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METHYLATION STATUS OF EPIDERMAL GROWTH FACTOR RECEPTOR GENE IN LUNG CARCINOMA CELLS

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The methylation status of the epidermal growth factor receptor (EGFR) gene was compared in cell lines from four major types of lung carcinoma, small cell lung carcinoma (SCLC), large cell lung carcinoma, squamous cell carcinoma and adenocarcinoma, in order to examine whether DNA methylation is responsible for the suppression of EGFR gene in SCLC cells. Southern blot analysis revealed that the structural region of the EGFR gene is methylated to various degrees regardless of the expression of EGF receptor on the surface. An 8-kilobase *EcoRI* fragment which contains the EGFR gene promoter region is readily digested with various methylation-sensitive restriction enzymes in all types of cells, indicating that the EGFR gene 5' region is barely methylated. Thus, a mechanism other than DNA methylation appears to control EGFR gene expression and the lack of EGFR gene expression in SCLC cells may be caused by a paucity of some transcription regulatory factor(s).

Key words: EGF receptor — Methylation — Gene expression

Small cell lung carcinoma (SCLC) is the most aggressive form of lung cancer with neuroendocrine properties including increased levels of creatine kinase-BB and

neuron-specific enolase and the secretion of gastrin-releasing peptide and calcitonin.¹⁾ We have previously demonstrated that none of the SCLC cell lines possesses the EGF receptor on the surface and that the EGFR gene is not transcribed in SCLC cells despite the presence of an intact and unarranged form of the EGFR gene.²⁾ We suggested that the lack of EGF receptor in SCLC cells is a distinguishing feature from the other types of lung cancer, large cell lung carcinoma (LCLC), adenocarcinoma (AdC) and squamous cell carcinoma (SqCC).²⁾ Thus, SCLC cells may have some active regulatory mechanism which prevents expression of the EGFR gene.

Recently, Baylin *et al.* demonstrated that the calcitonin gene 5' region in SCLC cells is hypermethylated at a high frequency as compared to other types of lung carcinoma cells and this hypermethylation parallels the presence of the neuroendocrine marker aromatic-L-amino acid decarboxylase.³⁾ This observation suggests that methylation may be involved in the regulation of SCLC-specific gene expression.

The EGFR gene 5' region contains neither a TATA box nor a CAAT box, has an extremely high G+C content and contains five CCGCCC repeats to which transcription factor Sp1 can bind.⁴⁾ However, whether methylation is involved in the regulation of EGFR gene expression is currently not known. In this communication, the methylation patterns of the EGFR gene are compared in four major groups of lung carcinoma.

Recently, we²⁾ and others⁵⁾ have found that none of the cell lines derived from SCLC possesses EGF binding activity on the surface, whereas cell lines derived from LCLC exhibited significant EGF binding activity. The EGF binding analysis was extended to two other types of lung cancer, AdC and SqCC. As summarized in Table I, various levels of EGF binding activity were seen in cell lines derived from LCLC, AdC and SqCC,⁶⁻¹¹⁾ but the SCLC cell lines had no activity. This result strongly suggests that the lack of EGF recep-

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Table I. EGF Binding Capacities in Lung Carcinoma Cell Lines

Cell Line	Receptor number ^{a)} ($\times 10^5$ sites/cell)	Affinity (nM) ^{a)}		Gene dosage ^{b)} (copy/haploid)	Reference
		Kd ₁	Kd ₂		
Adenocarcinoma					
OKD	0.17 – 0.33	0.19	2.6 – 4.4	1	This work
PC9	1.4	0.26 – 0.32	(ND) ^{c)}	1	"
RERF-LC-MS	2.0	0.4	1.2	1	"
Squamous cell carcinoma					
SK-MES-1	0.79 – 1.2	0.56	2.9	1	(11)
Calu-1	1.20 – 1.4	0.54	2.1	1	(11)
Large cell carcinoma					
NCC-Lu65	1.3	(ND)	4.9	1	(6)
NCC-Lu99	1.4	(ND)	4.4	1	(7)
Small cell carcinoma					
NCI-H69	0	(ND)	(ND)	1	(10)
NCC-Lu134	0	(ND)	(ND)	1	(8, 9)
NCC-Lu139	0	(ND)	(ND)	1	This work

The cells were collected in Earle's balanced salt solution containing BSA (0.1%), then incubated with ¹²⁵I-EGF (1 ng/ml) and various amounts of unlabeled EGF (0–200 ng/ml) at 4° for 2 hr. Cell-associated EGF was separated by filtration through a Millipore EG filter, and the filters were washed extensively with Earle's balanced salt solution containing BSA. Lung carcinoma cell lines NCC-Lu65, NCC-Lu99, NCC-Lu134, NCC-Lu139 and OKD were established at the National Cancer Center Research Institute (Tokyo). SCLC cell line NCI-H69 was provided by Dr. J. Minna (National Cancer Institute, Bethesda, MD). Lung AdC cell line PC9 was provided by Dr. Y. Hayata (Tokyo Medical College, Tokyo). Lung SqCC cell lines Calu-1 and SK-MES-1 were provided by Dr. M. Akiyama (Radiation Effects Research Foundation, Hiroshima). Lung AdC cell line RERF-LC-MS was provided by the Japanese Cancer Research Resources Bank (Tokyo).

a) EGF binding capacities were determined by Scatchard plot analysis.

b) Gene dosage was determined by Southern blot analysis as shown in Fig. 1 and DNA dot blot analysis.

c) (ND): Not detected.

tor in SCLC cell lines is a good marker for distinguishing SCLC from other types of lung cancer.

To examine whether methylation is involved in the mechanism to suppress the expression of the EGFR gene, the methylation status of the structural region of the EGFR gene was compared in cell lines from four different types of lung carcinoma. Cellular DNAs were isolated, cleaved with the CpG methylation-sensitive restriction enzyme *HpaII* and the methylation-insensitive enzyme *MspI*, electrophoresed on agarose gel (0.8%) and transferred to a nitrocellulose membrane. Southern blot hybridization was carried out using the EGF receptor cDNA probe pE7, a 2.4 kilobase-pair (kb) insert¹²⁾ labeled with [³²P]dCTP by an oligo-labeling kit as described.¹³⁾ The blot was washed with 0.1× SSC/0.1% SDS and subjected to autoradiog-

raphy. This analysis revealed that all the cell lines examined contained a normal dose of the EGFR gene in an apparently normal form and that methylation at CCGG sites was present in some of the SCLC cells as well as the EGF receptor-positive cells (Fig. 1). Hypomethylated and intermediately methylated EGFR genes were also found in SCLC, LCLC and AdC cell lines (Fig. 1). Therefore, the level of methylation at the EGFR gene structural region appears to be unrelated to the lung carcinoma type or to the level of EGF receptor on the surface.

Next, the methylation status of the 5' regulatory region of the EGFR gene was examined using the probe pERCAT-1 which contains 1.1 kb of genomic DNA upstream from the translation initiation site.¹⁴⁾ The 5' region from genomic DNA was detected as a 3.5-kb fragment in *HindIII* digests. This

Fig. 1. Methylation patterns of the structural region of EGFR gene in lung carcinoma cells. DNAs (10 μ g) were digested with *Hpa*II (lanes 1, 3 and 5) or *Msp*I (lanes 2, 4 and 6), electrophoresed on agarose gel (0.8%) and transferred to a nitrocellulose filter (Schleicher & Schull, BA85). Southern blot hybridization was carried out using EGF receptor cDNA probe pE7 (2.4 kb insert) labeled with [32 P]dCTP by using the oligo-labeling kit (Pharmacia-PL Biochem.). The blot was washed at 52 $^{\circ}$ with 0.1 $\times$ SSC/0.1% SDS. Size markers represent λ DNA cut with *Hind*III. (A) LCLC cell lines. Lanes 1 and 2, NCC-Lu65; lanes 3 and 4, NCC-Lu99. (B) AdC cell lines. Lanes 1 and 2, OKD; lanes 3 and 4, RERF-LC-MS; lanes 5 and 6, PC9. (C) SqCC cell lines. Lanes 1 and 2, Calu-1; lanes 3 and 4, SK-MES-1. (D) SCLC cell lines. Lanes 1 and 2, NCI-H69; lanes 3 and 4, NCC-Lu134; lanes 5 and 6, NCC-Lu139.

*Hind*III fragment was then further digested with either *Hpa*II or *Msp*I, electrophoresed on an agarose gel (2%) and transferred to a nylon membrane. Hybridization was carried out using the nick-translated pERCAT-1 insert. As shown in Fig. 2A, the 3.5-kb fragments from the EGF receptor-positive LCLC cell line NCC-Lu65 and the receptor-negative SCLC cell line NCI-H69 were readily digested by those enzymes and no fragment was seen under stringent conditions. After a long exposure, a weak 0.4-kb fragment could be detected which may be a 5'-half of the promoter region (Fig. 2B). These results together with the published sequence data⁴⁾ suggest that the 3'-half of the promoter region contains many unmethylated CCGG sites and thus is in a methylation-free HTF island (Fig. 2B).

To examine further the methylation status, we carried out Southern blot analysis using other methylation-sensitive enzymes.¹⁵⁾ For *Eco*RI, the EGFR 5' region was detected as an 8-kb fragment in genomic DNA digests (Fig. 3). This fragment was further digested with either *Not*I, *Sac*II, *Mlu*I or *Ava*I in separate reactions, electrophoresed on an agarose gel (0.8%) and transferred to a nylon membrane. Southern blot hybridization was carried out using the nick-translated pERCAT-1 insert. As shown in Fig. 3A, the 8-kb fragments from NCC-Lu65 and NCI-H69 cells were readily digested by these enzymes and were found as smaller fragments. Additional *Not*I and *Sac*II restriction sites have been found in the pERCAT-1 insert besides the sites expected from the sequence data⁴⁾ (data not shown). As all these sites are

Fig. 2. Methylation of the EGFR gene 5' region in NCI-H69 and NCC-Lu65 cells. (A) DNAs from NCI-H69 (lanes 1 through 4) and NCC-Lu65 (lanes 5 through 8) were first digested with *Hind*III (lanes 1 and 5) or *Msp*I (lanes 2 and 6). One part of *Hind*III digests was further digested with *Msp*I (lanes 3 and 7) or *Hpa*II (lanes 4 and 8). Those digests were electrophoresed on agarose gel (2%) and transferred to nylon membrane (NEN; Gene Screen Plus). Southern blot hybridization was carried out using the nick-translated pERCAT-1 (1.1 kb insert). Size markers represent λ DNA cut with *Hind*III and ϕ X174 cut with *Hae*III. (B) H and Y indicate the restriction sites by *Hind*III and *Msp*I, respectively, which were determined from sequence data and by restriction analysis. 0 indicates the translation initiation site. The closed bar indicates the region contained in pERCAT-1. Open bars indicate the fragments observed after digestion and hybridization to pERCAT-1. Broken line bars indicate the fragments with weak intensity.

located in close proximity, we have not identified which sites were cleaved in the restriction analysis. However, since the 3'-most fragment was not detected in *Not*I or *Sac*II digests, there appear to be additional *Not*I and

*Sac*II sites which are methylation-free and hence cleaved (Fig. 3B). We have confirmed these additional restriction sites by analyzing the genomic structure of the promoter region (Kawasaki *et al.*, unpublished results). We

Fig. 3. Methylation of the EGFR gene 5' region in lung carcinoma cells. (A) DNAs from NCI-H69 (lanes 1 through 5) and NCC-Lu65 (lanes 6 through 10) were first digested with *EcoRI* (lanes 1 and 6) and then digested with *NotI* (lanes 2 and 7), *SacII* (lanes 3 and 8), *MluI* (lanes 4 and 9) and *AvaI* (lanes 5 and 10). The digests were electrophoresed on agarose gel (0.8%) and transferred to a nylon membrane (NEN; Gene Screen Plus). Hybridization was carried out using the nick-translated pERCAT-1 (1.1 kb insert). (B) N, S and A indicate the restriction sites *NotI*, *SacII* and *AvaI* expected from the sequence data, respectively. The additional restriction sites described in the text are not shown in this diagram. E, M and A* indicate *EcoRI*, *MluI* and *AvaI* restriction sites determined by the restriction analysis. 0 indicates the translation initiation site. The closed bar indicates the region contained in pERCAT-1. Open bars indicate the fragments observed after digestion and hybridization to pERCAT-1 probe. Broken line bars indicate the fragments expected but not detected in (A).

further analyzed the methylation status of the promoter region in five cell lines: two SCLC cell lines (NCC-Lu134 and NCC-Lu139), two SqCC cell lines (SK-MES-1 and Calu-1) and an AdC cell line (PC9). The 8-kb fragments from these cell lines were cleaved by the methylation-sensitive enzymes (data not

shown). We have also obtained similar results using cDNA probe pE15 which contains a part of the promoter region, exon 1 and exon 2. These results suggest that there is little methylation in the EGFR gene promoter region regardless of the activity of the EGF receptor on the cell surface.

Methylation plays some important roles in gene expression.^{16,17)} However, the exact role of methylation in gene expression remains unclear. As mentioned, the calcitonin gene 5' region is hypermethylated in SCLC cells as compared to other types of lung cancer, and this same site is hypomethylated in the medullary thyroid carcinomas.³⁾ Although SCLC is known to produce calcitonin, the amount of calcitonin production is significantly lower than that in the medullary thyroid carcinoma. Chandler *et al.* investigated the methylation status of collagen, c-Ha-ras and thymidine kinase genes in several tumor cell lines, and found no generalized trend in gene hypomethylation in tumor cell lines as compared to fibroblasts.¹⁸⁾ Kaneko *et al.* examined the methylation status of the EGFR gene using a cDNA probe and found that the EGFR gene from hepatocellular carcinoma and fetal liver is substantially hypomethylated in comparison with the gene from normal liver.¹⁹⁾ In this communication, we demonstrate that the 5' region is methylation-free regardless of the expression status of the EGFR gene. Our observations indicate that gene methylation is not solely responsible for control of EGFR gene expression and that there may be some other regulatory mechanisms involved. Recently, deletion analysis has been performed using chimeric plasmids containing the chloramphenicol acetyltransferase gene and various deletions of the EGFR gene promoter. This analysis revealed that the 5' CpG-rich region plays an important role in the regulation of gene expression. Furthermore, it was demonstrated that Sp1 and trans-acting factor specific for the EGFR gene (ETF) bind to the 5' CpG-rich region and are required for the maximal expression.²⁰⁾ Recently we have confirmed the absence of EGF receptor by an immunostaining method in SCLC tissue sections (Gamou *et al.*, unpublished observation). Although the methylation status in these tumor samples remains to be investigated, the regulatory mechanism for the EGFR gene may be independent of methylation and dependent upon the action of trans-acting transcription factor(s). We are now exploring the possibility that the lack of EGFR gene expression in SCLC cells may be caused by a paucity of such regulatory factor(s).

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