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Study of the methylation patterns of the EGFR gene promoter in non-small cell lung cancer

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ABSTRACT. We investigated the methylation state of the epidermal growth factor receptor (EGFR) gene promoter in non-small cell lung cancer (NSCLC) and analyzed its effect on tumor biology. We enrolled 120 patients with NSCLC who had been confirmed by pathologic diagnosis and had been operated on. The methylation states of the EGFR gene promoter were detected and analyzed and a prognosis was given. NSCLC cell lines and nude mice were used to study the treatment reactivity of gefitinib (an EGFR inhibitor) with or without 5-aza-2'-deoxycytidine (5-aza-CdR) intervention. EGFR expression was high when the methylation degree was lower in patients with adenocarcinoma and poor pathological differentiation of tumor than in patients with squamous cell carcinoma and good pathological differentiation. NSCLC cells with low expression of EGFR and high methylation in the promoter region were insensitive to EGFR-targeted therapy. However, apoptosis and proliferation inhibition of cancer cells were even more pronounced when 5-aza-CdR was used to inhibit methylation. An *in vivo* study confirmed that methylation

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adjuvant therapy can improve the sensitivity of cancer to EGFR-targeted therapy. Application of a demethylating agent could be an important supplement for improving EGFR inhibition in the treatment of NSCLC, especially in those who are insensitive to the use of an EGFR inhibitor alone.

Key words: Non-small cell lung cancer; EGFR gene promoter; Epidermal growth factor receptor; Methylation

INTRODUCTION

Lung cancer is a malignant tumor and is currently responsible for a high mortality rate worldwide (Eisenberg-Lerner and Kimchi, 2012). Following the deterioration of the ecological environment and air pollution, the morbidity and mortality resulting from lung cancer keep on rising each year. Although there are many lung cancer lesion types, non-small cell lung cancer (NSCLC) is the most common one. At present, there are many different treatments for NSCLC. The response of individual patients to treatment mainly depends on the degree of tumor development, the pathological type, and their sensitivity to treatment (Zhu et al., 2012). Among the studies conducted on the treatment of NSCLC, studies involving the epidermal growth factor receptor (EGFR) target are widely respected. Although abnormal EGFR expression is ubiquitous in NSCLC patients (Easwaran and Baylin, 2012), there has been little investigation of the cause of the abnormal expression and its significance in clinical diagnosis and treatment (Li et al., 2013).

MATERIAL AND METHODS

Clinical samples

We enrolled 120 patients between January 2011 and January 2014 including 82 males and 38 females who had been confirmed as suffering from NSCLC by pathologic diagnosis and had been operated on. The patients' mean age was 56.9 years (42-71 years). The pathological TNM stage was I in 31 specimens, II in 43 specimens, III in 36 specimens, and IV in 10 specimens. There were 79 cases with squamous cell carcinoma and 41 cases with adenocarcinoma. Written informed consent was obtained from all participants before surgical sample collection. The specimens were cleaned with sterile phosphate-buffered saline and cryopreserved in liquid nitrogen. All patients were followed up for 1 year for their sensitivity to gefitinib after first-line chemotherapy.

EGFR expression detection

The tumor samples or cancer cell lines were ground and total RNA was extracted using TRIzol. The extracted RNA was reverse-transcribed to cDNA and real-time polymerase chain reaction (PCR) was performed to detect EGFR expression. The primer used was as follows: forward, 5'-CGCTGCTGCCTGCGCTCTG-3'; and reverse, 5'-AGCCACCTCCTGGATGGTC-3'.

EGFR methylation detection

cDNA was methylated using a Methylation-Gold Kit and PCR was performed with the primer focused on the EGFR promoter. The methylation primer used was as follows: forward, 5'-GGTTGGGTTTGTAAGTTCGC-3'; and reverse, 5'-ATAAACAACGATAACCCCCG-3'. The non-methylation primer was as follows: forward, 5'-GGTTGGGTTTGTAAGTTTGT-3'; and reverse, 5'-TAAACAACAATAACCCCCA-3'.

Methylation detection referred to the methylation fluorescence polarization measurement technology (CN 101736083 A). Each real-time reverse transcription PCR mixture (in 25 µL) contained 2.5 µL 10X PCR buffer, 2 µL 2.5 mM $MgCl_2$, 2.5 µL 2.5 mM dNTP, 0.5 µL primer, 1 µL Taq polymerase, and 2 µL template. The cycling conditions were 40 cycles of 30 s at 95°C, 30 s at 56°C, and 30 s at 72°C. Agarose gel electrophoresis was used to analyze the PCR products. The reaction was considered methylation-positive if specific products were amplified by the methylation primer, while the reaction was considered methylation-negative if specific products were amplified by the non-methylation primer but not by the methylation primer.

NSCLC cell line culture

EGFR mutant cell line HCC827 and EGFR wild-type cell line H1299 were maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal calf serum (Gibco, Gran Island, NY, USA) in a humid atmosphere containing 5% CO_2 at 37°C. Cells in the logarithmic phase of growth were used for all experiments.

Cell line stimulation and detection

Gefitinib of different concentrations was added to the cancer cell lines and the proliferation, apoptosis, and migration activity were tested. The EGFR-insensitive cancer cell line was further treated with 1 µM 5-aza-2'-deoxycytidine (5-aza-CdR).

Proliferation inhibition test

Cells were seeded on 96-well plates at a density of 1 x 10^4 cells/well and incubated overnight at 37°C. The cells were incubated with different concentrations of gefitinib with or without 5-aza-CdR for 48 h at 37°C. After addition of 10 µL CCK8 to each well, plates were incubated for 4 h at 37°C. The absorbance of each well, measured as optical density (OD), was read using a spectrophotometer. Cell viability = OD (test group) / OD (control).

Apoptosis test

Cells were seeded on 6-well plates at a density of 1 x 10^5 cells/well and incubated for 24 h at 37°C. The cells were incubated with different concentrations of gefitinib with or without 5-aza-CdR for 72 h at 37°C. After staining with propidium iodide for 30 min, flow cytometry was used to detect the cell cycle.

Animal experiments

Nude mice were injected with 2 x 10^7 H1299 cells in the logarithmic phase of growth at 6-7 weeks through the caudal vein and the intervention began after 1 week. The treatments included: A) 0.2 mL gefitinib at 40 mg/kg; B) 0.2 mL gefitinib at 40 mg/kg + 0.2 mL 5-aza-CdR at 0.25 mg/kg; and C) 0.2 mL normal saline, and they were injected once every 2 days for 2 weeks. The survival time of the mice was plotted.

Statistical analysis

Differences between groups were analyzed using the Student *t*-test or one-way analysis of variance. All statistical analyses were performed using the SPSS v17.0 software (Chicago, IL, USA). P values <0.05 were considered to be statistically significant.

RESULTS

EGFR expression and promoter methylation

Forty-five cases (37.5%) were positive for EGFR promoter methylation among the 120 enrolled NSCLC samples. The EGFR promoter methylation rate had a close relationship with the pathological differentiation and squamous cell carcinoma/adenocarcinoma classification, while there was a lack of significant correlation with TNM staging. The methylation rate in samples exhibiting poor differentiation was significantly lower than that in those showing strong differentiation. The EGFR expression rate was lower in the poorly differentiating NSCLC cells than in the strongly differentiating ones. Moreover, the EGFR promoter methylation level was significantly higher in patients that were insensitive to EGFR-targeted therapy than in the sensitive ones ($P < 0.05$; Table 1).

Table 1. EGFR expression and promoter methylation in NSCLC tissues.

Group	N	EGFR PCR intensity	Positive methylation (%)
TNM stage			
I+II	74	14.83 ± 2.42	27 (36.49)
III+IV	46	15.14 ± 2.66	18 (39.13)
Pathological stage			
Well	64	7.22 ± 5.61	30 (53.57)
Moderate and poor	56	19.32 ± 6.59*	15 (23.44)*
Tumor type			
Squamous carcinoma	89	13.14 ± 5.33	14 (45.16)
Adenocarcinoma	31	17.22 ± 4.87*	31 (34.83)*
Gefitinib sensitivity			
Sensitive	71	16.43 ± 6.26	34 (47.89)
Insensitive	49	12.14 ± 4.79*	11 (22.45)*

*P < 0.05 compared to the two projects with the same category.

For the two NSCLC cell lines, the EGFR expression level was relatively low in the EGFR wild-type cell line H1299, and EGFR promoter methylation was positive. In contrast, the EGFR mutant cell line HCC827 expressed higher levels of EGFR and had negative promoter methylation status. Both of the two cell lines exhibited demethylation after treatment with 5-aza-CdR (Table 2).

Table 2. EGFR expression and promoter methylation in NSCLC cell lines.

Cell type	EGFR PCR intensity	Methylation	5-aza-CdR treated
HCC827	0.521 ± 0.022	-	-
H1299	0.031 ± 0.002*	+	-

*P < 0.05 compared to HCC827.

Cell proliferation and apoptosis activity

After treatment with 0-20 μM gefitinib, the response from the EGFR mutant cell line H1299 was poor, while the response from the EGFR wild-type cell line HCC827 was notable. The sensitivity of H1299 to gefitinib increased significantly after treatment with 5-aza-CdR, while the sensitivity of HCC827 did not change greatly (Figure 1).

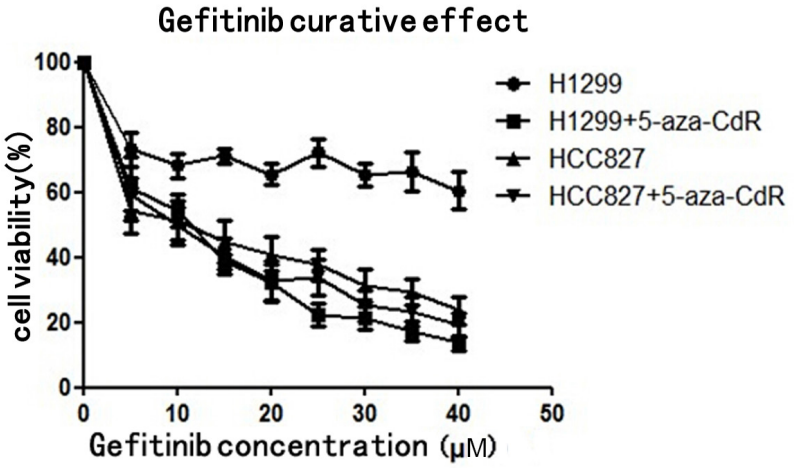

Figure 1. Gefitinib treatment response on EGFR mutant and wild-type cell line. H1299 was insensitive to gefitinib but can be improved by 5-aza-CdR. HCC827 was relative sensitive to gefitinib and the impact of 5-aza-CdR was less.

In contrast, gefitinib promoted EGFR mutant cell line apoptosis and the number of cells in the G_0/G_1 stage increased, as revealed by propidium iodide staining. However, gefitinib failed to exert a similar effect on the EGFR wild-type cell line H1299. The sensitivity of H1299 to gefitinib increased significantly after treatment with 5-aza-CdR (Table 3).

Table 3. NSCLC cancer cell changes in cell cycle after treatment with gefitinib.

Group	G_0/G_1	G_2	S
H1299	25.39 ± 1.7	22.04 ± 2.1	35.28 ± 1.9
H1299 + gefitinib	27.22 ± 2.1	23.54 ± 2.3	32.17 ± 2.5
H1299 + gefitinib + 5-aza-CdR	67.28 ± 5.2*	15.32 ± 1.3	16.25 ± 1.1
HCC827	29.29 ± 3.2	25.35 ± 1.9	36.79 ± 2.8
HCC827 + gefitinib	61.38 ± 2.6	12.42 ± 2.3	16.17 ± 1.2
HCC827 + gefitinib + 5-aza-CdR	60.03 ± 2.3	13.19 ± 1.5	17.92 ± 1.9

*P < 0.05 compared to the two projects with or without 5-aza-CdR with the same category.

Nude mouse transplantation tumor and treatment analysis

Gefitinib with or without 5-aza-CdR was used to treat nude mice that had been transfected with lung cancer (Figure 2). Compared with mice treated with gefitinib alone, the survival time of the nude mice that had been injected with the H1299 cell line, which was insensitive to gefitinib, was significantly longer after treatment with gefitinib + 5-aza-CdR.

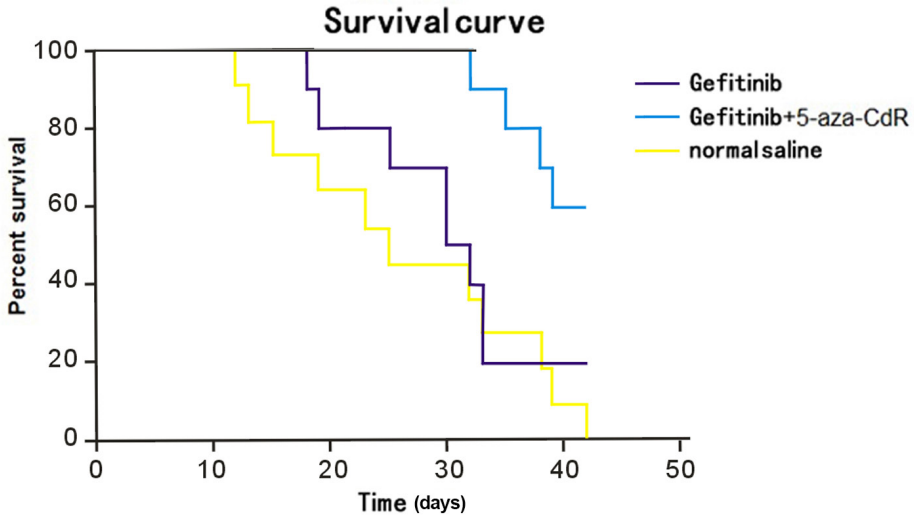

Figure 2. Nude mouse transplantation tumor survival curve. Ten nude mice in each group were injected by EGFR mutant cell line H1299 and treated by gefitinib with or without 5-aza-CdR. The observation time was 42 days.

DISCUSSION

Tumor development often involves multiple mutations that affect the intracellular signaling transduction process. In recent years, many intracellular signaling pathways have been found to be closely associated with cancer development. Among these, the EGFR pathway is currently the subject of intensive research (Johnson, 2012). As one of the members of the receptor tyrosine kinase super family, EGFR is coded by the proto-oncogene C-erbB1. It is a transmembrane receptor protein and is expressed on the cell surface. EGFR is the receptor for epidermal growth factor and is important in promoting oncogenesis (Shinjo et al., 2012). Abnormal expression of EGFR is ubiquitous in malignant tumors mainly originating in the glandular epithelium, such as NSCLC (Tasaki et al., 2011; Cui et al., 2012; Cumberbatch et al., 2014). Some researchers have reported that EGFR gene mutation in the encoding area produces EGFR structural changes that eventually lead to cells presenting the molecular biology of cancer (Tasaki et al., 2011).

EGFR expression can be regulated by methylation in the promoter region. Through methylation, cells can methylate CpG islands in the particular promoter region of the gene without changing the DNA sequence in the region of methylation, allowing it to combine with the DNA-binding protein and silence the expression (Watanabe et al., 2012). However,

in tumor cells, gene mutation in the related encoding area produces oncogenes and tumor suppressor gene methylation changes, leading to abnormal receptor protein expression and signaling pathways (Shinjo et al., 2012). EGFR overexpression, therefore, is one of the important characteristics of cancer cells, and is also an important target for cancer therapy.

Previous clinical and basic research has confirmed that EGFR overexpression is correlated with pathological severity and TNM stage in many tumors; the more EGFR is expressed, the greater the tumor pathological severity is. For example, research has revealed that EGFR expression is higher in patients' ovarian cancer specimens compared with the surrounding normal tissues, while EGFR mRNA expression is also higher in poorly differentiated and metastatic ovarian cancer (Cui et al., 2012). EGFR expression levels are also higher in high Dukes' stage colorectal cancer and gastric cancer patients with severe tumor invasion (Pass et al., 2013). In the present study, we found that EGFR expression was higher in NSCLC cells with poor differentiation, while the degree of invasion and malignancy of these cells was more marked. Conversely, these cells were almost all negative for EGFR methylation. However, unlike other tumors, EGFR expression in NSCLC does not seem to be correlated with the clinical stage, which suggests that EGFR expression may be more closely related to differentiation.

The current trend in oncotherapy is towards monoclonal targeted therapy (Oleksiewicz et al., 2011; Lee et al., 2013; Forde et al., 2014). The new chemotherapy drugs exhibit high specificity with significant cancer-killing effects and less drug toxicity. However, some patients have shown drug resistance in clinical treatment. Cytological studies have shown that tumor cells may have reduced susceptibility to the curative effects of targeted drugs arising from abnormal DNA methylation (Yanagawa et al., 2011; Yasuda et al., 2011; Dasgupta et al., 2013). For example, the breast cancer cell lines CAMA1 and MB453a with a high degree of EGFR gene methylation are insensitive to gefitinib (Pesek et al., 2011; Yamadori et al., 2012), and the drug sensitivity of these cells can be improved by demethylation (Toyooka et al., 2011). In the present study, we chose the EGFR wild-type cell line H1299 and EGFR mutant cell line HC837 to represent the clinical features. We confirmed the different EGFR expression statuses of the two cell lines by PCR and fluorescence polarization measurement technology. H1299 with low EGFR expression was insensitive to gefitinib, which cannot influence cell proliferation, apoptosis, or animal survival in the H1299 cell line. However, sensitivity to gefitinib in H1299 cells was increased after treatment with 5-aza-CdR. The curative effect of such a combination chemotherapy was better than that of the single targeted therapy. Thus, removing artificial EGFR inhibition may opened a door for targeted drugs, and this treatment is a promising new concept of multidrug therapy.

CONCLUSION

Our study showed that EGFR expression and methylation levels were closely related to cancer cell pathological type and stage in NSCLC patients. EGFR expression in adenocarcinoma or poorly differentiated carcinoma was low, while the rate of methylation of the promoter was high. The opposite was true in highly differentiated carcinoma or squamous cell carcinomas. Although the cell and animal experiment results showed that NSCLC cells with low EGFR expression and high methylation of the promoter region were insensitive to EGFR-targeted therapy, tumor cell apoptosis and proliferation inhibition were more significant following 5-aza-CdR adjuvant treatment. Further *in vivo* experiment results also confirmed that demethylation therapy can significantly improve the insensitivity of EGFR-

targeted therapy. Thus, demethylation adjuvant therapy for NSCLC, especially in cases of insensitivity to EGFR-targeted therapy, may be an important improvement in chemotherapy strategy. Further molecular methylation mechanisms and animal experimental research are needed to support the clinical application of the therapy.

Conflicts of interest

The authors declare no conflict of interest.

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