doi:10.4149/neo_2013_070

Individualized treatment of NSCLC: From research to clinical practice

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Received November 27, 2012/ Accepted March 13, 2013

The exact clinical significance of EGFR mutation status in NSCLC at the time of initial diagnosis remains disputable. The gene expression module in NSCLC for chemotherapy outcome prediction needs to be developed. We analyzed 56 patients with NSCLC received chemotherapy either with (n=20) or without EGFR-TKIs (n=36) between 2008 and 2012 in China. EGFR mutation test and gene expression profiling were performed in samples obtained before medication treatment by liquidchip platform. Significant association ($P = 0.028$) was seen between EGFR mutation status before first-line chemotherapy and EGFR-TKIs treatment outcomes, which even can be found from the status before second- or third-line treatment. A 14-gene expression profiling had been studied. Patients with low mRNA expression of ERCC1 or TYMS preferred higher DCR to cisplatin and pemetrexed than those with high expression ($P = 0.39$ and $P = 0.11$). Highly co-expression of TUBB3 and STMN1 gene has associated with the resistance to antimicrotubule drugs ($P = 0.03$). Our data suggest the EGFR mutations status, even at the time of initial diagnosis, is predictive of outcomes of TKIs treatment after chemotherapy. The mRNA expression profiling investigated in this study has a predictive value in NSCLC treatment, but further research with expanded samples is still required.

Key words: non-small-cell lung cancer, EGFR mutation, TKIs, gene expression profiling, chemotherapy

Lung cancer has been the most common cancer, and is the leading cause of death all over the world [1]. In recent years, along with the rapid development of life science, the knowledge and requirement of clinical medicine has enhanced. Furthermore, The Human Genome Project has promoted the progress in pharmacogenetics and pharmacogenomics, which makes the mode of medication treatment shift from diagnosis-directed drug therapy to gene-directed drug therapy on the basis of individual genetic characteristics. Oncology physicians are faced with the challenge of choosing the most effective anti-tumor agents for cancer patients to achieve the best possible outcome.

Previous studies on non-small-cell lung cancer (NSCLC) have identified the predictive value of EGFR (epidermal growth factor receptor) mutation for EGFR TKIs (epidermal growth factor receptor-tyrosine kinase inhibitors) - for example Gefitinib (Iresaa, AstraZeneca, UK). Determining optimal first-line therapy by these biomarkers is accepted as a standard for patients with NSCLC [2-7]. EGFR TKIs should be considered as the prior first-line treatments for the patients with EGFR mutation, while chemotherapy is superior to targeted therapy in the EGFR wild-type patients [8-9]. Because EGFR TKIs work only in some of the patients, the detection of EGFR mutation needs to be considered as essential routine before medication treatment. However, it remains unclear whether it is necessary

Abbreviations: NSCLC: non-small-cell lung cancer, EGFR: epidermal growth factor receptor, EGFR-TKIs : epidermal growth factor receptor-tyrosine kinase inhibitors, ERCC1: excision repair cross complementation 1, TYMS: thymidylate synthetase, RRM1: ribonucleotide reductase subunit M1, TUBB3: β-tubulin isotype III, STMN1: stathmin, ECOG: Eastern Cooperative Oncology Group, CAMS :Chinese Academy of Medical Sciences, CT : Computed tomography , CR :complete response , PR :partial response, SD : stable disease, PD :progressive disease, RECIST :Response Evaluation Criteria in Solid Tumors, BRCA1: breast cancer gene 1, TOP2A: Topoisomerase IIα

HER2: epidermal growth factor receptor 2, VEGFR2:Vascular endothelial growth factor receptor 2, PDGFR :platelet-derived growth factor receptor, IGF1R :Insulin-like Growth Factor receptor, FFPE :Formalin-fixed paraffin-embedded, B2M :beta-2-microglobulin, TBP :TATA box binding protein , TFRC :transferrin receptor, DCR: disease control rate

and which sample is chosen for detection for the decision on EGFR-TKIs in second- or third-line treatment

Besides targeted therapy, the response to chemotherapy of NSCLC can also be predicted by some biomarkers. The mechanisms of many chemotherapy agents revealed by relevant genes which may influence drug response have been studied. The prediction of chemotherapy outcome based on gene expression level is becoming possible for many classes of chemotherapy agents. For example, ERCC1(excision repair cross complementation 1) expression has been association with cisplatin in patients with NSCLC [10-14], gastric [15,16], colorectal [19]; TYMS (thymidylate synthetase) expression has been association with fluorouracil/pemetrexed in many cancers [20-22]; RRM1(ribonucleotide reductase subunit M1) expression is connected with gemcitabine [23,13], and TUBB3(β-tubulin isotype III) and STMN1(stathmin) are linked with paclitaxel/docetaxel [24-27]. In addition, multigene predictors for clinical outcome have been introduced to date, although none has become widely accepted [28-30]. However, now it is not possible to prospectively identify patients who can likely benefit from or suffer from certain chemotherapy in the clinical setting.

We retrospectively analyzed the data of NSCLC patients who received Gefitinib, as first-, second- or third-line treatment from January 2008 to May 2012 at our institution. We evaluated the predictive values of the EGFR mutation status at the time of initial diagnosis to Gefitinib treatment before and after chemotherapy. Meanwhile, we explored the association of a 14-gene expression profiling with the response of patients to Cisplatin-containing combination chemotherapy, pemetrexed-containing combination chemotherapy, or taxane-containing combination chemotherapy. The 14 genes which include TUBB3, EGFR, BRCA1(breast cancer gene 1), TOP2A (Topoisomerase IIα), RRM1, TYMS, STMN1, HER2 (epidermal growth factor receptor 2), VEGFR2 (Vascular endothelial growth factor receptor 2), KIT, PDGFR (platelet-derived growth factor receptor), VEGFR1, ERCC1 and IGF1R(Insulin-like Growth Factor receptor) have been reported as the biomarkers for NSCLC [10-16, 20-27, 42-49]. However, correlations of mRNA expression levels among these 14 genes and the efficacy of the 14-gene expression profiling on predicting chemotherapy outcomes have not been studied yet.

Patients and methods

Patients. We identified patients with NSCLC who had received chemotherapy and/or Gefitinib between January 2008 to May 2012 from the database of Cancer Institute and Hospital, Chinese Academy of Medical Sciences (CAMS). We analyzed the data of patients who were diagnosed with stage IV NSCLC. The performance status of all the patients was ECOG (Eastern Cooperative Oncology Group) grade 0-2. Finally, we gathered the data on 56 patients, of whom 52 patients had received chemotherapy as first-line treatment and 20 patients had received Gefitinib as first-, second- or third-line treatment. Thirty-one patients had metastasis, and fifteen from them had more than two metastatic lesions.

Cisplatin-containing combination chemotherapy was administered in 26 patients (ERCC1 group) as the first-line chemotherapy, pemetrexed-containing combination chemotherapy in 26 patients (TYMS group) too, and taxane-containing combination chemotherapy in 27 patients (TUBB3 & STMN1 group). Twenty patients received Gefitinib treatment. Computed tomography (CT) scans were performed at 4 weeks after treatment. The responses to treatment were classified as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. The institutional review board of CAMS approved this study.

EGFR mutation analysis and gene expression profiling. All the molecular analysis of this study were performed at SurExam Testing Center. The samples from enrolled patients were collected before medication treatment. Twenty-eight tumor samples were collected from the primary cancer by operation, twelve samples by transbronchial lung biopsy.

The presence of EGFR mutations in exons 18, 19 and 21 was investigated. We gathered the mutational data of 20 patients with their informed consent. DNA extraction from paraffin-embedded tissues was performed using the Maxwell® system (Promega, GA, USA). EGFR exons 18, 19 and 21 were screened using *SurPlex®*-xTAG70plex (SurExam, GZ, China). The method includes five steps published in ref [31].

The mRNA expression profiling of 14 genes was determined by multiplex. branched-DNA liquidchip technology. Formalin-fixed paraffin-embedded (FFPE) tissue samples were processed by following steps. First the sample was homogenized in a mixture of homogenizing solution at 65°C for 2 hours. The homogenate was centrifuged to remove residual paraffin and debris, and then the supernatant was transferred to a fresh microcentrifuge tube. Forty μl sample homogenate was added to each well of a 96-well plate that contains following reagents in each well: 18.5 μl of RNase-free water, 33.3 μl of lysis solutions, 2 μl of blocking reagent, 1 μl of capture beads and 5 μl of target gene-specific probe set. The plate was sealed and incubated for 18 hours at 54°C on a shaker with 750 rpm. The hybridization mixture was then removed to a filtered 96-well plate. The un-bound RNA and other debris in wells were removed by washing three times with 250 μl of wash buffer (0.1×SSC and 0.03% lithium lauryl sulfate) under a vacuum system. Signals for the bound targeted mRNA were developed by following steps: 1) incubate in 100μl of pre-amplifier solution for 1 hour at 50°C; 2) wash twice with 200μl wash buffer; 3) incubate in 100μl of amplifier solution for 1 hour at 50°C; 4) wash twice with 200μl wash buffer; 5) incubate in 100μl of the labeled probe for 1 hour at 50°C , and 6) wash twice with 200μl wash buffer. The samples were then incubated with 100μl SA-PE solution at 50°C for 30 min. The fluorescence value of each sample was analyzed by the Luminex 200 system. Control genes were beta-2-microglobulin (B2M), TATA box binding protein (TBP), and transferrin receptor (TFRC).

Table 1 Patient characteristics

	No. of Gefitinib-treated patients (N=20)	No. of chemotherapy patients (N=52)
Age, year		
Median	58.5	55
Range	44-81	31-90
<60	12	33
≥60	8	19
Gender		
Male	12	39
Female	8	13
Smoking history		
Smoker	11	30
Never-Smoker	8	18
Unknown	1	4
Histologic		
Adenocarcinoma	17	39
Squamous cell carcinoma	3	8
Unknown	0	9
Performance status		
0	2	7
1	10	29
2	4	9
Unknown	4	11

Statistical analysis. All clinical data were collected independently by three physicians. The median value was used to divide the patients into high expression group and low expression group based on the mRNA expression level. The Fisher's exact test was used to determine the significant differences of TKI treatment effect between EGFR-mutated and wild-type group. The predictive effect of ERCC1, TYMS, TUBB3 and STMN1 mRNA expression was also explored in the chemotherapy population by Fisher's exact test. Spearman rank correlation distant was used to perform hierarchical *clustering. Cluster analysis, heat-map and cluster-tree drawing was performed by R bioconductor package.* All other statistical analyses, performed using the SAS Business Analytics software 9.1, were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics. The patients consisted of 16 women and 40 men. There were 42 patients with lung adenocarcinomas; ten with squamous cell carcinomas; one adenosquamous carcinoma and three not clear. The baseline characteristics of the Gefitinib-treated and chemotherapy patients with NSCLC are listed in Table 1. There were 20 patients in Gefitinib-treated group. The median age was 58.5 years old and 12 of the patients were men. Seventeen patients had adenocarcinomas and eleven of the patients were smokers. Gefitinib was delivered as a first-line therapy in 8 patients, second-line therapy in 7 patients and third-line therapy in 5 patients. A total of 52 patients met the enrollment criteria and were entered into the chemotherapy group. Most of the patients received platinum-based doublet chemotherapy as first-line therapy. Two patients received cisplatin (1 patient) or pemetrexed (1 patient) monotherapy. Five patients received more than double chemotherapeutic agents.

EGFR mutation associated with response rate. Amongst the twenty cases in Gefitinib group, twelve patients had EGFR

Table 2. The tumor responsiveness and the patients' characteristics

patient ID	mutation status	response	smoking history	age	gender	histology	Therapy line
9	L858R	PR	never	76	female	Adeno	first
13	ΔE746-A750 (K745:AAA)	PR	never	58	female	Adeno	first
16	L858R	PD	Ex, 10 pack/yr	67	male	Adeno	second
17	unknown	PD	never	54	female	Adeno	first
26	ΔE746-A750 (K745:AAG)	PR	current	44	male	Adeno	second
27	ΔE746-A750 (K745:AAA)	SD	never	81	female	Adeno	first
28	ΔL747-S752 ins S	SD	never	69	female	Adeno	first
29	L858R	PR	Ex,30 pack/yr	60	female	Adeno	second
30	L858R	PR	current	59	female	Adeno	first
21	L858R	SD	current	59	male	Adeno	second
42	L858R	PR	current	55	male	Adeno	second
43	unknown	PD	never	47	female	Adeno	second
2	wild-type	SD	Ex, 23 pack/yr	49	male	Adeno	third
3	wild-type	SD	never	58	male	Adeno	third
24	wild-type	SD	Ex, 30 pack/yr	63	male	Squamous	first
35	wild-type	SD	current	47	male	Adeno	first
18	wild-type	PD	Ex, 30 pack/yr	55	male	Squamous	third
51	wild-type	SD	current	72	male	Adeno	first/second
4	wild-type	PD	current	52	male	Squamous	third
12	wild-type	PD	never	66	male	Adeno	second

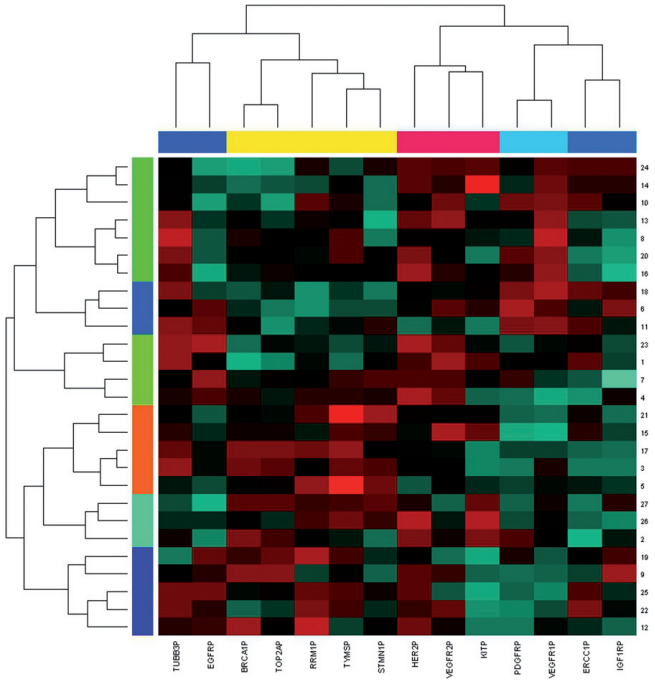

Figure 1. Identify tumor co-expression patterns based on 27 patients' gene expression modules.
The cells are colored according to Spearman's correlation coefficient values, with red indicating positive and green indicating negative correlations.

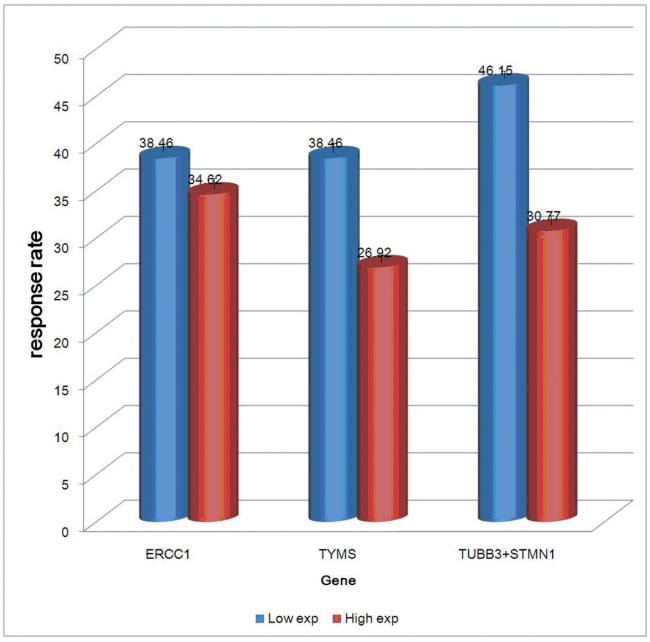

Figure 2. The comparison of DCR for high and low levels of ERCC1 and TYMS mRNA expression.
The disease control rate for high and low levels of ERCC1, TYMS and TUBB3 &STMN1 mRNA expression was described and compared, with red indicating high expression, blue indicating low expression and the error bar indicating standard deviation.

mutations. Six patients had EGFR exon 21 L858R point mutations and another four patients were exon 19 deletions. The tumor response was evaluated in 20 patients. Partial response (PR) was observed in six patients. There were eight patients with SD, and six patients with PD. Four of the six patients with PR had exon 21 L858R point mutations, and the other two had exon 19 deletions. However, three patients with PD also had EGFR mutations. Amongst the eight patients with SD, five cases had no mutations and three cases had either EGFR exon 21 point mutations or exon 19 deletions. Table 2 shows the results of the tumor responsiveness and the patients' characteristics. Here we found the EGFR mutation status is associated with the efficacy of EGFR TKIs, which is consistent with previous research.

Gene expression module correlations and classification. The mRNA expression profiling of the 14 genes was determined in 27 patients who were treated with chemotherapy. The correlations between gene modules and the classification of patients were showed in Figure 1. The twenty-seven patients were divided into two groups based on the gene expression module. One group had 13 patients with high expression of TUBB3, EGFR, BRCA1, TOP2A, RRM1, TYMS and STMN1 gene, while low expression of HER2, VEGFR2, KIT, PDGFR, VEGFR1, ERCC1 and IGF1R gene. The other group had 14 patients with the correspondingly opposite expressions. Patients with tumors expressing the TUBB3-set signature had a no significantly different response rate (P=0.109, Mcnemar Test) to cisplatin-containing combination chemotherapy than patients with tumors without TUBB3-set signature expression. That indicates this Gene Expression Classification may predict the cisplatin-based chemotherapy effect correctly.

The mRNA expression of four genes (ERCC1, TYMS, TUBB3 and STMN1) was detected in 52 patients who received chemotherapy as first-line treatment. The ERCC1, TYMS, TUBB3 and STMN1 status was considered high expression if the mRNA expression level was equal or higher than the cut-off value, provided by SurExam Testing Center; otherwise, considered as low expression. The cut-off values to identify the high and low mRNA expression levels were 0.683 (range 0.256-1.533) for ERCC1, 0.172 (range 0.010-0.619) for TYMS, 0.2070 (range 0.047- 1.346) for TUBB3 and 1.671 (range 0.869-4.784) for STMN1, respectively.

Within the ERCC1 group, the disease control rate (DCR, CR+PR+ SD) was 73.08% (19/26). There was no patient with CR, three patients with PR, and sixteen with SD. Twelve of the nineteen patients had a low expression of ERCC1. We found that the patients with low-ERCC1 expression had a higher DCR than the others (83.3% vs. 64.3%), but the difference was not statistically significant (P = 0.39, two-sided test). Similar results were observed in TYMS group. In the TYMS groups, the DCR were 65.38% (17/26). The patients with lower TYMS mRNA expression had a better outcome when treated with

pemetrexed-based chemotherapy. However, there was no statistically significant differences either ($P = 0.11$, two-sided test). Figure 2 showed the comparison of DCR for high and low levels of ERCC1 and TYMS mRNA expression. In the TUBB3 &STMN1 group, neither TUBB3 nor STMN1 mRNA expression demonstrated a predictive value to taxane-containing combination chemotherapy. However, patients with co-expression of the two genes showed a trend of worse DCR to the others. Furthermore, the DCR was lowest in patients with low expression of both genes.

Discussion

The present study investigates some molecular biomarkers and their response biomarkers in advanced NSCLC. Numerous studies reported that patients with EGFR mutation had a better response to EGFR TKIs. In this study, we found statistical association between the EGFR mutation status and the outcomes of patients treated with Gefitinib, which is consistent with previous studies [2-7].

With the development of clinical research and basic research in biology, the consensus of identifying EGFR mutation status before EGFR TKIs treatment has been accepted widely. The National Comprehensive Cancer Network has approved gefitinib/erlotinib to be a first-line treatment choice for patients with advanced NSCLC with EGFR mutation. They even defined the testing method for EGFR. However, the time of the testing sample to obtain has not been standardized. A recent study investigated the influence of chemotherapy on EGFR mutations in patients with NSCLC [32]. They analyzed two cohorts: advanced NSCLC who received first-line chemotherapy with matched pre- and post-chemotherapy blood samples, and IIB to IIIB NSCLC with pre- and post-neoadjuvant chemotherapy tumor tissues. The results showed that the EGFR mutation rates in both groups were decreased significantly. Therefore, they came to a conclusion that chemotherapy may alter the EGFR mutation status in patients with NSCLC. EGFR mutation status at the time of initial diagnosis couldn't predict the outcome of EGFR-TKIs as second- or third-line treatment. At last, they suggested that future prospective trials should consider analyzing biopsies taken immediately before second- or third-line EGFR-TKI therapy. Nevertheless, it's difficult to obtain tumor tissues from patients with advanced or recurrent NSCLC. Although several studies have focused on exploring the potential possibility of detecting EGFR mutation in circulating tumor DNA from plasma or other non-tissue samples, the concordance rate of EGFR mutation status between these samples and matched tumor tissue were not stable, varying from 59.1% to 92% [33-36]. It seems that assessing EGFR mutation status using blood sample is not absolutely accurate. This retrospective study showed that the EGFR mutation status at the time of initial diagnosis can predict the efficacy of EGFR-TKI as second- or third-line therapy. It may provide useful information for decision-making on EGFR-TKI therapy when the detection in blood sample is unstable. In addition, the blood-based testing need advanced technology and cost high, which limits the clinical application. For some medical institutions lacking of advanced technology, the tumor tissue obtained at the time of diagnosis is an alternative sample for molecular determining. Therefore, our current study suggests that the EGFR mutation status at the time of initial diagnosis is still useful to predict the efficacy of EGFR-TKI as second- or third-line therapy.

Previous study found that higher levels of ERCC1 expression are associated with poor response to platinum-based chemotherapy in advanced NSCLC [10]. Later studies confirmed the predictive role of ERCC1 in NSCLC [12,37] and other types of cancer, such as colorectal cancer [17], gastric carcinoma [16,38-39], and esophageal cancer [40]. Our study showed that patients with lower expression of ERCC1 had relatively higher DCR than patients with higher gene expression when they received platinum-based chemotherapy, which is consistent with previous studies. But the difference between the two groups did not reach statistical significance. Some researches linked the pemetrexed resistance to TYMS expression levels in NSCLC [20,41]. We also found that the patients in the low-TYMS group had a higher DCR compared with the ones in the high group. However, our results did not show any statistically significant correlation between TYMS expression and clinical outcome. As the sample size of this study is small and lacks of statistical significance, the cut-off value provided might not be suitable for it.

The mechanism of chemotherapy drug functioning in vivo is complex and multi-gene involved, and more and more genes are revealed the impact on chemotherapy outcomes. With the development of relevant researches and the deep understanding of correlation among genes, it is more valuable to use multi-gene module to predict the outcomes of chemotherapy than single gene, although the latter is applied widely in chemotherapy selection. In our study, we established a 14-gene expression module, and found it had a better guide to predict the chemotherapy outcomes. Multi-gene expression profiling may be a useful tool for chemotherapy and the tendency in future. In our opinion, multi-gene expression possesses the advantage of depicting the full-scale gene expression characteristics systemically. Further study is needed to optimize the module.

In conclusion, the present study did not reveal a significant clinical impact of the gene expression of ERCC1 or TYMS on advanced NSCLC patients treated with cisplatin-based or pemetrexed-based first-line chemotherapy. However, we reported significantly different DCR of first-, second- or third-line EGFR-TKI therapy according to the EGFR mutation status at the time of diagnosis in the patients with advanced stage, suggesting that the mutation of the EGFR gene determined using tumor tissue of diagnosis may predict the response to second- or third-line EGFR-TKI therapy. The 14-gene expression module established in this study might be useful for predicting chemotherapy outcomes.

Acknowledgements: The authors sincerely thank other physicians and investigators for their technical supports and contributions to this study.

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