Genetics and Molecular Research

Relationship of *EGFR* DNA methylation with the severity of non-small cell lung cancer

J. Li[1]*, X.F. Jia[1]*, J. Liu[2], J.J. Liu[3] and H.B. Zhao[1]

[1]Department of Oncology, First People's Hospital of Jining, Jining, China
[2]Department of Oncology, Shandong Cancer Hospital and
Institute Shandong Academy of Medical Sciences, Jinan, China
[3]Department of Respiration, First People's Hospital of Jining, Jining, China

*These authors contributed equally to this study
Corresponding author: H.B. Zhao
E-mail: ZHAOHaiBo103@163.com

Genet. Mol. Res. 14 (4): 11915-11923 (2015)
Received March 31, 2015
Accepted June 25, 2015
Published October 5, 2015
DOI http://dx.doi.org/10.4238/2015.October.5.5

ABSTRACT. The aim of this study was to study the relationship of *EGFR* DNA methylation with the severity of non-small-cell lung cancer (NSCLC). We enrolled 54 patients with NSCLC between March 2013 and June 2014 from Department of Cardiothoracic Surgery in our hospital. The methylation levels in the promoter region of the *EGFR* gene in cancerous and pericarcinomatous tissue were tested by pyrosequencing. *EGFR* mRNA expression levels were detected by real-time reverse-transcription polymerase chain reaction. The SPSS software was used for data analysis. We found that *EGFR* gene methylation levels showed no significant differences among patients of different gender, age, or smoking status. *EGFR* DNA methylation levels significantly increased ($P < 0.05$) following NSCLC malignancy upgrading, and showed negative correlation with mRNA expression ($P = 0.041$). DNA methylation levels of cancerous tissues were significantly higher compared to the corresponding pericarcinomatous tissues ($P < 0.05$) at stages I, II, and IIIA. The methylation levels at loci 3, 6, 9 among the detected CpG islands were higher in the cancer tissues at each stage ($P < 0.05$). In summary, our

results suggest that the DNA methylation levels of *EGFR* can be used as an important indicator for the stage of cancer tissue malignancy.

Key words: Epidermal growth factor receptor; DNA methylation; Nom-small cell lung cancer

INTRODUCTION

Epidermal growth factor receptor (EGFR), also called ErbB-1/HER1, is a member of the HER family. It regulates many kinds of biological functions such as cell proliferation, invasion, and migration through the Ras/Raf/MAPK and PI3K/AKT pathways (Hynes and Lane, 2005). It has been found that *EGFR* and *erb-2* genes were overexpressed in 60 patients with lung squamous cell carcinoma (Piyathilake et al., 2002). Furthermore, expression of these proteins gradually increased following the degree of malignancy. In addition, EGFR overexpression is associated with several tumor characteristics such as proliferation, blood vessel formation, cell adhesion, invasion, metastasis, and apoptosis (Oh et al., 2000). Thus, *EGFR* plays a vital role in tumorigenesis.

Many studies have shown that abnormal DNA methylation is closely related to tumorigenesis (Palii and Robertson, 2007). DNA methylation is an important component of epigenetic regulation. Normal DNA methylation is a key factor of cell function, genetic imprinting, embryonic development, and tumorigenesis in humans. Several studies have suggested that tumor suppressor gene promoter CpG methylation is associated with non-small cell lung cancer (NSCLC), including methylation of the DNA repair gene *hMLH1* (Kashiwababra et al., 1998) and the cell growth-related gene *P16* (Loeb et al., 2001). NSCLC is the most common type of lung cancer. Unfortunately, distant metastases are identified in most patients upon initial presentation (Jemal et al., 2010; Reungwetwattana et al., 2012).

The methods available for methylation research have advanced along with the development of biotechnology. Pyrophosphate sequencing is a new form of high throughput automatic sequencing technology that can accurately quantitate DNA methylation levels at a particular genomic locus in up to 96 samples concurrently (Tost et al., 2006). Therefore, the present study aimed to ascertain the methylation levels of the *EGFR* locus in carcinoma tissues at different degrees of malignancy through the use of pyrophosphate sequencing technology.

MATERIAL AND METHODS

Material

Reagents and instruments

The reagents utilized in this study included a genome-wide extraction kit (PureLinK Genomic DNA Mini Kit, Invitrogen, Carlsbad, CA, USA), a bisulfite modification kit (EpiTect Fast DNA Bisulfite Kit, QIAGEN, Venlo, the Netherlands), a polymerase chain reaction (PCR) amplification kit (PyroMark PCR Kit, QIAGEN), pyrophosphate sequencing analysis reagents (PyroMark CpG Assay 96 well, QIAGEN), TRIzol (Invitrogen), 3'-end DNA biotin-labelling kit (Beyotime, Shanghai, China), 2X SYBR Green Real-Time PCR Master Mix (Tiangen

Biotech, Beijing, China) and a real-time fluorescent quantitative RT-PCR kit (TaKaRa, Otsu, Shiga, Japan). Equipment included a PyroMark Q96 ID sequencer (QIAGEN), a Gel Doc imaging system (BioRad, USA), a ViiA7-type Fluorescent, and a quantitative PCR Amplifier (Applied Biosystems Inc., Foster City, CA, USA).

Methods

Patients' information and sample collection

The protocol of this study was approved by the Ethics Committee of the First People's Hospital of Jining (Jining, Shangdong, China). Informed consent was obtained from all subjects. We enrolled 54 patients with NSCLC diagnosed by pathologic examination and who received surgical treatment between March 2013 and June 2014 from the Department of Cardiothoracic Surgery in the First People's Hospital of Jining. The study group consisted of 39 men and 15 women with an average age of 67.23 ± 7.93 years. Smoking status was considered positive for at least one cigarette daily and continuous for over 1 year. NSCLC was divided into stages I, II, and IIIA according to the TNM-grading system. None of the patients received preoperative radiotherapy and/or chemotherapy. Tumor tissues and adjacent tissue specimen (about 1 cm^3) were collected during the surgery and frozen for further use.

DNA extraction and whole genome DNA bisulfite modification

The PureLinK Genomic DNA Mini Kit was utilized for DNA extraction from carcinoma or adjacent tissue samples (around 1.0 g) according to the manufacturer protocol. An ultraviolet spectrophotometer (Yuanxi Instrument, Shanghai, China) was used to detect the content and purity of isolated DNA. Bisulfite modification of 1 μg purified DNA was accomplished using the EpiTect Fast DNA Bisulfite Kit. Genomic DNA treated with the methyltransferase *Sss*I and converted by sodium bisulfite was considered as a positive control.

EGFR mRNA extraction and real-time reverse-transcription PCR (RT-PCR)

cDNA was synthesized using reverse transcriptase (TaKaRa), oligo (dT) primers, and 1 μg RNA extracted from tumor or adjacent tissue samples. Each real-time RT-PCR (in 10 μL) contained 2X SYBR Green Real-Time PCR Master Mix, 0.5 μL 10 μM primers, and 0.5 μL template cDNA. The cycling conditions consisted of an initial, single cycle of 5 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. PCR amplifications were performed in triplicates for each sample. Gene expression levels were quantified relative to the expression of β-actin using an optimized comparative $2^{-\Delta\Delta Ct}$ value method. The differences in gene expression levels between groups were compared using the Student *t*-test. P values < 0.05 were considered to be statistically significant. The primers used were as follows: β-actin: forward primer 5'-AAA CTG GAA CGG TGA AGG TG-3', reverse primer 5'-AGT GGG GTG GCT TTT AGG AT-3', product length: 166 bp. *EGFR*: forward primer 5'-GCC TCC AGA GGA TGT TCA ATA A-3', reverse primer 5'-TGA GGG CAA TGA GGA CAT AAC-3', product length: 132 bp.

Pyrophosphate sequencing

DNA that had undergone bisulfite modification and subsequent purification was used for *EGFR* gene amplification and electrophoresis. PCR products wer labeled with biotin at their 3'-end using a labeling kit and were mixed with microbeads containing streptavidin to separate the single-stranded amplified product. Single-stranded DNA was mixed with sequencing primers and the *EGFR* gene methylation status was detected on a Pyromark Q96 ID pyrosequencing system.

Statistical analysis

Differences between two paired samples were analyzed using the Student *t*-test. Enumeration data were analyzed by the chi-squared test with inspection level at $\alpha = 0.05$. All statistical analyses were performed using the SPSS13.0 software (SPSS, Chicago, IL, USA).

RESULTS

EGFR gene methylation levels in NSCLC and their correlation with clinical pathological characteristics

We enrolled 54 patients with NSCLC for this study, including 15 women (27.8%) and 39 men (72.2%); the proportion of men to women was 2.6:1. *EGFR* gene methylation levels showed no obvious differences among the patients with different gender, age, or smoking status. However, *EGFR* methylation increased significantly according to the upgrading clinical stage ($P < 0.05$; Table 1).

Table 1. *EGFR* gene methylation levels in NSCLC and their correlation with clinical pathological characteristics.

Clinicopathologic characteristics	Patient (N)	*EGFR* DNA methylation	P value
Gender			
Male	39	0.36 ± 0.21	0.587
Female	15	0.39 ± 0.22	
Age (years)			
<60	17	0.36 ± 0.23	0.212
≥60	37	0.38 ± 0.20	
Smoking status			
Yes	34	0.40 ± 0.23	0.893
No	20	0.37 ± 0.21	
Clinical staging			
Stage I	22	0.37 ± 0.23	<0.05
Stage II	14	0.41 ± 0.20	
Stage III	18	0.56 ± 0.11	

Data are reported as means ± standard deviation (SD). NSCLC = non-small cell lung cancer.

EGFR methylation and mRNA levels in cancer tissue

Pyrosequencing was utilized to detect the DNA methylation levels in cancer tissue from patients with NSCLC as follows: the methylation levels of 10 CpG loci were

equalized, and relative mRNA levels were determined using a real-time RT-PCR. As described previously, we observed that with the ascending degree of malignancy, the DNA methylation level increased (Figure 1). Accordingly, the DNA methylation levels in stage III cancer tissue were significantly higher than those in stage I ($P < 0.05$). On the other hand, the mRNA levels in stage III cancer tissue were markedly lower than those in stage I. The correlation coefficient was significantly negative between mRNA expression and DNA methylation levels ($R = -0.54$, $P = 0.041$).

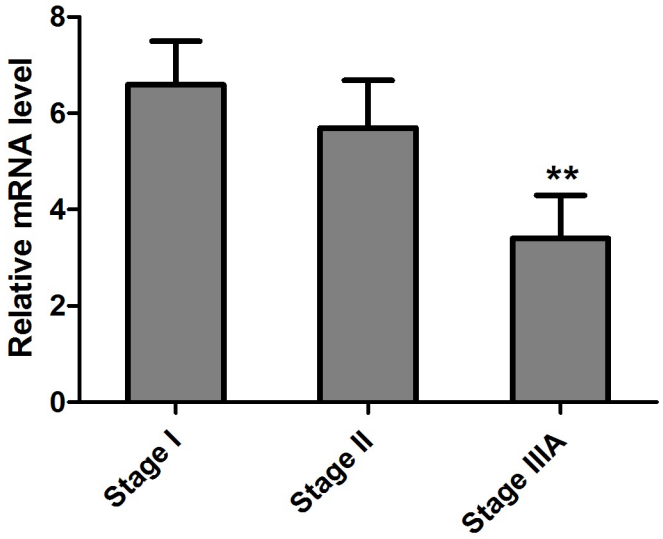

Figure 1. *EGFR* mRNA levels in different stage cancer tissues. Data are reported as means ± standard deviation (SD). **P < 0.05 compared with Stage I.

EGFR methylation levels in cancerous and pericarcinomatous tissue

DNA methylation analysis of the *EGFR* gene showed that the levels increased significantly in the cancerous compared with the corresponding pericarcinomatous tissue (Figure 2). Furthermore, as described previously, methylation levels were higher in stage III compared with stage I cancerous tissue. Together, these results suggested that *EGFR* methylation levels are upregulated following the malignancy of NSCLC, which might negatively affect *EGFR* expression.

Different CpG locus methylation levels in patients with different NSCLC stages

Upon further examination, we identified that the level of DNA methylation at various CpG loci in the *EGFR* gene differed between groups (Figure 3). Compared with the pericarcinomatous tissue, every *EGFR* locus examined in the cancerous tissue showed an increase in methylation level. Cancerous tissue from patients with different NSCLC stages all exhibited upregulated methylation at loci 3, 6, and 9 as compared with pericarcinomatous tissue at stages I and II ($P < 0.05$), whereas the methylation levels of cancerous tissue at loci 2, 5, 7, and 9 were higher in stage IIIA than those of pericarcinomatous tissue.

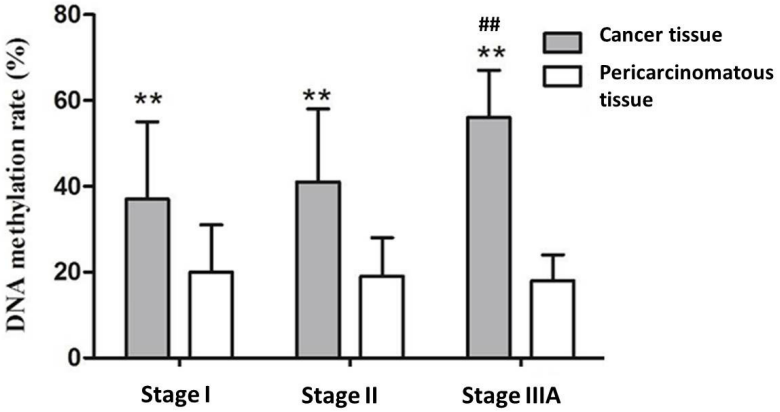

Figure 2. *EGFR* methylation level comparison in different stages of cancer and pericarcinomatous tissues. Data are reported as means ± standard deviation (SD). **P < 0.05 compared with pericarcinomatous tissue; ##P < 0.05 compared with Stage I.

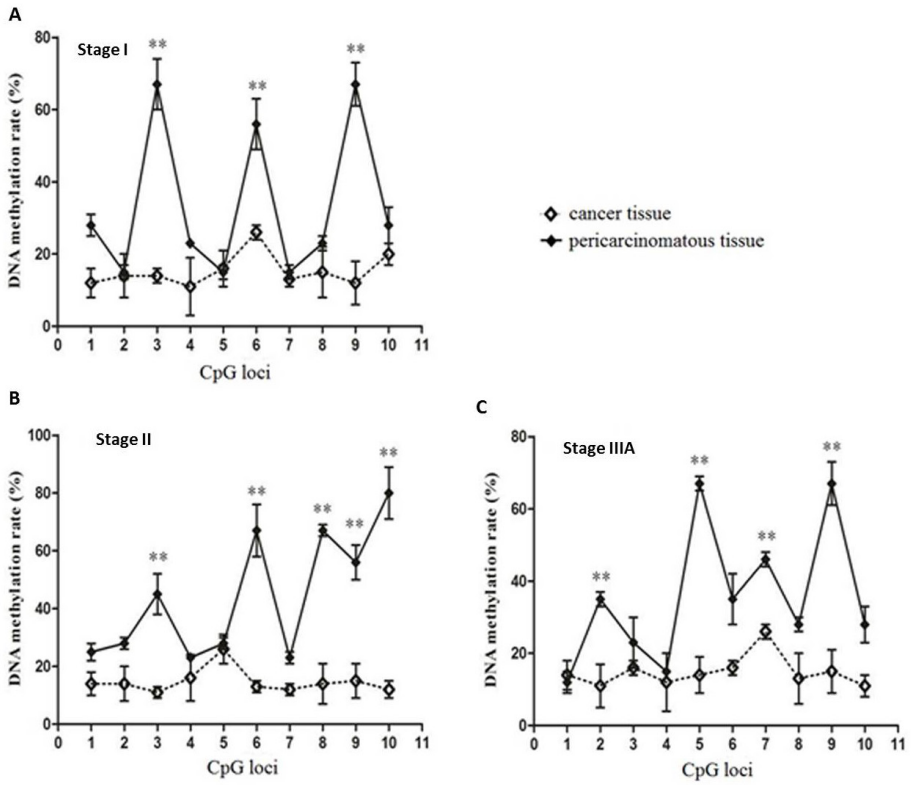

Figure 3. Methylation levels in patients with different NSCLC stages. The DNA methylation rate (%) at different CpG islands was described in both cancer and pericarcinomatous tissues in NSCLC patients at TNM stage I (**A**), stage II (**B**), and stage IIIA (**C**). Data are reported as means ± standard deviation (SD). **P < 0.05 compared with pericarcinomatous tissues. NSCLC = non-small cell lung cancer.

DISCUSSION

DNA methylation is one of mechanisms used in epigenetic regulation. Abnormal DNA methylation has been associated with most forms of tumorigenesis, and usually manifests as hypomethylation of the whole genome, in addition to methylation changes in certain areas of specific gene promoters. Thus, exploration of the association between DNA methylation state and disease occurrence, development, and prognosis assessment has considerable clinical significance (Palii and Robertson, 2007). In vertebrates, a DNA methyltransferase (DNMT) attaches a methyl group to the fifth carbon atom of the cytosine in a 5'-CpG-3' dinucleotide; the methylated CpG dinucleotide(s) inhibits gene expression, the recruitment of transcription factors, or hinders the interactions of transcriptional activators. In the human genome, 70-80% of CpG dinucleotides exist in a methylated state. Normally, DNA methylation plays an important role in regulating gene expression, the establishment and maintenance of chromosome structure and X chromosome inactivation, gene imprinting, embryonic development, normal cellular functions, and disease occurrence. In the embryonic period, abnormal DNA methylation patterns can lead to embryonic death (Li et al., 1992). In the adult, abnormal methylation can contribute to the etiology and progression of disease, and especially of tumors.

Recent technical developments have provided effective methodologies for DNA methylation analysis via sequencing. Pyrosequencing is a technique characterized by the rapid detection and effective quantification of gene methylation levels. It can detect up to 10 consecutive CpG loci (Tost and Gut, 2007). EGFR, the product of the proto-oncogene *HER-1*, is a preferred target for gene therapy and plays an important role in the treatment of NSCLC. Previous studies have mainly been focused on the relationship between *EGFR* gene mutation and the chemo-resistance. With the recent deepening understanding the relationship between cancer and DNA methylation, a greater number of studies are demonstrating that DNA methylation plays a considerable role in tumor development. Research from the laboratory of Métivier et al. (2008) has suggested that the methylation level of the *EGFR* promoter region is 90% in the breast cancer cell line CAMA1. Therefore, the aim of the present study was to investigate the potentially different methylation levels in NSCLCs with different malignancy stages using the fast and efficient pyrosequencing DNA methylation detection technology.

A previous study had shown that the *EGFR* promoter methylation status had no obvious correlation with the gender, age, smoking status, lymph node metastasis, or TNM staging of patients with lung cancer. This methylation change was speculated to be an early event in lung cancer progression. The study also demonstrated a significant correlation between *EGFR* methylation and gene mutations (Ohsaki et al., 2000). Our results also found that *EGFR* gene methylation levels failed to exhibit significant differences between patients with different gender, age, or smoking status. On the other hand, our study demonstrated that the level of *EGFR* gene methylation was positively correlated with the degree of NSCLC malignancy. Furthermore, this difference was also observed at individual sites of CpG methylation. The different results between these studies might be caused by the different selection of DNA fragments analyzed. In addition, the above-mentioned study was qualitative, focused on the positive rate of methylation in the TNM stage T1+T2 and T3+T4 groups, while our study reported the results of quantitative investigations of *EGFR* methylation.

EGFR is an important gene in the development of lung cancer: advanced lung cancer

often exhibits *EGFR* overexpression, and most such patients have the worst prognoses. Together, these characteristics reinforced EGFR as an important target for molecular therapy (Sharma et al., 2007). Other studies have shown that the expression rate of the EGFR protein in NSCLC is approximately 40-80%, while squamous carcinoma showed the highest positive rate at nearly 80%. The expression rate was close to 50% in adenocarcinoma and large cell carcinoma, while small cell lung cancer showed no EGFR expression (Raben et al., 2002; Hirsch et al., 2003; Mendelsohn and Baselga, 2003). Our results showed that *EGFR* expression decreased in conjunction with the rise of malignancy degree; however, it still remained higher than the expression level in normal (pericarcinomatous) tissue, which together suggested that *EGFR* is an important regulatory gene in NSCLC.

In the present study, we initially investigated the relationship between *EGFR* gene methylation and mRNA expression levels, and found that they were negatively correlated with each other in patients with different degrees of malignancy. This implied that *EGFR* expression in cancerous tissue might be regulated by methylation, although elucidation of the specific regulatory mechanism would still need further research. In addition, it has been shown that the median progression-free survival time in patients with or without methylation was 2.4 and 7.4 months, respectively, whereas the overall survival times were 6.1 and 17.8 months, respectively (Scartozzi et al., 2011). With the increase of malignancy staging, the methylation level in patients also rises. The combination of these two findings, therefore, leads to the suggestion that methylation detection might be useful for prognosis.

It had been shown that tumor cells carrying *EGFR* promoter methylation are resistant to EGFR-TKI. Methylation medicine can induce marked cell apoptosis and can be used to strengthen the TKI effect; for example, the methylation drug decitabine can cause *EGFR* to regain expression in the cell lines CAMA1 and MB453, while a combination of decitabine and gefitinib can induce pronounced apoptosis (Montero et al., 2006). Parra et al. (2004) found that high levels of EGFR on the cell membrane were not related to the sensitivity of a cell to TKI, although higher EGFR expression was associated with shorter survival times. Thus, the detection of *EGFR* methylation levels might be used instead to evaluate the efficacy of clinical drugs. However, CpG loci that are involved in the regulation of gene expression are usually located in the promoter region of a gene. As promoter regions are usually of considerable length, and pyrosequencing cannot analyze the entire region of interest, it is necessary to initially determine the CpG locus regulatory "HotSpot". Our study showed that methylation at loci 3, 6, and 9 of the *EGFR* promoter region was upregulated in cancerous compared to pericarcinomatous tissue at stages I and II, whereas loci 2, 5, 7, and 9 methylation levels were higher in stage IIIA. Our investigation therefore provides a theoretical basis for determining "HotSpots" for methylation analyses, although further identification and verification of this site in *EGFR* regulation is needed.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the anonymous reviewers for reviewing this manuscript.

REFERENCES

Hirsch FR, Varella-Garcia M, Bunn PA Jr, Di Maria MV, et al. (2003). Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. *J. Clin. Oncol*. 21: 3798-3807.

Hynes NE and Lane HA (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer* 5: 341-354.

Jemal A, Siegel R, Xu J and Ward E (2010). Cancer statistics, 2010. *CA Cancer J. Clin*. 60: 277-300.

Kashiwababra K, Oyama T, Sano T, Fukuda T, et al. (1998). Correlation between methylation status of the p16/CDK2 gene and the expression of p16 and Rb proteins in primary non-small cell lung cancers. *Ins. J. Cancer* 79: 215-220.

Li E, Bestor TH and Jaenisch R (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69: 915-926.

Loeb DM, Evron E, Patel CB, Sharma PM, et al. (2001). Wilms' tumor suppressor gene (*WT1*) is expressed in primary breast tumors despite tumor-specific promoter methylation. *Cancer Res*. 61: 921-925.

Mendelsohn J and Baselga J (2003). Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. *J. Clin. Oncol*. 21: 2787-2799.

Métivier R, Gallais R, Tiffoche C, Le Péron C, et al. (2008). Cyclical DNA methylation of a transcriptionally active promoter. *Nature* 452: 45-50.

Montero AJ, Diaz-Montero CM, Mao L, Youssef AM, et al. (2006). Epigenetic inactivation of *EGFR* by CpG island hypermethylation in cancer. *Cancer Biol. Ther*. 5: 1494-1501.

Oh MJ, Choi JH, Kim IH, Lee YH, et al. (2000). Detection of epidermal growth factor receptor in the serum of patients with cervical carcinoma. *Clin. Cancer Res*. 6: 4760-4763.

Ohsaki Y, Tanno S, Fujita Y, Toyoshima E, et al. (2000). Epidermal growth factor receptor expression correlates with poor prognosis in non-small cell lung cancer patients with p53 overexpression. *Oncol. Rep*. 7: 603-607.

Palii SS and Robertson KD (2007). Epigenetic control of tumor suppression. *Crit. Rev. Eukaryot. Gene Expr*. 17: 295-316.

Parra HS, Cavina R, Latteri F, Zucali PA, et al. (2004). Analysis of epidermal growth factor receptor expression as a predictive factor for response to gefitinib ('Iressa', ZD1839) in non-small-cell lung cancer. *Br. J. Cancer* 91: 208-212.

Piyathilake CJ, Frost AR, Manne U, Weiss H, et al. (2002). Differential expression of growth factors in squamous cell carcinoma and precancerous lesions of the lung. *Clin. Cancer Res*. 8: 734-744.

Raben D, Helfrich BA, Chan D, Johnson G, et al. (2002). ZD1839, a selective epidermal growth factor receptor tyrosine kinase inhibitor, alone and in combination with radiation and chemotherapy as a new therapeutic strategy in non-small cell lung cancer. *Semin. Oncol*. 29: 37-46.

Reungwetwattana T, Weroha SJ and Molina JR (2012). Oncogenic pathways, molecularly targeted therapies, and highlighted clinical trials in non-small-cell lung cancer (NSCLC). *Clin. Lung Cancer* 13: 252-266.

Scartozzi M, Bearzi I, Mandolesi A, Giampieri R, et al. (2011). Epidermal growth factor receptor (*EGFR*) gene promoter methylation and cetuximab treatment in colorectal cancer patients. *Br. J. Cancer* 104: 1786-1790.

Sharma SV, Bell DW, Settleman J and Haber DA (2007). Epidermal growth factor receptor mutations in lung cancer. *Nat. Rev. Cancer* 7: 169-181.

Tost J and Gut IG (2007). Analysis of gene-specific DNA methylation patterns by pyrosequencing technology. *Methods Mol. Biol*. 373: 89-102.

Tost J, El abdalaoui H and Gut IG (2006). Serial pyrosequencing for quantitative DNA methylation analysis. *Biotechniques* 40: 721-726.