Targeted Therapies Development in the Treatment of Advanced Nonsmall Cell Lung Cancer

Guest Editors: Cesare Gridelli and Enriqueta Felip



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Editorial

Targeted Therapies Development in the Treatment of Advanced Nonsmall Cell Lung Cancer

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Nonsmall cell lung cancer (NSCLC) is diagnosed in the majority of patients in advanced stage of disease. In this setting, the prognosis is very poor with median survival of 9-12 months and palliative chemotherapy being the standard of care. However, chemotherapy in advanced disease seems to have reached a plateau with no doublet combination shown to be clinically superior to the others. Advances in the knowledge of tumor biology and mechanisms of oncogenesis has granted the singling out of several molecular targets for NSCLC treatment. Targeted therapies are designed to interfere with specific aberrant biologic pathways involved in tumorigenesis. A large amount of preclinical in vivo and in vitro data have been gathered on the antitumor properties of a number of new biological agents, both as single agents and combined with other conventional treatment modalities such as chemotherapy. Several targeted therapies have been introduced in cancer treatment, and in particular gefitinib and erlotinib-two epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKI) and bevacizumab (an antiangiogen agent) have been introduced in advanced NSCLC, and others are in current clinical development.

The present issue includes 5 papers dedicated to targeted therapies of NSCLC, 2 on basic science and 3 focusing on medical treatment. The first paper by K. S. S. Enfield et al. deals with the role of microRNA gene dosage alteration assessment in order to define potential tumor drug resistance and response to chemotherapy. The authors, by instigating a logical stepwise strategy, have identified specific microRNAs that are associated with resistance to several chemotherapeutic agents and provide a proof-of-principle demonstration of how these various databases may be exploited to derive relevant pharmacogenomic results. The second paper is a complete review on targeted therapies in NSCLC. The authors describe the identified tumor target and biomarkers opening the field of personalized medicine. They deal with already registered drugs in current clinical practice and the new agents under current investigation. R. Costanzo et al. in the third paper reviewed the role of gefitinib in the treatment of advanced NSCLC harbouring an activating EGFR mutation. Gefitinib and erlotinib can be considered the standard first-line treatment in this subgroup of patients based on their superiority showed in terms of progressionfree survival, response rate, and quality of life as compared to chemotherapy. To date gefitinib has provided data on Asians only and has not been approved in the USA while erlotinib, with data on both Asians and Caucasians, has been approved worldwide.

When added to chemotherapy, continuous treatment with tyrosine kinase inhibitors (TKIs) offers no benefit in comparison with chemotherapy alone. This may be due to the antagonistic effect of TKIs and chemotherapy: cells are pushed into the G-0 phase of the cell cycle and are therefore resistant to cytotoxic drugs. In the fourth paper M. Zwitter et al. report the results of a phase II study in patients with advanced NSCLC with EGFR mutation using an intermittent schedule of erlotinib and chemotherapy with cisplatin plus gemcitabine in order to overcome the previously described negative interference of drugs. The authors observed even on a small patients' population interesting results and mild toxicity. In the last, paper Escobar M. et al. make a state-of-the-art review based on the available literature regarding the use of bortezomib as a single agent or in combination with chemotherapy in patients with lung cancer. The ubiquitin-proteasome system like other cellular pathways is critical for the proliferation and survival of cancer cells; thus, proteosome inhibition has become a very attractive anticancer therapy.

Cesare Gridelli Enriqueta Felip

Research Article

MicroRNA Gene Dosage Alterations and Drug Response in Lung Cancer

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Chemotherapy resistance is a key contributor to the dismal prognoses for lung cancer patients. While the majority of studies have focused on sequence mutations and expression changes in protein-coding genes, recent reports have suggested that microRNA (miRNA) expression changes also play an influential role in chemotherapy response. However, the role of genetic alterations at miRNA loci in the context of chemotherapy response has yet to be investigated. In this study, we demonstrate the application of an integrative, multidimensional approach in order to identify miRNAs that are associated with chemotherapeutic resistance and sensitivity utilizing publicly available drug response, miRNA loci copy number, miRNA expression, and mRNA expression data from independent resources. By instigating a logical stepwise strategy, we have identified specific miRNAs that are associated with resistance to several chemotherapeutic agents and provide a proof of principle demonstration of how these various databases may be exploited to derive relevant pharmacogenomic results.

1. Introduction

Lung cancer is the most common cause of cancer-related deaths worldwide, with a five-year survival rate of less than 15% [1]. The high incidence of late-stage diagnosis and a lack of efficient therapeutic strategies remain key contributors to the dismal survival statistics. Thus, to improve lung cancer patient outcome, improvement in early detection and a better understanding of the underlying tumor biology that governs response to therapy are necessary. Response to systemic therapy has been shown to be strongly associated with a variety of clinical and molecular features. For example, the chemotherapeutics Avastin and Permetrexed have shown differential response or adverse effects in different histological subtypes of lung cancer [2, 3]. Tyrosine kinase inhibitors (TKIs) targeting the epidermal growth factor receptor (EGFR) have shown preferential efficacy in Asian females who typically harbor sequence mutations in EGFR as well as those individuals who harbored EGFR amplifications, *EGFR* mutations, and the absence of *KRAS* mutations [4–6].

Very recently, inhibitors to ALK rearrangement also showed significant response in patients who harbor this genetic alteration [7].

In addition to molecular features that can predict sensitivity, there are also examples of features that can predict resistance. In ovarian cancer, resistance to therapy was observed in those individuals who carried amplifications of genes such as P-glycoprotein as well as specific regions in the genome such as 19q12 and 20q11.22-q13.12 [8, 9]. With respect to lung cancer, while there are individuals who do respond to TKIs, a large proportion will develop resistance to these therapies by acquiring an additional EGFR mutation (T790M), amplification of the c-MET oncogene, or hypermethylation of the PTEN locus [10-12]. High levels of ERCC1 mRNA and protein, a key player in nucleotide excision repair, have been associated with resistance to platinum-based chemotherapy [13]. Similarly, low levels of RRM1/2 mRNA and protein were associated with favorable gemcitabine response in NSCLC patients [14].

Although alterations in protein-coding genes remain a main focus to elucidate sensitivity or resistance to chemotherapy, deregulation of microRNAs (miRNAs) has recently been shown to play a role in chemotherapy response [15–17]. miRNAs are small noncoding RNAs approximately 18-25 nucleotides in length that negatively regulate gene expression posttranscriptionally [18, 19]. miRNA biogenesis begins with a long, double-stranded RNA known as a primiRNA, typically hundreds to thousands of nucleotides in length, which is processed into sequentially shorter doublestranded RNA sequences by the endonucleases Drosha and Dicer that are of 70 and 22 nucleotides in size, respectively [20, 21]. Dissociation of the duplex and incorporation of the mature strand into the RNA-induced silencing complex (RISC) guides RISC to the target mRNA, where the miRNA exhibits its effect [22]. miRNAs bind target transcripts based on sequence similarity-typically in the 3'UTR of the transcript and sometimes in the 5'UTR and the coding region-resulting in inhibition of translation or transcript degradation [18, 19, 23].

The relevance of miRNA deregulation to cancer biology arises because increased expression of certain miRNAs can result in downregulation of tumor suppressor genes, while decreased expression of other miRNAs can lead to increased expression of oncogenes [20, 21]. Often located at chromosomal breakpoint regions, fragile sites, and minimal regions of loss of heterozygosity or amplification, miRNA loci are highly susceptible to genomic alterations and subsequently, deregulated expression [24-27]. Aberrant miRNA expression is a common feature of both dysplasia and cancer, and miRNA expression profiles have been associated with prognosis, disease progression, survival, and outcome prediction [28, 29]. Further, miRNA expression profiles have been found to be superior to global mRNA expression profiles for the accurate definition of cancer types [30, 31]. Lung cancer drug response has been associated with the deregulation of several miRNAs. For example, sensitivity of nonsmall cell lung cancer (NSCLC) to cisplatin treatment was linked to upregulation of miR-181a, while resistance was conferred by upregulation of miR-630 [32]. Sensitivity to another chemotherapeutic agent, Gefitinib, was correlated with loss of miR-128b [33]. Several studies have shown that the overexpression of specific miRNAs, such as miR-134 and let-7a, can increase drug sensitivity, demonstrating the therapeutic potential of miRNAs [34, 35].

In this study, we sought to determine the role of DNA copy number alterations at miRNA loci in chemotherapy response. As a proof of principle, making use of datasets generated by multiple institutions, encompassing we performed an integrative and comparative DNA dosage and expression alteration analysis of miRNA loci in highly sensitive and resistant lung cancer cell lines for 18 different chemotherapeutics. Using a rigorous, stepwise analysis strategy, we identified four miRNAs which were frequently gained and overexpressed in lung cancer cell lines resistant to one or two of five different chemotherapeutic agents. Subsequent gene expression and gene network analyses for each set of mRNA targets of a given miRNA revealed functions such as DNA replication and repair and cellular assembly

and maintenance that were overrepresented in all four sets. These findings demonstrate the feasibility and the value of integrative analysis of multidimensional publicly accessible databases as a strategy for pharmacogenomics discovery.

2. Material and Methods

2.1. Drug Response Profiles of Cancer Cell Lines. Drug response IC₅₀ data for 18 different chemotherapeutics across 350 cancer cell lines (See Supplementary Material available online at doi: 10.1155/2011/474632 Supplemental Table 1) was generated as part of the Wellcome Trust Sanger Institute and Massachusetts General Hospital's (MGH) joint Genomics of Drug Sensitivity in Cancer Project. Data was downloaded from the following website: (http://www .sanger.ac.uk/genetics/CGP/translation/compound_sens_data.shtml). Briefly, IC₅₀ is the required concentration of a particular drug to cause in vitro growth to be inhibited by 50%, and thus, a measure of drug effectiveness. A low IC_{50} indicates that a drug is very effective at inhibiting growth while a high IC₅₀ indicates that a drug is less effective and thus requires a higher dosage to function. Of the 350 cancer cell lines, 73 cell lines were of lung origin.

2.2. Generation of DNA Copy Number Profiles for Cancer Cell Lines. Affymetrix SNP 6.0 data for the cancer cell lines were obtained from the Wellcome Trust Sanger Institute CGP Data Archive (http://www.sanger.ac.uk/genetics/CGP/Archive/). Of the 73 lung cancer cell lines with drug response data, 67 of them also had matching SNP array hybridization data (Supplemental Table 2). SNP array data were normalized using default parameters in *Partek Genomics Suite* (*PGS*, Partek Inc, St. Louis, MI). Whole genome copy number profiles were visualized using *SIGMA*² software [36].

2.3. miRNA and mRNA Expression Data for Cancer Cell Lines. The current annotation of autosomal miRNAs and their genomic coordinates were obtained from the UCSC Genome Browser (http://www.genome.ucsc.edu/) using the NCBI36/hg18 mapping [37]. miRNA and mRNA expression profiles for lung cancer cell lines were downloaded from the Broad Institute (http://www.broadinstitute.org/cgibin/cancer/datasets.cgi) under the "Sanger Cell Line Project." Affymetrix HG-U133A mRNA expression data were RMA-normalized using the "affy" package in *Bioconductor* in R [38–40]. Mapping of probes to genes was performed using the Affymetrix NetAffx annotation file (version NA31). Of the 73 lung cancer cell lines with drug response data, 64 had matching miRNA expression while 68 had matching mRNA expression data (Supplemental Table 2).

2.4. Determination of Predicted miRNA Targets. TargetSpy (version 1.0) and TargetScan (version 5.1) miRNA target prediction software were used to identify mRNA targets for further analyses [41–44]. For TargetSpy, the "no seed requirement, high sensitivity" set of targets were used, while for TargetScan, the nonconserved miRNA-mRNA targets

were used. For the miRNAs that were further assessed for target analysis, only miRNA-mRNA target pairs that were present in both databases were assessed for gene expression differences.

2.5. Statistical Analysis. For DNA alteration analysis, copy number profiles of the cancer cell lines were determined against a pooled reference comprised of 72 cytogenetically normal individuals in the HapMap collection. SNP 6.0 data for the HapMap individuals were obtained through Affymetrix. Subsequently, to determine copy number gains and losses, copy number profiles were subjected to segmentation analysis using the "Genomic Segmentation" algorithm in PGS with the following parameters: minimum genomic markers = 20, *P*-value threshold for adjacent regions having significantly different means = 1×10^{-6} , and *P*-value threshold for deviation from normal (diploid) copy number = 1×10^{-6} . In addition to meeting *P*-value thresholds, a region was deemed gained if the cell line had >2.3 copies while a region was deemed lost if the cell line has <1.7 copies. For each cell line, the copy number status for individual miRNA loci were determined by mapping the genomic coordinates of the miRNA loci to the identified regions of alteration.

To determine miRNA loci in differentially altered regions of copy number between highly resistant and sensitive cell lines, for each chemotherapeutic, cell lines were ranked based on their IC₅₀ value. The frequency of copy number gain, loss, and retention were compared between the top 1/3 and bottom 1/3 of cancer cell lines using a 3 by 2 Fisher's exact test. A miRNA was deemed significant if the *P* value from the Fisher's exact test was \leq .05.

For miRNA and mRNA expression analysis, similar to the differential copy number analysis, cell lines were ranked based on IC₅₀ for each drug. Subsequently, for each miRNA, the expression in the top and bottom tertiles of cell lines was compared using a nonparametric Mann Whitney U test. A miRNA was deemed significant if the P value from the Mann Whitney U test was \leq .05.

Upon identifying which lung cancer cell lines (LCCLs) contained matching DNA copy number and drug response profiles, for each chemotherapeutic, we compared the patterns of copy number alteration between the most sensitive and resistant LCCLs for 636 miRNA loci. Of the resulting differentially altered miRNA identified using the above statistical criteria, we filtered out those miRNAs which were both preferentially gained and lost in either highly resistant or highly sensitive LCCLs. We defined these variably altered miRNAs as those whose differential alteration frequency (DAF) of gain, frequency of gain in highly resistant minus the frequency of gain in highly sensitive, was within 10% of the DAF of loss, which is the frequency of loss in highly resistant minus the frequency of loss in highly sensitive. In parallel, upon identifying LCCLs with both miRNA expression and drug response profiles, we compared the miRNA expression profiles between the most sensitive and resistant LCCLs for 254 miRNAs using the above-mentioned statistical methods. Although 418 unique miRNAs are represented

on the microarray platform, we restricted this analysis to the 254 miRNAs that were expressed in at least 4 LCCLs. Subsequently, for each drug, we identified the miRNAs which were both significantly different at the DNA copy number and expression levels that matched in the same direction that is, if a miRNA had higher copy number in the highly resistant LCCLs as compared to the highly sensitive LCCLs, then the expression would also have to be higher, and vice versa. Next, for each significant miRNA, bioinformatic analysis was performed to identify target mRNAs, and mRNA expression profiles for these genes were compared in a similar manner to that performed in the differential DNA copy number and miRNA expression analyses (using TargetSpy and TargetScan; see above). Restricting to those targets whose mRNA expression profiles negatively correlate with miRNA expression profiles, we performed gene network and function analysis using Ingenuity Pathway Analysis to identify significantly overrepresented functions that were common to all sets of differentially expressed miRNA targets. A flow chart illustrating this strategy is shown in Figure 1.

2.6. Gene Network and Pathway Analysis. For each miRNA, the set of differentially expressed target genes were analyzed using *Ingenuity Pathway Analysis* (Ingenuity Systems, Redwood City, CA) to determine statistically overrepresented networks and pathways. Briefly, a right tailed Fisher's exact test was employed to calculate a *P*-value for the probability that enrichment of functions within the gene list of interest and the entire list of genes in the human genome is due to chance alone. Only the *Molecular and Cellular Functions* within the *Biological Functions* analysis were assessed.

3. Results

3.1. Copy Number Alterations of miRNA Loci Correlate with Drug Response in Lung Cancer Cell Lines. Sixty seven lung cancer cell lines with available IC50 data were used to analyze miRNA copy number alterations. For each drug, cell lines were sorted based on IC₅₀ values and the frequencies of DNA copy number gain, loss, and retention were compared between the highest (most resistant, n = 22) and the lowest (most sensitive, n = 22) tertile of cell lines. Of the 636 miRNAs assessed, 307 miRNAs (48.3%) were significantly different between high and low IC₅₀ for at least one drug, and 20 miRNAs (3.1%) were different for at least four drugs $(P \le .05 \text{ Fisher's exact test, Table 1, Supplemental Table 3}).$ In addition, among the 307 miRNAs, 58.4% were either more frequently gained in high IC₅₀ or more frequently lost in low IC₅₀ while 23.6% were either more frequently lost in high IC₅₀ or more frequently gained in Iow IC₅₀ lung cancer cell lines. The remaining 41 miRNAs (17.9%), although significantly different, had less than a 10% DAF difference between resistant and sensitive lines and were, therefore, deemed variably altered (see methods) and subsequently removed. This brought the total number of miRNAs with significant differences in copy number to 266 (Figure 2). In terms of the drug with the most striking pattern of



FIGURE 1: The search for drug response-related miRNAs began with data acquisition from several independent databases. Drug response data for lung cancer cell lines (LCCLs) was integrated independently with copy number and expression data, and unique filtering criteria were applied. The integration of all three dimensions applied further filtration criteria, and the remaining miRNAs underwent predicted target analysis. The resulting mRNA target expression was anticorrelated with miRNA expression, and cellular functions of the final mRNA target list were derived by *Ingenuity Pathway Analysis*.

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FIGURE 2: Flowchart summarizing the process for the identification of the four miRNAs which correlated significantly with drug response.

differential alteration between high and low IC_{50} cell lines, TAE684, a small molecule ALK fusion kinase inhibitor, had 66 miRNAs that were significantly different between the most resistant and most sensitive cell lines (Figure 3). Conversely, *miR-662* was the most frequently differentiallyaltered miRNA across all of the drugs, appearing significant in 6 of 18 drugs.

3.2. miRNA Expression Levels Correlate with Drug Response in Lung Cancer Cell Lines. miRNA expression was assessed in 64 lung cancer cell lines using a similar method to that applied for identifying copy number alteration differences, comparison of the highest and lowest tertile of cell lines (n =21) based on IC₅₀ values for each drug. miRNA expression profiles were available for 498 probes measuring 418 unique miRNAs. However, a number of miRNAs have little to no expression. To account for these cases, miRNAs with expression in less than four cell lines were removed, leaving 292 probes which corresponded to 254 unique miRNAs (Figure 2). One hundred thirty four miRNAs (represented by 146 probes) of the 254 (52.8%) miRNAs with available expression data were significant in at least one drug ($P \leq$.05, Mann Whitney U test) (Supplemental Table 4), with 18 miRNAs significant in at least four drugs (Table 2). Of the 134 differentially expressed miRNAs, 40% had higher expression in high IC50, while 60% had higher expression in low IC₅₀ lung cancer cell lines. HKI-272 had the most miRNAs [30] that were significantly different at the expression level (Figure 4), and miR-625 was the most frequently differentially expressed miRNA, appearing significant in 7 of 18 drugs.

TABLE 1: List of miRNA with most frequent differential copy number alterations.

miRNA	Significant drugs
hsa-mir-662	6 (AZ, Erl, Gel, Gö, HKI, MK)
hsa-mir-124-2	5 (Erl, HKI, Ra, Sor, Sun)
hsa-mir-1285-2	5 (MG, PF, PH, Ra, Sun)
hsa-mir-548h-2	5 (Gel, HKI, MK, PD, Sor)
hsa-mir-1208	4 (Cy, Erl, HKI, Sun)
hsa-mir-1225	4 (AZ, Gö, MK, Sor)
hsa-mir-1228	4 (Gel, MK, PF, TAE)
hsa-mir-1299	4 (AZD, Erl, MK, PH)
hsa-mir-147	4 (Erl, HKI, MK, TAE)
hsa-mir-181a-2	4 (Erl, HKI, MK, TAE)
hsa-mir-181b-2	4 (Erl, HKI, MK, TAE)
hsa-mir-1827	4 (AZ, Gel, Pac, PF)
hsa-mir-1972	4 (AZ, Gö, MK, Sor)
hsa-mir-492	4 (Gel, Im, Pac, PF)
hsa-mir-548c	4 (Gel, MK, PF, TAE)
hsa-mir-548d-1	4 (Cy, Gö, HKI, Sun)
hsa-mir-548f-2	4 (Gö, MG, PF, TAE)
hsa-mir-600	4 (Erl, HKI, MK, TAE)
hsa-mir-601	4 (Erl, HKI, MK, TAE)
hsa-mir-940	4 (AZ, Gö, MK, Sor)

3.3. Integrative Analysis of miRNA Gene Dosage and Expression Levels in Lung Cancer Cell Lines. To determine if miRNA dosage modulates expression, we compared the 266 miRNAs



FIGURE 3: Comparison of the frequency of alteration of 636 miRNA loci between highly sensitive and highly resistant lung cancer cell lines (LCCLs) to agent TAE684. Highly sensitive LCCLs were represented by the lowest tertile of IC_{50} while the highly resistant were represented by the highest tertile of IC_{50} miRNA genomic position information was obtained from the UCSC Genome Browser database [37], and miRNAs on chromosomes X and Y were excluded. Copy number alterations frequencies were plotted using *SIGMA*² software [36]. Vertical lines denote the frequency of alteration, where 1 or -1 signifies the alteration that occurs in 100% of samples. Horizontal bars depict miRNAs, with the frequency of copy gains and losses of each miRNA displayed to the right and left of 0, respectively. miRNAs disrupted in resistant lines are displayed in red, those occurring in sensitive lines are displayed in green, and regions of overlapping frequencies are shown in black.

differentially altered at the copy number level and the 134 miRNAs differentially expressed in at least one of the drugs analyzed. Considering only those miRNAs that were significant at both the copy number and miRNA expression level for the same drug, the intersection of these two lists yielded five miRNAs, *miR-10b*, *-191*, *-193b*, *-328*, and *-628* (Figure 2). Of these five, only expression of four miRNAs, *mir-10b*, *-193b*, *-328*, and *-628* matched the direction of their respective copy number alterations. For example, *miR-628* is more frequently gained in high IC₅₀ lines compared to low IC₅₀ lines treated with agent PF-2341066 and also shows higher expression in high IC₅₀ lines compared to low IC₅₀ lines when treated with TAE684, but shows higher expression in high IC₅₀ lines.

3.4. Gene Expression Analysis of mRNA Targets of miR-10b, miR-193b, miR-328, and miR-628. The target prediction software TargetScan and TargetSpy were used to identify putative mRNA targets of miRNAs found to be significantly different at the copy number and miRNA expression levels between high-IC₅₀ and low-IC₅₀ cell lines. For the four miR-NAs (miR-10b, miR-328, miR-193b, and miR-628) identified by integrative analyses, only miRNA-mRNA targets present in both databases were used for further analysis. miR-10b was identified as having a significant association with response to the proteosome inhibitor MG-132. In total, target prediction analysis found 636 genes that were deemed as putative targets of miR-10b (Supplemental Table 5). Comparison of the gene expression profiles between lung cancer cell lines with high and low IC₅₀ for MG-132 revealed 48 of these target genes to be differentially expressed ($P \leq .05$, Mann Whitney U test), with 32 of them showing the expected direction of differential expression (i.e., anticorrelated mRNA expression to miRNA expression) (Table 4).

Interestingly, *miR-193b* alteration was significantly associated with response to two therapeutics: AZ628 and MK0457 (RAF and aurora kinase inhibitors, resp.). When a similar analysis to *hsa-miR-10b* was performed for *miR-193b*, 518 genes were identified as putative targets of *miR-193b* (Supplemental Table 5). For the analysis of gene expression between highly sensitive and resistant AZ628 cells, 28 of these targets were differentially expressed, with ten of these genes matching the expected direction of differential expression. For MK-0457, 67 of these target genes were differentially expressed with over half (37) matching the expected direction (Table 4).

Alteration of *miR-328* was significantly associated with the response to Hsp90 inhibitor Geldanamycin in lung

TABLE 2: Most frequently different miRNAs at the expression level.

miRNA	Significant drugs
hsa-mir-625	7 (Erl, Gö, HKI, MG, Pa, PHA, Ra)
hsa-mir-130a	6 (Erl, Gö, HKI, MG, Pa, Sun)
hsa-mir-148a	6 (AZ, Erl, Gel, Gö, HKI, Pa)
hsa-mir-215	5 (Gel, Gö, MG, MK, Pa)
hsa-mir-518b	5 (Cy, PF, PHA, Ra, Sun)
hsa-mir-100	5 (Erl, Gö, HKI, Sun, TAE)
hsa-mir-192	5 (Gel, Gö, MG, MK, Pa)
hsa-mir-375	5 (Erl, Gel, Gö, HKI, Pa)
hsa-mir-503	5 (Cy, Erl, MK, PHA, Ra)
hsa-mir-193b	4 (AZ, MK, PHA, Ra)
hsa-mir-521	4 (Cy, Erl, Im, PHA)
hsa-mir-95	4 (Cy, Erl, MG, Sun)
hsa-mir-194	4 (Gel, Gö, MG, Pa)
hsa-mir-205	4 (Erl, Gel, Gö, HKI)
hsa-mir-222	4 (AZ, Erl, Gel, HKI)
hsa-mir-27a	4 (AZD, Erl, Gel, Gö)
hsa-mir-377	4 (MG, Pa, Sor, Sun)
hsa-mir-382	4 (AZD, MG, Pa, Sun)

cancer cell lines. Of the 437 genes targeted by *miR-328*, 49 of these genes were significantly differentially expressed between highly resistant and sensitive cell lines, with 31 of the genes matching the expected direction (Supplemental Tables 5 and 4). Finally, for *miR-628*, whose alteration was significantly associated with the MET inhibitor PF-2341066 response, 392 targets genes were identified with 49 of them being differentially expressed and 22 of those in the appropriate direction (Supplemental Tables 5 and 4).

4. Discussion

Chemotherapy response can be influenced by a number of clinicopathological and molecular factors. At the molecular level, while a large focus revolves around the role of activating and inactivating sequence mutations as well as copy number amplifications and deletions in protein coding genes, there has been an increasing emphasis on examining the role of miRNAs and response to chemotherapy. Recent studies have focused on differentially expressed miRNAs in conjunction to resistance and sensitivity to a variety of chemotherapeutics [32-35, 45, 46]. However, the influence of copy number alterations at miRNA loci (or gene dosage) in the context of drug response has not been thoroughly investigated. To this end, we have performed an integrative analysis of genomewide miRNA copy number, miRNA expression, mRNA expression, and drug sensitivity data from 18 different chemotherapeutics on a panel of lung cancer cell lines to identify miRNAs that are significantly different at the copy number and expression levels between the most sensitive and resistant cell lines for a given drug.



FIGURE 4: Heatmap visualization of the miRNA expression of the 254 miRNAs (represented by 292 unique probes) that passed expression filtering criteria for the 21 most sensitive (yellow bar, low IC₅₀) and 21 most resistant (blue bar, high IC₅₀) to drug HKI-272. In total, 30 miRNAs were found to be significantly differentially expressed between the most sensitive and resistant lung cancer cell lines (LCCLs, orange bar). For this visualization, since a value of 4 represented no expression, all expression values were subtracted by 4 such that baseline expression would be shown as 0 (black).

Upon comparison of the 636 annotated miRNAs throughout the human genome, it was found that 266 of them revealed significant differences in copy number alteration pattern between sensitive and resistant cancer cell lines for at least one drug (Supplemental Table 3). Moreover, of the 266 miRNAs, there were more miRNAs with increased copy number for the highly resistant versus the highly sensitive lung cancer cell lines than vice versa. The miRNA that was found to have a differential pattern of copy number alteration between sensitive and resistant cancer cell lines for the most drugs was *miR-662*, and, conversely, the drug with most significantly different miRNAs was TAE684. *miR-662* is

miRNA	Copy number alteration	Expression alteration	Drug in which significant
hsa-mir-10b	Gained, High IC ₅₀	Overexpressed, High IC ₅₀	MG-132
hsa -mir-193b	Gained, High IC ₅₀	Overexpressed, High IC ₅₀	AZ628, MK-0457
hsa-mir-328	Gained, High IC ₅₀	Overexpressed, High IC ₅₀	Geldanamycin
hsa-mire-628	Gained, High IC ₅₀	Overexpressed, High IC ₅₀	PF-2341066

TABLE 3: List of miRNAs with significant copy number and expression alterations in the same drug.

TABLE 4: Differentially expressed mRNA targets for the four identified miRNAs.

MG-132 (26S Proteasome inhibitor) Geldanamycin (HSP90 inhibitor) ZX628 (RAF inhibitor) MK-0457 (Aurora) kinase inhibitor) PF2-321(066 (MET, kinase inhibitor) SMARCC1 SLC16A1 PHF15 EH2S1 DCTD MKRN2 ANGRL1 RPP30 ARIH2 NUP188 CMTM6 UBS5 RRP1B TMEM231 ELAC1 CSDE1 SEMA3C EF4B GABBR1 DACT1 MAP4 MEIS2 WDR48 IKZF1 CYP7B1 MY010 TRIM32 PTPN21 FKTN RPA4 NKTR GMAA CLEC2D GPATCH8 GAR1 RYBP EH2S1 MRP516 EZH1 UCKL1 ZNF532 RAC2 NUDT15 OLFML2A IL7 SENP5 OLFML2A NECAP2 PSME3 ABCF1 CAL HIP1 AGTP8P1 CASP3 WHSC1 PRKD3 NMT2 WBP4 FCA2 VDR ADARB1 KLP9 ADOL1 YMEL1 GREB1 ACTN2	miRNA	10b	328	193b	193b	628
Trage Proteasome inhibitory (HSP90 inhibitor) inhibitor) Kinase inhibitor) ALK inhibitor) SMARCC1 SLC16A1 PHF15 EIES1 DCTD MKRN2 ANGEL1 RPP90 ARIN2 NUP188 CMTM6 UBR5 RRP1B TMEM2311 ELAC1 CSDE1 SEMA3C EIF4B GABR1 DACT1 MAP4 MES2 WDR48 IKZP1 CYP7B1 MY010 TRIM32 PTPN21 FKTN RPA4 NKTR GM2A CLEC2D GPATCH8 GAR1 ZNF532 RAC2 NUDT15 OLFM12A IL7 SENP5 OLFM12A NECAP2 PSME3 ABCE1 CRL HIP1 AGTPP1 CASP3 WH5C1 PKD3 NMC12 WBP4 FOX12 VDR ADARB1 KLF9 APOLD1 YME1L1 GREB1 ACTN2 PEA15 TFP63 SMC5 GPM6A RAD1 TEAP2C <td< td=""><td>Drug</td><td>MG-132 (26S</td><td>Geldanamycin</td><td>AZ628 (RAF</td><td>MK-0457 (Aurora</td><td>PF-2341066 (MET,</td></td<>	Drug	MG-132 (26S	Geldanamycin	AZ628 (RAF	MK-0457 (Aurora	PF-2341066 (MET,
SMARCCISLC16A1PHF15EH251DCTDMKRN2ANGELIRPP30ARH12NUP188CMTM6UBR5RP118TMEM231ELACICSDE1SEMA3CEIF48GABBR1DACT1MAP4MEIS2WDR48IKZ11CYP281MY010TRIM32PTPN21FKTNRP44NKTRGM2ACLEC2DGPATCH8GAR1RYBPEIF231MR916EZH1UCKL1ZSENP5OLEML2ANEC2PPSME3ABCE1CBLHIP1AGTP8P1CASP3WHSC1PRKD3NMT2WB94FOX12VDRADAR811KLP9APOLD1YME1L1GREB1ACTN2PEA15TF63SMC5GPM6ARAD1TEAP2CCCDC3ALSM12CDC6CH12RNMTPRKCAAPOLD1YME1L1GREB1ACTN2PEA15TF63SMC5GPM6ARAD1TEAP2CCDC372JAM11PAD68SERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOH8OLIFM2ARAB2APRKD3MAGOH8MMERP970ANKHHAGOH8MMERP970ANKE1SSX2IPMMERAB24SYT2CTDSPLHSM24KBTBD11CLEC2DMMERM162SETAD2KBM15BZNF510SS162KBM15BZNF510KBT6CK		Proteasome inhibitor)	(HSP90 inhibitor)	inhibitor)	kinase inhibitor)	ALK inhibitor)
MKRN2 ANCELI RPP30 ARH2 NUP188 CMTM6 UBR5 RRP1B TMEM231 ELACI CSDE1 SEMA3C ELF4B GABR1 DACT1 MAP4 MEIS2 WDR48 IKZF1 CYP7B1 MY010 TRIM32 PTPN21 FKTN RPA4 NKTR GA2A CLEC2D GPATCH8 GAR1 RTBP ELF2S1 MRP516 EZH1 UCKL1 ZNF532 RAC2 NUDT15 OLFML2A IL7 SENP5 OLFML2A NECAP2 PSME3 ABCE1 CBL HIP1 ACTP8P1 CASP3 WHSC1 PRKD3 NMT2 WBP4 FOX12 VDR ADAR81 KLP9 APOLD1 YME1L1 GRB1 ACTN2 PEA15 TP63 SMC5 GPM6A RAD1 TEAP2C CCDC28A ISM12 CDC6 CHP2 RNMT PRKC3 CDC6 CHP2 RNMT PRKC3 CDC6 JFP2 ADAT1 BNIP2 GPR165 SMC5 GPM6A RAD1 TEAP2C CCDC28A ISM12 CDC6 DSC2 UBA52 TRAIP PARD6B SERTAD2 AGPAT4 SP2 FCD6 DSC2 UBA52 TRAIP MMP14 PTGIS GOSR1 MAG0HB DIFM2A RA822A PRKD3 GNL31 FBXV2 SYF2 CTDSPL HS2ST1 SSX1P MMP14 PTGIS SKC5A GNL31 FBXV2 SYF2 CTDSPL HS2ST1 SSX1P MMP14 PTGIS ZETB39 MGPR MAN1A2 KRTFD11 KPNA6 TGIP2 SEC16A RM11 CLEC2D PHC3 RBM15B ZNC16A3 ZBTB39 MGPR MAN1A2 KRTFD11 KPNA6 TGIP2 SEC16A RBM15B ZNC16A3 ZBTB39 MGPR MAN1A2 KRTFD11 KPNA6 TGIP2 SEC16A CCT2 KDA34 BTFC CCT2 KDA35 BTFC CCT2 KDA55 BTFC CCT2 KDA		SMARCC1	SLC16A1	PHF15	EIF2S1	DCTD
CMTM6UBR5RP1BTMEM23ELAC1CSDE1SEMA3CEIF4BGABD31DACT1MAP4MEIS2WDR48IKZF1CYP7B1MY010TRIM32PTPN21FKTNRPA4NKTRGMAACLEC2DGPM7CH8GAR1RYBPEIF2S1MRPS16EZH1UCKL1ZNF552RAC2NUDT15OLFMI2AIL7SENP5OLFML2ANECAP2PSME3ABCE1CBLHIP1AGTPBP1CASP3WHSC1PRKD3NMT2WBP4FOXJ2VDRADARB1KLF9APOLD1YME1L1GREB1ACTN2PEA15TP63SMC5GPM6ARAD1TEAP2CCCDC8ALSM12CDC6CHP2RNMTPRKCATargetsUBE2KSTK24C15orf29ADAT1BNIP2GPR126RRP1BPBRM1PAD6BSERAD2AGP44SP2FGD6DSC2UBA52TRAIPMMF14PTGISGOSR1MACOHBOLFM12ARAB2APRKD3GN13LFBW2SYE10KDNA6TGIP2SEC16AKM11CLEC2DPHC3KBM15BZNF510SSI8L2RTF1CYLDWDR48HTRCCYLDKBM36HTRCCYLDKBM36		MKRN2	ANGEL1	RPP30	ARIH2	NUP188
CSDE1SEMASCEIF4BGABBR1DACT1MAP4MEIS2WDR48IKZF1CYP7B1MY010TRIM32PTPN21FKTNRPA4NKTRGM2ACLEC2DGPATCH8GAR1RYPPEIF2S1MRPS16EZH1UCKL1ZNP532RAC2NUDT15OLEML2AILTSENP5OLFML2ANECAPPSME3ABCE1CBLHIP1AGTPBP1CASP3WHSC1PRKD3NMT2WBP4FOXJ2VDRADARB1KLP9APOLD1YME1L1GRE81ACTN2PEA15TP63SMC5GPM6ARAD1TTAP2CCCDC28ALSM12CDC6CHP2RNP1BPBRM1BNIP2GPR126RRP1BPBRM1BNIP2GPR126GOSR1MAGOHBPARD6BSERTAD2ACRAT4SP2FGD6DSC2UB522TRAIPMMF14PTGISGOSR1MAGOHBMMF14RAB22APRKD3SYF2CTDSPLHS2T1SSF2GIN13LFBXW2SYF2CTDSPLMAN1A2KBTBD11KPNA6TGIP2SP163MGPRMAN1A2KBTBD11KPNA6TGIP2SI8L2KTF1CTDSFRM15BZNF510SI8L2KTF1KBM58ZNF510KBM58ZNF510KBM58ZNF510KBM58ZNF510KBM56KBTRCCTD2FRC<		CMTM6	UBR5	RRP1B	TMEM231	ELAC1
MAP4MEIS2WDR48IKZF1CYP31MYO10TRM32PTPN21RKTNRPA4NKTRGM2ACLEC2DGPATCH8GAR1NKTPEIP2S1MRN516EZH1UCKL1ZNF532RAC2NUDT15OLPML2AIL7SENP5OLFM12ANECAP2PSM53ABCE1CBLHIP1AGTPBP1CASP3WHSC1PRKD3NMT2WBP4FOXJ2VDRADARB1KLP9PGX12VDRCCDC2RALSM12CDC6CHP2RNMTPRKCARAD1TFA92CCDC6ALSM12CDC6CHP2RNMTPRKCABNIP2GPR126CBSR1MAC0HBPARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRMPMMP14PTGISGOSR1MAGOHBOLFM12ARAB2APRKD3FMMERP930ANKHFFFIBP1SLC16A3ZENT39GNL3LFBW2SEC16AKPNA6TGIP2SEC16AKPNA6TGIP2SEC16AKRM18CLEC2DPHC3KRM15BZNF510SS18L2KTF1KRM5BCVTDKRM5BKRB15BKRM5BKRF1KRM5BKRF1KRM5BKRF1KRM5BKRF1KRM5BKRF1KRM5BKCN72KRM55KR550KRM56KRF1KRM575KRF1 </td <td></td> <td>CSDE1</td> <td>SEMA3C</td> <td>EIF4B</td> <td>GABBR1</td> <td>DACT1</td>		CSDE1	SEMA3C	EIF4B	GABBR1	DACT1
MYO10TRM32PTPN21FKTNRPA4NKTRGM2ACLEC2DGRATCH8GAR1RYBPEIF2S1MRPS16EZH1UCKL1ZNF532RAC2NUDT15OLFML2AIE7SENP5OLFML2ANECAP2PSME3ABCE1CBLHIP1ATTPP1CASP3WHSC1PRKD3NMT2WBP4FOXJ2VDRADARBIKLP9APOLD1YME1L1GREB1ACTN2PEA15TF63SMC5GPM6ARAD1TEP2CRNMTPRKC3CDC6CHP2RNMTPRKC4BNIP2GPR126RRP1BPBRM1PARD6BSERTAD2GOSR1MAGOHBOLFML2ARAB2APRKD3TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB2APRKD3TRAIPMMERPP30ANKHFFIBP11KPNA6TGIP2SECI6ARM11CLEC2DPHC3KPNA6TGIP2SECI6ARM11CLEC2DPHC3KPNA6TGIP2SECI6AKRM15BZNF510SS18L2KTF1KNA56BTRCCVIDWDR48BTRCKNA56KNA56		MAP4	MEIS2	WDR48	IKZF1	CYP7B1
NKTRGM2ACLEC2DGPATCH8GARIRYBPEIF2S1MRPS16EZH1UCKL1ZNF532RAC2NUDT15OLFML2ALI7SENP5OLFML2ANECAP2PSME3ABCE1GBLHIP1AGTPBP1CASP3WHSC1PRKD3NMT2WBP4FOXJ2VDRADARB1KLP9APOLD1YME1L1GCDC6GPM6ARAD1TFAP2CCCDC28ALSM12CDC6CHP2RNMTPRKCACDC6SKC4GOSR1MAGOHBPARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBPARD6BSERTAD2SYF2FGD6DSC2UBA52TRAIPMMP14PTGISSS22IPMMERPP30ANKHPPFIBP1SLC16A3ZBTB39MMFMAI1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3KPNA6TGIF2SEC16ARM11CLEC2DPHC3KPN48BTRCKPN48BTRCKPN48BTRCKD510KDF40KD512KDF10KD514KDF10KD515KD510KD515KD510KD516KDF10KD517KDF10KD518KDF10KD518KDF10KD518KDF10KD518KDF10KD519KD510 </td <td></td> <td>MYO10</td> <td>TRIM32</td> <td>PTPN21</td> <td>FKTN</td> <td>RPA4</td>		MYO10	TRIM32	PTPN21	FKTN	RPA4
RYBPEIF2S1MRPS16EZH1UCKL1ZNF532RAC2NUDT15OLFML2AIL7SENP5OLFML2ANECAP2PSME3ABCE1CBLHIPCASP3MT2WB4CBLPRKD3NMT2WB4FOXJ2VDRADARB1KLF9APOLD1YME1L1GREB1ACTN2PEA15TP63SMC5GPM6ARAD1TFA92CCCDC28ALSM12CDC6CHP2RNMTPRCACDC6GPR126RRP1BPBM11BNIP2GPR126GGSR1MAGOHBPARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB2APRKD3TAIPMMF14RP30ANKHFF1PF1BP1SLC1643ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM1CLEC2DPHC3KBM15BZNF510SI8L2KBM15BCYLDKBM36KBTGKBA36		NKTR	GM2A	CLEC2D	GPATCH8	GAR1
ZNF532RAC2NUDTI5OLFML2AIL7SENP5OLFML2ANECAP2PSME3ABCE1CBLHIP1AGTPBP1CASP3WHSC1PKD3NDT2WPB4FOXJ2VDRADARB1KLF9APOLD1YME1L1GREB1ACTN2PEA15TP63SMC5GPM6ACDC6CHP2RNMTPRCACDC6CHP2RNMTPRCABNIP2GPR126RRP1BPBRM1PARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPOLFML2ARAB22APRCD3TRAIPMMP14PTGISSSX2IPMAGOHBOLFML2ARAB22ASYF2SYF2CTDSPLHS2ST1SSX2IPMAGOHBMPENSLC16A3ZETB39MGPRMMI1CLEC2DPHC3SEC16ARBM15BTGIF2SEC16ACN1DSI812SI812ZNF510SI812KTF1SK164BN175SI812STRCCN175SK164FTRCSI812KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF1KBM56KTF2 <td></td> <td>RYBP</td> <td>EIF2S1</td> <td>MRPS16</td> <td>EZH1</td> <td>UCKL1</td>		RYBP	EIF2S1	MRPS16	EZH1	UCKL1
SENP5OLFML2ANECAP2PSME3ABCEICBLHIP1AGTPNP1CASP3CBLPRKD3NMT2WBP4FOXJ2VDRADARB1KLF9APOLD1YMEIL1GREB1ACTN2PEA15TP63SMC5GPM6ARAD1TEAP2CCCDC28ALSM12CDC6CHP2RNMTPRKC3BNIP2GPR126RRP1BPBRM1PAD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB22APRKD3TAIPMMERPP30ANKHFUPFIBP1SLC16A3ZBTB39SEC16ARM11CLEC2DPHC3SEC16ARM115BCLEC2DPHC3FUKBM15BKTF1CYLDWDR48BM36FURCCNT2RM36FURCCNT2		ZNF532	RAC2	NUDT15	OLFML2A	IL7
CBLHIP1AGTPBP1CASP3WHSC1PRKD3NMT2WBP4FOX12VDRADARB1KLF9FOX12VDRGREB1ACTN2PEA15TF63SMCSGPM6ARAD1TEAP2CCCDC28ALSM12CDC6CHP2RNMTPRKCACDC6CHP2RNMTPRKCABNIP2GPR126RRP18PBRM1PARD6BSERTAD2AGPA74SP2FGD6DSC2UBA52TRAPMMP14PTGISGOSR1MAGOHBOLFML2ARAB2APRKD3HAGPMMERP930ANKHHPFIBP1SLC16A3ZBTB39HKPNA6TGIF2SEC16ALKPM15CLC2DPHC3CYLDKPM35ZNF510CYLDWDR48FBM15BCYLDWDR48BTRCCCNT2KBM15BCCNT2KDR48KBM15BKAB36KDR48KDR48		SENP5	OLFML2A	NECAP2	PSME3	ABCE1
WHSCIPRKD3NMT2WBP4FOXJ2VDRADARB1KLF9FOXJ2VDRGREB1ACTN2APOLD1YME1L1GREB1ACTN2PEA15TP63SMC5GPM6ARAD1TFAP2CCCDC28ALSM12CDC6CHP2RNMTPRKCABNIP2GPR126RRP1BPBRM1PARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB2APRKD3GN31GN31FBXW2SYF2SSX2IPMMERP930ANKHFUEPFIBP1SLC16A3ZETB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3KBN15BZNF510SS18L2KRM15BZNF510KBR4FTF1CYLDKDR48FTRCCNDKBA36KBTRCCNDKBA36		CBL	HIP1		AGTPBP1	CASP3
FOXJ2VDRADARB1KLF9APOLD1YMEIL1GREB1ACTN2PEA15TP63SMC5GPM6ARAD1TFAP2CCCDC28ALSM12CDC6CHP2RNMTPRKCABNIP2GPR126RRP1BPBRM1PARD68SERTAD2AGPA74SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB2APRKD3TGNL3LFBXW2SYF2TMMERP930ANKHTPFIBP1SLC16A3ZBTB39TKN11CLEC2DPHC3SC16AKBM15BZNF510SS18L2RTF1CVLDSS18L2RTF1GVLDKBM15BZNF510STRCGCNT2KAB36KAB36		WHSC1	PRKD3		NMT2	WBP4
APOLD1YMEIL1GREB1ACTN2PEA15TP63SMC5GPM6ARAD1TFAP2CCCDC28ALSM12CDC6CHP2RNMTPRKCADBE2KSTK24C15orf29ADAT1BNIP2GPR126RRP1BPBRM1PAR06BSERTAD2UB452TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB22APRKD3TATSGN13LFBXW2SYF2TCDSPLMMERP930ANKHTGIF2KPNA6TGIF2SEC16ATGIF2KPNA6TGIF2SEC16ATGIF2KBM15BZNF510SS18L2RTF1SS18L2RTF1CYLDWDR48BTRCCXDBTRCGCN72KAB36TTATS		FOXJ2	VDR		ADARB1	KLF9
PEA15TP63SMC5GPM6ARAD1TFAP2CCCDC28ALSM12CDC6CHP2RNMTPRKCATargetsUBE2KSTK24C15orf29ADAT1BNIP2GPR126RRP1BPBRM1PARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBGNL3LFBXW2SYF2SYF2CTDSPLHS2ST1SSX2IPMMERP930ANKHPPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3SS18L2RTF1SS18L2RTF1KBM15BZNF510SS18L2RTF1KBR05SI8L2KBR05BTRCCCNT2RAB36		APOLD1	YME1L1		GREB1	ACTN2
RAD1TFAP2CCCDC28ALSM12CDC6CHP2RNMTPRKCATargetsUBE2KSTK24C15or/29ADAT1BNIP2GPR126RRP1BPBRM1PARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB22APRKD3SEX1PGNL3LFBXW2SSX2IPITMMERP930ANKHITPPFIBP1SLC16A3ZBTB39ITM6PRMAN1A2KBTBD11ITKPNA6TGIF2SEC16AITSI8L2RTF1CYLDWDR48BTRCCYLDBTRCCN12KBM15BFRCCYLDWDR48BTRCCCN72KAB36IT		PEA15	<i>TP63</i>		SMC5	GPM6A
CDC6CHP2RNMTPRKCATargetsUBE2KSTK24C15orf29ADAT1BNIP2GPR126RRP1BPBRM1PARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB22APRKD3GNL3LFBXW2SYF2CTDSPLHS2ST1SSX2IPMMERP930ANKHPPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3SI8L2RTF1SI8L2RTF1GTCCYLDWDR48BTRCGCNT2KBA36		RAD1	TFAP2C		CCDC28A	LSM12
TargetsUBE2KSTK24C15orf29ADAT1BNIP2GPR126RRP1BPBRM1PARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB22APRKD3GNL3LFBXW2SYF2CTDSPLHS2ST1SSX2IPMMERPP30ANKHPPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3SS18L2RTF1SS18L2RTF1KDP48BTRCCCN72RAB36		CDC6	CHP2		RNMT	PRKCA
BNIP2GPR126RRP1BPBRM1PARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB22APRKD3GN13LFBXW2SYF2CTDSPLHS2ST1SSX2IPMMERP930ANKHPPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARBM15BZNF510SS18L2RTF1CYLDKDR48BTRCCCNT2RAB36CNT2	Targets	UBE2K	STK24		C15orf29	ADAT1
PARD6BSERTAD2AGPAT4SP2FGD6DSC2UBA52TRAIPMMP14PTGISGOSR1MAGOHBOLFML2ARAB22APRKD3GNL3LFBXW2SYF2CTDSPLHS2ST1SSX2IPMMERPP30ANKHPPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3SS18L2RTF1CYLDKDR48BTRCCNT2RAB36CNT2		BNIP2	GPR126		RRP1B	PBRM1
FGD6 DSC2 UBA52 TRAIP MMP14 PTGIS GOSR1 MAGOHB OLFML2A RAB22A PRKD3 Image: Comparison of the compariso		PARD6B	SERTAD2		AGPAT4	SP2
MMP14 PTGIS GOSR1 MAGOHB OLFML2A RAB22A PRKD3 GNL3L FBXW2 SYF2 CTDSPL HS2ST1 SSX2IP MME RPP30 ANKH PPFIBP1 SLC16A3 ZBTB39 M6PR MAN1A2 KBTBD11 KPNA6 TGIF2 SEC16A RM11 CLEC2D PHC3 SS18L2 RTF1 KPN48 FTRC GN14 KPK48 KPK48 KTRC KPK59 KAB36 KPK59		FGD6	DSC2		UBA52	TRAIP
OLFML2ARAB22APRKD3GNL3LFBXW2SYF2CTDSPLHS2ST1SSX2IPMMERPP30ANKHPPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARMI1CLEC2DPHC3SS18L2RTF1SS18L2CYLDWDR48BTRCCCNT2RAB36		MMP14	PTGIS		GOSR1	MAGOHB
GNL3LFBXW2SYF2CTDSPLHS2ST1SSX2IPMMERPP30ANKHPPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3SS18L2RTF1SS18L2CYLDWDR48BTRCCCNT2RAB36		OLFML2A	RAB22A		PRKD3	
CTDSPLHS2ST1SSX2IPMMERPP30ANKHPPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3RBM15BZNF510SS18L2RTF1CYLDWDR48BTRCCCNT2RAB36SA36		GNL3L	FBXW2		SYF2	
MMERPP30ANKHPPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3RBM15BZNF510SS18L2RTF1CYLDWDR48BTRCCCNT2RAB36KAB36		CTDSPL	HS2ST1		SSX2IP	
PPFIBP1SLC16A3ZBTB39M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3RBM15BZNF510SS18L2RTF1CYLDWDR48BTRCCCNT2RAB36		MME	RPP30		ANKH	
M6PRMAN1A2KBTBD11KPNA6TGIF2SEC16ARM11CLEC2DPHC3RBM15BZNF510SS18L2RTF1CYLDVDR48BTRCCNT2RAB36		PPFIBP1	SLC16A3		ZBTB39	
KPNA6TGIF2SEC16ARM11CLEC2DPHC3RBM15BZNF510SS18L2RTF1CYLDCYLDWDR48BTRCCCNT2RAB36		M6PR	MAN1A2		KBTBD11	
RMI1CLEC2DPHC3RBM15BZNF510SS18L2RTF1CYLDWDR48BTRCCCNT2RAB36		KPNA6	TGIF2		SEC16A	
RBM15B ZNF510 SS18L2 RTF1 CYLD WDR48 BTRC CCNT2 RAB36		RMI1	CLEC2D		РНС3	
SS18L2 RTF1 CYLD WDR48 BTRC CCNT2 RAB36		RBM15B			ZNF510	
CYLD WDR48 BTRC CCNT2 RAB36		SS18L2			RTF1	
WDR48 BTRC CCNT2 RAB36					CYLD	
BTRC CCNT2 RAB36					WDR48	
CCNT2 RAB36					BTRC	
RAB36					CCNT2	
					RAB36	

located on chromosomal region 16p13.3 and was found to be more frequently gained in cell lines highly resistant to AZ628, Erlotinib, Geldanamycin, Gö-6976, HKI-272 (Neratinib), and MK-0457. All of these drugs, except for Geldanamycin, which is an antibody that targets HSP90, are kinase inhibitors [47]. While not much is known of *miR*-662, it was recently shown that it is transiently upregulated in response to high doses of X-ray radiation in human fibroblasts [48]. It should also be noted that *miR*-124-2, *miR*-1285-2, and *miR*-548h-2 were significantly altered for five drugs (Table 1). Similar to *miR*-662, little is known of *miR*-548h-2. However, *miR*-124 has been shown to play a tumor suppressive role in cervical cancer, hepatocellular carcinoma, and glioblastoma, while *miR*-1285 inhibits p53 and p21 expression by targeting the 3'UTR of *p53* transcript [49–52].

To identify differentially expressed miRNAs in our dataset, we employed the same approach used to identify differential copy number alterations by assessing the expression of 254 miRNAs in 64 lung cancer cell lines. We identified 134 unique miRNAs significantly different at the expression level between resistant and sensitive lines in at least one drug. Of which, 40% overexpressed in highly resistant and 60% overexpressed in highly sensitive lung cancer cell lines. Of these 134 miRNAs, miR-625, about which little is known regarding function, was the most frequently differentially expressed. It was significantly differentially expressed in the analyses of agents Paclitaxel, HKI-272, Gö-6976, Erlotinib, Rapamycin, PHA665752, and MG-132. In terms of the drug comparisons with the highest number of differentially expressed miRNAs, the comparison between LCCLs highly sensitive and resistant to HKI-272, an irreversible tyrosine kinase inhibitor of HER2, revealed 30 differentially expressed miRNAs.

Previous studies of miRNA deregulation with respect to response of some of the drugs used in our analyses have identified a number of miRNAs whose expression correlates with drug sensitivity. For example, underexpression of *miR*-34a and overexpression of *miR-125b*, 2-21, -222, and -923 confer Paclitaxel resistance in prostate cancer [53] and breast cancer [54], respectively, while for hepatocellular carcinomas expression of let-7c [55], miR-122 [56] and miR-193b [57] confer sensitivity to Sorafenib. Notably, Sorafenib is a multikinase inhibitor with highest potency for RAF; this is consistent with our findings that link mir-193b with resistance to the RAF inhibitor AZ628. In addition, underexpression of miR-130a and -126 was correlated with resistance to Paclitaxel [58] and Imatinib [59], respectively. From our analyses, we observed miR-130 and -126 to be overexpressed in lung cancer cell lines sensitive to Paclitaxel and Imatinib.

Cancer genomes are characterized by widespread genetic aberrations including high-level amplifications, deletions, DNA methylation, mutations, and chromosomal rearrangements. Within the hundreds of alterations in a cancers genome, only a small subset of these alterations drive tumor initiation and progression and DNA alterations with corresponding expression alterations are more likely to contribute to tumorigenesis [60, 61]. To identify miRNAs likely implicated in drug resistance, we integrated the 266 miRNAs that were significantly different at the copy number level and the 134 miRNAs that were significantly different at the expression level and subsequently filtered for those miRNAs that were differentially expressed and altered in the same drug. Our analysis identified four miRNAs, miR-10b, -193b, -328, and -628, that met these criteria. While the overlap of significant miRNAs in the same drug is minimal, stringent selection criteria such as $P \leq .05$ for both copy number and expression alterations, and limited miRNA expression data, likely contributed to the small number of overlapping miRNAs. Importantly, many of the miRNAs most frequently differentially altered at the copy number level (128 of 266, 48.1%) were not represented on the microarray platform. Moreover, when we factored in our expression criteria of expression in at least four cell lines, the number of miRNAs with expression profiles and significantly different copy number alterations was reduced to 66. The copy number profiles of these miRNAs suggest they may play an important role in drug resistance, dictating the importance and need to assess these uninvestigated miRNAs at the expression level.

The observation that *miR-10b* is differentially gained and overexpressed in resistant cell lines treated with MG-132 is consistent with previous findings (Figures 5(a) and 5(b)). *miR-10b* is an oncomir whose overexpression has been identified in a variety of cancers [62-67]. Specifically, overexpression of miR-10b has been demonstrated to promote the development of metastatic disease in breast cancer and correlate with clinical breast cancer progression, poor overall survival in gastric cancer, and higher grades of malignant glioma. It was also found to be an effective therapeutic target by using antagomirs to reduce expression of HOXD10, subsequently suppressing breast cancer metastasis [62, 64, 66, 68]. Bioinformatic and gene expression analysis of mRNA targets of miR-10b revealed 32 of 636 target genes that were underexpressed in highly resistant cell lines, which have high expression of miR-10b. Amongst the identified genes was RAD1 (Figure 5(c)). RAD1 is part of a complex of proteins known as the 9-1-1 complex, which functions as a heterotrimeric cell cycle checkpoint [69]. The complex, which functions in DNA repair, is recruited to the site of DNA damage or incomplete replication where it recruits DNA polymerases and DNA repair enzymes. RAD1 has been shown to be important in preventing tumor development in response to DNA damage in mice, whereas deletion of RAD1 greatly increased the susceptibility for skin tumor development [70]. In addition, RAD1 is an important component of nucleotide excision repair (NER) which can have drastic effects on chemotherapy drug response. In drugs that instill double stranded DNA breaks, such as the platinum based treatment Cisplatin, upregulation of NER increases drug resistance while in certain non-DNA damage based chemotherapies, downregulation of NER has been shown to increase resistance [71, 72]. In NSCLC patients that have low expression of ERCC1, a gene also involved with NER, have decreased survival when compared to patients with high ERCC1 expression [73], and in both murine and human cells, low XPC expression, another gene involved in NER, correlated with resistance to the Doxorubicin derivative, Nemorubicin [72]. Intriguingly, one of the overrepresented



FIGURE 5: Example of a miRNA showing differentially copy number alteration, differential miRNA expression, and differential target gene expression. (a) Copy number alteration comparison between cell lines which are highly resistant and sensitive to agent MG-132 revealed that the hsa-miR-10b locus, on chromosomal region 2q31.1, is more frequently gained in the highly resistant cell lines (P < .05, Fisher's exact test). (b) miRNA expression analysis of miR-10b shows that expression is significantly higher in highly resistant cell lines as compared to sensitive cell lines to MG-132 (P = .03, Mann Whitney U test). (c) mRNA expression analysis of *RAD1*, a gene identified by bioinformatics prediction analysis as a putative target of miR-10b, shows anticorrelative expression to miR-10b expression. Specifically, decreased expression of RAD1 in highly resistant cell lines to MG-132 relative to highly sensitive lines is observed.

functions identified by *Ingenuity Pathway Analysis* of the 32 differentially expressed target genes was *DNA Replication*, *Recombination*, *and Repair* (Figure 6).

Expression patterns of *miR-193b* in human cancers, unlike *miR-10b*, are largely variable. High expression of *miR-193b* is frequently observed in head and neck squamous cell carcinomas and is associated with a high risk of metastatic disease in uveal melanoma [74, 75]. Conversely, in other cancer types, overexpression of *miR-193b* has elicited increased tumor suppression as well as sensitivity to specific chemotherapeutics [57, 76, 77]. Moreover, conflicting results have also been observed within a given cancer type. In malignant cutaneous melanoma, overexpression of *miR-193b* predicts disease outcome and is associated with poor survival, while induced overexpression in cell lines repressed proliferation through the downregulation of Cyclin D1 [78, 79]. Subsequent gene expression analysis of target mRNAs of *miR-193b* revealed 10 genes that were underexpressed in cell lines highly resistant to AZ628and 37 genes that were underexpressed in cell lines highly resistant to MK-0457. One of the target genes that was also differentially expressed was IKAROS family zinc finger 1 (IKZF1). This transcription regulating gene functions through associations with complexes that are both histone deacetylase (HDAC)dependent and HDAC-independent [80]. Previous studies have shown that nonhigh-risk ALL9 patients with IKZF1 deletions show a 12-fold higher rate of relapse compared to patients without IKZF1 deletions and IKZF1 deletion has also been implicated in tyrosine kinase inhibitor (TKI) resistance and disease progression in patients with chronic phase- (CP-) chronic myeloid leukemia (CML) [81, 82]. Overexpression of an Isoform of IKZF1 lacking a DNA binding domain, IK6, in acute lymphoblastic leukemia (ALL) patients with the Philadelphia chromosome has also been associated with TKI resistance [83]. Interestingly, MK-0457 is a small molecule inhibitor chemotherapy drug that targets



FIGURE 6: Overrepresented cellular and molecular functions that are common to all four sets of differentially expressed target genes. A "Core Analysis Comparison" was performed using Ingenuity Pathway Analysis and within the *Biological Functions*, only functions within *Molecular* and *Cellular Functions* were assessed. In total, eight of these functions were significant in all four sets. The orange threshold line corresponds to a *P*-value of .05.

aurora kinase. Underexpression of *IKZF1* as a result of *miR-193b* targeted degradation may increase resistance to MK-0457 in a similar mechanism to TKI resistance.

Similar to miR-193b, evidence supporting the role of *miR-328* in cancer is also unclear. In lung adenocarcinoma, miR-328 has been shown to be overexpressed in tumor tissue relative to matched nonmalignant tissue regardless of EGFR or KRAS mutation status [28]. However, in other cancer types, miR-328 underexpression, for example, enables drug resistance through the upregulation of ABCG2 and correlates with cancer progression [84-86]. Our analyses revealed miR-328 to be gained and overexpressed in lung cancer cell lines resistant to Geldanamycin, an antibody against HSP90. Target and gene expression analysis of miR-328 identified 31 genes underexpressed in cell lines highly resistant to Geldanamycin, with one of the targets being the Vitamin D receptor (VDR). VDR and its downstream components, have been previously shown to have antiproliferative effects in a wide variety of cancer types. The anticancer effects of VDR signaling are mostly mediated through its active metabolite, 1,25-dihydroxyvitamin D (calcitriol), which has been shown to exhibit anti-inflammatory effects as well as the suppression of tumor angiogenesis, invasion, and metastasis [87, 88]. Expression of VDR has also been shown to be associated with increased survival in breast, colorectal cancer, and cholangiocarcinoma. It has been recently shown that nuclear VDR status may be a prognostic marker of improved survival in patients with NSCLC [88]. Another intriguing finding for miR-328-associated mRNAs is the implication of both H(+)-monocarboxylate cotransporter (MCT) proteins 1 and 4 (SLC16A1/MCT1 and SLC16A3/MCT4). MCT1 and 4 are involved in lactate uptake and pH balance. Inhibition of MCT1 in tumors can shift aerobic cancer cells from oxidative phosphorylation (lactate metabolism) to glycolysis (glucose), resulting in the death of hypoxic tumor cells due to glucose deprivation [89].

Relatively little has been reported with regard to the role of miR-628. A recent study revealed that miR-628 was expressed in neuroblastomas with favorable prognosis, while those with unfavorable prognosis were devoid of expression, suggesting a tumor suppressive role in this type of cancer [90]. From our analyses, we identified miR-628 to be gained and overexpressed in resistant lung cancer cell lines treated with agent PF-2341066, a MET and ALK kinase inhibitor which has recently shown tremendous efficacy in a subset of lung cancer patients [7]. While the direction of expression contradicts the findings in neuroblastoma, miRNA tissue specificity may play a role in differential expression patterns. Regardless, further analysis of miR-628 is required to better elucidate its role in human cancers. Target and gene expression analysis of *miR-628* revealed 22 genes which were underexpressed in cell lines highly resistant to PF-2341066, with one of these differentially expressed being caspase 3 (CASP3). CASP3 is a gene involved in the caspase apoptosis cascade by activating caspases 6, 7, and 9 through cleavage [91]. Moreover, it is also used as a general indicator of cell death and apoptosis. Notably, PF-2341066, which functions as a TKI inhibitor, was found to induce the caspase cell death cascade in vitro through increased levels of CASP3 [92]. Thus, CASP3 downregulation, as a result of miR-628 targeting, may play a significant role in resistance to PF-2341066.

While all involved in response to different drugs, the targets of these miRNAs share certain biological functions. Figure 6 illustrates the functions in which the targets of all four miRNAs participate at a statistically significant level. Broadly, if roles such as cellular maintenance and DNA repair were compromised, such cell populations could develop tolerance to the accumulation of mutations, some of which could dictate resistance. Participation in small molecule biochemistry has implications in the alteration of how these administered drugs are processed. Cellular organization and cell-to-cell signaling, if altered, could confer a more invasive phenotype, contributing to drug resistance.

5. Conclusions

In conclusion, we have demonstrated our method of integrative analysis of multiple dimensions of data including genome-wide miRNA copy number, miRNA expression, mRNA expression, and drug sensitivity data, all available in the public domain, can be a powerful tool to identify miRNAs and genes involved in drug sensitivity. Through these initial analyses, we have identified miRNAs that may have a role in conferring chemoresistance to a number of drugs. Further in vitro and in vivo analyses of the miRNAs and their respective mRNA targets will be necessary to confirm the findings from this study. In addition, given that nearly half of the miRNAs that were differentially altered were not even represented on the miRNA platform analyzed, evaluation of these miRNAs may prove fruitful when new data becomes available. It should also be noted that miRNA target prediction approaches and algorithms are constantly evolving and increasing number of miRNAmRNA interactions being experimentally validated, potentially revealing important target genes that are not currently implicated. Finally, since the MGH/Sanger collaboration aims to generate drug response profiles for a large number of chemotherapeutics in over 1000 cancer cell lines, as more data becomes available, our approach could identify candidate miRNAs that are associated with multiple drugs which have similar mechanisms of action. Moreover, our strategy could also be repeated in a more specific and clinically relevant manner, which could ultimately lead to the identification of prognostic biomarkers and therapeutic indicators for better disease management and patient outcome.

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CGP Data Archive (http://www.sanger.ac.uk/genetics/CGP/ Archive/). Authors declare that those carried out the original analysis and collection of the data bear no responsibility for the further analysis or interpretation of it by the authors. Contributed equally to this work. K. S. S. Enfield, G. L. Stewart, and L. A. Pikor.

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Review Article **Target Therapies in Lung Cancer**

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Targeting intracellular signaling molecules is an attractive approach for treatment of malignancies. In particular lung cancer has reached a plateau regarding overall survival, and target therapies could offer the possibility to improve patients' outcome beyond cytotoxic activity. The goal for target therapies is to identify agents that target tumor-specific molecules, thus sparing normal tissues; those molecules are called biomarkers, and their identification is recommended because it has a predictive value, for example, provides information on outcome with regard to a specific treatment. The increased specificity should lead to decreased toxicity and better activity. Herein we provide an update of the main target therapies in development or already available for the treatment of nonsmall cell lung cancer.

1. Introduction

Nonsmall cell lung cancer (NSCLC) remains a leading cause of death worldwide among patients diagnosed with malignancy [1]. Despite new chemotherapy regimens and new cytotoxic combinations investigated in multiple randomized clinical trials in recent years, no significant improvement in the prognosis of patients with lung cancer was achieved. The five-year survival rate for all patients diagnosed with NSCLC is about 15%, only 5% better than 40 years ago [2]. Significant progress has been made in the recent years in understanding the molecular mechanism of lung cancer. Multiple pathways that are active in NSCLC progression and growth were identified [3]. New therapeutic approaches that target various different aspects of tumor progression and metastasis have been intensively investigated in NSCLC, with benefit/advantage on median overall survival, recently increased to more than one year.

Many drugs that block tumor vascularization (angiogenesis) or interfere with the activity of growth factor receptors and molecular pathways downstream triggered are already used in clinical practice, and more are on study. In this paper we will discuss the basic mechanism of activity and rationale for using those new drugs.

2. Tumor Angiogenesis

In 1971, Dr. Judah Folkman put forward the theory that malignant tumors cannot grow beyond a certain size without recruiting their own blood vessels (tumor angiogenesis) through a process that involved production of a soluble growth factor that was secreted by the tumor itself [4]. He also proposed that the local tumor growth and formation of metastases could be prevented by inhibiting the tumor angiogenesis. Among the list of factors that induce tumor angiogenesis, the most important is vascular endothelial growth factor A (VEGF), discovered in 1983 [5]. VEGF is the primary survival factor of vascular endothelial cells, stimulates proliferation, and migration and inhibits apoptosis and modulates their permeability. Those biological functions are mediated upon binding to receptor tyrosine kinases: vascular endothelial growth factor receptors 1, 2, and 3 (VEGFR 1,2,3) [6-9].

Expression of VEGF within tumors is regulated by multiple factors including the level of oxygen within the tumor, growth factors and cytokines produced by the tumor, and mechanism involving oncogene/tumor suppressor inactivation [10]. Hypoxia and Hypoxia-inducible factor (HIF) in the microenvironment are the most important There are two major way of blocking the VEGF pathways blocking the activation of extracellular part of VEGF receptor by inhibiting antibodies against VEGF molecule or blocking the activation of tyrosine kinase within the intracellular part of VEGF receptor by tyrosine kinase inhibitors [12, 13].

Bevacizumab is a humanized, monoclonal antibody that binds to VEGF. In 2004 a phase II trial investigated the use of bevacizumab in advanced NSCLC patients [14]. This trial highlighted the most important side effect of bevacizumab, the bleeding events. In particular the majority of patients having tumors with squamous histology and centrally located in close vicinity to major blood vessels had serious pulmonary bleeding. Following this trial, Eastern Cooperative Oncology Group conducted a phase III trial (E4599) comparing paclitaxel and carboplatin chemotherapy alone and the same chemotherapy combined with bevacizumab [15]. After completion of 6 cycles of treatment, patients receiving bevacizumab with chemotherapy continued on bevacizumab as single agent until disease progression or intolerable toxicity occurred. Patients with squamous histology, brain metastases, and central localization were excluded from the study.

The combination of chemotherapy and bevacizumab resulted in the significant improvement in median survival by 2 months when compared with chemotherapy alone group, 12.3 versus 10.3 months, respectively.

The AVAiL trial investigated similar approach as ECOG 4599 study in advanced NSCLC patients, comparing cisplatin and gemcitabine alone versus the same chemotherapy combination with bevacizumab in two different doses [16]. Although the study was powered for overall survival (OS), the primary endpoint was changed from OS to progression-free survival (PFS) during accrual. Median PFS improved upon adding Bevacizumab to chemotherapy both with 7.5 mg/kg dose and 15 mg/kg when compared with chemotherapy alone. However, no survival benefit was observed with adding bevacizumab to standard chemotherapy as shown in ECOG trial. There are multiple different reasons for this different result including insufficient statistical power of the study or the different platinum-based doublet combined with bevacizumab that may matter.

There are ongoing trials with new antiangiogenic molecules as the vascular disrupting agent ASA404, just concluded and press-released to be negative, and oral smallmolecule tyrosine kinase inhibitors. Among those TKIs, Vandetanib (ZD6474, AstraZeneca), an inhibitor of VGFR2/3, RET, and EGFR, has the more advanced development program; in second-line, phase III trial Zodiac [17], Vandetanib showed a slightly improvement of PFS when combined with Docetaxel, 4 and 3.2 months, respectively; there was no statistical difference in the PFS when combined with Pemetrexed in another randomized phase III trial (ZEAL) for second-line treatment [18]. Vandetanib was compared to Erlotinib in a phase III trial for pretreated patients affected by advanced NSCLC, Zest trial [19]. The study did not meet its primary objective of demonstrating PFS prolongation. In the Zephyr trial Vandetanib was compared to placebo in patients resistant to chemotherapy and EGFR inhibitors; any statistically significant advantage was reported neither for the progression-free survival nor for the overall survival [20]. Many other trials are ongoing with Sunitinib, multityrosine kinase inhibitor of VEGF, Kit, FLT3, PDGFR, and Raf, Sorafenib, inhibitor of PDGFR- β , Raf, c-Kit, FLT3, and all VEGFRs, BIBF1120, a potent triple inhibitor of VEGFR 1,2,3, fibroblastic growth factor, and PDGFR, Axitinib, a potent inhibitor of all three VEGFRs [21]. In particular the results of the SUN 1087 trial have been recently reported; in this phase III trial Sunitinib in combination with Erlotinib was compared to Erlotinib in patients with previously treated advanced NSCLC, bringing a statistically significant improvement in PFS but not in OS [22]. NExUS, a phase III, randomized, double-blind, placebo-controlled study evaluated Sorafenib versus placebo in combination with two chemotherapeutic agents, gemcitabine and cisplatin, in treatment-naive nonsmall cell lung cancer patients [23]. No advantage in OS was demonstrated; however, a slight improvement in PFS was shown, although this was not the primary endpoint of the study.

3. EGFR Pathway

The Epidermal Growth Factor (EGF) pathway was discovered by Stanley Cohen in the sixties [24]; later in 1980 involvement of its receptor, EGFR, in the tumor genesis was demonstrated. The EGFR pathway can be modulated by monoclonal antibodies that block EGFR (Cetuximab, Panitumumab) or by small molecule tyrosine kinase (TKIs) (Erlotinib, Gefitinib) that interfere with activation of EGFR. The first important trials were designed with TKI Gefitinib, Ideal 1 and 2, two large Phase II trials, demonstrating an antitumoral activity of Gefitinib in the treatment of advanced NSCLC, in particular in adenocarcinoma, females, nonsmokers and Asian population [25, 26]. Although two North American groups reported the importance of EGFR mutations (exon 19 and exon 21 L858R) for prediction of higher response rate and their prevalence in nonsmoker, Asian, female population with adenocarcinoma [27, 28], two large randomized clinical trials, placebo-controlled, phase III were already started, assessing Gefitinib or Erlotinib in second or further line of therapy, respectively, the ISEL and BR.21 trials [29, 30]. Response rate was similar in both trials, 8%; however, only the Erlotinib trial reached a significant impact on overall survival. Later on, clinically or molecularly enriched trials confirmed the role of mutations and as predictive and prognostic positive biomarker. In the IPASS trial, East Asian patients who were never or light smokers were randomized to receive chemotherapy or gefitinib as first-line treatment [31]. Patients who were EGFR mutation positive benefited more from gefitinib, whereas the mutation-negative patients did better with chemotherapy. The same result was obtained from a Korean trial, First Signal, showing the consistence of those results [32].

The West Japan and North East Japan groups conducted parallel trials, where molecularly selected population for EGFR mutations was randomized to receive chemotherapy of Gefitinib as first-line treatment. Both trials demonstrated the significant superiority in time to progression of the patients receiving Gefitinib [33, 34]. Overall survival did not differ between the two arms, likely for a crossover effect. The same result, for example, no difference in overall survival despite the significant benefit in PFS, was obtained in the IPASS trial [35].

Cetuximab as an antibody to EGFR may work differently from the TKIs. Two phase III trials, FLEX and BMS 099, combined chemotherapy with or without Cetuximab in the treatment of chemo-naive patients with advanced NSCLC [36]. Patients on the FLEX trial had to be EGFR positive by immunohistochemistry (IHC), and patients who received the Cetuximab had a modest but significant survival benefit. On the BMS 099 trial, there was no patient selection and no survival advantage for the Cetuximab arm [37]; however, the lack of a significant survival advantage could be due to the small sample size of the study.

There are now a number of new-generation EGFR inhibitors. BIB9229 (Afatinib) is an oral irreversible TKI of both EGFR and HER2, and it demonstrates activity in EGFR mutants resistant to Erlotinib, Gefitinib, and Lapatinib. It has demonstrated single agent activity in patients with EGFR mutations (LUX-Lung2) and in EGFR TKIs failures [38].

IMC-11F8 is a fully human IgG1 antibody with an epitope similar to Cetuximab. It is currently being evaluated in clinical trials in colon and lung cancer.

4. KRAS

KRAS mutations are found predominately in the adenocarcinoma histologic subtype of NSCLC (approximately 30%) and less frequently in the squamous cell carcinoma subtype (approximately 5%) [39]. KRAS mutations are associated with a history of tobacco use, and the frequency of KRAS mutations varies among different ethnic groups [40, 41]. The mutant KRAS genes in human cancers encode mutated proteins that harbor single amino acid substitutions, in lung cancer primarily at codons 12 and 13. Mutant KRAS proteins are constitutively activated, leading to stimulusindependent, persistent activation of downstream effectors, in particular, the Raf-MEK-ERK cascade [42, 43]. It has been recently investigated the role of KRAS mutations and EGFR in 1081 patients, and those patients with KRAS mutations had a shorter survival than patients with EGFR mutations or EGFR/KRAS wild type [44]. Although there is a reasonable biologic rationale to support the hypothesis that NSCLC tumors with KRAS mutations are resistant to EGFR-TKIs, the clinical data confirming it have been elusive. This might be a result of the very low prevalence of KRAS and EGFR mutations in NSCLC [45] and the low rate that tumor tissue has been available for KRAS mutational analysis from trials.

5. MET Receptor Tyrosine Kinase

The c-MET (hereafter referred as MET) receptor tyrosine kinase was originally identified as the cellular homologue of

the TPR-MET oncoprotein [46]. MET can be overexpressed in a number of malignancies, sometimes mutated, or sometimes even amplified. MET located on chromosome 7 encodes for a single precursor that is posttranscriptionally modified, forming a transmembrane protein. The ligand for MET has been identified as hepatocyte growth factor (HGF). Ligation of MET receptor to HGF leads to activation of its intrinsic tyrosine kinase. Activating mutations of MET have been reported in a variety of cancers such as lung cancer, melanoma, mesothelioma, and pancreatic cancer; MET can also be amplified in lung cancer.

Several MET inhibitors are currently under evaluation, like ARQ 197 or PF 23411066; promising results of a phase II trial with ARQ 197 associated to chemotherapy were recently presented at the ASCO meeting [47].

6. ALK

A new fusion oncogene, named EML4-ALK, has been described in about 4% of NSCLC patients, mostly in never smokers, young, male, usually not harboring EGFR mutations. The oncogene is due to a translocation within chromosome 2 bringing to a fusion between the N-terminus of the echinoderm microtubule-associated protein-like 4 (EML4) and the intracellular domain of anaplastic kinase (ALK), and its tyrosine kinase activity can be triggered by ALK, MET, and HGF. The activity of EML4-ALK can be abolished by an oral compound, PF 02341066 (Crizotinib, Pfizer) [48]. EML4-ALK can be tested by FISH, the recommended dose is 250 mg twice daily and after the promising results of a phase II trial, a phase III trial is ongoing.

7. Insulin Growth Factor Pathway

The insulin growth factor receptor (IGFR) is involved in essential steps of cancer development such as survival, proliferation and metastases [49]. Predictive factors, that is, predictive biomarkers, are yet not identified, although it has been suggested that pretreatment levels of circulating free IGF1 could help in selecting responsive patients [50].

Several compounds, including monoclonal antibodies and tyrosine kinase inhibitors, are currently under clinical investigation in NSCLC. The major toxicity is hyperglycemia and fatigue, as class effect. The figitumumab (CP-751,871) is the only anti-IGF1R monoclonal antibody whose phase III trial has already finished, and no statistical improvement was demonstrated by adding figitumumab to standard chemotherapy in advanced NSCLC patients [51].

More trials are ongoing with other antibodies with different affinity to IGF1R, like IMC-A12 and MK-0646.

8. Conclusions

Although a platinum doublet remains the standard treatment for advanced NSCLC patients and histology drives the choice of the drugs, biomarkers are useful for prognostic and predictive information. Up to now, the lack of established predictive biomarker to select patients for the antiangiogenic EGFR mutations are present in 35% of the Asian population and in 15% of the Caucasian population; patients affected by advanced NSCLC with sensitizing mutations in the EGFR gene are highly responsive to EGFR-TKIs with dramatical improvement of their OS, and they should receive those drugs during their treatment. EML4-ALK and EGFR mutations are reported to be mutually exclusive; therefore, EML4-ALK should be checked in patients EGFR negative, for the outstanding results obtained with Crizotinib in the phase II trial, to be confirmed. Other molecular markers and target drugs are advancing rapidly, so the molecular analysis of tumor tissue for molecular characterization is a crucial step in defining the best treatment strategy.

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Review Article Gefitinib in Non Small Cell Lung Cancer

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Gefitinib is an oral, reversible, tyrosine kinase inhibitor of epidermal growth factor receptor (EGFR) that plays a key role in the biology of non small cell lung cancer (NSCLC). Phase I studies indicated that the recommended dose of gefitinib was 250 mg/day. Rash, diarrhea, and nausea were the most common adverse events. The positive results obtained in early phase 2 clinical trials with gefitinib were not confirmed in large phase 3 trials in unselected patients with advanced NSCLC. The subsequent discovery that the presence of somatic mutations in the kinase domain of EGFR strongly correlates with increased responsiveness to EGFR tyrosine kinase inhibitors prompted phase 2 and 3 trials with gefitinib in the first line-treatment of EGFR-mutated NSCLC. The results of these trials have demonstrated the efficacy of gefitinib that can be now considered as the standard first-line treatment of patients with advanced NSCLC harbouring activating EGFR mutations.

1. Introduction

Gefitinib (ZD1839, Iressa) is an orally administered, reversible tyrosine kinase inhibitor (TKIs) of epidermal growth factor receptor (EGFR), belonging to the smallmolecule class (quinazoline-derivative molecule) [1]. The EGFR family includes four different tyrosine kinase receptors: EGFR (ErbB-1), ErbB-2, ErbB-3, and ErbB-4 [2]. Each of these proteins has an extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a cytoplasmic tyrosine kinase-containing domain. The receptors of the ErbB family are activated following binding to peptide growth factors of the EGF-family. Upon ligand binding, the ErbB receptors form either homo- or heterodimers and, following dimerization, auto- and transphosphorylation in tyrosine residues of the ErbB receptors occurs [3]. EGFR signaling plays a key role in promoting the growth and survival of various types of solid tumors, including non small cell lung cancer (NSCLC) [4, 5].

Gefitinib has an inhibitory effect both on the autophosphorylation and downstream signaling, competing reversibly with the adenosine triphosphate (ATP) for the catalytic domain of EGFR. *In vitro* studies indicated that gefitinib potently inhibited EGFR tyrosine kinase activity at low concentrations that did not significantly affect other kinases tested [6]. *In vivo* studies showed that gefitinib had a favourable tolerability profile and an antitumor activity in various xenograft models and enhanced the antitumor activity of a variety of cytotoxic drugs, including platinum compounds [7, 8]. Gefitinib was well tolerated in healthy volunteers and showed a terminal half-life of 28 hours, supporting the once-daily oral administration [9].

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This paper focuses on the clinical development of gefitinib in NSCLC, discussing the causes of its failure in unselected NSCLC patients and summarizing the available evidence coming from the randomized phase 3 trials that support the use of gefitinib as the standard first line treatment of patients with advanced NSCLC harbouring EGFR mutations.

2. Phase I Clinical Studies

Gefitinib has been evaluated as single agent in four phase 1 clinical trials, including patients with advanced refractory solid tumors. In the first study, conducted in UK and USA, gefitinib was administered once daily for 14 consecutive days, followed by 14 days off treatment [10]. Dose escalation started at 50 mg and continued to 925 mg or until consistent dose-limiting toxicity (DLT). Sixty-four patients were entered at eight dose levels. The most frequent dose-related grade 1 and 2 adverse events were acne-like rash, nausea, and diarrhea. Three of 9 patients treated at 700 mg/day developed DLT (reversible grade 3 diarrhea). Four of 16 patients with NSCLC had partial responses (observed from 300 to 700 mg/day). In the second study, including 88 patients in Europe and Australia, gefitinib was administered at dose ranging from 150 to 1000 mg/day in 28-day cycles to patients with either advanced non small cell lung, ovarian, head and neck, prostate, or colorectal cancer [11]. At 1000 mg/day, 5 of 12 patients experienced DLT (grade 3 diarrhea in four patients and grade 3 somnolence in one patient). The most frequent adverse events were acne-like rash (64%) and diarrhea (47%), which were generally mild (grade 1/2) and reversible on cessation of treatment. Nineteen patients had stable disease and received gefitinib for >3 months. In the third study, conducted in USA, 71 patients were enrolled at seven dose levels (ranging from 150 to 1000 mg/day in 28day cycles) and most had NSCLC (n = 39) [12]. Diarrhea and rash, the primary DLTs, occurred at 800 mg. Frequent treatment-related grade 1-2 adverse events were diarrhea (55%), asthenia (44%), and acne-like follicular rash (46%). At doses >800 mg, 45% of patients required dose reductions. One partial response and 6 prolonged stable disease were observed in patients with NSCLC. The fourth phase 1 study investigated the tolerability and toxicity of gefitinib in Japanese patients with solid tumors [13]. Thirty-one patients were included and received oral gefitinib on 14 consecutive days, every 28 days. Dose escalation was from 50 mg/day to a maximum of 925 mg/day or DLT. The most frequent adverse events were an acne-like rash and gastrointestinal side effects. Two of 6 patients at 700 mg/day had DLT; no further dose escalation occurred. A partial response was observed in 5 of the 23 patients with NSCLC (duration 35-361 days) over a range of doses (225-700 mg/day), and 7 patients with various tumors had disease stabilization. Therefore, gefitinib showed a favourable tolerability profile and antitumor activity also in Japanese patients. Moreover, pharmacokinetic analyses from all these studies confirmed the feasibility of the once daily schedule.

The preclinical evidence of synergism between gefitinib and chemotherapy provided the rationale for a feasibility study designed to assess the tolerability and antitumor activity of the combination of two doses of gefitinib (250 and 500 mg/day), gemcitabine and cisplatin (at standard doses) in chemotherapy-naïve patients with advanced or metastatic solid tumors, and to assess whether there was a pharmacokinetic interaction between these drugs when administered concurrently [14]. Eighteen patients were entered, 9 at each gefitinib dose level. Two patients developed DLT: one grade 3 convulsion (250 mg/day dose group) and one grade 3 rash (500 mg/day dose group). The most common grade 3/4 adverse events were vomiting (7 patients), asthenia (6 patients), thrombocytopenia (6 patients), diarrhea (5 patients), and anorexia (5 patients). Pharmacokinetic analyses showed no apparent pharmacokinetic interaction between gefitinib and cisplatin or gemcitabine, with the exception of a possible small increase in the geometric mean exposure to gemcitabine seen on day 8 of therapy when given alone with the higher dose of gefitinib. Of 10 evaluable patients with NSCLC, 5 had confirmed partial response, 4 had stable disease and 1 had progressive disease.

3. Development of Gefitinib in "Unselected" Patients

3.1. Phase II Clinical Studies. Two randomized phase 2 clinical studies evaluated the safety and the activity of two doses of gefitinib (250 mg or 500 mg) as second- or third-line therapy of NSCLC patients (IDEAL 1 and IDEAL 2) [15, 16]. The IDEAL-1 (Iressa Dose Evaluation in Advanced Lung cancer) study recruited 210 patients who were pretreated with one or two chemotherapy regimens, at least one containing platinum [15]. The IDEAL-2 study included 221 patients who were pretreated with two or more regimens containing platinum and docetaxel [16]. In both studies, the two doses of gefitinib produced similar results in terms of objective responses (approximately 20% in IDEAL-1 and 10% in IDEAL-2), disease control rate (about 50% in IDEAL 1 and 40% in IDEAL 2), and overall survival (about 8 months in IDEAL 1 and 7 months in IDEAL 2). Overall, the incidence of toxic effects, including skin rash and diarrhea, was lower in patients treated with 250 mg/day as compared with patients treated with 500 mg/day. These results led to choosing the lower dose for subsequent development of the drug in NSCLC. In both trials, an attempt has been made to identify predictive factors for objective response to gefitinib. In the IDEAL-1 study, a multivariate analysis showed that performance status, previous immuno/hormonal treatment, histology, and female gender were significantly associated with a higher response rate, while in the IDEAL-2 study only female gender was significantly predictive of response to gefitinib. The promising results of these trials led, in 2003, the Food and Drug Administration (FDA) to grant an accelerated approval for gefitinib as monotherapy treatment for patients with locally advanced or metastatic NSCLC after failure of both platinum-based and docetaxel chemotherapies.

The results of other phase 2 studies conducted with gefitinib as single agent in unselected patients with advanced NSCLC are summarized in Table 1 [15–23].

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TABLE 1: Phase II clinical trials with Gefitinib as single agent in "unselected" NSCL

Author (yr)	Setting	Design	Pts	Gefitinib dose	Results	Toxicity
Fukuoka et al. (2003) [15]	Pretreated with 1-2 lines	Randomized phase 2	210	250 mg versus 500 mg	RR: 18.4% (250 mg) versus 19% (500 mg); PFS: 2.7 versus 2.8 months	Diarrhea, rash and other skin events
Kris et al. (2003) [16]	Pretreated with 2-3 lines	Randomized phase 2	221	250 mg versus 500 mg	RR: 12 versus 9%; OS: 7 versus 6 months	Diarrhea, rash and other skin events
D'Addario et al. (2008) [17]	Chemonaive	Phase 2, single arm	63	250 mg	RR: 9.5 %; DSR at 12 weeks: 38%	Rash and other skin events, hepatotoxicity
Wan et al. (2006) [18]	Not fit for chemo or pretreated	Phase 2, single arm	151	250 mg	RR: 29.8%; TTP: 12 months; 1 yr OS: 57%	Rash, diarrhea, nasal/oral mucosa bleeding
Lin et al. (2006) [19]	Chemonaive	Phase 2, single arm	53	250 mg	RR: 32.1%; TTP: 12 months; OS: 15.3 months; 1 yr OS: 57%	Skin toxicity, diarrhea, nail change, ILD
Niho et al. (2006) [20]	Chemonaive	Phase 2, single arm	42	250 mg	RR: 30%; OS: 13.9 months; 1 yr OS: 55%	Rash and other skin events, ILD
Reck et al. (2006) [21]	Chemonaive	Phase 2, single arm	58	250 mg	RR: 5%; TTP: 1.8 months; OS: 7.3 months	Skin toxicity and diarrhea
Suzuki et al. (2006) [22]	Chemonaive	Phase 2, single arm	34	250 mg	RR: 26.5%; OS: 14 months; 1 yr OS: 58.2%	Rash, fatigue, hepatotoxicity
Spigel et al. (2005) [23]	Chemonaive	Phase 2, single arm	70	250 mg	RR: 4%; TTP: 3.7 months; OS: 6.3 months; 1 yr OS: 24%	Rash and diarrhea

RR: response rate; PFS: progression-free survival; OS: overall survival; DSR: disease stabilization rate; TTP: time to progression; ILD: interstitial lung disease.

3.2. Phase III Clinical Studies in First-Line Therapy. The encouraging results obtained in early clinical trials and the preclinical evidence of synergism between gefitinib and chemotherapy prompted two large randomized phase 3 clinical trials examining the role of gefitinib in combination with standard chemotherapy (cisplatin plus gemcitabine in INTACT-1 and carboplatin plus paclitaxel in INTACT-2) for the first line treatment of advanced NSCLC [24, 25]. Both of these studies failed to demonstrate any advantage in overall survival for patients treated with chemotherapy in combination with gefitinib. Moreover, subgroups analyses of predictive factors of sensitivity to gefitinib did not demonstrate any survival advantage for specific subgroups when gefitinib was added to chemotherapy. Negative results were similarly observed with the combination of another tyrosine kinase inhibitor, erlotinib, with chemotherapy (TALENT and TRIBUTE studies) [26, 27]. Several explanations regarding the lack of an additive effect between tyrosine kinase inhibitors and chemotherapy have been proposed: a mechanistic interaction between gefitinib or erlotinib and chemotherapy, for which the antiproliferative effects of anti-EGFR agents may render tumor cells less sensitive to cytotoxic agents, as suggested by preclinical studies; the possibility that patients who benefit from EGFR-targeted treatments are the same who likely respond to chemotherapy: in this case, the effect of tyrosine kinase inhibitors can be masked by the effect of chemotherapy; finally, the lack of patient selection based on the expression of EGFR [28].

Because no additive effect was observed by administering gefitinib in combination with chemotherapy, a phase 3 trial was conducted to evaluate the efficacy of a sequential strategy, with gefitinib given after first line platinumdoublet chemotherapy for NSCLC, which might have avoided problems of drug interference or antagonism [29]. Unfortunately, sequential gefitinib therapy after three cycles of standard platinum doublet chemotherapy showed no survival benefit over platinum doublet chemotherapy up to six cycles (HR 0.86, 95%CI 0.72–1.03, P = .11), although sequential gefitinib was associated with significantly prolonged progression-free survival (HR 0.68, 95%CI 0.57–0.80; P < .001). An exploratory subset analysis demonstrated a possible survival prolongation for sequential therapy of gefitinib, for patients with adenocarcinoma (HR 0.79, 95%CI 0.65–0.98, P = .03).

Table 2 summarizes the results of the randomized clinical trials with gefitinib in first- and second-line therapy, in locally advanced disease and adjuvant setting and in special populations [24, 25, 29–38].

3.3. Phase III Clinical Studies in Second-Line Therapy. A multicenter phase 3 study compared gefitinib as monotherapy at the dose of 250 mg/day to placebo in 1692 pretreated patients with NSCLC [30]. Patients treated with gefitinib reported significantly higher response rate (8% versus 1.3%) and longer time to treatment failure (3.0 versus 2.6 months). However, treatment with gefitinib was not associated with significant improvement in survival in the overall population (5.6 versus 5.1 months in the gefitinib and placebo arms, resp.) nor in the subgroup of patients with adenocarcinoma. There was pronounced heterogeneity in survival outcomes between groups of patients, with some evidence of benefit among never-smokers (median survival of 8.9 versus 6.1

		IABLE 2:	Kandomiz	ed clinical trials with Gentinio	in unselected inscript.		
Author (yr)	Setting	Design	Pts	Treatment	RR (%)	PFS (mos)	OS (mos)
Giaccone et al. (2004) [24]	Chemonaive	Phase 3	1093	CIS/GEM + gefitinib 500 versus CIS/GEM + gefitinib 250 versus CIS/GEM + placebo	50.3 versus 51.2 versus 47.2 P ns	5.5 versus 5.8 versus 6.0 <i>P</i> = .7633	9.9 versus 9.9 versus 10.9 $P = .4560$
Herbst et al. (2004) [25]	Chemonaive	Phase 3	1037	Carbo/taxol + gefitinib 500 versus Carbo/taxol + gefitinib 250 versus Carbo/taxol + placebo	30 versus 30.4 versus 28.7 P ns	4.6 versus 5.3 versus 5.0 P = .0562	8.7 versus 9.8 versus 9.9 P = .64
Takeda et al. (2010) [29]	Chemonaive	Phase 3	604	CIS/GEM × 3 → gefitinib versus CIS/GEM × 6	34.2 versus 29.3 P = .20	4.6 versus 4.3 HR: 0.68, 95% CI 0.57–0.80, <i>P</i> = .001	13.7 versus 12.9 HR 0.86, 95% CI 0.72–1.03, <i>P</i> = .11
Thatcher et al. (2005) [30]	Pretreated with 1-2 lines	Phase 3	1692	Gefitinib 250 mg + BSC versus placebo + BSC	8 versus 1.3 <i>P</i> < .0001	3 versus 2.6 HR: 0.82, 95% CI 0.73–0.92, P = .0006	5.6 versus 5.1 HR: 0.89, 95% CI 0.77–1.02, P = .087
Cufer et al. (2006) [32]	Secondline	Randomized phase 2	133	Gefitinib 250 mg versus Docetaxel 75 mg/mq	13.2 versus 13.7 HR 0.98, 95% CI 0.47–2.03	3 versus 3.4 HR: 0.94, 95% CI 0.64–1.39	7.5 versus 7.1 HR: 0.97, 95% CI 0.61–1.52
Kim et al. (2008) [33]	Pretreated with 1-2 lines	Phase 3	1466	Gefitinib 250 mg versus Docetaxel 75 mg/mq	9.1 versus 7.6 $P = .33$	2.2 versus 2.7 HR: 1.04, 95% CI 0.93–1.18, P = .47	7.6 versus 8.0 HR: 1.020, 95% CI 0.905–1.150
Maryuama et al. (2008) [35]	Pretreated with 1-2 lines	Phase 3	489	Gefitinib 250 mg versus Docetaxel 60 mg/mq	22.5 versus 12.8 $P = .009$	2 versus 2 HR: 0.90, 95% CI 0.72–1.12, P = .0335	11.5 versus 14 HR: 1.12, 95% CI 0.89–1.40, <i>P</i> = .330
D. H. Lee et al. (2010) [36]	Second line	Phase 3	161	Gefitinib 250 mg versus Docetaxel 60 mg/mq	28.1 versus 7.6 $P = .0007$	3.3 versus 3.4 HR 0.729, 95% CI 0.533–0.998, <i>P</i> = .0441	HR: 0.870, 95% CI 0.613–1.236, <i>P</i> = .4370
Crinò et al. (2008) [40]	Chemonaive ≥70 yrs	Randomized phase 2	196	Gefitinib 250 mg versus Vinorelbine 30 mg/mq 1–8	3.1 versus 5.1	2.7 versus 2.9 HR:1.19, 95% CI 0.85–1.65	5.9 versus 8 HR: 0.98, 95% CI 0.66–1.47
G. Goss et al. (2009) [41]	Chemonaive PS 2-3	Randomized phase 2	201	Gefitinib 250 mg + BSC versus Placebo + BSC	6% versus 1% OR: 6.57, 95% CI 0.74–58.17	1,4 versus 1,3 HR: 0.82, 95% CI 0.60–1.12	3.7 versus 2.8 HR:0.84, 95% CI 0.62–1.15
Kelly et al. (2008) [39]	Maintenance after chemo-radio Inoperable stage III	Phase 3	243	Gefitinib 500/250 mg versus Placebo	I	8.3 versus 11.7 HR: 0.80, 95% CI 0.58–1.10, P = .17	23 versus 35.0 HR: 0.633, 95% CI 0.44–0.91, <i>P</i> = .013
G. D. Goss et al. (2010) [38]	Adjuvant	Phase 3	503	Gefitinib 250 mg versus Placebo	I	4.2 yrs*versus nr HR: 1.22, 95% CI 0.93–1.61, P = .15	5.1 yrs* versus nr HR: 1.24, 95% CI 0.94–1.64, P = .14
CIS: cisplatin; Gem: gem	citabine; RR: response rate; F	FS: progression-free	survival; OS	: overall survival; ns: not significa	nt; nr: not yet reached; *d	lisease-free survival.	

TABLE 2: Randomized clinical trials with Gefitinib in unselected NSCLC.

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months; HR 0.67; 95% CI 0.49-0.92, P = .012) and Asian ethnicity (9.5 versus 5.5 months; HR 0.66; 95% CI 0.48-0.91, P = .01). Explanations of the negative results of this trial could be the large number of chemotherapy refractory patients (90%), a suboptimal dose of gefitinib and the lack of selection based on potential molecular markers, associated with clinical outcome. In addition, exploratory biomarker analyses, including the assessment of EGFR gene copy number by FISH, EGFR and p-AKT protein expression by IHC, EGFR, K-RAS and D-RAF mutational status, showed a trend towards a better survival outcome for gefitinib in patients with high EGFR-gene-copy number (HR 0.61 for high copy number and HR 1.16 for low copy number, P = .045), while patients with EGFR mutations obtained higher RR than wild-type patients (37.5% versus 2.6%) [31]. No relationship was observed between p-AKT protein expression and survival outcome. On the basis of the lack of survival benefit in the ISEL study, in 2005 the FDA restricted the use of gefitinib to patients continuing to benefit from treatment already initiated or participating in clinical trials.

Four randomized trials compared gefitinib versus docetaxel as a second-line therapy of advanced NSCLC patients.

An open-label randomized phase 2 study (SIGN trial— Second line Indication of Gefitinib in NSCLC) compared gefitinib (250 mg/day) with docetaxel (75 mg/mq every 3 weeks) in 135 patients with advanced pretreated NSCLC [32]. Primary objective of this trial was symptom improvement using the FACT-L questionnaire. Gefitinib and docetaxel showed similar activity (symptom improvement rates of 36% and 26%, response rate of 13.2% and 13.7%, median progression-free survival of 3 and 3.4 months, median overall survival of 7.5 and 7.1 months, with quality of life improvement rates of 33.8% and 26% for gefitinib and docetaxel, resp.). However, gefitinib had a more favorable tolerability profile than docetaxel (adverse events of all grades: 51.5% versus 78.9%; grade 3-4: 8.8% versus 25.4%).

The INTEREST trial was the largest study comparing gefitinib to docetaxel as second- or third-line therapy in 1466 patients with advanced NSCLC treated with prior platinumbased chemotherapy [33]. The coprimary endpoints were the noninferiority of gefitinib in comparison with docetaxel in terms of overall survival in the total population and the superiority in patients expressing a high EGFR gene copy number. The study demonstrated the noninferiority of gefitinib (OS 7.6 versus 8.0 months, with a 1-year survival of 32% versus 34%, in the gefitinib and docetaxel arms, resp., HR 1.02, 96% CI 0.905-1.150, meeting the pre-defined non inferiority criterion of 1.154), while failed to demonstrate the superiority of gefitinib in the subgroup of 174 patients with high EGFR gene copy number: in this setting, median survival was 8.4 months in the gefitinib group and 7.5 months in the docetaxel group, and 1-year survival was 32 and 35%, respectively (HR 1.09; 95% CI 0.78–1.151, P =.620). The most common adverse events in the gefitinib group were skin reactions (49% versus 10%) and diarrhea (35% versus 25%), whereas in docetaxel group neutropenia (5% versus 74%), asthenia (25% versus 47%), and alopecia (3% versus 36%). Significantly more patients had sustained clinically relevant improvement in quality of life with

gefitinib than with docetaxel, as assessed by FACT-L total score (OR 1.99, 95% CI 1.42–2.79; P < .0001) and the FACT-L-TOI (OR 1.82, 95%CI 1.23–2.69; P = .0026). Similar proportions of patients had improvements in lung cancer symptoms (FACT-L LCS) with gefitinib and docetaxel (OR 1.29, 95%CI 0.93–1.79; P = .013). Moreover, a biomarkers analysis was conducted in this trial on 453 patients (31%) who had tissue samples evaluable for at least one biomarker (EGFR copy number by fluorescent in situ hybridization, EGFR protein expression by immunohistochemistry, and EGFR and KRAS mutations) and showed no difference in overall survival between treatments for any biomarker [34]. However, notably, among patients with EGFR mutationpositive tumors, PFS was longer (HR 0.16; 95% CI 0.05-0.49, P = .001) and objective response was higher (42.1%) versus 9.8%) for gefitinib as compared to docetaxel. Overall survival was longer in patients with EGFR mutation-positive tumors in both gefitinib and docetaxel subgroups (median survival 14.2 and 16.6 months, resp.) than in the overall population (7.6 and 8.0 months, resp.) and in the population with wild-type EGFR (6.4 and 6.0 months, resp.), but there was no difference between treatments. Finally, exploratory analyses showed no difference between patients with high and low EGFR copy number within the gefitinib arm (high versus low HR, 1.02, 95% CI 0.74–1.41, P = .914) and no significant differences in survival outcome between the study arms according to KRAS mutation status.

Two further randomized phase 3 clinical trials (conducted in Japan and Korea, resp.) compared gefitinib versus docetaxel in patients with locally advanced or metastatic NSCLC, pretreated with one or two chemotherapy regimens [35, 36]. The Japanese trial did not meet the primary objective (non inferiority of gefitinib versus docetaxel) in terms of overall survival (11.5 months for gefitinib versus 14 months for docetaxel), although fewer severe adverse events (40.6% versus 81.6%) and benefits in terms of quality of life improvement occurred with gefitinib compared with docetaxel [35]. In the Korean study, gefitinib improved significantly objective response rate (28.1 versus 7.6%) and PFS (HR 0.73, 90% CI 0.53–0.98, P = .0441) than docetaxel [36]. However, no differences were observed in terms of OS (14.1 versus 12.2 months in the gefitinib and docetaxel arms, resp.) and quality of life or symptom improvement rates. A meta-analysis of the randomized clinical trials comparing gefitinib to docetaxel was presented at 2009 ASCO Meeting and showed similar overall and progressionfree survival between the two drugs and superior response rate with gefitinib [37]. Therefore, given the similar efficacy demonstrated by gefitinib, its favorable tolerability profile, the quality of life benefits, and the oral administration, the Authors concluded that gefitinib has a favorable benefitrisk profile compared with docetaxel in a broad pretreated advanced NSCLC patient population.

3.4. Phase III Clinical Studies in Adjuvant and Locally Advanced Setting. A single phase 3 trial of adjuvant gefitinib has been conducted to date (the BR.19 trial), starting in the early 2000s, when a great enthusiasm existed for exploring the potential of this drug in NSCLC treatment [38].

In the BR.19 trial, patients with completely resected stage IB to IIIA NSCLC were randomly assigned to receive daily gefitinib 250 mg or placebo, for two years. They could also receive adjuvant chemotherapy as appropriate. The primary end-point was overall survival. The study planned to enrol 1160 patients, but it was stopped prematurely in 2005, following the negative results of the ISEL study [30] and the SWOG S0023 trials [39]. At the time of study closure, 503 patients had been enrolled. Data were presented at 2010 ASCO Annual Meeting. Median age of patients was 67; 54% were male, 54% PS 0 and most of them were ever smoker (89%); most of tumors were adenocarcinoma (59%); only 17% of patients received chemotherapy. Median followup was 4.7 years. Median time on treatment was 4.8 months in both arms. Differences were not significant for both overall survival (HR 1.24; 95% CI 0.94-1.64; P = .14) and disease-free survival (HR 1.22, 95% CI 0.93-1.61; P = .15), with a negative trend for gefitinib treatment. The toxicity analysis excluded the possibility of attributing this disadvantage to a higher incidence of fatal toxicity in the gefitinib arm. Preplanned subgroup analyses according to EGFR mutational status (357 evaluable patients, 76 of whom with mutation) demonstrated no benefit for gefitinib treatment in both wild-type and mutant NSCLC patients, with a more evident negative trend just in patients with EGFR mutations (HR 1.58, 95% CI 0.83–3.00; P = .16). Although all the comparisons have weak power due to the small number of the patients, these results are very striking and preclude the use of adjuvant gefitinib outside from clinical trials.

The above-mentioned SWOG S0023 trial [39] compared maintenance gefitinib to placebo after concurrent chemoradiotherapy and docetaxel consolidation in inoperable stage IIIA and IIIB NSCLC patients. Overall survival was the primary end-point. This study also closed prematurely, on the recommendation of an unplanned interim analysis that was prompted by the results of the ISEL trial. Of the 571 eligible patients registered at the time of the interim analysis (against the 840 planned), 234 were randomized to receive gefitinib 500 mg or placebo, daily for five years. The interim analysis of this study showed that the hypothesized alternative of a 33% improvement in survival with gefitinib over placebo was ruled out with a one-sided P = .0015. Updated results, after a median followup of 27 months, were successively published, showing that patients receiving gefitinib had a worse survival than patients on placebo, with a median survival of 23 compared with 35 months (HR 0.63, 95% CI 0.44–0.91; P = .013). As in the BR.19 trial, the analysis of cancer-related and toxic death revealed that the inferior survival was due to tumor progression and not to gefitinib toxicity. Unfortunately, molecular features of the tumors, including EGFR mutations, were not recorded in this study. The detrimental effect of maintenance gefitinib after optimal cytoreduction with chemoradiotherapy in stage III NSCLC reported by the S0023 trial excludes the use of the drug in this setting of disease.

The evidence coming from these two randomized trials do not support the use of gefitinib in the localized stages of NSCLC patients, even with tumors carrying EGFR mutations. The intrathoracic disease could have a different biologic behavior that should be further explored.

3.5. Randomized Clinical Studies in Special Populations. In consideration of its good toxicity profile, gefitinib has been tested as an alternative to a single-agent chemotherapy in elderly and poor performance status (PS) NCSLC patients. A randomized phase II trial was conducted by Crinò et al. with gefitinib (250 mg daily) versus vinorelbine in 196 untreated elderly (\geq 70 years) NSCLC patients [40]. The trial was designed to determine the superiority of gefitinib versus vinorelbine in terms of progression-free survival. The results showed no statistical difference in progressionfree survival (2.7 versus 2.9 months, HR 1.19, 95% CI, 0.85-1.65, P = .310, overall survival (5.9 versus 8.0 months; HR 0.98, 95% CI, 0.66-1.47), and response rate (3.1 versus 5.1%) between gefitinib and vinorelbine, respectively. However, gefitinib showed a better toxicity profile. Most of the enrolled patients were male (77%), smokers (82%), and with squamous cell carcinoma, thus without clinical features conferring sensitivity to gefitinib, and this may explain the low percentage of responders in this study.

Goss et al. compared gefitinib to BSC in 201 untreated NCSLC patients with PS \geq 2, not eligible for chemotherapy, in a randomized phase II trial [41]. Primary endpoint was PFS and, nevertheless the results showed no statistical difference, there was a trend toward improved progression-free survival (HR 0.82, 95% CI, 0.60–1.12, *P* = .217), overall survival (HR 0.84, 95% CI, 0.62–1.15, *P* = .272) and response rate (6% versus 1 % placebo) in favor of gefitinib.

4. Development of Gefitinib in "Selected" Patients

4.1. *Phase II Clinical Studies.* The recent discovery that some somatic mutations in the tyrosine kinase domain of the EGFR gene are associated with a high response to EGFR tyrosine kinase inhibitors in NSCLC highlighted the need for patient selection through molecular screening [42, 43].

Several phase 2 studies showed a high response rate (55-90%) and a prolonged progression-free survival (of approximately 9 months) with first-line gefitinib in Asiatic patients selected on the basis of the presence of activating EGFR gene mutations [44-56]. The results of these studies are summarized in Table 3. Yang and colleagues observed in 43 patients with exon 19 deletions and L858R mutations a response rate of 95% and 73.9% and a progressionfree survival of 8.9 and 9.1 months, respectively [46]. The iTARGET trial selected chemo-naïve patients with nonsquamous histology who had one or more clinical characteristics associated with activating EGFR mutations, such as low or never smoking history, adenocarcinoma histology, female gender, and East Asian ethnicity [47]. In this study, mutations were identified in 35% of patients and 31 patients received gefitinib: the response rate was 55%, the median progression-free survival was 9.2 months, and overall survival was 17.5 months. Actually, the response rate was 78% and 59% for patients carrying L858R mutation and exon 19 deletion, respectively, which are activating

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TABLE 3: Phase II trials with Gefitinib as single agent in "selected" patients with NSCLC.

Author (yr)	Setting	Pts	Treatment	Results	Toxicity
Asahina et al. (2006) [44]	Chemonaive, EGFR mutation	16	Gefitinib 250 mg	RR: 75%; PFS: 8.9 months, 1 yr OS: 88%	Rash, hepatotoxicity
Inoue et al. (2006) [45]	Chemonaive, EGFR mutation	16	Gefitinib 250 mg	RR: 75%; PFS: 9.7 months; 1 yr OS: 88%	Skin toxicity, stomatitis, diarrhea
Yang et al. (2008) [46]	Chemonaive, EGFR mutation	55	Gefitinib 250 mg	RR: 84.2%; PFS: 8.9 months, OS: 24 months	Skin toxicity, hepatotoxicity, diarrhea
Sequist et al. (2008) [47]	Chemonaive, EGFR mutation	31	Gefitinib 250 mg	RR: 55%; PFS: 9.2 months, OS: 17.5 months	Skin toxicity, diarrhea, nausea, fatigue
Sutani et al. (2006) [48]	1st-2nd line, EGFR mutation	27	Gefitinib 250 mg	RR: 78%; PFS: 9.4 months, OS: 15.4 months	Diarrhea, skin toxicity
Yoshida et al. (2007) [49]	Chemonaive, EGFR mutation	21	Gefitinib 250 mg	RR: 90%; TTP: 7.7 months	Skin toxicity, diarrhea, hepatotoxicity
Sunaga et al. (2007) [50]	Chemonaive, EGFR mutation	19	Gefitinib 250 mg	RR: 76%; DSR: 90%; TTP: 12.9 months	Skin toxicity
Tamura et al. (2008) [51]	Chemonaive, EGFR mutation	27	Gefitinib 250 mg	RR: 75%; DSR: 96%; PFS: 11.5 months,1 yr OS: 79%	Skin toxicity, hepatotoxicity, stomatitis, diarrhea
Sugio et al. (2009) [52]	Chemonaive, EGFR mutation	19	Gefitinib 250 mg	RR: 63.2%; PFS 7.1 months, OS: 20 months	Skin toxicity, nail change
Inoue et al. (2009) [53]	Chemonaive, EGFR mutation and poor PS	30	Gefitinib 250 mg	RR: 66%, DSR 90%, PFS 6.5 months, OS 17.8 months, PS improvement rate: 79%	Hepatotoxicity, anemia, skin toxicity
Cappuzzo et al. (2007) [54]	FISH positive or never smokers	42	Gefitinib 250 mg	RR: 47.6%; PFS: 6.4 months; 1 y OS: 64%.	Skin toxicity, diarrhea
West et al. (2006) [55]	Brochoalveolar] carcinoma, 1st-2nd line	91	Gefitinib 500 mg	RR: 9% and OS 13 months in 2nd line; RR 17% and OS 13 months in 1st line	Skin toxicity, diarrhea
D. H. Lee et al. (2005) [56]	Adenocarcinoma and never smokers	37	Gefitinib 250 mg	RR: 69%; PFS: 33 weeks; 1 yr OS: 73%	Skin toxicity, diarrhea

RR: response rate; PFS: progression-free survival; OS: overall survival; DSR: disease stabilization rate; TTP: time to progression.

mutations, predictive for response to gefitinib, whereas it was 0% in patients with atypical mutations. Therefore, this study has demonstrated that genotype-directed EGFR-TKI therapy with gefitinib for patients with previously untreated NSCLC is feasible also in a Western population. Inoue et al. tested gefitinib in a phase II trial in NSCLC patients harbouring EGFR mutations and with poor PS, not eligible for chemotherapy [53]. To note, 22 of 30 patients had very poor PS (3 or 4). The overall response rate was 66%, with disease stabilization rate of 90%. PS improvement rate was 79%. The median progression-free and overall survival were 6.5 and 17.8 months, respectively. This is the first report indicating that EGFR mutation-positive patients with poor PS can benefit from front line gefitinib treatment. Others phase II trials have selected patients on the basis of clinical, pathological, or molecular features. The ONCOBELL trial enrolled 42 patients who were never smokers or who had evidence of a high EGFR gene copy on FISH and were p-AKT positive [54]. The response rate was 47.6%, the median time to progression was 6.4%, and 1-year survival rate was 64.3%. In EGFR-mutated patients (66.8%), the response rate was 62.5%. The Southwest Oncology Group performed a phase II trial for pretreated (n = 22) or untreated (n = 69)patients with bronchioalveolar carcinoma [55]. The dose of gefitinib used in this trial was 500 mg/day. The response rate in the pretreated and untreated patients was 9% and 17%, respectively. Finally, a phase II trial investigated the activity of gefitinib 250 mg daily in 37 chemo-naïve Korean patients with adenocarcinoma and a never-smoking history [56]. The response rate was 69% with a disease stabilization rate of 81%; median progression-free survival and 1-year survival rate were 33 weeks and 73%, respectively.

4.2. Phase III Clinical Studies. Four randomized phase III clinical trials evaluated the role of gefitinib as first line therapy of patients with advanced NSCLC, selected on the basis of clinical or molecular features (Table 4) [57–60]. The first evidence of efficacy of a therapeutic strategy based on an EGFR tyrosine kinase inhibitor as a first-line treatment of patients with advanced NSCLC derived from a large, randomized phase 3 clinical trial conducted in Asian patients, the IPASS study [57]. The IPASS (IRESSA Pan Asia Study) trial randomized 1217 patients with advanced adenocarcinoma, non-smoker or former light smoker, to receive gefitinib, 250 mg daily until progression or unacceptable toxicity, or carboplatin (AUC 5-6) plus paclitaxel (200 mg/m²) for a maximum of 6 cycles. The study met the primary objective (non inferiority of gefitinib) and

Author (yr)	Study	Setting	Pts	Treatment	RR (%)	PFS (mos)	OS (mos)
Mok et al. (2009) [57]	IPASS	1st line, clinically selected	1217	Gefitinib versus Carboplatin + Paclitaxel	43 versus 32.3 (<i>P</i> = .0001)	5.7 versus 5.8 HR: 0.74, 95% CI: 0.65–0.85, <i>P</i> < .0001	18.6 versus 17.3 HR: 0.91, 95% CI: 0.76–1.10
		Subgroup of EGFR mutated	261		71.2 versus 47.3 (<i>P</i> < .001)	9.5 versus 6.3 HR: 0.48, 95% CI: 0.36–0.64, <i>P</i> < .001	HR: 0.78, 95% CI 0.50–1.20
J. S. Lee et al. (2009) [58]	FIRST SIGNAL	1st line, clinically selected	309	Gefitinib versus Cisplatin + Gemcitabine	53.5 versus 45.3 (<i>P</i> = .153)	6.1 versus 6.6 HR: 0.813, 95% CI: 0.641–1.031, <i>P</i> = .044	21.3 versus 23.3 HR: 1.003, 95% CI: 0.749–1.343, P = .428
		Subgroup of EGFR mutated	42		84.6 versus 37.5 (<i>P</i> = .002)	8.5 versus 6.7 HR: 0.613, 95% CI: 0.308–1.221, P = .084	30.6 versus 26.5 HR: 0.823, 95% CI: 0.352–1.922, <i>P</i> = .648
Mitsudomi et al. (2010) [59]	WJTOG 3405	1st line, EGFR mutated	172	Gefitinib versus Cisplatin + Docetaxel	62.1 versus 32.2 (<i>P</i> < .0001)	9.2 versus 6.3 HR 0.489, 95% CI: 0.336–0.710, <i>P</i> < .0001	30.9 versus nr HR: 1.638, 95% CI 0.749–3.582, <i>P</i> = .211
Maemondo et al. (2010) [60]	NEJ002	1st line, EGFR mutated	230	Gefitinib versus Carboplatin + Paclitaxel	73.7 versus 30.7 (<i>P</i> < .001)	10.8 versus 5.4 HR 0.30, 95% CI: 0.22–0.41, P < .001	30.5 versus 23.6 $P = .31$

TABLE 4: Phase III trials with Gefitinib in "selected" NSCLC.

RR: response rate; PFS: progression-free survival; OS: overall survival; nr: not reached.

also demonstrated the superiority of gefitinib compared to carboplatin and paclitaxel in terms of progression-free survival in intention-to-treat analysis (HR 0.74, 95% CI: 0.65-0.85, P = .001). Because of the crossing of the curves, the median progression-free survival is similar with both treatments: however, the pattern of progression-free rates favors chemotherapy for the first 6 months and gefitinib for the remaining 16 months. The initial superiority of chemotherapy was attributed to the benefit that the EGFRmutation-negative subgroup received from chemotherapy but not from gefitinib, whereas prolonged progression-free survival in the EGFR-mutation-positive subgroup explained the subsequent improvement favoring gefitinib. Crossing of the curves did not occur in the mutation-positive subgroup or the mutation-negative subgroup. Another important finding of this study was the significant interaction between treatment efficacy and EGFR mutational status. In the subgroup of patients with EGFR mutation (261 of 437 available samples), progression-free survival was significantly longer (HR 0.48; 95% CI 0.36-0.64, P = .001), and the response rate was significantly higher with gefitinib than with carboplatinpaclitaxel (71.2% versus 47.3%, P = .001). On the contrary, in the mutation-negative subgroup, progression-free survival was significantly shorter (HR 2.85; 95% CI 2.05–3.98, P <.001) and response rate was significantly lower with gefitinib (23.5% versus 1.1%, P = .001). Overall survival data were immature, based on only 37.0% of events, and showed a similar overall survival between the two groups: 18.6

months with gefitinib and 17.3 months with carboplatinpaclitaxel (HR for death in the gefitinib group, 0.91; 95% CI, 0.76 to 1.10). Final overall survival data confirmed no difference between gefitinib and chemotherapy, in the whole population (18.8 months with gefitinib versus 17.4 months with chemotherapy, HR 0.90, 95% CI: 0.79-1.02, P = .11) and in the mutation positive subgroup (HR 1.00, 95% CI: 0.76–1.33) [61]. Patients in the gefitinib group had a clinically relevant improvement in quality of life, as assessed by FACT-L questionnaire (OR 1.34; 95% CI 1.06-1.69, P = .01) and by TOI (Trial Outcome Index) scores (OR 1.78; 95% CI 1.40–2.26; P < .001). Moreover, gefitinib was associated with a lower rate of grade 3 or 4 adverse events compared to chemotherapy. The incidences of rash or acne, diarrhea, and elevated aminotransferase levels were significantly higher with gefitinib, whereas neurotoxic effects, nausea and vomiting, and hematologic toxic effects were significantly higher with carboplatin-paclitaxel. Interstitial lung disease events (i.e., the acute respiratory distress syndrome, interstitial lung disease, pneumonitis, or radiation pneumonitis) occurred in 16 patients treated with gefitinib (2.6%) and in 8 patients treated with chemotherapy (1.4%).

A second randomized phase 3 clinical trial compared gefitinib (250 mg daily) with cisplatin-gemcitabine as a firstline treatment in 309 Asian, never smokers patients, with advanced adenocarcinoma [58]. The study failed to reach its primary endpoint, overall survival, even if gefitinib allowed the achievement of a favorable response rate: 53.5% for gefitinib versus 45.3% for chemotherapy (OR 1.385, 95% CI 0.885–2.167, P = .153). The overall mutation rate in this study was 43.8%: in mutation positive patients, the response rate was 84.6% for gefitinib versus 37.5% for chemotherapy (P = .002), while, in mutation negative subgroup, the response rate was 29.9% for gefitinib versus 51.9% for chemotherapy (P = .051). Median overall survival and progression-free survival were similar between the two groups. There was some difference in progression-free survival favoring gefitinib in mutation positive patients (8.5 versus 6.7 months; HR 0.613, 95% CI 0.308–1.221, P = .0849). There was no difference in overall survival by mutation status, both in the overall and EGFR-mutated populations: it could be due to the poststudy use of EGFR TKIs in 80.7% of chemotherapy arm.

Two randomized phase 3 studies have been performed in Japanese, EGFR-mutated patients with advanced NSCLC, to compare the efficacy of gefitinib versus chemotherapy in the first-line setting. In the open label phase III WJTOG3405 trial, 172 EGFR mutated patients were randomly assigned to receive gefitinib (250 mg daily) or chemotherapy (cisplatin 80 mg/m² plus docetaxel 60 mg/m² adminstered every 21 days for three to six cycles) [59]. The primary endpoint was progression-free survival. The study met its endpoint, showing a median progression-free survival of 9.2 months in the gefitinib group versus 6.3 months in the chemotherapy group (HR 0.489, 95% CI: 0.336-0.710, P = .0001). In this molecularly selected population, progression-free survival curves did not cross, unlike IPASS trial, being the benefit of gefitinib over chemotherapy consistent at any time of treatment. Response rate was 62.1% and 32.2% with gefitinib and chemotherapy, respectively (P < .0001). Myelosuppression, alopecia and fatigue were more frequent in the cisplatin-docetaxel group, while skin toxicity, liver dysfunction, and diarrhea in the gefitinib group.

Another prospective phase III study, the NEJ002 Trial, compared gefitinib to chemotherapy with carboplatin and paclitaxel as a first-line treatment in advanced NSCLC patients selected for EGFR mutation [60]. The study was stopped by independent data and safety monitoring committee after the preplanned interim analysis, conducted 4 months after the 200th patient enrolled, because it showed a significant difference in progression-free survival between the two treatment groups. The median progression-free survival was 10.4 months versus 5.5 months for gefitinib and chemotherapy, respectively (HR 0.36, 95% CI: 0.25-0.51, P < .001), and the final analysis confirmed these results, showing a median PFS of 10.8 versus 5.4 months for gefitinib and chemotherapy, respectively (HR 0.30, 95% CI 0.22-0.41, P < .001). The response rate was significantly higher in the gefitinib than chemotherapy arm (73.7% versus 30.7%, P < .001). The median progression-free survival and overall survival did not differ significantly between patients with exon 19 deletion and those with L858R point mutation (11.5 months versus 10.8 months, resp.). The overall survival did not differ significantly between the two treatment groups (median survival time and the 2-year survival rate were 30.5 months and 61.4% for gefitinib group as compared with 23.6 months and 46.7% for the chemotherapy, resp., P = .31).

Importantly, among 112 patients who had completed firstline carboplatin-paclitaxel, 106 (94.6%) received second-line gefitinib and 58.5% of these patients had a response. The most common adverse events in the gefitinib group were rash and elevated levels of aspartate aminotransferase or alanine aminotransferase and, in the chemotherapy arm, appetite loss, neutropenia, anemia, and sensory neuropathy. Interstitial lung disease was reported in 6 patients (5.3%) in the gefitinib arm, with one of these fatal. In general, the incidence of severe toxic effects (NCI-CTC \geq 3) was significantly higher in the chemotherapy group than in the gefitinib group (71.7% versus 41.2%, *P* < .001).

Therefore, these both studies confirmed gefitinib to be superior to chemotherapy in terms of response rate and progression-free survival in patients with EGFR mutations.

5. Ongoing Phase III/IV Studies in NSCLC

Several phase III/IV studies are currently ongoing with gefitinib in NSCLC in different clinical settings (Table 5).

A double-blind, multicenter, randomized, placebocontrolled phase III study is evaluating the efficacy, safety, and tolerability of gefitinib as a maintenance therapy in 296 patients with locally advanced or metastatic NSCLC (INFORM trial, ClinicalTrials.gov ID: NCT00770588). Patients must have completed 4 cycles of platinum-based first-line doublet chemotherapy without experiencing disease progression or unacceptable toxicity and are randomized to gefitinib or placebo at 1:1 ratio. The primary endpoint is progression-free survival; secondary endpoints are overall survival, objective tumor response, quality of life, and safety profile in terms of adverse events.

Another randomized phase III trial is evaluating the efficacy of a maintenance therapy with gefitinib compared with placebo in 600 Japanese patients treated with first-line chemotherapy for stage IIIB or IV NSCLC (ClinicalTrials.gov ID: NCT00144066). The primary aim of the study is to determine if gefitinib improves overall survival of the patients that did not progress on prior first line induction chemotherapy. Secondary objectives are progression-free survival and safety profile.

A phase IV study is investigating the activity and safety of gefitinib as first-line therapy for 100 Caucasian patients with EGFR-positive mutations (ClinicalTrials.gov ID: NCT01203917). The primary endpoint is the objective response rate; secondary endpoints are disease control rate, safety data, and overall survival.

A randomized phase III clinical study will compare gefitinib versus pemetrexed in never-smoker patients with adenocarcinoma histotype, previously treated with platinumbased chemotherapy (ClinicalTrials.gov ID: NCT01066195). The estimated enrollment is of 129 patients, and the main endpoints are progression-free survival, overall survival, objective response rate, and toxicity.

A randomized, open label, phase III study is enrolling 226 East Asian never or light ex-smoker patients with locally advanced or metastatic nonsquamous NSCLC, with the aim to compare first line cisplatin + pemetrexed for 6 cycles

Study phase	Line of treatment	ClinicalTrials.gov ID	Setting	Estimated sample size (pts)	Treatment	Primary endpoint
III	1st	NCT00770588	Maintenance after first line platinum-based chemotherapy	296	Gefitinib 250 mg versus Placebo	Progression-free survival
III	1st	NCT00144066	Maintenance after first line platinum-based chemotherapy	600	Gefitinib 250 mg versus Placebo	Overall survival
IV	1st	NCT01203917	Selected Caucasian pts	100	Gefitinib 250 mg	Objective response rate
III	≥2nd	NCT01066195	Never smoker pts with adenocarcinoma	129	Gefitinib 250 mg versus Pemetrexed	Progression-free survival
III	1st	NCT01017874	Selected East Asian pts	226	Gefitinib alone versus Cisplatin- Pemetrexed → Gefitinib	Progression-free survival
IV	1st	NCT00173524	First line Asian pts	200	Gefitinib versus Platinum-based chemotherapy	Cost- effectiveness
					Systemic and radiant	
III	1st-2nd	NCT00955695	Never smoker adenocarcinoma pts treated with prophylactic cranial irradiation	242	Prophylactic whole brain radiation therapy during gefitinib 250 mg or erlotinib 150 mg	Incidence of symptomatic brain metastases

TABLE 5: Ongoing phase III/IV studies in NSCLC.

followed by gefitinib for 6 courses (each of 21 days) versus gefitinib alone for 6 courses (each of 21 days) (ClinicalTrials.gov ID: NCT01017874). Primary endpoint is progression-free survival; secondary endpoints are overall survival, tumor response rate, disease control rate, time to progression, duration of response, and time to worsening of health-related quality of life. The trial should be completed in 2013.

A multicenter randomized phase III study is investigating the efficacy of whole brain radiation therapy compared with observation in preventing brain metastases in 242 patients with advanced NSCLC responding to first- or second-line gefitinib (250 mg/day) or erlotinib (150 mg/day) administered continuatively until disease progression or unacceptable toxicity (ClinicalTrials.gov ID: NCT00955695). Patients must be never smoker, with a diagnosis of adenocarcinoma with the EGFR-positive mutations on exon 19 or 21. Prophylactic cranial irradiation consists of 25 Gy cumulative dose over 10 fractions. The primary endpoint is the incidence of symptomatic brain metastases. Secondary endpoints are overall survival, progression-free survival, safety, psycho-neurological effects, and quality of life.

A phase IV pharmacoeconomics study will have the objective to analyze the cost-effectiveness and the cost-utility of gefitinib as a first-line treatment for 200 patients affected by stage IIIB or IV NSCLC, compared with the conventional first-line platinum-based chemotherapy (ClinicalTrials.gov ID: NCT00173524).

6. Discussion and Conclusions

The development of gefitinib in NSCLC is a clear example of the difficulties in designing and conducting of clinical trials with new molecular-targeted agents and of the uncertainty about predictive factors and selection criteria [62]. Crucial points, regarding the methodology of clinical research with target-based agents, especially for phase 3 trials, are how should patients be selected and which patients are expected to benefit from a targeted agent [63]. The proper characterization of a molecular target that allows the identification of responding versus nonresponding patients to a moleculartargeted agent could have important implications for the design of randomized trials evaluating the efficacy of the drug. In fact, the presence of unrecognized molecular heterogeneity can result in a falsely negative study that could be underpowered and may fail to detect a truly effective new therapy, leading to the rejection of a potentially useful drug [64].

Gefitinib was the first targeted drug that entered into clinical practice for the treatment of lung cancer: however, the positive results obtained in early clinical trials were not confirmed in large phase 3 trials, testing the efficacy of gefitinib in unselected patients with advanced NSCLC and, therefore, the use of gefitinib in clinical practice was stopped for several years.

It has been then shown that the presence of somatic mutations in the kinase domain of EGFR strongly correlates with increased responsiveness to EGFR tyrosine kinase inhibitors in patients with advanced NSCLC and that a substantial percentage of tumors with objective response to gefitinib or erlotinib harbours somatic mutations in the EGFR gene [42, 43, 65]. Moreover, clinical and demographic factors, including female sex, nonsmoking status, adenocarcinoma histotype, and Asian race have been identified as potentially predictive of the efficacy of EGFR tyrosine kinase inhibitors.

Currently, 4 randomized clinical trials have demonstrated the efficacy of gefitinib as a first-line treatment of NSCLC patients harbouring EGFR mutations: the IPASS and the First-SIGNAL studies, conducted in Asian patients selected for clinical factors; the WJTOG 3405 and the NEJ002 studies, conducted in patients selected for the presence of EGFR-activating mutations [57-60]. All these trials have demonstrated a statistically significant increase in progression-free survival with gefitinib compared to platinum-based chemotherapy in patients with EGFRmutated advanced NSCLC. Moreover, treatment with gefitinib was associated with evidence of high objective response rate, better quality of life and more favourable toxicity profile. On these bases, in July 2009 gefitinib received from EMEA the authorization for the treatment of locally advanced or metastatic NSCLC with activating mutations of EGFR, across all lines of therapy and, currently, it can be considered the standard first-line treatment of patients with advanced NSCLC harbouring EGFR mutations.

Similar results have been recently obtained with erlotinib in a phase 3 clinical trial conducted in China (the OPTIMAL trial), comparing erlotinib to gemcitabine plus carboplatin, in EGFR-mutation-positive tumors in terms of progressionfree survival [66]. The OPTIMAL study showed that erlotinib was significantly superior to chemotherapy in terms of progression-free survival (13.1 versus 4.6 months, HR 0.16, 95% CI: 0.10–0.26, P < .0001) and also in terms of objective response rate (83% versus 36%).

On the contrary, a treatment strategy based on a tyrosine kinase inhibitor (erlotinib) as a first-line therapy, followed at progression by chemotherapy in unselected patients with advanced NSCLC, is inferior to standard treatment with a first-line platinum-based doublet, followed at progression by erlotinib and cannot be recommended in clinical practice [67].

Several questions need to be addressed, regarding the reproducibility of these results in Western patients with NSCLC and EGFR mutations, the proper tyrosine kinase selection (gefitinib versus erlotinib), the lack of a survival benefit with first-line gefitinib in all these studies, the most appropriate clinical use of TKIs in mutated patients (first versus second line), the efficacy of gefitinib as neoadjuvant therapy or in combination with radiotherapy in patients with locally advanced NSCLC and EGFR mutations, and overcoming resistance to tyrosine kinase inhibitors. A large randomized phase 3 trial (the EURTAC trial, ClinicalTrial.gov ID NCT00446225) testing erlotinib in Western patients harbouring EGFR mutation is addressing the first question. Conversely, there are no ongoing phase 3 trials that directly compare gefitinib with erlotinib: therefore, the choice of the tyrosine kinase inhibitor to use in clinical practice should

be based on evidence coming from these randomized trials. Whether progression-free survival prolongation translates into survival gain is not yet clear: mature data from the IPASS trial showed no survival difference between first-line gefitinib and chemotherapy, probably due to treatment cross-over of patients with tumor harbouring EGFR mutation [61].

The lack of a survival benefit with first-line gefitinib raises the question regarding its use as first- or secondline therapy, in patients selected by the presence of EGFR mutation. Data from Western and Asian patients suggest that there was no statistically significant difference in overall survival between patients receiving EGFR inhibitors as a first-line therapy or after failure of previous chemotherapy [68, 69]. However, these analyses were not based on a prospective comparison between the two strategies (firstversus the second-line therapy with EGFR inhibitors). Moreover, it should be considered that, for patients who do not receive first-line tyrosine kinase inhibitors, there is the risk of never receiving an EGFR inhibitor at the time of disease progression, due to a rapid worsening of clinical conditions. Finally, the better quality of life and more favourable toxicity profile with first line gefitinib, in addition to the prolonged progression-free survival, compared to chemotherapy, strongly support the use of gefitinib as a firstline therapy in patients with activating EGFR mutations.

The final results of ongoing clinical trials should define the efficacy of gefitinib also as neoadjuvant therapy or in combination with radiotherapy in patients with locally advanced NSCLC and EGFR mutations, while the development of irreversible inhibitors of EGFR tyrosine kinases [70] may have the potential to overcome the resistance to tyrosine kinase inhibitors.

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Research Article

Intermittent Chemotherapy and Erlotinib for Nonsmokers or Light Smokers with Advanced Adenocarcinoma of the Lung: A Phase II Clinical Trial

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Background. Intermittent application of chemotherapy and tyrosine kinase inhibitors may avoid antagonism between the two classes of drugs. This hypothesis was tested in a Phase II clinical trial. *Patients and Methods*. Eligible patients were nonsmokers or light smokers, chemo-naïve, with metastatic adenocarcinoma of the lung. Treatment: 4 to 6 cycles of gemcitabine 1250 mg/m² on days 1 and 4, cisplatin 75 mg/m² on day 2, and erlotnib 150 mg daily on days 5–15, followed by erlotnib as maintenance. *Results*. 24 patients entered the trial. Four pts had grade 3 toxicity. Complete remission (CR) and partial remission (PR) were seen in 5 pts and 9 pts, respectively (response rate 58%). Median time to progression (TTP) was 13.4 months and median overall survival (OS) was 23 months. When compared to patients with negative or unknown status of EGFR mutations, 8 patients with EGFR gene activating mutations had significantly superior experience: 4 CR and 4 PR, with median TTP 21.5 months and OS 24.2 months (P < .05). *Conclusions*. Intermittent schedule with gemcitabine, cisplatin and erlotnib has mild toxicity. For patients who are positive for EGFR gene activating mutations, this treatment offers excellent response rate, time to progression and survival.

1. Introduction

To the surprise and deep disappointment of all involved in the treatment of lung cancer, several large trials did not demonstrate any benefit of tyrosine kinase inhibitors (TKIs) as an addition to chemotherapy [1–3]. Virtually all further clinical research on combinations of TKIs and chemotherapy was then abandoned. Basic and clinical research then focused on mutations of the gene for epidermal growth factor receptor (EGFR) as a predictive factor for response to monotherapy with TKIs and to development of new compounds with broader and/or irreversible inhibition.

The biological basis for the negative experience with combined treatment was never given proper attention. Gefitinib and erlotinib met all three standard criteria for inclusion in a combination with chemotherapy: activity as monotherapy, different mechanism of action, and different toxicity. Why, then, did the combination not work? As explained in a recent editorial [4], we believe that the cells of tumors sensitive to TKIs are pushed into the G-0 phase of the cell cycle and therefore become resistant to cytotoxic drugs. If antagonism between the two classes of drugs is really the biological basis for the aforementioned negative experience, then an optimal combination of TKIs and chemotherapy should be in an intermittent, rather than a continuous schedule.

This brief report presents a single-institution experience on intermittent chemotherapy and TKI in a small series of patients with advanced adenocarcinoma of the lung. Our hypothesis was that intermittent treatment would lead to superior time to progression, when compared to experience with chemotherapy alone. If confirmed, such a result would be a solid basis for a randomised clinical trial.

2. Patients and Methods

2.1. Inclusion Criteria. Patients eligible for the trial were chemonaïve with microscopically confirmed adenocarcinoma of the lung, had stage III B (wet) or IV according to UICC-TNM classification (6th edition), had smoking history of less than 10 packs in years, had an ECOG performance status 0 or 1, and had adequate parameters of hematological, liver, and renal function to receive cisplatin-based chemotherapy. In the absence of neurological symptoms, patients with brain metastases were eligible and were treated with brain irradiation only in case of intracranial progression. All patients were fully informed and gave written consent to participate in the trial.

2.2. *Initial Diagnostics*. All patients had their diagnosis confirmed by biopsy or cytology. At the time when the trial was initiated, testing for EGFR mutations was not available.

Within three weeks prior to treatment, the precise extent of the disease was determined by chest X-ray and CT scanning of the chest, upper abdomen, and brain. Since 2008, PET-CT scanning has been available and included in the initial diagnostics and in followup.

2.3. *Treatment*. The treatment started with four cycles of intermittent chemotherapy and erlotinib according to the following schedule:

day 1: gemcitabine 1250 mg/m^2 in 30-minute infusion,

day 2: cisplatin 75 mg/m^2 , with appropriate hydration and antiemetics,

day 4: gemcitabine 1250 mg/m^2 in 30-minute infusion,

days 5–15: erlotinib 150 mg daily p.o.

Cycle was repeated on day 22.

Patients received 4 to 6 cycles of intermittent treatment. The number of cycles depended on tolerance to cisplatinbased chemotherapy and was determined individually. Immediately after the last cycle, patients continued with erlotinib 150 mg/m^2 daily continuously until progression or unacceptable toxicity.

2.4. Monitoring for Response, Time of Progression, and Followup. Definition of complete response (CR), partial response (PR), stable disease (SD), and progression followed the RECIST criteria [5].

The first evaluation of response was done during the third cycle of intermittent therapy, with confirmation of response during the fifth cycle. After 4 cycles, patients were seen every second month. Control radiological examinations were repeated every 2 months for chest X-ray, every 4 months for CT, and at 6 and 12 months for PET-CT (only patients who had this examination during their initial diagnostics).

2.5. Posttreatment Analysis of Archived Bioptic Material. In October 2010, all biopsy samples were reviewed, and specimens with more than 10% of tumour tissue were analyzed. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue sections using QIAAmp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantification of extracted DNA was done on Qubit Fluorometer (Invitrogen, Carlsbad, USA). To detect EGFR gene-activating mutations, we used TheraScreen EGFR29 Mutation Kit (DxS Diagnostics, Qiagen, Manchester, UK). All realtime PCR reactions were performed in a $25 \,\mu$ L final volume on ABI 7500 instrument (Applied Biosystems, Carlsbad, USA).

2.6. Endpoints and Statistical Planning. The primary endpoint was time to progression. Secondary endpoints were response rate, toxicity, and overall survival.

After standard chemotherapy for metastatic nonsmall cell carcinoma, the expected TTP is 5 months. The size of this single-arm nonrandomised trial of intermittent therapy was based on the assumption of 9 months as the median time to progression (TTP). To obtain such a result with a confidence interval of 6–12 months, we planned to recruit 40 patients.

2.7. Ethical Considerations. The investigators strictly followed recommendations of the Helsinki Declaration (1964, with later amendments) and of the European Council Convention on Protection of Human Rights in Bio-Medicine, as accepted in Oviedo in 1997. The protocol was approved by the Institutional Review Board (Institute of Oncology, Ljubljana) and by the National Committee for Medical Ethics, Ministry of Health, Republic of Slovenia.

3. Results

3.1. Patient Population. Between September 2005 and July 2010, 25 patients were recruited into the trial. One patient was later found to have metastatic carcinoma of the pancreas rather than primary lung cancer and was excluded from all further analyses.

With 12 patients each, male and female patients were equally represented. Median age was 50 years (range: 25 to 73 years). Twelve patients were never-smokers, and most were in good general condition (PS 0-1 for 21 patients). With the exception of a single patient with "wet" stage III B, all other patients had stage IV disease. Bone metastases were the most common site of metastatic disease, followed by pleura/pericardium, contralateral lung metastases, and liver. Two or more sites of metastatic disease were documented in 4 and 12 patients, respectively (Table 1).

3.2. Analysis of EGFR Mutations in Bioptic Material. Analysis of the archived bioptic material was completed in October 2010.

Three patients had only cytological diagnosis, and an additional 3 had biopsy samples too small to allow for analysis of EGFR mutations in tumor cells. Of the 18 adequate samples, 8 were positive for EGFR gene-activating mutations.



FIGURE 1: A woman, 39, never-smoker, developed adenocarcinoma of the right lower lobe, T4 N2 M1b with metastases in bone and liver. 18F-FDG PET-CT before treatment (a) and after 4 months (b). Complete remission was confirmed also a year later.



FIGURE 2: The same patient as on Figure 1. Bone metastasis in the right iliac bone (a) and complete response after treatment (b).

3.3. Treatment. The actual number of cycles of intermittent therapy was from 1 to 6 cycles (median: 4 cycles). Due to early progression, one patient did not receive erlotinib as maintenance treatment. In October 2010, 7 patients were still on maintenance treatment with erlotinib, and an additional patient stopped treatment with erlotinib after 12 months in PET-CT confirmed complete remission (Figures 1 and 2). For the remaining patients, median total duration of treatment was 10 months.

3.4. Toxicity. During the initial phase, 3 pts had grade 3 toxicity (2 neutropenia, 1 thrombocytopenia). Side effects of maintenance with erlotinib were skin toxicity (grade 3: 1 pt; grade 2: 11 pts) and diarrhea (grade 2 in 1 pt).

No patient experienced grade 4 or greater toxicity.

3.5. Response to Treatment, Time to Progression, and Survival. All patients are evaluable for response, and no patient has been lost to followup. For the whole group of 24 patients, complete remission (CR) was seen in 5 pts; partial remission (PR) in 9 pts (response rate 58%), minimal response or stable disease (SD) in 8 pts, and progression in 2 pts. A clear and statistically significant (P < .05) correlation was seen between the presence of activating EGFR mutations and response. Among the 8 patients who were positive for EGFR gene-activating mutations, 4 complete and 4 partial remissions were seen. On the other hand, no CR and only 2 PR were seen among the 10 patients negative for mutations (Table 2).

For the whole group, median time to progression (TTP) was 13.4 months, and median overall survival (OS) was 23 months. Again, patients positive for EGFR gene-activating mutations had-superior experience. Median TTP and OS for this group was 21.5 months and 24.2 months, respectively. For patients without EGFR mutations, TTP was 5 months, and OS was 7 months (Table 2 and Figures 3 and 4).

4. Discussion and Conclusions

This clinical trial was launched at a time when routine testing for EGFR gene-activating mutations was not yet available. Selection of patients for a combination of chemotherapy and erlotinib was made on the basis of classical histopathology (adenocarcinoma) and smoking status.

Recent developments led to premature closure of our trial. Since testing for EGFR gene mutations is now available,

Age 50 Range 25–73 Gender 12 Male 12 Female 12 Smoking 12 Never-smoker 12 Light smoker (<10 pack years) 8 Performance status 8 EGOG PS 0 5 1 16 2 3 Stage 1 III B "wet" 1 IV 23 Site(s) of metastatic disease 7 Bone 17 Pleura and pericardium 11 Distant lung 11 Liver and/or suprarenals 10 Distant lymph nodes and/or soft tissues 6 Brain 2 Number of metastatic sites 1 1 8 2 4 3 or more 12		No. of patients
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Never-smoker12Light smoker (<10 pack years)	Smoking	
Light smoker (<10 pack years)8Performance statusEGOG PS 0511623StageIII B "wet"1IV23Site(s) of metastatic disease17Pleura and pericardium11Distant lung11Liver and/or suprarenals10Distant lymph nodes and/or soft tissues6Brain2Number of metastatic sites118243 or more12	Never-smoker	12
Performance statusEGOG PS 0511623Stage1III B "wet"1IV23Site(s) of metastatic disease7Bone17Pleura and pericardium11Distant lung11Liver and/or suprarenals10Distant lymph nodes and/or soft tissues6Brain2Number of metastatic sites118243 or more12	Light smoker (<10 pack years)	8
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Distant lung11Liver and/or suprarenals10Distant lymph nodes and/or soft tissues6Brain2Number of metastatic sites8243 or more12	Pleura and pericardium	11
Liver and/or suprarenals10Distant lymph nodes and/or soft tissues6Brain2Number of metastatic sites818243 or more12	Distant lung	11
Distant lymph nodes and/or soft tissues6Brain2Number of metastatic sites118243 or more12	Liver and/or suprarenals	10
Brain2Number of metastatic sites818243 or more12	Distant lymph nodes and/or soft tissues	6
Number of metastatic sites18243 or more12	Brain	2
1 8 2 4 3 or more 12	Number of metastatic sites	
2 4 3 or more 12	1	8
3 or more 12	2	4
	3 or more	12

TABLE 1: Demographics, prognostic factors, and extent of disease.

it is clear that patients with activating mutations are those who really benefit from TKIs. In addition, standard firstline treatment for patients with activating EGFR mutations is now monotherapy with a TKI [6, 7]. Since continuing a trial with the same selection criteria and without considering the status of EGFR gene activating mutations was not justified, the research group made a decision to close the trial and analyse the experience.

In order to get a longer interval for intermittent erlotinib, gemcitabine was given on days 1 and 4 of the cycle. When compared to the standard day 1 and day 8 schedule, this minor modification in timing of cytotoxic drugs did not have any adverse effect on the tolerance to treatment. Clearly, other platin-based schedules which apply chemotherapy on a 3-weekly basis (such as pemetrexed-cisplatin or paclitaxel-carboplatin) can offer an even longer interval for TKIs and might be considered for future trials of intermittent treatment.

Two other groups recently reported promising experience with intermittent chemotherapy and TKIs. In a trial from the USA, two schedules of intermittent treatment were tested [6]. In combination with pemetrexed (500 mg/m^2 on day 1), erlotinib was given either as a pulse application in a high dose (range: 800 to 1400 mg) given on days 2, 9



FIGURE 3: Progression-free survival in relation to the status of EGFR gene activating mutations.



FIGURE 4: Overall survival in relation to the status of EGFR gene activating mutations

and 16, or in lower doses (150–250 mg daily) on days 2 to 16. Patients had various advanced malignancies, most of which were pretreated. While tolerance to this treatment was good, the small number and heterogeneity of patients recruited into this trial do not allow for any clear conclusion regarding the effectiveness of intermittent treatment. Of more importance is a randomised Phase II trial by Mok

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			EGFR mutations		
		Positive	Negative	Unknown	All
	CR	4		1	5
Response to treatment	PR	4	2	3	9
Response to treatment	SD	—	6	2	8
	Progression	_	2	_	2
Time to progression (months)	Median (95% CI)	21.5 (14.8–27.2)	5.0 (0.9–9.1)	5.0 (3.9–4.1)	13.4 (5.4–20.6)
Survival (months)	Median (95% CI)	24.2	7.0 (0.1–13.9)	11.0	23.0 (10.9–35.2)

TABLE 2: Response to treatment, time to progression, and survival in relation to EGFR mutations.

et al. [7]. This study from Asia compared gemcitabine and either cisplatin or carboplatin to a schedule with addition of intermittent application of erlotinib (150 mg on days 14 to 28 of the cycle) and reported significantly superior TTP with the intermittent schedule. Their experience is most valuable but may not be of direct relevance for the rest of the world, due to the well-known differences in sensitivity of lung cancer to TKIs between Asian and Caucasian patients.

Despite its small size, our trial can offer valuable experience for further research on optimisation of treatment with combinations of chemotherapy and TKIs. Looking at the whole series of patients, we can conclude that intermittent chemotherapy and erlotinib is a treatment of very low toxicity. It is also clear that the efficacy of treatment is closely related to the presence or absence of EGFR gene-activating mutations.

The most important finding is the excellent response rate with a substantial proportion of complete responses and prolonged TTP and OS for patients positive for EGFR gene-activating mutations. For many years, the maximal expectation of a patient with metastatic nonsmall cell lung cancer was a partial remission of relatively short duration in the range of 5 to 9 months. With intermittent treatment, we now see durable complete remissions in a subpopulation of patients. While the number of patients in our trial is small and any definitive conclusion would be premature, we nevertheless believe that further research of intermittent therapy for patients positive for EGFR gene-activating mutations is warranted. A randomised trial comparing first-line TKI as monotherapy to the intermittent schedule should clarify the real value of this new approach.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article **The Role of Proteasome Inhibition in Nonsmall Cell Lung Cancer**

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Lung cancer therapy with current available chemotherapeutic agents is mainly palliative. For these and other reasons there is now a great interest to find targeted therapies that can be effective not only palliating lung cancer or decreasing treatment-related toxicity, but also giving hope to cure these patients. It is already well known that the ubiquitin-proteasome system like other cellular pathways is critical for the proliferation and survival of cancer cells; thus, proteosome inhibition has become a very attractive anticancer therapy. There are several phase I and phase II clinical trials now in non-small cell lung cancer and small cell lung cancer using this potential target. Most of the trials use bortezomib in combination with chemotherapeutic agents. This paper tends to make a state-of-the-art review based on the available literature regarding the use of bortezomib as a single agent or in combination with chemotherapy in patients with lung cancer.

1. Introduction

One of the common strategies for cancer therapy is the targeting of cell homeostasis leading to deregulation of cell processes necessary for survival. In recent years, one of the novel approaches has been the deregulation of protein homeostasis through the obstruction of intracellular protein degradation. This has been done by targeting the ubiquitinproteasome system (UPS). The UPS plays a central role in the targeted destruction of cellular proteins, including cell cycle regulatory proteins. Because these pathways are critical for the proliferation and survival of all cells, and in particular cancerous cells, proteasome inhibition is a very attractive anticancer therapy [1].

The first element of this pathway being investigated as a target is the proteosome. Because the proteasome degrades about 80% of all intracellular proteins [2], the use of a proteasome inhibitor triggers a mixed repertoire of tumor-suppressing and prosurvival pathways in cancer cells [3]. Its inhibition disturbs the critical intracellular balance between proapoptotic and antiapoptotic signals shifting it towards

tumor growth inhibition, apoptosis, and decreased metastasis.

The proteasome inhibitor PS-341 (bortezomib), an already approved agent for the treatment of multiple myeloma, is under evaluation in clinical trials against various malignancies. Here we will review preclinical and clinical data involving this novel anticancer mechanism focusing primarily in the work that has been done in lung cancer. Bortezomib has been tested as single agent and most recently in combination with chemotherapeutic and targeted agents. Multiple targets that directly interact with the proteasome have been described and may represent future focuses of more research and possibly therapeutic development.

2. Action of the Ubiquitin-Proteasome System

The UPS regulates many normal cellular processes including signal transduction, cell cycle control, transcriptional regulation, inflammation, and apoptosis through protein degradation [4]. It requires a series of highly regulated and complex intracellular activities that have not been completely TABLE 1: Antitumor and autoprotective mechanisms triggered by proteasome inhibition. Possible antitumor mechanisms of proteasome inhibitors.

(i) Accumulation of p53, p21, and p27

(ii) Differential effects on pro- and antiapoptotic members of Bcl-2 family

(iii) Downregulation of XIAP and survivins

(iv) Inhibition of inducible NF-kB activity

(v) Accumulation of misfolded proteins and endoplasmic reticulum stress

(vi) Induction of oxidative stress

(vii) Activation of bone morphogenetic protein signaling

(viii) Inhibition of protein translation

(ix) Inhibition of telomerase activity

(x) Downregulation of PI3 K/Akt signaling

(xi) Upregulation of death receptor

(xii) Histone acetylation

(xiii) Repression of E2F

(xiv) Inhibition of IL-6-mediated signaling

(xv) Suppression of FoxO and FoxMl proteins

(xvi) Tubulin stabilization

(xvii) Induction of mitotic catastrophe

(xviii) Inhibition of epithelial-mesenchymal transition

(xix) Inhibition of angiogenesis

(xx) Immunosensitization of cancer cells to the cytotoxicity of lymphocytes

(xxi) Increased genomic instability after exposure to ionizing radiation

(xxii) Overcoming multidrug resistance by inhibition of pglycoprotein

Autoregulatory mechanisms against proteasome inhibition

(i) Induction of macroautophagy

(ii) Activation of constitutive NF-kB activity

(iii) Activation of EGFR signaling

(iv) Stat3 phosphorylation

- (v) Akt phosphorylation
- (vi) Induction of hsp72 and AKR1B10

(vii) Upregulation of glutathione synthesis

Adapted from Wu et al. [3].

elucidated. In general, proteins are targeted for recognition and for subsequent degradation by the proteasome via the attachment of multiple ubiquitin molecules. In order to do this, there are several preparatory steps before proteins are presented to the proteasome. The first step involves the activation of ubiquitin by the formation of a thioester bond with the ubiquitin-activating enzyme (E1) in an ATP-dependent reaction. Then, E1 delivers the activated ubiquitin to the E2 ubiquitin-conjugating enzyme. Finally, E3 ligases transfer ubiquitin from E2 to a lysine residue in the substrate protein [5]. An ubiquitin chain subsequently forms and presents the protein to the 26 S proteasome. It is important to note, however, that these preparatory steps are not used for the degradation of all proteins. Some proteins such as calmodulin and troponin C undergo degradation by the proteasome via ubiquitin-independent pathway [6]. Ultimately, the protein enters the proteasome, ubiquitin is released (if the protein required preubiquination), and the protein is degraded.

The degradation of proteins inside the proteasome is similar to the degradation of proteins by intestinal digestive enzymes. In fact, the proteasome is considered to have chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing- (PHGH-) like activity. The 26 S proteasome is a large multicatalytic complex that is comprised of a 20 S core catalytic component (the 20 S proteasome) capped at one or both ends by a 19 S regulatory component [1]. The 19 S lid serves as an entry portal for the proteins, which are then subjected to adenosine triphosphate (ATP) hydrolysis within the base. ATPases unfold and linearize large proteins before they undergo catalysis within the core. Allosteric interactions guide the intricate sequencing of proteolytic reactions within the core, which ultimately produces oligopeptides that can be recycled within the cell [6].

3. Bortezomib's Inhibition of the Proteasome

Because peptide boronic acids inhibit serine proteases such as chymotrypsin by mimicking substrate binding at the active site, it was postulated that they might inhibit the proteasome by binding to the chymotrypsin-like site in the 20 S core [1]. Adams synthesized 13 boronic acid proteasome inhibitors and tested them for their ability to inhibit cell growth against the panel of 60 cell lines from the National Cancer Institute. One compound, bortezomib, the boronic acid derivative which was later called bortezomib, was potent and was active against a broad range of cancer cell lines, including nonsmall cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast cancers, and had a unique cytotoxicity profile, compared with the NCI's historical file of 60,000 compounds [1]. Since the publication of this study in 1999, bortezomib has been tested in numerous in vitro and in vivo models of several cancers including NSCLC [6].

4. Results of the Inhibition of the Ubiquitin-Proteasome System by Bortezomib

Numerous proteins are degraded by the proteasome, so multiple cellular processes are affected by proteasome inhibition. Therefore, the activity of bortezomib in different cancers may involve a variety of molecular mechanisms (see Table 1) [3]. Nevertheless, one protein that has been clearly implicated in the efficacy of bortezomib is NF- κ B.

The proteasome has a direct role in allowing the cell to progress through the cell cycle by degrading cell cycle regulatory proteins and an indirect role by regulating the availability of transcriptional activators [1]. One transcriptional activator believed to have a central role in mediating many of the effects of proteasome inhibition is the transcriptional activator NF- κ B [1]. This transcriptional activator is involved

Carcinoma	Cell lines	Effects	References
Multiple myeloma	MM.1S, MM.1R, Dox40, MR20, LR5, RPMI8226, IM-9, U266, ARH-77, Hs Sultan	IkBa degradation, inhibited IL-6-triggered activation of p42/44 MAPK as well as TNF-a induced activation of NF-κB,	Hideshima et al., 2001 [7]
Mantle cell lymphoma	Mino, DB (sp53), Molt-4, L-428	NF-κB activation, bcl-xL and bfl/A1 inhibition, and bcl-2 cleavage	Pham et al., 2003 [8]
NSCLC	H460, H322, H358, H157, A549	Cell cycle arrest at G2-M; Bcl-2 phosphorylation and cleavage; p53 stabilization; induction of p21Cip; increase in cyclins A and B; activation of CDKs; mitochondrial cytochrome c release; activation of caspase pathway; apoptosis; NF-κB Downregulation	Ling et al., 2002 [9], 2003 [10], and 2003 [11]; Denlinger et al., 2004 [12]
Prostate	PC-3 (p53 null)	Cell cycle arrest at G2-M; increase in p21Cip; inhibition of CDK4 activity; PARP cleavage; apoptosis	Adams et al., 1999 [13]
	LNCaP-Pro5	Activation of caspase-3; apoptosis	Williams et al., 2003 [14]
Pancreatic	MIA-PaCa-2	Enhanced cytotoxic effects of gemcitabine; reduced NF-κB activation; reduced Bcl-2 expression without affecting Bax or Bak; PARP cleavage; apoptosis	Bold et al., 2001 [15]
	BxPC3	Cell cycle arrest in G0–G1; increase in p21Cip; caspase-3 activation; apoptosis	Shah et al., 2001 [16]
SCCHN	UM-SCC-9, UM-SCC-11B	Cell cycle arrest in G2-M and S phases; increase in p21Cip; apoptosis; (PARP cleavage shown in murine SCCHN lines); NF-κB Downregulation	Sunwoo et al., 2001 [17]
Ovarian	SKOV 3	Induction of p21Cip; inactivation of Bcl-xL; Downregulation of XIAP; PARP cleavage; activation of caspase pathway; apoptosis	Frankel et al., 2000 [18]
Breast	MCF-7	Cytotoxicity (molecular markers not determined)	Teicher et al., 1999 [19]
Colorectal	LOVO, KM12L4, WiDR	Inhibits chemotherapy-induced NF-κB activation; enhances chemotherapy-induced apoptosis; stabilizes p53, p21Cip; p27Kip	Cusack Jr. et al., 2001 [20]

TABLE 2: In vitro studies with bortezomib.

Adapted from Ludwig et al. [21].

in inflammatory and immune responses, and its signaling pathways are implicated in tumor development [1].

This proto-oncogenic NF- κ B pathway requires proteasomal activity. Under normal conditions, NF- κ B factors are retained in an inactive state in the cytoplasm by the inhibitors of NF- κ Bs (I κ Bs). In order to be freed from this inhibition, I κ Bs need to be phosphorylated, polyubiquitylated, and degraded by the proteasome. Bortezomib downregulates NF- κ B signaling by blocking I κ B degradation [5], and this seems to be its prevalent mechanism of action, especially in multiple myeloma and certain solid tumors [21]. Inhibition of NF- κ B reduces the expression of proinflammatory response genes and upregulates the cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1, resulting in increased apoptosis in tumor cells [5].

Other important ways in which apoptosis is induced by bortezomib in various models was the induction of phosphorylation and subsequent cleavage of the antiapoptotic factor Bcl-2, the Upregulation of CDK inhibitors, such as p21Cip, stabilization of p53 [21], and interference with the unfolded protein response (UPR) leading to endoplasmatic reticulum stress and thus increased apoptosis [22]. Additionally, bortezomib sensitizes resistant solid tumor cells to TNF-like apoptosis, inducing ligand- (TRAIL-) induced apoptosis, probably by increasing the levels of death receptors DR4 and DR5 [23].

5. In Vitro Studies Showing the Effect of Bortezomib in Cancer

Extensive preclinical research has been conducted with bortezomib to elucidate its mechanism of action and to examine its activity. In cell culture, bortezomib induces apoptosis in both hematologic and solid tumor malignancies (see Table 2).

6. Proteasome Inhibitor Targets in Lung Cancer

As in part mentioned above, multiple targets of proteasome inhibition with different cellular effects have been identified, among those the very important transcription factor directly involved in apoptosis resistance and expression of adhesion molecules is NF- κ B. Usually inactive intracellularly due to binding to IkBa, it becomes activated after exposure to cytokines, stress, and receptor signaling, leading to apoptosis resistance, increase in growth factors, angiogenesis, and possible tumor metastasis. NF-kB activation is blocked via proteasome inhibition decreasing downstream signaling thus decreasing cell survival and growth [17]. Overexpression of the antiapoptotic protein Bcl-2 leads to chemoresistance; bortezomib causes downregulation of Bcl-2 via phosphorylation in NSCLC [9, 24], as well as decreased transcription of the Bcl-2 promoter, decreased Bcl-2 level, and induced apoptosis in SCLC [25]. An upregulation of Bax a proapoptotic mediator has proven beneficial leading to an increase benefit from proteasome inhibitor by decreasing Bcl-2/Bax ratio [15].

Cell cycle arrest in G2M phase can be induced by bortezomib in NSCLC which is in part due to accumulation of P53, which is crucial for transcription of genes involved in cell cycle and DNA synthesis [36]. The absence of cyclindependent kinase inhibitor p27 acts as poor prognostic factor in NSCLC, bortezomib causes upregulation of p21 and p27 kinase inhibitor leading to arrest of cell cycle inhibiting cyclin A and cyclin E [10, 24, 36].

Bortezomib has been also shown to enhance tumor necrosis factor related apoptosis inducing ligand (TRAIL) induced apoptosis in human cancer cells, bortezomib induced caspase 8 dependent apoptosis, cooperated with trail to induce apoptosis and up-regulated death receptor 5 (DR5) expression in NSCLC cells, which correlated with increased apoptosis by PS-341 and enhancement of TRAIL-induced apoptosis in NSCLC. On the other hand, c-FLIP and surviving levels were elevated after exposure to bortezomib, which in turn protects cells from bortezomib-induced apoptosis [37].

6.1. Phase I Single Agent Proteasome Inhibitors in Lung Cancer. Aghajanian et al. evaluated the safety and pharmacodynamic behavior of bortezomib in patients with histologically confirmed solid tumors who had been heavily pretreated and for which no other therapeutic options were available [38]. Forty-three patients were enrolled after eligibility criteria were met, and informed consent was signed; patients with 14 histologically different tumor types entered the study; among those, 8 patients had documented NSCLC. Prior treatment included a median number of 4 prior chemotherapy regimens, and 12 subjects had received radiation therapy as primary treatment for their malignancy. Forty-three patients received a total of 89 cycles of therapy given twice weekly for 2 consecutive weeks and followed by 1-week recovery period, doses ranged from 0.13 to $1.56 \text{ mg/m}^2/\text{dose}$ (9 dose levels), with a median number of 2 courses given per patient.

Toxicities were minimal in the first five dose level groups; no hematological dose limiting toxicity was reported, with an increase in the incidence of thrombocytopenia and neutropenia at higher doses. Dose limiting nonhematological toxicities were reported and consisted mainly of diarrhea and painful sensory neuropathy; 2 out of 12 patients treated at the $1.56/m^2$ dose developed grade 3 diarrhea and also another 2 out of 12 patients in the same dose group and one in a lower dose group (1.30 mg/m^2) developed grade 3 painful sensory neuropathy which had worsened from prior preexisting symptoms. All these patients had been exposed to taxanes and either carboplatin or cisplatin as prior therapies.

Pharmacodynamic studies revealed a dose-related inhibition of 20 S proteasome activity at higher doses, no significant difference in the mean percentage of inhibition at the 4 different dosing days after 1 hour of drug administration; complete recovery of proteasome activity to baseline was evident prior to drug administration on days 4, 8, and 11 indicating no apparent change to drug sensitivity towards bortezomib-induced proteasome inhibition. Proteasome activity also evaluated at 24 h after day 1 and day 8 dosing which showed recovery but not back to baseline values.

One partial response was seen in a patient with NSCLC who had received prior therapy with six cycles of paclitaxel and carboplatin, two cycles of gemcitabine, three of mitomycin and vinblastine, four weekly docetaxel, and eight weekly methotrexate doses, with disease progression on all of the above regimens; a 50% reduction in the size of bilateral pulmonary nodular infiltrate was seen, with a duration of three months, patient symptoms improved as well, but had to discontinue treatment after three cycles due to painful sensory neuropathy. Stable disease was seen in 3 patients with other tumor types with a mean duration of 4 months.

Dy et al. conducted another phase I and pharmacologic trial of two schedules of bortezomib in patients with advanced cancer [39]; the trial enrolled a total of 44 patients with multiple different tumor types. Of those 2 patients had lung cancer, most of them consisted of colorectal and kidney tumors followed by pancreatic and prostate cancer. 73 courses of therapy with 6 different dose levels (ranging from 0.5 to 1.70 mg/m²) were administered; 28 patients received study treatment twice weekly for 4 out of 6 weeks, but due to increased toxicity on this schedule, 16 additional patients received study treatment only twice weekly for 2 out of every three weeks. The median number of courses given per patient was 2 in both schedules.

Hematological toxicities related to treatment grade >2 were anemia and thrombocytopenia, most of them occurring in schedule one. Reversible thrombocytopenia was dose limiting for both schedules at 1.60 and 1.70 mg/m² dose, no bleeding complications were associated with such nor need for platelet transfusion. Mild leukopenia was observed in one patient in schedule two. Most nonhematological toxicities were reported as mild to moderate consisting of fatigue, diarrhea, nausea, anorexia, sensory neurotoxicity, rash, and vomiting for schedule one; sensory neurotoxicity was dose limiting in one patient in this schedule. Similar side effects were reported in schedule two with the exception of rash and sensory neuropathy; two cases of grade 3 diarrhea were reported in schedule two which improved with dose reductions and the use of loperamide.

Forty-one patients out of the 44 enrolled were assessable for antitumor activity; partial regression (>50%) of a perinephric plasmacytoma was observed in one patient before cycle 2 of treatment and was sustained for 4 months; five patients had stable disease in at least one evaluation. There was as in the previously described study a dose-dependant increase in the degree of proteasome inhibition after 1 hour of drug administration with a recovery of proteasome activity of 85% at 24 hours except in those receiving 1.50 mg/m² on schedule one where a 35% inhibition was still observed at 24 hours. A 549 human NSCLC cells showed a marked increase in p53 levels for 24 hours after exposure to bortezomib.

6.2. Phase II Single Agent Proteasome Inhibitor in Lung Cancer. Stevenson et al. conducted a phase II pharmacodynamic study using single agent bortezomib in patients with advanced stage NSCLC who had received less or equal to one prior regimen [40]. 23 patients were enrolled and received bortezomib at 1.3 to 1.5 mg/m² dosing on days 1, 4, 8 and 11 every 21 days; results revealed one patient having partial response, and 9 patients had stable disease, lasting more than 4 cycles in 5 of the patients. Most common grade 3 toxicities included nausea and vomiting, sensory neuropathy, constipation, rash, and thrombocytopenia. Evaluation of p65 and phosphorylated p65 (pp65) by western blot analysis in 12 patients revealed no change in total p65, the ratio of p65/pp65 was also unaffected across the entire group, but significantly decreased in patients with grade 3 toxicity at 30 minutes with nadir at 4 hours and recovery at 24 hours. They were unable to achieved clinical significance with these results.

The role of bortezomib was evaluated in relapsed or refractory extensive stage small cell lung cancer (SCLC) by Lara et al. in the Southwest Oncology Group (SWOG) phase II trial (S0327) [41]; 56 patients with histologically or cytologically confirmed diagnosis or SCLC with evidence of measurable disease, good performance status, and adequate end organ function who had received prior platinum containing regimens and who had not received prior bortezomib were enrolled. Treatment was administered on days 1, 4, 8, and 11 every 21 days at a dose of 1.3 mg/m² with dose reductions to 1.0 mg/m² if toxicities graded at 3 or 4 based on the National Cancer Institute Common Toxicity Criteria (CTC) version 2.0. Primary end point was response rate (RR); secondary end points included time to progression (TTP) and overall survival (OS). In terms of sensitivity to platinum-based therapy, the patients were well distributed: 28 with platinum sensitive (relapse >90 days after platinum) and 28 with platinum refractory (progression during or < or equal to 90 after platinum). Partial response was observed in one patient and stable disease in two patients in the platinum refractory group; most patients (83%) had disease progression and/or developed symptomatic deterioration; early death was observed in one patient on each group. Three patients were not assessable for response due to other reasons. Median progression-free survival (PFS) and OS for the platinum refractory group were 1.1 and 3.1 months, respectively; in the platinum sensitive group, median PFS was 1.2 months and OS 2.9 months. The 6-month PFS rate was 10% and 0% for the platinum refractory and platinum sensitive group, respectively, and overall 6-month survival

was 25% for both strata. Side effects exceeding grade 2 were fatigue and thrombocytopenia; one death possibly related to bortezomib was reported consisting of dyspnea which led to respiratory failure. Pretreatment samples were analyzed via immunhistochemistry; two out of eight patients had abnormally low p27 levels, five had low BAX levels, and six had abnormally high Bcl-2. Bcl-xl was abnormally expressed in a high percentage in all 8 specimens. Patients had at least two of these markers abnormally expressed in their tumors with five patients having 3 proteins abnormally expressed.

These and other studies showed that bortezomib as a single agent has limited activity with single agent responses up to 8% only [42].

7. Bortezomib Combinations in NSCLC

More recently in combination with chemotherapy, bortezomib has shown its most encouraging activity [42]. Recent phase I studies have shown that bortezomib combinations are generally well tolerated and have little addition in toxicity as compared to chemotherapy alone (Table 3). More importantly, there has been a significant increase in survival observed with the use of bortezomib in combination. Work from Davies et al. showed that bortezomib plus gemcitabine/carboplatin resulted in a notable survival benefit (11 months overall survival) in patients with advanced NSCLC [32].

Work remains to be done to determine if more combinations of bortezomib with other chemotherapy regimens or with targeted therapies will yield further survival advantages. Thus far, results with docetaxel, docetaxel + cetuximab, pemetrexed, and erlotinib show modest results at best (Table 3). There are interesting results for example about the combination of erlotinib and bortezomib. Piperdi et al. [43] found that in H358 bronchoalveolar cells, the combination is neither additive nor synergistic in the NSCLC cell lines studied. The choice of schedule may be very important in combining erlotinib with bortezomib, and further *in vivo* studies are required to further evaluate this combination.

Also there is ongoing research looking for predictive markers of bortezomib sensitivity. Voortman et al. [44] showed that the proteasomal as well as apoptotic phenotype determines bortezomib sensitivity in NSCLC cells. There is a preclinical rationale to combine proteasome inhibition with proapoptotic agents as well as agents promoting a more favorable proteasomal phenotype to overcome this resistance.

8. Conclusion

Ubiquitin-proteasome system is critical for the proliferation and survival of cancer cells, and its inhibition by proteasome inhibitors such as bortezomib has become a very attractive anticancer therapy. Bortezomib has proven to be active against a broad range of cancer cell lines including NSCLC, and it has been tested in numerous *in vitro* and *in vivo* NSCLC models. Current phases I and II studies are showing the possibility to have a new targeted therapy for NSCLC

		TABLE 3: Chemotherapy combinations with bor	tezomib.	
Combination	Study	Dose/schedule	Results	Reference
Docetaxel	Bortezomib Plus Docetaxel in NSCLC and Other Solid Tumors: A Phase I California Cancer Consortium Trial	Patients with NSCLC and r solid tumors were enrolled in cohorts of three over six dose levels. Each cycle was 3 weeks and consisted of one docetaxel infusion (day 1) and four bortezomib injections (days 1, 4, 8, and 11)	The MTD was 1.0/75 mg/m ² . The combination was well tolerated. Two patients with NSCLC achieved a PR (6%), and seven (19%) patients achieved SD (6 patients with NSCLC)	Lara Jr. et al. 2006 [26]
	Randomized Phase II Study of Bortezomib Alone and Bortezomib in Combination with Docetaxel in Previously Treated Advanced Nonsmall-Cell Lung Cancer	Patients were assigned to bortezomib 1.5 mg/m ² (arm A) or bortezomib 1.3 mg/m ² plus docetaxel 75 mg/m ² (arm B). A treatment cycle of 21 days comprised four bortezomib doses on days 1, 4, 8, and 11, plus, in arm B, docetaxel on day 1	RORR were 8% in arm A and 9% in arm B. DCR rates were 29% in arm A and 54% in arm B. Median TTP was 1.5 months in arm A and 4.0 months in arm B. One-year survival was 39% and 33%, and OS was 7.4 and 7.8 months in arms A and B, respectively	Fanucchi et al. 2006 [27]
Docetaxel + Cetuximab	Randomized Phase II Trial of Docetaxel Plus Cetuximab or Docetaxel Plus Bortezomib in Patients with Advanced Nonsmall-Cell Lung Cancer and a Performance Status of 2: CALGB 30402	Docetaxel 30 mg/m ² on days 1, 8, and 15 every 28 days in combination with either cetuximab 400 mg/m ² loading dose followed by 250 mg/m ² weekly (D + C) or bortezomib 1.6 mg/m ² on days 1, 8, and 15 every 28 days (D + B) for up to 4 cycles. Patients with responding or stable disease continued cetuximab or bortezomib until progression	ORR response rates were 13.3% and 10.3% for $D + C$ and $D + B$, respectively. Median PFS was 3.4 months in the $D + C$ arm and 1.9 months in the $D + B$ arm. 6-month PFS were 27.8% and 13.8% and 5.0 and 3.9 months for median survival, respectively. Grade 3/4 hematologic toxicity was 16% for $D + C$ and 21% for $D + B$, whereas nonhematologic toxicities were observed in 63% and 44% of patients, respectively. Neither combination met the prespecified PFS end point to justify further research in this setting	Lilenbaum et al. 2009 [28]
Carboplatin + Paclitaxel and XRT	Phase I Trial of Carboplatin/Paclitaxel/Bortezomib and Concurrent Radiotherapy followed by Surgical Resection in Stage III Nonsmall Cell Lung cancer	Bortezomib was administered on days 1, 4, 15, and 18 during the 6-week induction chemoradiotherapy. Cohorts of three patients were entered. All patients were to receive consolidation chemotherapy with carboplatin AUC = 6 and paclitaxel 200 mg/m^2	12 patients in three cohorts were enrolled. The addition of bortezomib was well tolerated, with no unexpected toxicities during the induction phase. However, there were 3 postoperative deaths (two pneumonitis and one from failure of the bronchopulmonary flap). The trial was halted as a consequence of these toxicities	Edelman et al. 2010 [29]

		TABLE 3: Continued.		
Combination	Study	Dose/schedule	Results	Reference
Gemcitabine + Cisplatin	A Parallel Dose-Escalation Study of Weekly and Twice-Weekly Bortezomib in Combination with Gemcitabine and Cisplatin in the First-Line Treatment of Patients with Advanced Solid Tumors (Phase I study)	Patients were assigned to increasing doses of bortezomib days 1 and 8 (weekly schedule) or days 1, 4, 8, and 11 (twice-weekly schedule), in addition to gemcitabine 1,000 mg/m ² days 1 and 8 and cisplatin 70 mg/m ² day 1, every 21 days. Maximum of six cycles	Weekly bortezomib 1.0 mg/m² plus gemcitabine 1,000 mg/m² and cisplatin 70 mg/m² is the recommended phase II schedule. Of 34 evaluable patients, 13 achieved PR, 17 SD, and 4 PD	Voortman et al. 2007 [30]
Gemcitabine + Carboplatin	The Proteasome Inhibitor Bortezomib in Combination with Gemcitabine and Carboplatin in Advanced Nonsmall Cell Lung Cancer: A California Cancer Consortium Phase I Study	Bortezomib was administered on days 1, 4, 8, and 11, after gemcitabine on days 1 and 8, and carboplatin on day 1 of a 21-day cycle. Three escalating dose levels were evaluated: bortezomib 1.0 mg/m ² /gemcitabine 800 mg/m ² , bortezomib 1.0 mg/m ² /gemcitabine 1000 mg/m ² , and bortezomib 1.3 mg/m ² /gemcitabine 1000 mg/m ² , in combination with carboplatin AUC 5.0	The MTD was defined as bortezomib 1.0 mg/m ² , gencitabine 1000 mg/m ² , and carboplatin AUC 5.0. The most common grade $3/4$ toxicities were thrombocytopenia (rarely associated with bleeding), and neutropenia. Nine of 26 patients (35%) achieved PR, and eight patients had SD	Davies et al. 2008 [31]
	Bortezomib Plus Gemcitabine/Carboplatin As First-Line Treatment of Advanced Nonsmall Cell Lung Cancer A Phase II Southwest Oncology Group Study (S0339)	Stage IIIB/IV NSCLC, performance status 0-1, and no history of brain metastasis received up to six 21-day cycles of gemcitabine 1000 mg/m ² , days 1 and 8, carboplatin area under curve 5.0, day 1, and bortezomib 1.0 mg/m ² , days 1, 4, 8, and 11	 114 patients (52% adenocarcinoma, 85% stage IV) OS was 11 months; 1-year and 2-year survival rates were 47% and 19%, respectively. Median PFS was 5 months; 1-year PFS rate was 7%. ORR was 23%, and DCR rate was 68% 	Davies et al. 2009 [32]
Pemetrexed	Phase I Study of Two Different Schedules of Bortezomib and Pemetrexed in Advanced Solid Tumors with Emphasis on Nonsmall Cell Lung Cancer	Two separate dose-escalating arms (arm A and arm B) were conducted simultaneously. Patients received pemetrexed on day 1 (D1) ($500-600 \text{ mg/m}^2 \text{ IV}$) every 21 days. In arm A, bortezomib was given twice weekly (0.7–1.3 mg/m ² on D 1, 4, 8, and 11). In arm B, bortezomib was given weekly (1.0–1.6 mg/m ² on D 1 and 8)	Of 26 evaluable patients, 2 patients had PR (1 in arm A and 1 in arm B), 13 had SD (7 in arm A and 6 in arm B), and 11 had PD (6 in arm A and 5 in arm B). Of the 16 patients with NSCLC, 2 (12.5%) had PR and 9 had SD, for a DCR of 68.8%. Phase II dose for arm A is pemetrexed 500 mg/m ² and bortezomib 1.3 mg/m ² twice weekly. For arm B, the recommended dose is pemetrexed 500 mg/m ² , bortezomib 1.6 mg/m ² weekly	Davies et al. 2007 [33]

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		TABLE 3: Continued.		
Combination	Study	Dose/schedule	Results	Reference
	A Randomized Phase II Study of Bortezomib and Pemetrexed, in Combination or Alone, in Patients with Previously Treated Advanced Nonsmall-cell Lung Cancer	Pemetrexed (500 mg/m ²) on day 1 plus bortezomib (1.6 mg/m ²) on days 1 and 8 (Arm A) or pemetrexed (500 mg/m ²) on day 1 (Arm B) or bortezomib (1.6 mg/m ²) on days 1 and 8 (Arm C) of a 21 day cycle	In previously treated NSCLC the addition of bortezomib to pemetrexed was well tolerated but offered no statistically significant response or survival advantage versus pemetrexed alone, while bortezomib alone showed no clinically significant activity	Scagliotti et al. 2010 [34]
Erlotinib	A Randomized Phase II Study of Erlotinib Alone and in Combination with Bortezomib in Previously Treated Advanced NSCLC	Erlotinib 150 mg/d alone (arm A; n = 25) or in combination with bortezomib 1.6 mg/m ² , days 1 and 8 (arm B; $n = 25$) in 21-day cycles	ORR were 16% in arm A and 9% in arm B; DCR were 52 and 45%, respectively. The study was halted at the planned interim analysis due to insufficient clinical activity in arm B. Median PFS and OS were 2.7 and 7.3 months in arm A, and 1.3 and 8.5 months in arm B. Six-month survival rates were 56.0% in both arms; 12-month rates were 40 and 30% in arms A and B, respectively. ORR to erlotinib \pm bortezomib was significantly higher in patients with EGFR (50 versus 9% for wild type). Insufficient activity was seen with erlotinib plus bortezomib in patients with relapsed/refractory advanced NSCLC to warrant a phase III study of the combination	Lynch et al. 2009 [35]
PR: partial response	, CR: complete response, ORR: overall response rate.	PFS: progression-free survival, OS: overall survival, I	DCR: disease control rate (CR + PR + SD), MTD: max	cimum tolerated dose.

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combining this bortezomib with available chemotherapeutic agents. Prospective phase III trials are needed to validate the use of this agent in NSCLC.

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