Kosin Medical Journal 2015;30:141-147
http://dx.doi.org/10.7180/kmj.2015.30.2.141

KMJ
Original Article

Analysis of an EGFR mutation by PNA clamping method in lung carcinoid tumors

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폐유암종에서 PNA-clamping 방법을 이용한 EGFR 돌연변이 분석

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Background: Pulmonary carcinoid tumors consisting of typical carcinoid tumors (TC) and atypical carcinoid tumors (AC) are rare, accounting for 2-5% of all lung tumors. TC is considered a low-grade tumor with a rate of distant metastasis up to 12%. In contrast, ACs are more aggressive tumors, displaying a metastatic rate up to 70%. Surgery is the treatment of choice; however, the current treatment outcomes of metastatic lung carcinoids are discouraging. This study aimed to investigate the EGFR mutation using the PNA-mediated clamping method and to provide basic data for using EGFR-TK1 and its clinical implications.
Materials and Methods: A total of 14 cases that underwent surgery were diagnosed as carcinoid tumors and pathologically classified as TC and AC. The paraffin-embedded tissues were analyzed for EGFR mutations using the PNA-mediated PCR clamping technique. The mutant type was noted in the cases with a $^{\triangle}$Ct greater than 2.0.
Result: Of 14 cases, eight were AC and six cases were TC. No known EGFR mutation was detected with a $^{\triangle}$Ct less than 2.0.
Conclusion: The EGFR genotype determined using the PNA-mediated PCR clamping method was wild-type in all pulmonary carcinoid tumors. Therefore, the application of EGFR-TK1 is limited in pulmonary carcinoid tumors.

Key Words: carcinoid, EGFR mutation, Lung, PNA clamping

The pulmonary carcinoid tumor is a rare tumor derived from neuroendocine origins, accounting for 2~5% of all lung tumors and classified as typical or atypical.[1] In the WHO classification, the number of mitosis events, a tumor size of 2~10/㎟ and the existence of necrosis are key factors differentiating typical tumors from atypical tumors.[2]

Epidermal growth factor receptor (EGFR) is a protein with tyrosine kinase self-activating factor derived from the surface of epidermal cells. Overexpression of this protein affects intracellular signals inducing cell proliferation, neo-vascularization and metastasis.[3] It is

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Received: Oct. 21, 2014
Revised: Mar. 3, 2015
Accepted: Apr. 1, 2015

known that over expression of EGFR is common in non-small cell lung cancer.[4] Some studies have reported that, when EGFR was suppressed by the EGFR tyrosine kinase inhibitors(TKI) gefinitib or erlotinib, apoptosis was induced, and cell growth was reduced due to the suppression of neovascularization.[4-8] Interestingly, due to the correlation between a specific mutation in the ATP-binding domain of the EGFR gene and the responserate to EGFR-TKI, mutation detection is now an important factor for selecting candidate patients and predicting drug response.[4-8] Based on these results, knowledge of the EGRF mutation is essential for determining the efficacy of EGFR target therapy in lung cancer patients and plays a leading role in the prediction of treatment and prognosis. However, there have not been any reports on EGFR mutations in Korean carcinoid tumors. In current reports, direct sequencing is used to detect EGFR mutations,[6-8] but its sensitivity is too low to detect mutant DNA with concentrations lower than 20%.[9,10]

Recently the PNA-mediated clamping method was developed. This method is a PCR-based technique, amplifying mutations exclusively by suppressing wild-type DNA using the addition of a peptide nucleic acid (PNA) probe complementary to wild-type during the PCR amplification process11). Also, this method is characterized by high sensitivity and the ability to accurately and rapidly detect a small amount of mutated DNA.

This study aimed to demonstrate the clinical significance of EGFR mutations in primary pulmonary carcinoid tumors using the PNA-mediating clamping method and by analysis of the clinicopathologic characteristics of tumors.

Materials and Methods

1. Samples

For the 10-year period from 1991 to 2000, 14 microscopic slides and paraffin sections collected from pulmonary carcinoid tumors were enrolled in this study.

2. Pathologic examination

Count of mitosis events: The average value among three sites was calculated by counting the number of mitosis events of the No. 10 nucleus in the region where mitosis most frequently occurs.

Identification of necrosis: We included punctate necrosis and necrosis similar to infarction but excluded tissue denaturation due to an ulcer or laceration.

Neuroendocrine marker immunostaining: We performed immunostaining using the avidin-biotin conjugation method with synaptophysin, chromogranin, CD56 and NSE as the markers.

3. Gene mutation analysis

3-1. DNA extraction

The pathologist marked territories containing greater than 50% tumor cells under microscopic examination. After removal of the cover glass using xylene, cancer cells were extracted using a 26-gauge needle. We used the PNA-mediated clamping method, a PCR technique that amplifies and then detects mutant DNA by suppression of wild-type DNA using the addition of a peptide nucleic acid (PNA) probe, which is complementary to the wild-type, during the PCR amplification process.

We added 200ug/mL of cancer cells into 50-100uL DNA extracts (50mM Tris-Cl pH 8.5, 1mM EDTA

pH 8.0, 0.5& Tween 20) containing 10% resin and proteinase K. We applied treatment at 56℃ and heated to 100℃ for 10 minutes. Then we collected the supernatant free from resin mixture for the PCR reaction using centrifugation at 12,000rpm for 10~15minutes.

3-2. PNA-mediated PCR clamping

We utilized a PNA Clamp™(PANAGENE, Daejeon, Korea) EGFR mutation detection kit for exons 18, 19, 20, and 21 and used a CFX 384 (Bio-Rad, Hercules, CA, USA) real-time PCR machine. The PNA mixture was prepared using a vortex mixer and centrifuged for 1~2 seconds. Thirteen microliters of each centrifuged specimen was sampled into eight PCR tubes of 0.2 ml (G719x, deletion, S7681, Insertion 3 duplication, Insertion, L858R & L861Q , 1790M). The specimen was titrated to 50 ng in 7 ul by addition of dry water without DNase/RNase. At that time, 7 ul of clamping control reagent was added for clamping control. The PCR tube containing the specimen was analyzed by real-time PCR. Forty cycles of PCR were performed: denaturation at 94℃ for 30seconds, PNA clamping at 70℃ for 20seconds, annealing at 63℃ for 30seconds, and extension at 72℃ for 30seconds. The clamping reaction of PNA Clamp™ contains an additional step of PNA annealing, allowing PNA to bind to the target site. This mutation detection method utilizes Amplicon

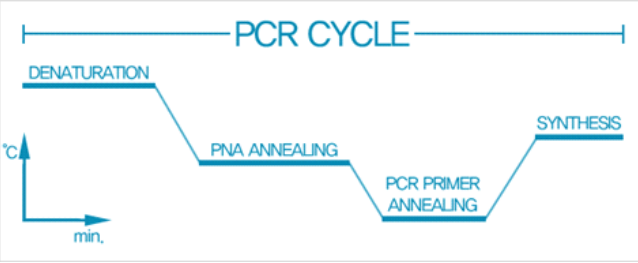

Fig. 1. The four steps of the PCR cycle in PNA Clamp™

or not after PCR amplification usingreal-time PCR.

3-3. Analysis

Mutation detection is achieved by preferential amplification using suppression of wild-type by the addition of a compensatory PNA probe during the PCR amplification process. We determined that a positive amplification occurred when the threshold cycle ('Ct') value was 30 or lower in the non-PNA mixing reaction, and a mutation occurred when the *Q value (standard Ct −specimen Ct = *Q) of each gene position was 2.0 or higher.

Results

1. Clinicopathologic analysis

In a total of 14 cases of pulmonary carcinoid tumors, six cases were TC and eight cases were AC. The mean age of enrolled patients was 52.6 (±4.6) years, for TC patients it was 49.2 (±6.7) years, and for AC patients it was 55.4 (±3.7) years. Though the AC group was older than the TC group, this difference was not statistically significant. Both females in the study were in the TC group, and males had a higher occurrence of carcinoid tumors (p=0.032) (Table 1). The overall tumor size ranged from 0.5 to 5cm, and the mean TC and AC tumor size was 2.9cm (±2.1) and 3.1cm (±3.3), respectively. Though AC tumors were larger, this was not statistically significant. Lymph node metastasis and necrosis were detected in two cases of AC. Mitosis events counted at 10x magnification with a high-powered field numbered 5.5/10HPF on average, 0-1 in TC patients and 3-10 in AC patients. All 14

cases were positive for the neuroendocrine marker but ranged from strongly positive to weakly positive(Table 1).

2. EGFR mutation analysis (Fig. 1)

According to the EGFR mutation analysis of DNA extracts in each position by the PNA Clamp™ EGFR mutation detection kit, the △C t value was below 2.0 in all 14 cases. Thus, we determined that all cases were wild-type according to the mutation criterion of a △Ct value greater than 2.0 (Fig. 2).

Discussion

EGFR mutations are frequent in adenocarcinomas, females, Oriental people and non-smokers, but they also occur in non-adenocarcinoma, males and smokers. In addition, because of the exclusive relationship between EGFR mutations and EGFR-TK1 reactions in several reports on Oriental patients, the EGFR mutation test has been an essential study tool for treatment of lung cancer in Koreans. Currently, direct sequencing[11-13] isused for detecting mutations. However, this method has a low detection acuity and cannot detect mutant DNA with concentration slower than 20%. Therefore a more sensitive test is required.[13]

PNA is a kind of artificial DNA with a higher gene recognition ability and stability against enzymes like nuclease. The PNA clamp method has been developed and used in routine studies of lung cancer as well as in clinical experiments in Japan.[14] In recent years, there have been reports about better diagnostic performance of PNA clamp method comparing to direct sequencing in Korea.[15,16] In this study, we used PNA-mediated PCR, a more sensitive method compared to direct sequencing, but could not detect any known EGFR mutations. Rickman[17] et al. reported that the EGFR

Table 1. The clinical and pathological characteristics of 14 cases of pulmonary carcinoid tumors

Case	Dx	Age	Gender	Tumor Size (cm)	No of mitosis events (/10HPF)	Necrosis	Lymph node metastasis	Neuroendocrine markers
1	T	63	F	2.8	0	−	−	+
2	T	41	F	3	1	−	−	+
3	T	48	M	5	0	−	−	+
4	T	53	M	3.5	1	−	−	+
5	T	31	M	1.8	0	−	−	+
6	T	59	M	1.2	0	−	−	+/−
7	A	34	M	4	6	−	−	+
8	A	55	M	5	7	−	+	+
9	A	47	M	4.8	5	−	−	+
10	A	61	M	4.5	10	+	−	+
11	A	54	M	3.3	4	−	+	+
12	A	72	M	5	4	+	−	+
13	A	62	M	2.3	3	−	−	+
14	A	57	M	0.5	5	−	−	+

A: atypical carcinoid, T: typical carcinoid, M: male, F: female, +: present, −: absent

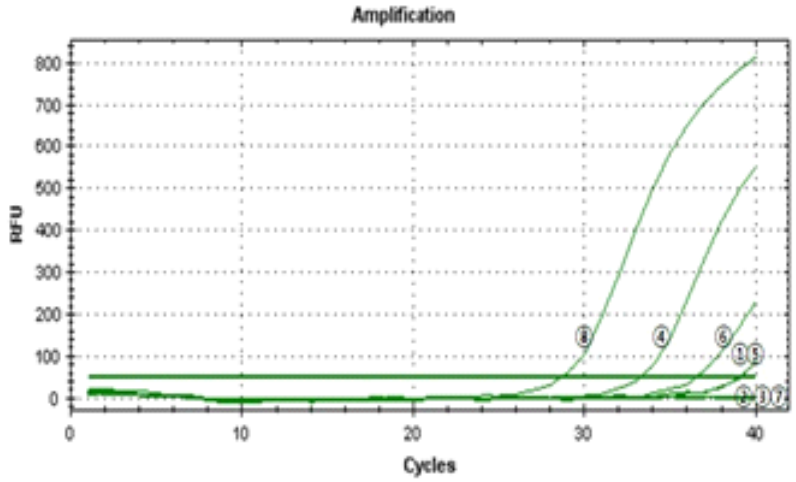

Fig. 2. The wild type was detected in PNA-mediated clamping real-time PCR for EGFR.

Table 2. The results of analysis of EGFR PNA-mediated clamping using real-time PCR

Sample No(Ct)	G719X (①)	E19 del. (②)	T790M (③)	S768I (④)	Ins. 3 dup (⑤)	Ins.3 (⑥)	L858R & L861Q(⑦)	Non-PNA (⑧)
15	39.11	38	38	33.24	39.02	36.5	38	28.63
Clamping control	38	36.16	39.79	31.82	30.42	35.92	34.42	23.66
standard Ct	36	33	33	30	27	30	33	
△Ct	-2	-0.06	-0.37	-3.91	-3.45	-8	-6.62	
determine	wild	wild	wild	wild	wild	wild	Wild	

Criterion: EGFR mutation is noted when the △Ct value, standard Ct minus sample Ct, is 2.0 or above. [△Ct = standard ct - sample ct]

expression ratio of carcinoid tumors is 45% for TC tumors and 28.6% for AC tumors by immunostaining, however, there were no EGFR mutations in exons 18-21 according to direct sequencing. This study supposed that EGFR immunostaining was not correlated with EGFR mutations so there were several mechanisms affecting EGFR protein expression. However, Wheler et al[18] reported a EGFR mutation at exon 21 in one of eight carcinoid tumors (1/8) by direct sequencing. Otherwise, Gilbert[19] et al. suggested that there were no EGFR mutations in the fresh neuroendocrine tumor tissue of the gastrointestinal duct and pancreas. Additionally, mutations of EGFR exons 18, 19, 21 of neuroendocrine tumors containing carcinoid tumors were not detected in reports by Ameur[20] et al. and Jack man[21] et al. These studies correspond with the results of our more sensitive method, and we suggest that there is little possibility to detect mutations of carcinoid tumors, one of the neuroendocrine tumors, because Yeo[16] reported that although there was concordance between PNA clamp and direct sequencing, PNA clamp had higher sensitivity to detect EGFR mutation.

EGFR mutations are classified as sensitive and resistant. The most important sensitive mutations are an exon 19 deletion and a L858R spot mutation,

accounting for 85-90% of all mutations. Sensitivity to TK1 is known to be higher in exon 19 deletions.[14,20]

Tsao[7] et al. and Zhu[8] et al. reported that the absolute value of EGFR is an independent factor, important to EGFR-TK1 reactions unless EGFR mutation. Rickman[17] suggested that the therapeutic sensitivity to EGFR-TK1, like that of erlotinib, is can be influenced by EGFR expression measured by Western blotting or immunostaining, showing single nucleotide polymorphism (SNP) in TC and AC and significant reduction of cell proliferation of TC cell line by erlotinib. There is no report about the association of SNP with EGFR-TK1 effect in carcinoid tumors .

TC tumors are tumors with relatively good prognoses with 12% distant organ metastasis, and AC tumors are aggressive with greater than 70% metastasis.[1,22] However, there is currently no effective treatment for advanced pulmonary carcinoid tumors with metastasis. Therefore, targeted therapy with surgery for these patients is required.

The purpose of this study was to determine the clinical significance of detecting EGFR mutations using the PNA-mediated PCR method. However, no EGFR mutations were detected. This study has limitations that small number of cases was studied and concordance between PNA clamp and direct sequencing was not studied. Further study with larger scales is needed for verifying the results of this study, in the light of rarely identified EGFR mutation in large cell neuroendocrine carcinoma of the lung[23] and previous report of a mutation in carcinoid tumors.[18]

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