*Pharmacogenomics and Targeted Therapy of Cancer: Focusing on Non-Small
Cell Lung Cancer*

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Abbreviation list:

ALK:	Anaplastic Lymphoma Kinase
BRAF:	v-Raf murine sarcoma viral oncogene homolog B1
EGFR:	Epidermal growth factor receptor
EGFR-TKIs:	EGFR tyrosine kinase inhibitors
EGFRvIII:	EGFR variant III
EML4:	Echinoderm microtubule-associated protein-like 4
FISH:	Fluorescence in situ hybridization
FDA:	America Food and Drug Administration
G6PD:	Glucose -6- Phosphate Dehydrogenase
HGFR:	Hepatocyte Growth Factor Receptor
IACR:	International Agency for Research on Cancer
IHC:	Immunohistochemistry
JAK/STAT:	Janus kinase/Signal Transducer and Activator of Transcription
K-RAS:	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LOH:	Loss of Heterozygosity
MAPKs:	Mitogen-activated protein kinases
NSCLC:	Non-Small Cell Lung Cancer
NTRK1:	Neurotrophic tyrosine kinase receptor type 1
PI3K/AKT:	Phosphatidylinositide 3-kinases/Protein kinase B
RET:	Rearranged during transfection
ROS1:	c-ros oncogene 1
RT-PCR:	Reverse Transcription Polymerase Chain Reaction
SCC:	Squamous Cell Carcinoma
SCLC:	Small Cell Lung Cancer
SNPs:	Single Nucleotide Polymorphisms
VEGF:	Vascular Endothelial Growth Factor
RTK:	Receptor Tyrosine Kinase
CSC:	Cancer Stem Cell
ALDH:	Aldehyde dehydrogenase

Abstract:

Recent studies have been established high degree of genetic diversity in solid organ tumors' cells and also among individuals. This intratumor and intertumor genetic diversity lead to a heterogeneous tumor with special characteristics, and therefore, drug efficacy can be affected. Nowadays, personalized medicine is a powerful strategy for the cancer treatment and overcoming drug resistance. The main goal of pharmacogenomics is clarifying complex genetic network behind drug efficacy and drug resistance. In this regard, targeted therapy is one the most important tools which can be more effective when used along with pharmacogenomics data. The accumulation of knowledge about the differences between normal and cancer cells and differences among cancer cells has allowed for the development of new treatments which target the key molecules involved in cancer initiation, proliferation, differentiation, angiogenesis, survival, and invasion. For some of the most common cancers, notably lung cancer, due to high incidence, prevalence, mortality, and greater tendency to drug resistance; personalized medicine can play an important role to overcome these problems. In this review we summarized the history of pharmacogenomics and then, we discussed the place of pharmacogenomics in cancer targeted therapy with focusing on non-small cell lung cancer (NSCLC).

1. Introduction

William Bateson was the first person to use the term of “**genetic**” in his studies of Mendelian inheritance in human populations in 1900 (1, 2). Two years later, Archibald Grove and colleagues observed that diseases such as Pentosuria and Alkaptonuria were inherited in an autosomal recessive manner and they used the concept of “**chemical Individuality**” for the first time (1, 2). Nevertheless, the beginning of pharmacogenetics is attributed to Laurence Snyder’s study on “Inheritance of phenylthiocarbamide taste recognition” (1). He indicated that only some people were able to taste **phenylthiocarbamide** which is also inherited in an autosomal recessive manner (1). In fact, Snyder demonstrated that there was a relationship between inheritance and response to an intervention.

The clinical importance of inheritance and the response to treatment was demonstrated in the 1950s with the appreciation of the relationship between a glucose-6-phosphate dehydrogenase (G6PD) defect and the occurrence of hemolysis during treatment with primaquine (an antimalarial drug) (3-5). Simultaneously, a link between the response to isoniazid (an anti-tuberculosis drug) and an autosomal recessive defect in enzymatic acetylation was established (6-9). Also, at this time, genetic defects in other drug metabolizing enzymes were realized as being important in the cause of death in affected individuals (1, 10, 11). Friedrich Vogel and colleagues applied the term “pharmacogenetics” to such studies. The term “Pharmacogenomics” was introduced in 1989 to encompass the involvement of complex genetic networks behind drug resistance, efficacy, and side effects (1, 2, 12-15).

It has become clear that cancers, particularly solid organ tumors, have a high degree of genetic diversity (16, 17). Indeed, solid tumors may have up to 100 mutated genes which vary between individual cells within the tumor and it is generally unclear what the driver mutations are. The realization that many driver mutations are linked to a smaller number of pathways which are critical for oncogenesis has highlighted the need for tumor analysis at the molecular level in order to increase our understanding of the basis for NSCLC pharmacogenomics.

Targeted therapy is a powerful strategy for cancer treatment and overcome drug resistance (18). The accumulation of knowledge about the differences between normal and cancer cells and differences among cancer cells has allowed for the development of new anticancer agents which target key molecules involved in cancer initiation,

proliferation, differentiation, angiogenesis, survival, and invasion. (18-20). In this review we summarized the history of pharmacogenomics and personalized medicine. Then, we discussed the place of pharmacogenomics in cancer targeted therapy with focusing on NSCLC, approved devices and methods in NSCLC pharmacogenomics study and new promising targeted therapy agents.

2. Pharmacogenomics and cancer therapy

Cancer progression is related to the combined effects of cell membrane receptors and intracellular signaling pathways which modulate cell proliferation, apoptosis, motility, adhesion, and angiogenesis (19). Human cancer genome sequencing has detected a series of genetic changes that occur in different cancers. Single nucleotide polymorphisms (SNPs), haplotypes, microsatellites, insertion or deletion of nucleotides (Ins/Del), copy number variations, aneuploidy, and loss of heterozygosity (LOH) are the most common genetic changes reported to be associated with uncontrolled growth and metastasis (21-23). In addition, the resistance of cancer cells to various drugs has been characterized by the increasing expression of cell membrane transporter proteins, changes in the activity of cellular proteins involved in detoxification, DNA repairing, apoptosis and activation of oncogenes/inactivation of tumor suppressor proteins (20).

The high prevalence of drug resistance in NSCLC, especially in advanced stages, has driven the increase in pharmacogenomics studies in this cancer (24-27). The American Food and Drug Administration (FDA) strongly recommends pharmacogenomics testing before the prescription of several anticancer agents to avoid, or at least minimize, possible life-threatening side effects and to reduce the costs of ineffective treatment (10, 28).

3. Non-Small Cell Lung Cancer

Lung cancer is the leading cause of cancer-related death worldwide (29, 30). The International Agency for Research on Cancer (IACR) has estimated that the number of deaths due to lung cancer will increase to ten million deaths per year by 2030 (31). The main risk factor for lung cancer is smoking to the extent that 75-90% of patients have a history of smoking (30). The term lung cancer usually refers to tumors that originate from the lining cells of the respiratory tract (epithelial cells) (31). Based on

differences in biological characteristics, lung cancer is classified into two types, namely non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for approximately 85% of lung cancer cases (31, 32). Platinum-based chemotherapy is prescribed as the standard first-line therapy in patients with advanced NSCLC (31). However, resistance to platinum-based drugs reduces the survival rate which has not reduced as dramatically as seen in other cancers. Recent data, however, suggest that targeted therapy linked to analysis of predictive biomarkers may be one way to overcome drug resistance. Considering the prominent role of receptor tyrosine kinase (RTK) such as epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) in NSCLC pathogenesis and progression, small molecule tyrosine kinase inhibitors and the approval of a number of these agents for the treatment of NSCLC represented a landmark in drug development and a significant step towards the goal of personalized medicine in lung oncology (18, 33, 34).

3.1. EGFR mutation

Among the key pathways implicated in NSCLC, the importance of the EGFR signaling pathway has recently become evident (35). EGFR is a member of the ErbB family of RTK (35). Ligand binding to EGFR induces receptor dimerization and auto-phosphorylation of its intracellular domain which subsequently leads to activation of downstream signaling cascades such as mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) and janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways and increased cell proliferation and survival (Figure-1) (36, 37). The EGFR gene is located on the short arm of chromosome 7 (7p12) and the kinase domain is encoded by exons 18-24 (Figure-2) (35). To date, dozens of mutations in the kinase domain have been described which affect the response to treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs) (38).

Mutations usually occur in exons 18-21 and cause either resistance or sensitivity to EGFR-TKIs (Figure-2). Mutations in exons 18, 19, and 21 are predominantly associated with enhanced sensitivity to EGFR-TKIs whilst mutations in exon 20 (e.g. T790M) are mainly associated with resistance to these drugs (38). The first generation EGFR-TKIs such as Erlotinib (Tarceva®) and Gefitinib (Iressa®) compete with ATP for binding at the tyrosine kinase domain (39) and are licensed for use in patients with

tumors positive for drug susceptibility mutations. However, the T790M mutation is identified in 50% of patients who acquire resistance to EGFR-TKIs. Mutations associated with EGFR-TKI resistance are rare before treatment and generally occur after the start of treatment (25, 40, 41). This acquired resistance to EGFR-TKIs is common in cancer and highlights the ability of tumors to adapt to pathway inhibition using a variety of processes to bypass the blockade. In recent years, Lux-Lung clinical trial program has been set up to test Afatinib in patients with advanced NSCLC (harboring either exon 19 or 21 mutation in EGFR gene) as first line treatment. Its findings can be useful for determining the best TKI for NSCLC (42, 43).

Studies conducted 10-15 years ago indicated the presence of two mutations in exons 19 and 21 of the EGFR gene, deletion (Δ E746-A750) and point mutations (L858R), (44, 45) that are common and which can predict the response to treatment with EGFR-TKIs in patients with NSCLC. These mutations are observed in 10-40% of cases of NSCLC (46). Third generation irreversible EGFR-TKIs such as CO-1686, WZ-4002, and AZD-9291 have been developed which act as mutant-selective agents against both T790M and the initial EGFR mutations but not against wild-type EGFR. These may be used as monotherapies or as combination therapy (47-49).

Mutations associated with sensitivity to EGFR-TKIs cause structural changes at the protein level and reduce the affinity of ATP for the active site of the kinase thereby augmenting TKI sensitivity (48). Another hypothesis proposed by Bernard Weinstein in 2000 suggests "oncogene addiction" as the reason for enhanced susceptibility to EGFR-TKIs. Oncogene addiction refers to the phenomenon by which a cancer cell, despite many other genetic changes, can become completely depend on one oncogenic pathway for its proliferation and survival. Determination of the EGFR mutations present in an individual cancer allows clinicians to predict the response to treatment with EGFR-TKIs (50-52).

The standard method for the detection of these mutations is direct sequencing. Recently, two FDA-approved PCR-based companion diagnostic assays (Roche cobas and QuiagenTherascreen) have been validated for use on formalin fixed paraffin embedded (FFPE) tissue (53, 54). Furthermore, highly selective monoclonal antibodies have been developed that can detect mutations associated with EGFR-TKI sensitivity and these may be used in a primary screening test to assess potential treatment responses. However, there are some limitations in that not all mutations are

detected and there is no well-defined cut-off point to determine the presence or absence of mutations (55, 56).

3.1.1. EGFR variant III

EGFR variant III (EGFRvIII) results in deletion of exons 2-7 in EGFR gene which characterized by loss of 268 amino acids (6 to 273). This mutation is commonly associated with squamous cell carcinoma (SCC). Deletion of exons 2-7 results in a protein product with profound functional changes including the loss of the ability to bind to EGF and a constant activation of the tyrosine kinase domain. EGFRvIII has also been reported in other cancers such as glioblastoma, breast cancer, ovarian cancer and prostate cancer. The clinical importance and incidence rate of this EGFRvIII is not clear although studies indicate that EGFRvIII is associated with resistance to Erlotinib and Gefitinib but a susceptibility to irreversible EGFR-TKIs such as Afatinib (57-59).

3.2. K-Ras mutations

K-Ras (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is a membrane-bound oncoprotein and a member of the Ras protein superfamily. The *K-Ras* gene is located on the short arm of chromosome 12 (12p12.1) and its protein product plays an important role in several cell signaling pathways including the activation of the MAPK and PI3K/AKT pathways. K-Ras is a GTPase and binding of GTP to the active site acts as a molecular on/off switch. Normally K-Ras binds GTP and hydrolyzes it to GDP and phosphate. K-Ras is turned off upon conversion of GTP to GDP leading to regulation of cell proliferation and growth (60, 61). Mutations in the *K-Ras* gene can result in GTP being permanently bound and consequently uncontrolled proliferation and growth of tumor cells (40, 62).

Approximately 25-35% of NSCLC patients are diagnosed with activating mutations in K-Ras. K-Ras mutations are more common in patients with adenocarcinoma, who have a history of smoking, than in any other subtype of NSCLC. The most common mutations occur in codons 12 and 13 of exon 2 (63, 64). Studies have shown that K-Ras mutations can also be associated with resistance to EGFR-TKIs (Figure-3) (65-67). However, generally EGFR and K-Ras mutations are mutually exclusive and resistance to first generation EGFR-TKIs may be due to the presence of wild type

EGFR (68). Furthermore, K-Ras mutations are associated with other problems such as thrombosis in cancer patients and in different studies it has been introduced as a prognostic biomarker for NSCLC (69, 70). Although, K-Ras appears to be a logical druggable target, but no drug has been approved for inhibition of mutant K-Ras and alternative strategies such as inhibition of heat shock protein 90 have been suggested (71, 72).

3.3. EML4-ALK rearrangement

Anaplastic lymphoma kinase (ALK) is another tyrosine kinase receptor and its gene is located on the short arm of chromosome 2 (2p23) (Figure-4) (73). Fusion of the ALK gene with the echinoderm microtubule-associated protein-like 4 (EML4) gene, located on short arm of chromosome 2 (2p21), is observed in 5% of NSCLC cases (73). The first report of an EML4-ALK fusion gene in NSCLC was published in 2007 (74). The EML4-ALK fusion gene results in a protein with persistent ALK kinase activity and uncontrolled cell growth, proliferation and survival (73-76)(Figure-4). The gold standard method for detection of EML4-ALK rearrangement is FISH (fluorescence in situ hybridization) (77, 78) and a diagnostic test, the Vysis break apart FISH assay, has got pre-marketing approval from the FDA for use in formalin fixed paraffin embedded tissue (79).

Crizotinib (Xalkori®) was approved for the treatment of ALK positive NSCLC patients in 2013 (80-83) and is significantly more effective than chemotherapy in previously untreated patients (84). However, a minority of NSCLC patients who are EML4-ALK positive do not response to Crizotinib and patients who initially respond to Crizotinib often have a transient response rather than a cure. The reasons for the lack of response and the drug-acquired failure are unknown. Mutations such as C1156Y and L1196M can occur in the ALK kinase domain but a link to drug responsiveness has not been reported (85-88). Ceritinib (Zykadia®) (an ALK inhibitor) has been approved by the FDA in April 2014 for the treatment of patients with ALK positive, metastatic NSCLC with disease progression or for those who are intolerant to Crizotinib (89). It is noteworthy that initial studies with second generation ALK tyrosine kinase inhibitors including LDK-378 and AP-26113 have shown high response rates in patients with acquired Crizotinib resistance (90, 91).

3.4. BRAF mutation

Other genetic changes that occur in NSCLC which are associated with resistance to EGFR-TKIs are activating mutations in the BRAF (v-Raf murine sarcoma viral oncogene homolog B1) gene that increase its kinase activity. This protein is a serine/threonine kinase downstream of K-Ras signaling (Figure-3) (92, 93). BRAF mutations usually occur with low incidence in adenocarcinomas (94). In 50% of cases, a mutation in exon 15 leads to the substitution of valine for glutamic acid at position 600 in BRAF (V600E). Substitutions of glutamine for alanine (mutation in exon 11) and substitution of aspartate for glutamine (mutation in exon 15) are observed in 39% and 11% of cases respectively (95). The majority of clinical trials to date have focused on melanoma since >90% of melanoma cases have the V600E mutation (92, 94).

The FDA has approved BRAF-specific inhibitors such as Vemurafenib (Zelboraf®) and Dabrafenib (Tafinlar®) for the treatment of metastatic melanoma (96). These drugs show clinical efficacy with acceptable safety profiles, although drug-acquired loss of efficacy does occur which can be prevented using combination therapy with the MEK inhibitor Trametinib (Mekinist®) for example (97). Clinical trials in NSCLC patients enriching for BRAF mutations are needed.

3.5. MET amplification

MET also called hepatocyte growth factor receptor (HGFR) is another cell surface tyrosine kinase receptor (98). When oncogene addiction occurs in tumor cells, inhibition of the EGFR-mediated downstream proteins result in a “kinase switch” to ensure their survival. One of the main switch pathways activated is the tyrosine kinase MET (98, 99). Increased expression of MET leads to acquired resistance to the EGFR-TKIs and ~20% of EGFR-TKI-resistant patients have increased MET amplification (100). MET inhibitors such as Cabozatinib (Cometriq®, formerly known as XL184) has been approved by the FDA for the treatment of some cancers, but there is no clinical data is available to date on cabozatinib in patients with NSCLC (101, 102).

3.6. Rare genetic alterations in NSCLC

Recently, several genetic alterations have been discovered in NSCLC which have clinical importance in targeted therapy. C-ros oncogene1 (ROS1) and rearranged during transfection (RET) are tyrosine kinase receptors and associated genes located on chromosomes 6 (6q22) and 10 (10q11.2) respectively (103-106). ROS1 and RET

rearrangements result in formation of fusion kinases capable of oncogenic transformation (107, 108). These rearrangements seldom occur simultaneously with other genetic alterations like EGFR, K-Ras, BRAF or ALK. This finding suggests that ROS1 and RET are independent oncogenic drivers and could be potential druggable targets (105, 109-112).

ROS1 and RET rearrangements were identified in 1%–2% of NSCLC cases (105). Patients with ROS1 rearrangement have many similar features to that seen in ALK and EGFR positive patients. For example ROS1 rearrangements were associated with younger age, non-smoking history, Asian ethnicity, advanced stage and adenocarcinoma. Patients with RET rearrangement have fewer shared features with ALK or EGFR positive patients than ROS1 patients (109-114). Detection of these rearrangements can be performed by using FISH, reverse transcription polymerase chain reaction (RT-PCR), and immunohistochemistry (IHC). However, to date, no gold standard screening technique is available (105, 109-111, 114).

Since ROS1 and ALK share a high degree of homology within their tyrosine kinase domains, it is possible to hypothesize that ALK tyrosine kinase inhibitors may also inhibit ROS1. Accordingly, current studies have shown that Crizotinib can be useful in patients with NSCLC, but clinical trials enriching for patients with RET rearrangements are clearly needed (105, 113, 115-117). Other rearrangements in RTK such as AXL and NTRK1 (neurotrophic tyrosine kinase receptor type 1) have been discovered, but the incidence of these alterations are quite rare and their clinical importance may be indicated in the near future (118-120).

3.7. Targeting of angiogenesis

Bevacizumab (Avastin), a humanized monoclonal antibody against Vascular endothelial growth factor-A (VEGF-A), is used to prevent angiogenesis in several human cancers include colorectal, lung, breast, and ovarian cancer (121). VEGF-A is a multi-functional cytokine which plays a major role in both inflammation and angiogenesis, the expression of which is elevated in different types of neoplasms (122). There is no reliable marker that predicts the response to Bevacizumab (121).

3.8. Targeting cancer stem cells

Cancer stem cells (CSCs) refer to subset of cancer cells which may share many properties with normal stem cells such as self-renewal and capacity of differentiation. CSCs have also a potential to form tumors following transplantation (123-125). Recently, there are several in-vitro and clinical evidences of support for the critical role of CSCs in tumor initiation, heterogeneity, metastasis, relapse and drug resistance (125-127). Therefore, it can be reasonable to choose these small population as a new target for cancer treatment.

Currently, we can see the increasing number of evidences supporting CSC phenotype in NSCLC (128). Expression of CD133, CD166 and CD44, elevation of aldehyde dehydrogenase (ALDH) activity and elevated nuclear β -catenin has been introduced as the markers of CSCs in NSCLC (128-133). However, these markers have been found in other tumors and also in the normal stem cells (128). It is noteworthy to point the crucial signaling pathways in CSCs including Wnt, Hedgehog, and Notch pathways. All of these signaling pathways have important roles in controlling of self-renewal and developmental pathways in normal and cancer stem cells (134-139). Accordingly, the concept of CSC can inspire the development of therapeutic strategy. Nonetheless, existence of some properties such as quiescence, expression of ATP binding cassette transporters (ABC), resistance to DNA damage, high expression of antiapoptotic proteins and broadly expression of CSC identification marker in normal cells complicate targeting of these small population (126). Targeting CSCs with specific antibodies or small molecules are promising, but we need to well characterize them and also we must discover the unique genetic changes and key metabolic pathway to target CSCs more effective than before (126).

4. Conclusion

Genetic changes occur in cancer cells enabling the dysregulation of oncogenic and suppressor genes and overexpression or activation of genes that drive cancer growth. These genetic changes can even vary between cells within a single cancer but frequently target a relatively few oncogenic driver pathways. The resulting structural changes in membrane receptor tyrosine kinases caused by genetic changes and by oncogene addiction in tumor cells drive cell proliferation. These selected changes in receptor tyrosine kinases have provided opportunities for targeted therapy and along with pharmacogenomics has been a powerful strategy towards personalized medicine.

391 Selected drug therapy linked to molecular analysis has resulted in a remarkable
392 increase in the the survival rate and quality of life for many patients.

393 However, resistance to targeted therapy exists and is often acquired after the onset of
394 therapy and highlights that cancer is a moving target. This emphasizes that we should
395 define and target the critical nodes within each cancer cell that controls proliferation,
396 differentiation, apoptosis, and growth pathways. The advent of next generation
397 sequencing linked to the development of better drugs should enable the selective
398 treatment of patients using drug combinations which prevent the onset of therapy
399 resistance. Future studies will further increase our understanding of cancer biology
400 and the mechanisms of resistance. It is only by being armed with this knowledge that
401 we will be able to defeat cancer by predicting its next move.

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Figure legends

Figure-1: Epidermal Growth Factor Signaling (EGFR) Pathway. Binding ligand such as Epidermal Growth Factor (EGF), TGF- α (Transforming growth factor alpha), and AREG (Amphiregulin) to receptor can activate its downstream pathways including PI3K/AKT, STAT, and MAPKs cascade. Activating downstream signaling can lead to cell proliferation, survival, adhesion, migration, and also lead to angiogenesis. Some drugs like monoclonal antibodies such as Cetuximab can bind to extracellular region of EGFR and another one like TKIs (Tyrosine Kinase Inhibitors) can inhibit tyrosine kinase domain in intracellular region.

Figure-2: Exons 18-24 encode tyrosine kinase domain of Epidermal Growth Factor Receptor (EGFR). Mutations which affected resistance and susceptibility to EGFR Tyrosine Kinase Inhibitors (EGFR-TKIs) occur in exons 18-21. Mutations that lead to susceptibility to EGFR-TKIs commonly located on exons 18, 19, and 21. Mutations that lead to resistance to EGFR-TKIs commonly located on exon 20. Point mutations in exon 18 cause substitution of Leucine for Arginine (L858R) in protein product. Deletion of 746-750 amino acids occur due to deletion mutation in exon 21. These two mutations observed in approximately 90% of patients with NSCLC.

Figure-3: EGFR signaling pathway. EGFR-TKIs can bind to EGFR intracellular region and preventing binding of ATP to tyrosine kinase domain. Occurring mutation in downstream protein like K-Ras and BRAF, due to constant activation, can lead resistance to EGFR-TKIs.

Figure-4: ALK receptor and EML-4 genes located on short arm of chromosome 2. Due to translocation, a fusion gene is created and this gene encode a fusion protein with constant kinase activity. Then, ALK-EML-4 fusion protein can leads to activating downstream pathways in cell including MAPKs cascade, PLC-γ, STAT, and PI3K. The results of activating pathways are increasing cell proliferation, growth, and survival.

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