is indefinite (Matthew J. and Jeffrey A., 2013).

2.3.2.3.3 Second-Generation ALK Inhibitors:

New cohort of ALK and HSP90 inhibitors corresponds to two diverse and potential tactics to surmount the “ALK-dominant” resistance. These inhibitors during preclinical studies potently stalled ALK kinase and demonstrated action against various mutations including CNS as discussed below. Both are under current phase I/II retrospective analysis (Mark M. Awad, and Alice T. Shaw, 2014).

(A) Ceritinib (LDK378)

Ceritinib is unique inhibitor that precisely targets ALK TKD. Preliminary outcomes of current phase I/II trials are available for this agent together for group devoid of resistance and other group with crizotinib resistant patients. Daily dosage of 750 mg was reported for this compound but dosage linked toxicity such as hypophosphatemia and increased aminotransferase levels were observed. NSCLC patients administered with minimum dose of 400mg daily, showed response of 60% and PSF of nearly 8 months. Response rate of 55% was visualized in patients who had received crizotinib as prior regimen. Patients harboring several resistant mutations counting 1196M were also marked with better response (Mark M. Awad, and Alice T. Shaw, 2014). Owing to these stupendous outcomes FDA sanctioned ceritinib as second-line therapy for ALK mutant NSCLC subjects with subsequent crizotinib intolerance/collapse. However, numbers of clinical studies are ongoing. These incorporate in total 2 phase II clinical investigational studies of ceritinib for crizotinib resistant population and one for patients untreated with crizotinib. Another phase II trial is exclusively conducted on the patients harboring ROS1 mutation. Two more retrospective phase III analysis are comparing ceritinib with chemo in patients,
those never exposed to chemotherapy with those who had received platinum based standard therapy earlier. HSP90 inhibitors are also being explored for their efficacy in combination with ceritinib in clinical trials (Mark M. Awad, and Alice T. Shaw, 2014; Azusa Tanimoto et al., 2014).

(B) Alectinib (CH5424802/RO5424802)

Alectinib is ALK inhibitor that specifically works against resistance developed due to gatekeeper “L1196M” mutation in addition to various secondary mutations (R1275Q/F1174L). Outcomes of current phase I/II trials executed with alectinib in Japanese patient demonstrated the MTD of 300 mg two times a day devoid of any toxic side effects.

![Chemical structure of various ALK inhibitors.](image)

Partial response of 85% was observed just in short time span of 5-6 weeks in phase II analysis with the exception of two patients who responded absolutely.
Patients with Brain metastasis were also reported to show response. But, unfortunately, 25% of patients suffered with adverse effect of grade III/IV such as elevated creatine phosphokinase levels. Altogether, multiple ALEXA phaseII/III clinical studies are currently being carried out with alectinib pertaining to crizotinib-naïve as one group and crizotinib-resistant patients in another group (Matthew J. and Jeffrey A., 2013).

(C) AP26113

Another novel ALK inhibitor, AP26113 is found to be capable to circumvent various mutations including gatekeepers like T790M, L1196M and ROS1. It has been verified from current clinical research that daily dose of 180 mg enhanced anti-cancerous action in ALK + patients. Better ORR of about 65% was observed in resistant population and unexpectedly patients with brain metastasis also responded. Fatigue, nausea and diarrhea are the drug associated adverse effects that are commonly noticed in patients. An assenting phase II investigational trial conducted on crizotinib resistant groups is presently under advancement (Susan Devine et al., 2008).

Multiple other new-generation ALK agents are currently under phase I study. These include TSR-001, ASP3026, PF-06463922, as well as X-396 (Khashayar Esfahani, 2014). Details about these studies can be found in Table11.

(D) Hsp90 Inhibitors

Experimental studies pertaining to inhibitors of HSP90 confirmed their role in reducing level of oncogenic ALK fusion protein leading to cancer retrogression. There are numerous HSP90 inhibitors under recent investigation in clinical phase I/II trials considering either as monotherapy or in permutation with others, in ALKC NSCLC population. Preliminary data obtained from phase II trial concerned with IPI-504 provided that 3 subjects showed partial response while 1 patient appeared with extended disease stability. It was remarked from phase II trial of STA-9090, that half of patient showed partial response and few were encountered with steady disease (Damien Kee and John R. Zalcberg, 2012). Anorexia, fatigue, diarrhea and nausea are the frequently perceived adverse
Table 11: Ongoing clinical trials involving novel ALK inhibitors.

events but regrettably 2 subjects died due to failure of kidney functioning and cardiac arrest during this study. Another study undertaken to evaluate with AUY922 as monotherapy revealed that improved ORR of 30% was achieved in both subsets, crizotinib naïve and resistant patients (Johanna N. Spaans and Glenwood D. Goss, 2014).

Similar to EGFR-TKIs crizotinib inevitably develops acquired resistance during the treatment. The matchless invention of ALK and Hsp90-inhibitory molecules exemplify assuring substitutes for patients harboring resistant mechanism so called “ALK-dominance”. Diverse targeted molecules may be believed to prevail over the commencement of other signaling circuits (EGFR,
KRAS), when combined together. But unfortunately development of resistance is observed regardless of the improved effectiveness and precision of next-generation ALK TKIs. Consequently, explicating mechanism of acquired resistance and formulating satisfactory curatives are the critical problems to be countered back by fanatical transformational analysis in field of lung cancer therapeutics (Wenfang Tan et al., 2012).

2.3.3 Mutations In Downstream RAS/RAF Pathway:

The resistance to EGFR TKIs is also owing to presence of other mutated proteins like KRAS and BRAF in downstream of EGFR signaling pathway (Wendy A. Cooper et al., 2013) (Fig. 26). Irrespective of ubiquitous expression of EGFR, investigations demonstrated that anti-EGFR therapeutics are only effective and advantageous to population harboring KRAS and BRAF in wild forms, which are chief intermediaries of MAP kinase signaling cascade (figure 26). Furthermore, a number of studies states that, KRAS/EGFR mutations occurs in mutually exclusive manner, pin pointing their perceptible functional surplus in NSCLC.

Figure 26: The EGFR mutation and EML4–ALK fusion protein and their downstream pathway in NSCLC (Yongjun Li et al., 2011). Mutation in tyrosine kinase EGFR and as well as in signaling molecules downstream to this receptor (Ras and Raf); have been implicated in non-small-cell lung cancer (NSCLC). The mutant proteins are thought to stimulate the proliferation of cancer cells and/or prevent their programmed death. A chromosomal rearrangement results in a fusion gene, the product of which is EML4–ALK is also reported in NSCLC. This fusion protein also functions as an activated tyrosine kinase, and thus might stimulate the EGFR-mediated signaling pathway.
2.3.3.1 KRAS Mutations:

KRAS is an established G-protein encoding proto oncogene which is significantly associated with RAF/MAPK/MEK/ERK signaling cascade. Mutant KRAS hydrolyses the RAS bound GTP to GDP resulting into stimulus independent constitutive activation RAS/RAF/MAPK downstream pathway. KRAS mutation comprises of approximately 30% of NSCLC patients with substitution of single amino acid mostly at codon 12, 13 and 61 within exon 2 and 3 being the most common mutations. Predominant association with adenocarcinoma histology over squamous cell subtype and an increased prevalence in patients having smoking history and tobacco exposure makes KRAS the most common driver mutation in lung cancer patients. KRAS mutations are generally not found to co-exist along with EGFR mutations or ALK fusions in same NSCLC tumor apart from some exceptions (Grzegorz J. Korpanty et al., 2014; P J Roberts and C J Der, 2007). Although with lot of advancement in field of targeted therapeutic research, till date there are no direct anti-KRAS therapies available for cancer associated with KRAS mutations. Since EGFR is present in the upstream of KRAS, there have been attempts to target these mutations with anti-EGFR TKIs and MAbs. Various clinical investigations conducted so far have confirmed that patients with positive KRAS mutation confer resistance to EGFR inhibitors such as gefitinib, erlotinib and/or chemotherapy, resulting in faster disease progression rate and reduced overall survival (AF Gazdar, 2009). In Phase II trials, NSCLC Patients with wild type KRAS when treated with erlotinib showed about 30% response rate in contrast to merely 5% in KRAS mutated patients (Corey J. Langer, 2011). Further, anti-EGFR monoclonal antibodies, cetuximab or panitumumab also failed to curb these mutations (Shalini Sree Kumar et al., 2014). Thus, collectively these studies indicate that somatic mutations present in KRAS oncogene results in poor efficacy and are negative predictor of response of these anti EGFR-TKIs & MAbs. Various other approaches have been adopted to indirectly inhibit KRAS by targeting molecules of pathway downstream to KRAS. One of these includes MEK inhibitor selumetinib when given along with docetaxel in Phase II randomized trial, showed good efficacy with higher response rate and progression free survival. Another way of targeting KRAS mutant which is in clinical trials is by utilizing PIK3CA/mTOR/AKT pathway inhibitors combined with selumetinib to obstruct the KRAS downstream signaling (Javier de C and Cristóbal B, 2013).
2.3.3.2 **BRAF Mutation:**

BRAF is a serine/Threonine protein kinase which acts as a downstream molecule of KRAS that is activated upon phosphorylation in a GTP dependent manner, thus mediating essential functions of cell including survival and proliferation by stimulating MEK/MAPK cascade. These mutations approximately account for about 3-5% in smoking habituated adenocarcinoma patients and amongst them papillary phenotypic histology is commonly reported as compared to lepidichistiotype in NSCLC. Majority of BRAF mutations are found in exon 15 (V600E:50%, D594G:11%) and exon 11(G469A:39%) occurring in a mutually exclusive manner with KRAS mutations excluding some exceptions (Helen Davies et al., 2002). These driver mutations were targeted with cetuximab in colon cancer patients which demonstrated deteriorated response rates while dabrafenib treatment in randomized phase II trials responded in partial manner in lung adenocarcinoma patients. Recently, BRAF mutants in melanoma were treated with vemurafenib or dabrafenib depicting a relatively good response rate as compared to other drugs in clinical trials (I V Fedorenko et al., 2015). Presence of characteristic V600E mutation in NSCLC is directly co-related with poor prognosis, lack of response and development of resistance for EGFR-TKIs, thus limiting utilization of BRAF inhibitors. Apparently several lead compounds such as MEK inhibitors are under experimental trial, demonstrating good clinical response initially but their approval for commercial use is still awaited (Helen Davies et al., 2002).

Disappointingly, regardless of the progress offered by these therapeutic approaches, the “addicted” tumors showed increased prevalence of regression owing to acquisition of resistance, hampering the “success” of these small molecule inhibitors in delaying the mean survival period just by few months. For this reason, there is prerequisite to discover new compounds and/or examining the present agents in combinations with new molecules to alleviate drug acquired resistance, is extremely crucial to any imminent success in management of lung cancer.

2.3.4 **Natural Products from Plants as New Treatment Modalities in Cancer:**

Natural compounds are always found to be conventional elementary resources of anti-tumor agents, but pharmaceutics industries declared their annihilation owing to appearance of targeted therapeutics In spite of the extraordinary growth in molecular
and biological perpectives of cancer, the dearth of dependence of the majority cancers to a sole ‘druggable’ target has showed improvement in the management of some cancers but overall advantage remained insufficient which revitalized the consideration towards natural therapeutics (Javad Tavakoli et al., 2012; S.M. Sagar et al., 2006).

![Chemical structures of various phytochemicals showing anticancerous properties.](image)

Figure 27. Chemical structures of various phytochemicals showing anticancerous properties.
Table 12: Molecular targeting mechanisms of various phytochemicals in the cancer cell (Min-Yu Chung et al., 2013).

In contrast with targeted inhibitors, phytochemicals have discrete benefits like non-ample availability, non-toxicity, cost effective etc. Synchronized with the advancement of inimitable targeted therapies that precisely aims the intricate
networks of signaling trails, natural supplements from food products are also progressively recognized as powerful inhibitors of these pathways. Table 12 outlines the specific molecular pathways targeted by these agents. Along with strong anticancerous qualities as mono therapy, these phytochemicals have also confirmed probable synchronism with conventional cytotoxic treatments in experimental studies. They manipulate carcinogenesis progression and alter the response to conventional therapy which assures further efficient and meticulous exploration in randomized clinical trials (David M. Lucas et al., 2010).

The major fact that natural compounds generally target multiple pathways simultaneously, which might contributes to lung carcinogenesis. From various experimental studies it is evident that, some of novel natural products show their probable implication during preclinical and clinical investigation against lung cancer. Epigallocatechin-3-gallate, EGCG can hinders TGF-β-induced EMT by down-regulation of phosphorylated Smad2 and Erk1/2 in A549 cells. Curcumin blocks proliferation of NSCLC cell lines by arresting cell cycle at G0/G1 phase thus resulting in tumor shrinkage (Stephen S. Hecht et al., 2009). Additionally, it appreciably augments the cytotoxicity of erlotinib/gefitinib, down regulates the expressions of EGFR and its phosphorylation, stimulates apoptosis, inhibits invasion by MTA-1 mediated inactivation of Wnt/β-catenin signaling pathway represses the NF-κB activation in erlotinib/gefitinib-resistant NSCLC cells (Nina Gottschalk et al., 2012). These findings indicate that curcumin is a potential adjuvant during TKIs treatment in adenocarcinoma patients. An oral, water soluble silibinin (milk thistle) extract considerably abrogates the tumor volume by preventing the loss of EMT markers and repressing the synthesis of mesenchymal markers leading to inhibition of invasive potentials. Similarly, Withaferin A which repressed the proliferation and apoptosis of NSCLC cells by deactivating PI3K/Akt pathways (Srinivas Koduru et al., 2010). Another compound called apigenin inhibited HIF-1 and VEGF expression suggesting suppression of angiogenesis in lung cancer cell lines. Other natural compounds including flavonoid, Luteolin, resveratrol, harmol, Emodin, Trilinolein and many more proven to be promising chemopreventive agent against NSCLC cells. Various traditional Chinese medicines like Scutellaria barbata, Saikosaponin D, Bupleurum scorzonerifolium, Marsdenia tenacissima extract, etc have been shown to inhibit A549 cell growth by inducing apoptosis and blocking the cell cycle progression.