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EGFR, *KRAS*, and *BRAF* mutational profiles of female patients with micropapillary predominant invasive lung adenocarcinoma

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Background/aim: This study aimed to analyze *EGFR*, *KRAS*, and *BRAF* mutations in females with micropapillary predominant invasive lung adenocarcinoma and their relationships with immunohistochemical and clinicopathological patterns.

Materials and methods: A total of 15 females with micropapillary lung adenocarcinoma were selected. Mutational analysis of the *EGFR*, *KRAS*, and *BRAF* genes was carried out. Information regarding the demographic data, tumor size, treatment, and survival time for each patient was collated, and the predominant cell type, secondary architectural growth patterns, psammoma bodies, necrosis, and visceral pleural and angiolymphatic invasions were evaluated.

Results: We identified *EGFR* mutation in six cases, *KRAS* mutation in three cases, and *BRAF* mutation in one case. EGFR, c-kit, VEGFR, and bcl-2 positivity was observed in ten, seven, four, and six cases, respectively. All cases were positive for VEGF (strong positivity in 11 cases and weak positivity in four cases) and bcl-2 (strong positivity in nine cases and weak positivity in six cases). Seven (46.6%) cases were positive for c-kit and 10 (66.6%) cases were positive for EGFR.

Conclusion: *EGFR* mutation occurred at a higher incidence rate in micropapillary predominant invasive adenocarcinoma than has previously been found in conventional lung adenocarcinomas. *KRAS* mutation was observed as having a similar frequency to what was previously observed, but the frequency of *BRAF* mutation was lower than previously reported.

Key words: *BRAF*, *EGFR*, *KRAS*, micropapillary dominant invasive adenocarcinoma, mutation

1. Introduction

Although lung cancer is not the most frequently observed type of cancer, it is the cause of the most cancer-related deaths in both males and females worldwide (1). Adenocarcinomas, which have different clinical, radiological, molecular, and pathological properties, are the most commonly seen histological type of lung cancer, and lung adenocarcinomas have a heterogeneous morphology (2). Micropapillary dominant invasive adenocarcinoma was defined as a new subtype in the most recent classification of lung adenocarcinomas (3), and diagnosis is associated with poor prognosis and a high grade (4). Therefore, targeted therapy may represent the best hope in the treatment of this type of tumor. Nowadays, the most popular therapeutic target is the tyrosine kinase receptor, epidermal growth factor receptor (EGFR). Asian, nonsmoking female patients are particularly suitable for this type of treatment. The *KRAS* and *BRAF* mutations are nonresponsive to treatment (5).

Few molecular studies investigating micropapillary lung adenocarcinomas have previously been conducted (6–10). Therefore, we studied the *EGFR*, *KRAS*, and *BRAF* gene mutations and their relationships with the immunohistochemical expressions of the EGFR, vascular endothelial growth factor (VEGFR), c-kit, and bcl-2 oncoproteins in women with micropapillary dominant invasive adenocarcinomas.

2. Materials and methods

This study was approved by the Ethics Committee of Atatürk Chest Diseases and Thoracic Surgery Education and Research Hospital in Ankara, Turkey. A total of 745 patients underwent complete surgical resection of primary lung adenocarcinoma in this hospital between January 2000 and December 2010, and a group of 15 women with micropapillary dominant invasive adenocarcinoma were retrospectively investigated. All specimens were fixed in 10% buffered formaldehyde and embedded in paraffin,

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and between four and eight slides, including hematoxylin and eosin-stained pleural surfaces, were reviewed. The histological type of the tumor was assigned according to the American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma. Micropapillary components consisted of papillary tufts without a fibrovascular core, and the tumor cells were small and cuboidal with minimal nuclear atypia (Figure 1). The smaller components were semiquantitatively recorded using 5% increments (3). The following histopathological factors were evaluated: tumor diameter, necrosis, mitotic count, vascular invasion, perineural invasion, and predominant cell type (hobnail vs. columnar). Vascular and pleural invasion was evaluated using Verhoeff–van Gieson staining, and mucicarmine staining was used to detect cytoplasmic mucin production. Tumor size was measured as the largest diameter of the cut sections of tumor mass, and the pathological stage was determined based on the criteria of the 7th TNM classification of the Union of International Cancer Control (11).

Clinical data were obtained from the case histories of the patients. These histories were analyzed with regard to age, smoking status, asbestos exposure, biomass history, tumor location, TNM stage, survival data, and chemotherapy.

In order to carry out immunohistochemistry analysis, 6-μm-thick sections were obtained from micropapillary areas of the paraffin block. The antibodies used were as follows: bcl-2 mouse monoclonal antibody clone 124 (Dako, Denmark), VEGF mouse monoclonal antibody clone VG1 (Thermo-Shandon Lab Vision, USA), c-kit oncoprotein polyclonal antibody (Thermo-Shandon Lab Vision), and EGFR mouse monoclonal clone 2-18C9 (Dako). VEGF, c-kit, bcl-2, and EGFR analysis was performed via the immunoperoxidase method, using the avidin-biotin complex (Thermo-Shandon Lab Vision). Positive controls included lymphocytes for bcl-2, angiosarcoma for VEGF, tonsils for c-kit, and squamous cell carcinoma for EGFR. Negative controls included omission of the primary antibody. Sections were deparaffinized with xylene and dehydrated in a graded alcohol and endogenous peroxidase, using 3% hydrogen peroxide. Antigen retrieval was performed using EDTA for VEGF and bcl-2, citrate for c-kit, and proteinase for EGFR. The reaction products were visualized with DAB, and the samples were semiquantitatively evaluated according to the percentage of cytoplasmic staining for bcl-2 and VEGFR and membranous staining for c-kit and EGFR. Immunostaining with all antibodies was evaluated by dividing the staining reactions into four groups according to cytoplasmic staining intensity: 1 = weak, 2 = moderate, 3 = strong, and 4= very strong. Immunostaining quantity was evaluated as the percentage of tumor cells showing cytoplasmic or membranous positivity as follows: 0 = negative, 1 = <25%, 2 = 25%–50%, 3 = 50%–75%, and 4 = >75%. They were then divided into three groups according to their summed scores: 0 = no immunostaining (a score of 0), 1 = weak immunostaining (scores between 1 and 4), and 2 = strong immunostaining (scores between 5 and 8) (12).

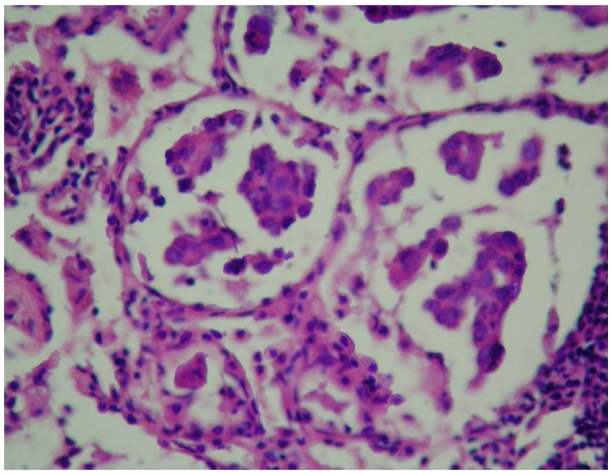

Figure 1. The tumor cells were grown in papillae-lacking fibrovascular cores (H&E, 400×).

Formalin-fixed paraffin embedded tissue blocks that were made up of more than 50% tumor cells were used for mutation analysis. For each patient, three 10-μm-thick sections of a block were used for DNA extraction, and the DNA was prepared from formalin-fixed, paraffin-embedded tissues using a DNA FFPE tissue kit (QIAGEN, Valencia, CA, USA). Microarray-based technology was used to carry out the mutational analysis. The system performs automated, allele-specific, primer extension hybridization on a biofilm chip microarray, as well as fluorescence detection and data analysis. This analysis detects 50 *EGFR* mutations at exons 18, 19, 20, and 21; 19 *KRAS* mutations at codons 12, 13, and 61; and seven *BRAF* mutations at codon 13.

Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and McNemar's test was used to assess associations between categorical variables. Two-tailed P-values of <0.05 were considered statistically significant.

3. Results

The clinicopathological features of 15 females (between the ages of 37 and 76 years old; mean: 50; median: 55) with micropapillary dominant invasive adenocarcinoma are summarized in Table 1. Of the 15 patients, two (13.2%) had a history of smoking, and nine (60%) were younger

Table 1. Clinicopathological features of our case studies.

Case no.	Age, years	Smoking	BM and asbestosis	Stage	Tumor location	Size	Necrosis	Mitosis	Cell type	LVI	PNI	Psammoma bodies
1	51	Yes	Yes	1A	LLL	20	No	6	Hbn	No	No	No
2	37	No	No	3A	RUL	15	No	11	Col	yes	No	No
3	69	No	No	1A	RUL	20	No	5	Col	Yes	No	No
4	62	No	No	3B	RML	30	Yes	4	Col	Yes	Yes	Yes
5	63	No	Yes	2A	RLL	50	No	8	Hbn	Yes	No	Yes
6	59	No	No	1B	RUL	40	No	10	Col	No	No	No
7	48	No	Yes	1A	LUL	25	No	12	Pol	Yes	No	No
8	42	yes	No	1A	LLL	15	No	5	Col	No	No	No
9	37	No	No	3B	RLL + ML	12	Yes	19	Col	No	No	No
10	67	No	No	3A	RUL	45	No	14	Hbn	No	No	No
11	46	No	Yes	2B	RUL	60	No	2	Col	No	No	No
12	76	No	Yes	2B	RUL	100	Yes	1	Col	No	No	No
13	54	No	No	3A	RUL	40	No	6	Pol	Yes	No	No
14	71	No	No	1B	LLL	40	No	8	Hbn	Yes	No	No
15	52	No	Yes	1B	RLL	40	No	2	col	No	No	No

BM: Biomass; LLL: left-lower lobe; RUL: right-upper lobe; RML: right-middle lobe; RLL: right-lower lobe; LUL: left-upper lobe; ML: middle lobe; Hbn: hobnail; Col: columnar, Pol: polygonal.

than 60 years old. Six cases involved asbestos and biomass exposure, and seven tumors were located in the right upper lobe. Tumor diameter ranged from 15 to 100 mm (mean: 44 mm, median: 40 mm). Following resection, disease stages were identified as follows: stage 1A, four patients; stage 1B, three patients; stage 2A, one patient; stage 2B, one patient; stage 3A, three patients; and stage 3B, two patients.

A micropapillary pattern (MPP) was found in 45%–80% of the tumor mass (Figure 1), while secondary minor patterns included a papillary pattern in six (40%) cases, an acinar pattern in five (33.4%) cases, a lepidic pattern in three (20%) cases, and a solid pattern in one (6.6%) case. Cytologically, the tumors revealed a predominant cell type of hobnail in four (26.6%) cases, columnar in nine (60%) cases, and polygonal in two (13.2%) cases. Psammoma bodies were observed in four (27%) cases. Only one case showed a signet ring change (case 7).

Table 2 summarizes the mutation analysis and immunohistochemical findings. All cases were positive for VEGF (strongly positive in 11 cases, weakly positive in four cases) and bcl-2 (strongly positive in nine cases, weakly positive in six cases). A total of seven (46.6%) cases were positive for c-kit, and 10 (66.6%) cases were positive for EGFR (Figures 2–5).

Molecular analysis showed that nine (60%) cases had mutations involving *EGFR*, *KRAS*, and *BRAF*; six (40%) cases showed *EGFR* mutation, including at exon 19 in five cases and at exon 20 in one case; three (20%) cases had *KRAS* mutation, including at codons 12, 13, and 61. One (6.6%) case had both *KRAS* (codons 12 and 61) and *BRAF* (V600E) mutations (case 11). Tables 3 and 4 show the nucleotide changes in *EGFR*, *KRAS*, and *BRAF* in our case studies.

Of the cases showing *EGFR* mutation, three were positive for c-kit, five were positive for EGFR, and four contained a papillary pattern as a secondary pattern. Of the cases with *KRAS* mutation, only one showed positivity for c-kit immunostaining, and only two cases showed positivity for EGFR immunostaining. The case that showed both *KRAS* and *BRAF* mutations was weakly positive for EGFR, VEGF, and c-kit. The secondary patterns observed in cases with *KRAS* mutations were papillary, acinar, and lepidic patterns. A secondary papillary pattern correlated with smoking ($P = 0.002$), psammoma bodies ($P = 0.002$), and perineural invasion ($P = 0.001$). Immunohistochemically, EGFR positivity correlated with perineural invasion ($P = 0.004$). There was no significant correlation between mutation, morphological, and clinical features. It was very difficult to reach targeted therapy between January 2000 and December 2010, so only one patient with *EGFR* mutation in exon 19 was treated with EGFR tyrosine kinase inhibitor and survived 50 months. Others were treated with platinum-based chemotherapy,

Table 2. Mutation analysis and immunohistochemical findings.

Case no.	VEGF IHC	Bcl-2 IHC	c-kit IHC	EGFR IHC	*EGFR* mutation	*KRAS* mutation	*BRAF* mutation	Secondary pattern
1	2	2	2	2	Exon 19	No	No	PAP
2	2	2	0	0	No	No	No	AC
3	1	1	2	0	No	No	No	PAP
4	2	2	2	2	Exon 19	No	No	PAP
5	1	1	0	2	Exon 19	No	No	PAP
6	2	2	2	2	No	No	No	LEP
7	2	2	0	2	No	No	No	SOL
8	1	2	0	0	No	Codon 12	No	PAP
9	2	1	0	2	Exon 20	No	No	AC
10	2	2	2	0	Exon 19	No	No	AC
11	2	1	0	2	No	Codon 12 and 61	V600E	LEP
12	1	2	1	1	No	Codon 13 and 61	No	AC
13	2	1	0	2	Exon 19	No	No	PAP
14	2	2	0	0	No	No	No	AC
15	2	1	2	2	No	No	No	LEP

PAP: Papillary; AC: acinar; SOL: solid; LEP: lepidic.

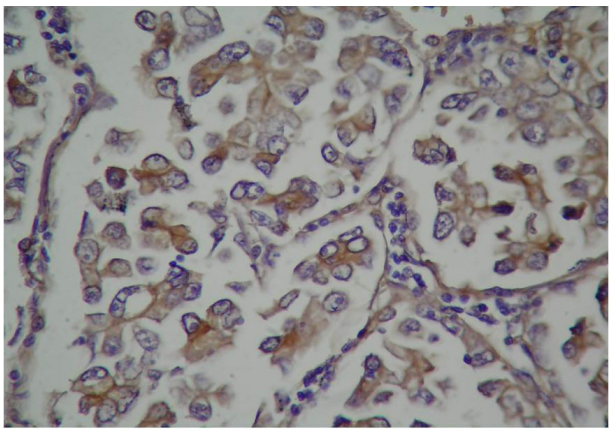

Figure 2. Immunohistochemistry for vascular endothelial growth factor receptor (VEGFR, 400×).

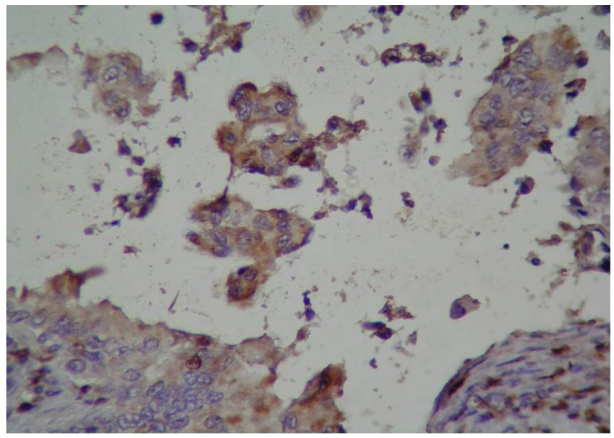

Figure 3. Bcl-2 positivity for tumor cells (bcl-2, 400×).

and the mean survival time of all these patients was 30.5 months. The overall survival of the patient that showed both *KRAS* and *BRAF* mutations was 14 months.

4. Discussion

Although micropapillary carcinoma had previously been reported in several other organs, including the breast, ovary, and urinary bladder, the first subtype of pulmonary adenocarcinoma was described by Amin et al. in 2002 (13). Pulmonary micropapillary adenocarcinoma is associated with a high risk of lymph node metastasis and a poor prognosis, in a similar manner to other anatomical locations (14,15). This subtype was not defined in the 1999 and 2004 World Health Organization (WHO)/ International Association for the Study of Lung Cancer Classifications (16). However, micropapillary lung adenocarcinoma was classified as micropapillary dominant in the proposed new lung adenocarcinoma classification of resected tumors, in which the use of comprehensive histological subtyping to semiquantitatively assess histologic patterns in 5% increments, choosing a single

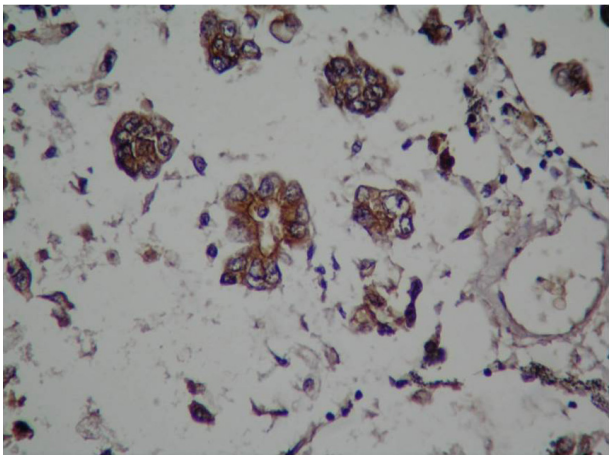

Figure 4. Immunohistochemistry for epidermal growth factor receptor (EGFR, 400×).

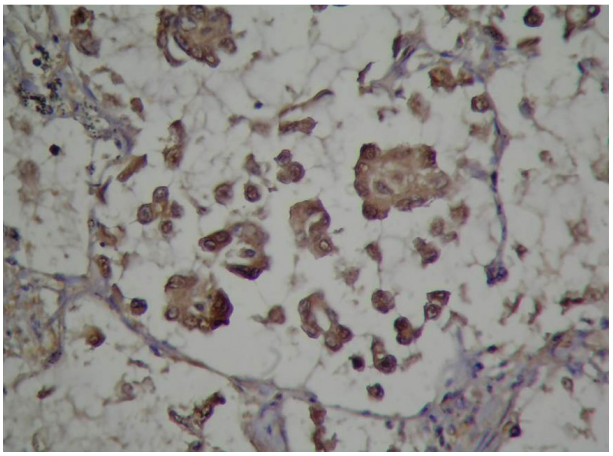

Figure 5. Immunohistochemistry c-kit (c-kit, 400×).

predominant pattern, was suggested (3). In the present study, we used the latter adenocarcinoma classification. To date, different definitions of micropapillary lung carcinoma have been used in molecular and immunohistochemical studies of this tumor subtype. For example, Ninomiya et al. defined micropapillary pulmonary adenocarcinoma as an adenocarcinoma in which 25% of the tumor has an MPP (17). However, Achcar et al. used the established definition made by Silver and Askin, whereby a papillary growth of greater or equal to 75% must be observed (6). Zhang et al. classified these tumors into two groups: MPP-negative (<1%) and MPP-positive (≥1%) (7), while Ohe et al. divided these tumors into those with an erogenous micropapillary component and those with a stromal invasive micropapillary component (8). In the present study, we used the most recent adenocarcinoma classification to identify females with micropapillary dominant adenocarcinoma (Table 5).

Our patients were younger than those studied by Achcar et al. (6), and we used a larger patient sample. Only two patients had a history of smoking. Although we found that the most common secondary pattern in our cases was the papillary pattern, the lepidic pattern was the most frequently observed secondary pattern found in the study by Achcar et al. We found that a secondary papillary pattern correlated with smoking (P = 0.002), psammoma bodies (P = 0.002), and perineural invasion (P = 0.001).

Driver genes are those that encode the signaling proteins that are critical for cellular proliferation and survival. Lung adenocarcinoma driver genes include *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, and *EML-ALK*. Initiation and maintenance of malignancy are the result of mutations of these genes (18). Different studies of driver gene mutations in nonsmall-cell lung carcinoma (NSCLC) have led to varying results because of differences in patient ethnicity or clinical information. *EGFR* and *KRAS* mutations were observed in 10%–30% of cases, with a higher frequency of *EGFR* mutations being found in Asians, in people who had no history of smoking, and in nonmucinous tumors (5). However, it has been found that the level of *KRAS* mutation is associated with smoking, being non-Asian, and invasive mucinous adenocarcinoma (19). A *BRAF* mutation rate of 1.9% was reported in nonsmokers with adenocarcinoma (18). With regard to the correlation between histological subtype and driver mutations, Li et al. found a significantly higher frequency of *EGFR* mutations in micropapillary dominant adenocarcinoma (11 of 13 cases, 84.6%), while

Table 3. *KRAS* and *BRAF* nucleotide changes in our case studies.

Codon	Amino acid changes	Base sequences	Cases
KRAS-Codon 12	Gly12Asp	GGT > GAT	Case 8
KRAS-Codon 12 KRAS-Codon 61	Gly12Asp Gln61Hys	GGT > GAT CAA > CAC	Case 10
KRAS-Codon 13 KRAS-Codon 61	Gly12Asp Gln61Pro	GGC > GAC CAA > CCA	Case 11
BRAF-Codon 600	Val600Glu	GTG > GAG	Case 10

Table 4. Epidermal growth factor receptor nucleotide changes in our case studies.

Exon	Amino acid change	Effect	Case
Exon 19	Glu746_Thr751delinsVal Glu746_Thr751delinsValAla Glu746_Ser751delinsVal	Activating	Case 1
Exon 19	Lysin739_ll744dupLysllProValAlalle Lysin745_Glu746del Glu746_Ala750del Glu746_Thr751delinsAla Glu746_Thr751delinslle	Activating	Cases 4, 5, 10, and 13
Exon 20	Leu792Pro		Case 9

Table 5. Features of research related to micropapillary pulmonary adenocarcinoma in previous studies.

	MP cases' election criteria	Method of mutation analysis	*EGFR* mutation results	*KRAS* mutation results	*BRAF* mutation results
Motoi et al. (9)	Major histological component	Oligonucleotide microarray	4 cases	Absent	Not studied
Achcar et al. (6)	MP pattern greater than or equal to 75%	Sequencing	Exon 19: 2 cases Exon 21: one case	4 cases	Three cases
Ohe et al. (8)	IASLC/ATS/ERS classification, AMPC, and SMPC	Sequencing	Exon 19: 7 cases Exon 21: 13 cases	Not studied	Not studied
Li et al. (10)	IASLC/ATS/ERS classification	Sequencing	11 cases	Absent	Absent
Zhang et al. (21)	MP pattern positive ≥1%	Allele-specific real-time PCR	Exon 18: no cases Exon 19: 15 cases Exon 20: 3 cases Exon 21: 18 cases Exons 20–21: 1 case	Not studied	Not studied
Current study	IASLC/ATS/ERS classification,	PCR microarray-based technology	Exon 19: 5 cases Exon 20: 1 case	3 cases	1 case

MP: Micropapillary; IASLC/ATS/ERS: International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society; SMPC: stromal invasive micropapillary component; AMPC: aerogenous micropapillary component.

no other driver mutations were found in this subtype (10). Achcar et al. reported an *EGFR* mutation rate in 10%, a *KRAS* mutation rate in 23%, and a *BRAF* mutation rate in 5.5% of cases (6). In our study, *EGFR*, *KRAS*, and *BRAF* mutation rates were 40%, 20%, and 6.6%, respectively, and the frequency of *EGFR* mutation was higher than that observed by Achcar et al. These results may be related to the sex of the patient. However, recent studies have shown that the *EGFR* mutation frequency of the micropapillary dominant subtype is higher that of other subtypes (20,21). The types of *EGFR* mutations reported in pulmonary adenocarcinoma are multiple deletions in exon 19 in 45% of cases, missense point mutations in exon 21 in 40% of cases, and missense or insertion mutations in exons 18–21 in 15% of cases (22). In our study, *EGFR* mutation in exon 19 was most frequently observed (5 cases, 83.3%), and EGFR immunostaining was not correlated with *EGFR* mutation.

KRAS mutation is found in approximately 30% of adenocarcinomas, and single amino acid substitutions in codons 12, 13, and 61 are the most frequently observed *KRAS* mutations in NSCLC. Negative predictive markers of *KRAS* mutation are most commonly found in the white population and in smokers.

EGFR and *KRAS* mutations are mutually exclusive. An et al. reported two cases of concurrent *EGFR* and *BRAF* mutations (18), while we identified one case that showed both *KRAS* and *BRAF* mutations. This is different from mutually exclusive *KRAS* and *EGFR* mutations.

Our study was associated with a higher rate of *EGFR* mutation than previous studies. When a cancer comprises more than 5% MMP, the prognosis is poor, even with regards to pathological stage I disease. However, patients with *EGFR*-mutated adenocarcinomas had improved survival rates. This dilemma was explained via the strong positivity of VEGF; angiogenesis is very important for tumor progression. Previous studies have shown that strong VEGF immunostaining is correlated with postoperative relapse and survival in NSCLC (23).

Kit (CD117), the product in the protooncogene *c-kit*, is a tyrosine kinase transmembrane receptor that regulates the development and growth of some human cell types. Inhibitors of c-kit have been used in the treatment of gastrointestinal stromal tumors. In our study, c-kit positivity was observed in three cases with *EGFR* mutation. Data on c-kit immunostaining in NSCLC is limited, but Butnor et al. detected c-kit positivity in 17% of adenocarcinomas (24), while 46.6% of our cases were positive for c-kit.

In this study *EGFR* mutation occurred at a higher incidence in micropapillary predominant invasive adenocarcinoma than has previously been found in conventional lung adenocarcinomas. Our data highlight the national mutation profile of micropapillary predominant invasive lung adenocarcinoma, which is associated with a poor prognosis and a high grade. We must ascertain the factors that influence targeted mutation with further studies.

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