Genome-wide DNA Methylation Analysis Reveals GABBR2 as a Novel Epigenetic Target for EGFR 19 Deletion Lung Adenocarcinoma with Induction Erlotinib Treatment

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Abstract

Purpose: The past decade has witnessed the rapid development of personalized targeted therapies in lung cancer. It is still unclear whether epigenetic changes are involved in the response to tyrosine kinase inhibitor (TKI) treatment in epidermal growth factor receptor (EGFR)-mutated lung cancer.

Experimental Design: Methyl-sensitive cut counting sequencing (MSCC) was applied to investigate the methylation changes in paired tissues before and after erlotinib treatment for 42 days with partial response (PR) from stage IIa (N2) lung adenocarcinoma patients (N = 2) with EGFR 19 deletion. The Sequenom EpiTYPER assay was used to validate the changed methylated candidate genes. Up- or downregulation of the candidate gene was performed to elucidate the potential mechanism in the regulation of erlotinib treatment response.

Results: Sixty aberrant methylated genes were screened using MSCC sequencing. Two aberrant methylated genes, CBF1, and GABBR2, were clearly validated. A same differential methylated region (DMR) between exon 2 and exon 3 of GABBR2 gene was confirmed consistently in both patients. GABBR2 was significantly downregulated in EGFR 19 deletion cells, HCC4006 and HCC827, but remained conserved in EGFR wild-type A549 cells after erlotinib treatment. Upregulation of GABBR2 expression significantly rescued erlotinib-induced apoptosis in HCC827 cells. GABBR2 was significantly downregulated, along with the reduction of S6, p-p70 S6, and p-ERK1/2, demonstrating that GABBR2 may play an important role in EGFR signaling through the ERK1/2 pathway.

Conclusions: We demonstrated that GABBR2 gene might be a novel potential epigenetic treatment target with induction erlotinib treatment for stage IIa (N2) EGFR 19 deletion lung adenocarcinoma. Clin Cancer Res; 1–12. ©2017 AACR.

Introduction

Lung cancer has been the leading life-threatening cancer in the world (1). With the development of molecular biology, targeted therapy has been more and more involved in anticancer treatment. In the clinical treatment of non–small cell lung cancer (NSCLC), targeted therapy against epidermal growth factor receptor (EGFR) has gradually matured and significantly contributed to the improvement of patient outcomes and quality of life (2). The results of the IRESSA Pan Asia Study (IPASS) make it common practice to screen for EGFR mutations before NSCLC treatment, as 75% of NSCLC patients carrying EGFR mutation benefit from tyrosine kinase inhibitor (TKI) treatment (3). However, the benefit of EGFR-TKI regimens for patients with stage IIa NSCLC remains uncertain. The goal of the treatment in locally advanced NSCLC is to cure without long-term therapy related complications. With the successful integration of TKIs in the treatment of advanced NSCLC with mutated EGFR, it is reasonable to wonder whether we could extend this benefit to locally advanced NSCLC and actually improve long-term survival rates for these patients. There are some clinical trials (NCT01822496, NCT00600587) evaluating the value of induction erlotinib therapy before thoracotomy or radiotherapy in stage IIa (N2) EGFR-mutated NSCLC. However, an important question is why the recurrence rates after complete surgical resection still remain as high as 70% (4).
Translational Relevance

The is the first study to investigate the changes in DNA methylation before and after induction tyrosine kinase inhibitor (TKI) treatment for epidermal growth factor receptor (EGFR)-mutated lung adenocarcinoma at the whole-genome level. A differential methylated region (DMR) of GABBR2 gene was confirmed in the two patients involved in the present study. Upregulation of GABBR2 expression significantly rescued erlotinib-induced apoptosis, which indicated that GABBR2 gene might be a novel potential epigenetic treatment target with induction erlotinib treatment for stage IIIa (N2) EGFR 19 deletion lung adenocarcinoma. Our research provides a new theoretical basis for the epigenetic study of EGFR-mutated lung adenocarcinoma treatment and suggests that targeting GABBR2 together with EGFR inhibition may improve clinical outcomes in patients with EGFR-mutated lung adenocarcinoma.

Considering the current clinical issue, our research is to investigate whether epigenetic changes such as DNA methylation are involved in the response to induction TKI treatment in EGFR-mutated lung cancer.

Recent studies have demonstrated that DNA methylation plays a role in the targeted therapy of NSCLC (5–7). DNA methylation status of Wnt antagonist genes from 155 NSCLC patients who received EGFR-TKI treatment were investigated using methylation-specific PCR, showing that DNA methylation status of Wnt antagonist SFRP5 can predict the response to EGFR-TKI therapy in NSCLC, and methylated SFRP5 may contribute to shorter progression-free survival (PFS; ref. 8). The EGFR 19 deletion cell line PC-9, with the unmethylated promoter region of EGFR gene, was more sensitive to gefitinib compared with the other EGFR 19 deletion cell line H1650 with the methylated promoter region, which was “resistant” to gefitinib, suggesting that EGFR gene promoter methylation may be a potential mechanism for acquired resistance to gefitinib (9). Methylation of death-associated gene protein kinase (DAKP) is reported to be an important target with cetuximab and erlotinib resistance (10). DNA methylation regulates gene expression without altering the nucleotide sequence, thereby potentially influencing the sensitivity of EGFR-mutated lung cancer to targeted drugs. Currently, there is an ongoing phase I clinical trial testing the use of vorinostat–gefitinib combined therapy on resistance due to BIM polymorphism (BH3-only proapoptotic member of the Bcl-2 protein family) in EGFR-mutant lung cancer (NCT02151721). Vorinostat, a small-molecule inhibitor of histone deacetylase (HDAC), can epigenetically restore BIM function and death sensitivity to EGFR-TKIs, which may provide some direction for the epigenetic treatment of EGFR-mutated lung cancer.

Now the popularity of next-generation sequencing (NGS) technology makes it possible to study the epigenetic mechanism of targeted therapy at the genome-wide level, while previous studies were limited to only one or several genes (11, 12). In this study, one of the most commonly used whole-genome DNA methylation sequencing technologies, methyl-sensitive cut counting sequencing (MSCC), was used to investigate the changes in DNA methylation before and after induction TKI treatment for EGFR-mutated lung cancer at the whole-genome level, which may help to discover how epigenetic changes are involved in the response to TKI therapy and further indicate new potential combined induction therapy in EGFR-mutated NSCLC.

Materials and Methods

Patients and samples

The patients from Shanghai Chest Hospital were confirmed to have locally advanced stage IIIa-N2 lung adenocarcinomas with mediastinal lymphadenopathies confirmed by mediastinoscopy that were unresectable at the first diagnosis, carrying EGFR 19 deletion mutations by direct sequencing. After two cycles of induction erlotinib treatment (21 days for one cycle of treatment, a total of 42 days), the patients exhibited a partial response (PR) according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (13) and then subsequently received a radical sleeve lobectomy after multidiscipline discussion on tumor board. The current study conformed to the Declaration of Helsinki and was performed after approval by the Institutional Review Board (IRB) of Shanghai Chest Hospital (No. of ethics approval: KS1307).

The two patients signed the IRB-approved written informed consents and were further enrolled for this study, allowing for the collection and genomic analysis of archived tissue specimens. The metastatic mediastinal lymph nodes before TKI treatment were collected using mediastinoscopy, and post-TKI treatment metastatic mediastinal lymph nodes were collected during the sleeve lobectomy operation (N = 2, a total of 4 samples). After surgery, the samples were collected and stored at −80°C immediately. A total of 4 samples (2 matched pairs) were used for whole-methylome screening and were then verified by a Sequenom Epityper assay to double confirm the changed methylated candidate genes.

Genomic DNA extraction

Genomic DNA was isolated from the four samples using the QIAamp DNA Mini Kit (Qiagen). A Thermo NanoDrop 2000 (Thermo Scientific) and a Qubit 2.0 fluorometer (Life Technologies) were used to detect the DNA concentrations. An Agilent 2100 Bioanalyzer (Thermo Scientific) was used to determine the DNA length and ensure the integrity.

MSCC library construction

For each of the samples, two MSCC libraries were constructed according to the description of the procedure by Guo and colleagues published in Nature Neuroscience (14). A detailed experimental procedure is provided in the Supplementary Material (Supplementary File S1).

MSCC sequencing and data analysis

After the MSCC libraries were pooled, an Illumina HiSeq2000 sequencing system (Illumina) was used to perform the sequencing. A detailed procedure of the NGS data analysis is provided in the Supplementary Material (Supplementary File S1).

Basic units of 200-bp nonoverlapping windows were used to look for differential methylated regions (DMR). For each of these units (≥2 sequenced CpGs), the average methylation level of the CpGs involved was calculated. A P value was also assigned using the Fisher exact test. Regions were identified as DMRs if their detection P value was <0.05 and the changed average methylation level (ΔMSCC, either increase or decrease) was ≥20%.
and gene ontology (GO) analyses were performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 software (15, 16). Gene structure was predicted by WebScipio 2.0 (17).

Sequenom Epityper assay

After the MSCE screening sequencing, the methylation of GABBR2 and CBFA2T3 exhibited changes post-erlotinib treatment (please see the Results section), which needed further validation. A detailed experimental procedure of Sequenom Epityper assay and the primers of the two genes, GABBR2 and CBFA2T3, were provided in the Supplementary Material (Supplementary File S1).

Cell culture and treatment by erlotinib

A549 (EGFR wild-type), HCC4006 (EGFR 19 deletion, DelE746-E749, i.e., A750P), and HCC827 (EGFR 19 deletion, DelE746-A750, i.e., A750P) cell lines were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI1640 medium (HyClone) supplemented with 10% FBS (HyClone) and 100 U/mL of penicillin and streptomycin (Gibco) in a 37° C incubator with a humidified atmosphere of 5% CO2. Erlotinib powder was purchased from Santa Cruz Biotechnology. The cells were then incubated with or without erlotinib for 48 hours.

Plasmid construction and transfection

According to the cDNA fragments within the coding region of the GABBR2 gene (GenBank: BC035071), the sequence was cloned into a pCMV-HA plasmid to generate GABBR2 overexpression using Lipofectamine 2000 Transfection Reagent (Life Technologies). Western blot analysis was applied to validate the gene expression level.

To knock out the GABBR2 gene, two shRNAs were designed and synthesized. The shRNAs were then inserted into the core plasmid of the FG12 lentiviral system labeled with green fluorescent protein (GFP). Along with the other three packaging plasmids, of the FG12 lentiviral system labeled with green expression level. Western blot analysis was applied to validate the gene expression level.

Cell lysis and Western blotting

Cells were rinsed with ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, #9806), containing complete protease inhibitors (Roche), phosphatase inhibitors (Roche), 5 mmol/L DTT (Sigma), and 1 mmol/L PMSF for 15 minutes on ice. Cells were then centrifuged at 15,000 × g for 10 minutes at 4°C. After the supernatant was collected, the protein concentration was measured using the Bio-Rad protein assay (Bio-Rad). Afterwards, the proteins were separated using SDS-PAGE and blotted onto a PVDF membrane (Millipore). The antibodies were obtained from Santa Cruz Biotechnology, Inc.

Flow cytometric assays for apoptosis

After being transfected with plasmid and incubated with or without erlotinib, cells were digested by trypsin/EDTA (Gibco) and washed twice with ice-cold PBS. Following fixation with 70% ethanol overnight at 4°C, the cells were washed twice with PBS and then digested by 50 μg/mL RNase A in 500 μL of PBS at 37°C for 30 minutes. Next, the cells were stained with 20 μg/mL propidium iodide (PI) for 30 minutes at 37°C. For apoptosis, cells were harvested using trypsin/EDTA and washed with PBS, and then binding buffer was added to resuspend the cells. Following incubation with Annexin-V and PI staining according to the manufacturer’s protocol (Bio-Vision), cells were analyzed by FACSCanto II flow cytometer (BD Biosciences).

IHC

IHC staining was performed using freshly cut paraffin-embedded tissues. Slides were incubated for 12 hours at 4°C with GABBR2 antibody (at a dilution of 1:10, Ab75838, Abcam). The resulting mixture were then washed by PBS and incubated with K5007 antibody (DAKO) for 30 minutes at room temperature. Signals were then visualized (DAKO DAB detection kit, K5007) as per the manufacturer’s instructions.

Results

Patient characteristics and therapeutic effects of TKI treatment

These two patients both had locally advanced IIa-N2 lung adenocarcinoma with mediastinal lymph node metastases at diagnosis. The pathology and staging were diagnosed on the basis of the metastatic mediastinal lymph nodes using mediastinoscopy at baseline, and EGFR 19 deletion in metastatic mediastinal lymphadenopathies was detected by direct sequencing for these two patients. The clinical characteristics of these two patients were summarized in Table 1. One patient, patient A, was a 54-year-old nonsmoking male with a 7.6 × 5.6 cm mass in the right upper lobe (RUL) and an enlarged 1.8 cm right lower paratracheal lymphadenopathy. The RUL mass shrank significantly from 7.6 × 5.6 cm to 3.4 × 3.0 cm (Fig. 1A), with PR consisting of a 55.3% reduction after 42 days of induction erlotinib treatment. The other one, patient B, was a 59-year-old non-smoking male, with a 6.1 × 4.0 cm RUL consolidation with greater involvement of the right lower paratracheal lymphadenopathy measuring 1.5 cm on the baseline chest CT (Fig. 1B), who was also diagnosed with EGFR exon 19 delE750-E758 stage IIIa lung adenocarcinoma (Fig. 1C) by mediastinoscopy sampling with a right lower paratracheal lymphadenopathy. The RUL mass shrank significantly from 6.1 × 4.0 cm to 2.2 × 2.9 cm (Fig. 1B) with PR consisting of a 52.5% reduction after 42 days of induction erlotinib treatment. These two patients were further treated with a RUL sleeve lobectomy based on the multidisciplinary discussion on tumor board considering the

Table 1. Clinical characteristics of the two patients for the MSCE screening study

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age</th>
<th>Gender</th>
<th>Smoking status</th>
<th>Primary tumor location</th>
<th>N2 station</th>
<th>Pathology</th>
<th>EGFR exon 19 deletion status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient A</td>
<td>54</td>
<td>Male</td>
<td>Non-smoker</td>
<td>RUL</td>
<td>4R</td>
<td>Adenocarcinoma</td>
<td>delE750-E758</td>
</tr>
<tr>
<td>Patient B</td>
<td>59</td>
<td>Male</td>
<td>Non-smoker</td>
<td>RUL</td>
<td>4R</td>
<td>Adenocarcinoma</td>
<td>delE746-A750</td>
</tr>
</tbody>
</table>

Abbreviations: RUL, right upper lobe; 4R, right lower paratracheal lymphadenopathy.
marked induction erlotinib response achieved. The four tissue samples of pre- and posterlotinib treatment from these two patients were received and stored.

Global properties of DNA methylation in lung cancer

We performed MSCC sequencing and obtained an average of 49.3 (44.1–52.9) million uniquely mapped reads for each sample (Supplementary Table S1). According to previous research, the sequencing depth of every CCGG site has a significant effect on the accurate estimation of the methylation level, namely, the accuracy increases with sequencing depth (14). The distribution of CCGG sites with high sequencing depth of 40+ reads in our study was very similar to that of all the CCGG sites in lung cancer tissues (Supplementary Fig. S1A), showing that the CCGG data with high sequencing depth of 40+ reads could well reflect the data of all CCGG sites. Therefore, we chose CCGG sites with 40+ reads to proceed with the following analysis. After excluding CCGG sites with a low sequencing depth (<40 reads), we profiled 265,020 and 314,366 CCGGs for the two patients, respectively, representing approximately 1% of all CpGs in the whole genome. The moving average methylation levels of each region of genes were calculated according to the relative location of the genes. Mapping of the CCGG sites to their relative location to associated genes showed that most regions of genes, including the gene body, had similar methylation levels, while the transcription starting site (TSS) region was hypomethylated (Supplementary Fig. S1B).
which is similar to the findings from the human methylome (18, 19). We meanwhile mapped the CCGG sites to the known CpG islands (CGI), which were highly clustered CpGs located at gene promoter regions (20). The methylation level of the CCGG sites inside of CGIs was significantly lower than those outside of CGIs (Supplementary Fig. S1C), which is similar to previous reports (21). All of these results showed that the whole-genome DNA methylation sequencing results were accurate and reliable from multiple aspects.

Using MSCC screening, 60 aberrant methylated genes were demonstrated to be related to TKI treatment in both patients. Using MSCC screening on tissue samples from patient A and patient B, there were 621 and 220 genes that acquired TKI-induced methylation changes for these two patients, respectively, according to the DMRs with different levels of methylation changes ($\Delta$MSCC $\geq$ 20%). The results revealed that the genes were enriched in several different types of pathways, including endocytosis, long-term depression, Wnt signaling pathway, Cadherin signaling pathway, regulation of beta-cell development, and others, among which Wnt signaling pathway, Cadherin signaling pathway, and regulation of beta-cell development were enriched in both patients. C, The results of the gene ontology (GO) enrichment analysis of the 60 genes with aberrant methylation levels in both patients indicated that the 60 genes were over-represented (or under-represented) in pathways related to sequence-specific DNA binding, transcription factor activity, transcription regulator activity, DNA binding and RNA polymerase II transcription factor, and others.
methylation changes for these two patients, respectively, according to the DMRs with different levels of methylation changes (ΔMSCC ≥ 20%). We conducted pathway analysis based on the two sets of genes with methylation changes for each patient, showing that the genes were enriched in several different types of pathways, among which genes in the Wnt signaling pathway, Cadherin signaling pathway, and regulation of beta-cell development were enriched in both patients (Fig. 2A and B).

Notably, there were 60 genes with aberrant methylation levels found in both patients using whole-genome methylation screening. Supplementary Table S2 lists these 60 aberrant genes and the detailed information of the DMRs, including the chromosomal locations and average methylation changes. The result of the GO enrichment analysis based on these 60 genes with aberrant methylation levels revealed that these genes were enriched in pathways involving sequence-specific DNA binding, transcription factor activity, transcription regulator activity, DNA binding and RNA polymerase II transcription factor, etc (Fig. 2C). This indicated that the genes with changed methylation levels might have an effect on the expression of more genes by transcription regulation.

Two differential methylated genes were validated using the Sequenom EpiTYPER assay in both patients

By using the Sequenom EpiTYPER assay, the methylation level of the 60 genes (Supplementary Table S2) was further validated. The methylation state of the 60 genes from the MSCC sequencing was reanalyzed in both patients using the Sequenom EpiTYPER assay.
assay. Among the 60 genes screened, the methylation of GABBR2 and CBFA2T3 were clearly verified, with hypermethylation in red and hypomethylation in yellow, demonstrating that the adjacent CG sites exhibited increased methylation levels consistent with the MSCE screening results (Fig. 3A). The genomic locations of the 60 genes screened with aberrant methylation levels in both patients indicated that the methylation changes were at the whole-genome level, with the two validated genes, GABBR2 and CBFA2T3, marked by arrows in Fig. 3B. The relative locations of the DMRs in the two genes were presented in Fig. 3C, showing that the DMR of the GABBR2 gene was located exactly in the same location between exon 2 and exon 3 in both patients, while the DMRs of the CBFA2T3 gene were located in the TSS region in one patient and intron 1 in the other. Supplementary Table S2 showed the detailed information about these two genes, illustrating the exact same DMR location (chr_start: chr9_101338201) and the same direction of the methylation change for GABBR2 gene in both patients, with an average change of methylation in the DMRs at 42.35% for one and 23.50% for the other patient. The DMRs of CBFA2T3 were located in the TSS region (chr16_89045001) in one patient and intron 1 (chr16_89035601) in the other patient, and the average methylation change of the DMRs was 26.88% and 25.95%, respectively.

GABBR2 was verified to be a potential treatment target in vitro

We validated the role of GABBR2 and CBFA2T3 by treating lung cancer cells with erlotinib in vitro. After 1 μmol/L erlotinib treatment for 48 hours, GABBR2 expression was significantly decreased in EGFR-mutated lung cancer cells, HCC4006 and HCC827 cells, but remained conserved in EGFR wild-type A549 cells, while CBFA2T3 expression was not as significantly reduced as GABBR2 expression in HCC827 cells (Fig. 4A).

We then studied whether a reduction in the expression of protein GABBR2 is induced by methylation-related enzymes at the protein level. The expressions of DNMT1, TET2 and TET3 were significantly downregulated in HCC4006 and HCC827 cells compared to those in A549 cells, while DNMT3A expression did not show a significant change (Fig. 4A). The results illustrated that not only erlotinib induced a direct epigenetic effect on the GABBR2 gene, the downregulation of methylation-related enzymes may also regulate the methylation of GABBR2, these two aspects together leading to the reduction of GABBR2 expression.

We meanwhile detected the expression of GABBR2 in the tissues before and after induction erlotinib treatment in these two patients by IHC. The IHC results showed that the expression of GABBR2 was detected in the tissues before erlotinib treatment, while decreased dramatically after erlotinib therapy (Fig. 4B).

Considering there are two classes of GABA receptors: GABA_A and GABA_B, with 19 subtypes of GABA_A receptors in humans and only 2 subtypes of GABA_A receptors, GABBR1 and GABBR2, we reanalyzed erlotinib-related methylation changes of the whole GABA receptors families, 19 subtypes of GABA_A receptors, and 2 subtypes of GABA_B receptors, generated by MSCE sequencing. The methylation status of GABBR1 was not scrubbed by MSCE method because there were not enough CCGG sites in GABBR1 gene for analysis. Furthermore, the methylation status of 19 subtypes of GABA_A receptors were determined by MSCE, and the methylation of GABA_A receptor families was relatively conservative before and after erlotinib treatment (no 200-bp windows with >20% methylation change; Supplementary Table S3).

On the basis of all these results, together with the fact that the same DMR location and methylation change direction of GABBR2 gene were exhibited in both patients, the GABBR2 gene was selected as the targeted gene in the following studies.

Knockdown of GABBR2 did not further increase erlotinib-induced apoptosis in HCC4006 and HCC827 cells

After treatment with erlotinib, the apoptosis percentages of EGFR 19 deletion cells, HCC4006 and HCC827, were significantly higher compared with that of EGFR wild-type cells A549. The apoptosis percentages were 21.9%, 32.7%, and 5.54% in HCC4006, HCC827 and A549 cells, respectively (P < 0.05 for A549 compared with HCC4006, P < 0.01 for A549 compared with HCC827). After knockdown of the GABBR2 gene, apoptosis did not increase further in the cells harboring EGFR 19 deletion mutations, HCC4006 and HCC827 (P > 0.05, Fig. 4C and D).

GABBR2 overexpression rescued cleaved PARP and erlotinib-induced apoptosis in HCC827 cells, and the effect of GABBR2 in combination with erlotinib may play an important role in EGFR signaling

In our previous study, GABBR2 was found significantly decreased in HCC4006 and HCC827 cells when treated with erlotinib (Fig. 4A), and the apoptosis rate was the highest at 32.7% in HCC827 cells (P < 0.01 compared with A549) after treatment with erlotinib (Fig. 4C and D). So the role of GABBR2 was further investigated in HCC827 cells by cloning GABBR2 into the pCMV-HA plasmid to demonstrate whether overexpression of GABBR2 could rescue erlotinib-induced apoptosis in EGFR 19 deletion cells. The level of cleaved PARP, a marker of apoptosis, was obvious when treated with erlotinib, while upregulation of GABBR2 significantly rescued the apoptosis of HCC827 cells, reflected by the rescued level of cleaved PARP (Fig. 5A). The apoptosis results showed that the apoptosis percentage of HCC827 cells after treatment with erlotinib significantly increased from 32.2% to 54.3% compared with the control group and was rescued from 54.3% back to 36.8% with overexpression of GABBR2 (P < 0.05 when compared with the "control + erlotinib" group, and P > 0.05 when compared with the "control" group, Fig. 5B and C). These results showed that GABBR2 overexpression rescued erlotinib-induced apoptosis back to control levels in HCC827 cells, supporting the hypothesis that erlotinib-induced downregulation of GABBR2 which played a significant role in apoptosis of EGFR-mutated lung cancer cells.

To characterize whether the apoptosis induced by GABBR2 in combination with erlotinib is involved in EGFR signaling, we examined the expression of several key regulators, p-ERK1/2, t-ERK1/2, S6, and p-p70 S6, which function within EGFR signaling pathway (22). The caspase family proteins and cleaved PARP were also examined to investigate the underlying mechanism of apoptosis induced by erlotinib. The lysates were analyzed using antibodies against caspase-3 and its active cleaved form, cleaved caspase-3. As shown in Fig. 5D, after treatment with erlotinib, the expression of cleaved caspase-3 and cleaved PARP proteins, together with the decreased GABBR2 expression, were observed in both EGFR 19 deletion cell lines, HCC4006 and HCC827, but not in EGFR wild-type A549 cells. These results verified that GABBR2 was involved in the apoptotic effects of EGFR signaling when treated with erlotinib. Meanwhile erlotinib promoted a reduction in the expression of the phosphorylated proteins p-ERK1/2 in HCC4006 and HCC827 cells, with a minimal effect on the total protein levels of t-ERK1/2, and a reduction in the...
expression of S6 and p-p70 S6 which are the upstream of ERK1/2 pathway, but not in A549 cells, suggesting that GABBR2 may play an important role in EGFR signaling through the ERK1/2 pathway.

**Discussion**

Patients with advanced stage NSCLC harboring activating EGFR mutations tend to respond well to EGFR-TKIs. However, the benefit of EGFR-TKI regimens for locally advanced NSCLC patients still remains uncertain, and there have been some clinical trials (NCT01822496, NCT00600587) trying to apply this benefit to locally advanced stage NSCLC. On the basis of the current background, our research is to investigate whether epigenetic changes are involved in the response to induction TKI treatment in locally advanced EGFR-mutated lung cancer. Our research demonstrated that methylation of GABBR2 gene might play a role in the treatment of EGFR-mutated lung adenocarcinoma with induction erlotinib, suggesting that targeting GABBR2 together with EGFR inhibition may improve clinical outcome in patients with locally advanced NSCLC.

Changes in methylation patterns and levels have been associated with different types of cancers and could be used to predict response to chemotherapy. In our study, we observed changes in methylation patterns and levels in the GABBR2 gene before and after induction erlotinib treatment in locally advanced EGFR-mutated lung cancer. Our research showed that methylation of GABBR2 gene changed significantly after induction erlotinib treatment in HCC4006 and HCC827 cells, while it remained conserved in A549 cells. These results suggest that targeting GABBR2 gene together with EGFR inhibition may improve clinical outcome in patients with locally advanced NSCLC.
treatment response or outcomes in malignancies (23, 24), including lung cancer (23). However, the role of DNA methylation in the response to TKI therapy for the treatment of lung cancer has remained unclear. In our study, two lung adenocarcinoma patients with EGFR 19 deletion showed a marked response to TKI treatment, indicating a good foundation for the following whole-methylome screening. Hypomethylation of the TSS region and CGI region in our study was also consistent with previous studies (14, 18, 19). The pathway analysis of the differential methylated genes indicated that Wnt signaling pathway, Cadherin signaling pathway, and other pathways enriched in both patients are related to the EGFR pathway. Many studies have proven that Wnt signaling pathway has a role in EGFR-mutated lung cancer (8, 25, 26). Inhibition of various components of Wnt pathway with shRNAs or small molecules significantly increased the efficacy of EGFR inhibitors both in vitro and in vivo (25). The DNA methylation status of the Wnt antagonist SFRP5 can predict the response to EGFR-TKI therapy in NSCLC (8). Wnt inhibitory factor 1 (Wif1) promoter methylation is an early and frequent event as an epigenetic field manner, and Wif1 hypermethylation can be an unfavorable prognosis marker of NSCLC with EGFR mutation (26). The cadherin signaling pathway has also been well studied in NSCLC with EGFR mutation. E-cadherin, a hallmark of epithelial–mesenchymal transition (EMT), plays a significant role in the sensitivity regulation of EGFR molecular targeting treatment (27). EMT-induced cells by E-cadherin depletion facilitate invasion in a matrix metalloproteinase-2 (MMP2)-dependent manner with aberrant activation of EGFR signaling (28).

In our study, genes with altered DNA methylation in lung adenocarcinoma patients with EGFR 19 deletion before and after TKI treatment were first screened using an epigenetic genome-wide analysis and then verified by Sequenom EpiTYPER assay.

Figure 5.
GABBR2 overexpression rescued cleaved PARP and erlotinib-induced apoptosis in HCC827 cells, and the effect of GABBR2 in combination with erlotinib may play an important role in EGFR signaling. A, The apoptosis represented by cleaved PARP was obvious when HCC827 cells were treated with 2 μmol/L erlotinib for 48 hours, while upregulation of GABBR2 significantly rescued the apoptosis of HCC827 cells, which was reflected by the rescued level of cleaved PARP. B and C, The apoptosis percentage of HCC827 cells after treatment with 2 μmol/L erlotinib for 48 hours significantly increased from 32.2% to 54.3% compared with the “control” group and was rescued from 54.3% back to 36.8% when the GABBR2 gene was overexpressed by cloning into a pCMV-HA plasmid (*P < 0.05 when compared with the “control–erlotinib” group, and P > 0.05 when compared with the “control” group), showing that GABBR2 overexpression rescued erlotinib-induced apoptosis, reverting to the level found in the “control” group in HCC827 cells. D, The expression of cleaved caspase-3 and cleaved PARP proteins, together with the decreased GABBR2 expression, was observed in both EGFR 19 deletion cell lines (HCC4006 and HCC2827), but not in EGFR wild-type A549 cells, with treatment of 1 μmol/L erlotinib for 48 hours. At the same time, erlotinib promoted a reduction in the expression of the phosphorylated proteins p-ERK1/2 in HCC4006 and HCC827 cells, with minimal effect on the total protein levels of t-ERK1/2, and a reduction in the expression of S6 and p-p70 S6, which are the upstream of ERK1/2 pathway, but not in A549 cells, suggesting that GABBR2 may play an important role in EGFR signaling through the ERK1/2 pathway.
with the result showing that the DMR of GABBR2 gene was located in the same region of the two patients. The IHC results showed that the expression of GABBR2 was decreased dramatically after erlotinib therapy. The following in vitro experiments also suggested that TKI treatment induced downregulation of GABBR2 and regulated the expression of methylation-related enzymes DNMT1 and TET, which may, in turn, result in the aberrant methylation and downregulation of GABBR2. There are three enzymatically active mammalian DNMTs-DNMT3A, DNMT3B and DNMT1. DNMT3A and DNMT3B are de novo methyltransferases that establish DNA methylation patterns by targeting unmethylated CpG sites. DNMT1 acts primarily as a maintenance methyltransferase. TET proteins are α-ketoglutarate-dependent dioxygenases involved in the conversion of 5-methylcytosines (5-mC) to 5-hydroxymethylcytosine (5-hmC) (29, 30). DNMTs and TETs play crucial roles in the cytosine methylation and demethylation process. The intronic DMR may block transcription elongation of GABBR2 through affecting histone deacetylation and chromatin structure (31, 32) and downregulate gene expression by regulating the methyl-sensitive binding of transcription factors (33). The relationship between GABBR2 and cancer development and prognosis has been considered in several studies (34–36). The possible role of GABBR2 in cancer progression was first reported by Stein and colleagues and increased expression of GABBR2 was demonstrated in papillary thyroid carcinomas (PTC) using gene expression profiling (34). Similarly, by using a whole-transcript expression array, Schulten and colleagues found that expression of GABBR2 was significantly upregulated in the follicular variant of PTC (35). Notably, the gene expression levels of GABBR2 were also significantly higher in NSCLC tissue than in the paired paracancerous tissue, and patients with high expression of GABBR2 gene had a better prognosis (36). However, the relationship between the methylation status of GABBR2 gene and EGFR-TKI response in lung cancer has not been well investigated before.

Our in vitro function study demonstrated that overexpression of GABBR2 rescued the TKI-induced cell apoptosis, suggesting that GABBR2 may be involved in EGFR-mutated lung cancer–targeted therapy. Erlotinib downregulated GABBR2 expression in EGFR 19 deletion cells, along with a reduction in the expression of S6, p-p70 S6, and p-ERK1/2, suggesting that GABBR2 may play an important role in EGFR signaling through the ERK1/2 pathway which may occur in two ways. First, GABBR2 may promote phosphorylation of ERK directly. One research revealed that the GABA(B) receptor can induce ERK1/2 phosphorylation in the CA1 area (region I of the hippocampus proper) of the mouse hippocampus (37). Activation of the GABA(B) receptor by either GABA or the selective agonist baclofen can induce ERK1(2) phosphorylation in cultured cerebellar granule neurons (38). Second, we observed the aberrant methylation of the cadherin signaling pathway, and as mentioned above, ERK is known to be involved in cadherin signaling (28, 39); therefore, the phosphorylated proteins, p-ERK1/2, may be downregulated by the aberrant methylation of the cadherin signaling pathway. In addition, we also demonstrated the reduction of S6 and p-p70 S6 along with the downregulation of p-ERK1/2. Cross-talk between ERK and P70 S6 signaling has long been considered as early as in the year 2001 (40). In cancer, growth factors, including epidermal growth factors (EGF), can activate ERK and PI3K pathways (22), while either activation of ERK or PI3K pathway can contribute to the activation of S6 and protein synthesis via upregulation of P70 S6 (22). Overall, our study demonstrated that GABBR2 may play a role in EGFR signaling through the ERK1/2 pathway and its cross-talk pathway, P70 S6 signaling.

Although the epigenetic mechanism of TKI therapy in lung cancer was studied for the first time, there are still some shortcomings in our research. First, multiple tools and methods have been developed and used for differential methylation data preprocessing and data analysis, such as whole-genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS). Thus, the question that remains to be answered is which platform should be used to better fit the needs of this study. MSCC sequencing technology could quantify approximately 1.1% of all CG sites in humans (14). The CCGG sites sequenced in the present study could well reflect the methylation status of all CG sites; and the number of CpGs with sufficient coverage from each appears more efficient for CpG capture, making sequence-based MSCC technology adequate and reliable for methylation experiments. Second, we investigated the possible function of GABBR2 in cell lines and noticed the relationship to the ERK signaling pathway. However, a more detailed mechanism, including whether overexpression of GABBR2 could rescue the expression of S6, p-p70 S6, and p-ERK1/2, requires further study. In addition, xenograft preclinical studies in vivo may be more helpful to elucidate the tumor-related function of GABBR2. Third, there were only two patients in our study, which could limit our final results in some way. Considering this limitation, we first used MSCC to screen for changes in methylation, then used a Sequenom Epityper assay to validate our screening results, and finally confirmed our results with the functional experiments in vitro. Importantly, we used high quality, fresh surgical tumor specimens to provide accurate and adequate molecular diagnoses, which are necessary for biomarker discovery and validation. Although it is difficult to collect the tumor tissues before and after induction TKI treatment with remarkable response, our report still describes our experience and challenges in obtaining fresh lung tumor specimens for the utilization in EGFR-related biomarker discovery. In addition, the ideal duration for induction treatment with EGFR-TKI drugs is still uncertain. Some clinical trials (NCT01822496, NCT00600587, NCT01407822) were performed to evaluate the value of induction erlotinib therapy for uninterrupted 42 days before thoracotomy or radiotherapy in EGFR-mutated IIIa-N2 NSCLC, which were the same design with our research. But caution should be used when interpreting the epigenetic mechanism of TKI therapy for different induction treatment durations and different generation TKI drugs.

In summary, we performed a genome-wide DNA methylation analysis in EGFR-mutated lung adenocarcinoma with induction erlotinib treatment and found that the GABBR2 gene plays a role in targeted therapy. Our findings provide a new theoretical basis for the application of epigenetic studies in EGFR-mutated lung adenocarcinoma and suggest that targeting GABBR2 together with EGFR inhibition may improve clinical outcomes in patients with locally advanced NSCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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DNA Methylen Screen for Lung Adenocarcinoma with Erlotinib

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