Clinicopathological features of nonsmall cell lung carcinomas with BRAF mutations

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Background: Recently, driver tyrosine kinase gene mutations have been detected in malignant tumors, including lung tumors. Notwithstanding their attractiveness as targets for molecular therapy, limited information is available regarding BRAF-mutated lung carcinomas.

Materials and methods: BRAF mutation status was determined in 2001 surgically resected nonsmall-cell lung cancer (NSCLC) cases using high-resolution melting analysis (HRMA) followed by Sanger sequencing and/or deep sequencing using next generation sequencer.

Results: BRAF mutations were detected in 26 (1.3%) of 2001 NSCLC cases (25 adenocarcinomas and 1 squamous cell carcinoma). In the 26 cases, 13 mutation genotypes were identified, including V600E (8 of 26; 30.8%), G469A (6 of 26; 23.1%), K601E (4 of 26; 15.4%), and other residual mutations (1 of 26; 0.04%). Of the 13 genotypes, 4 genotypes (G464E, G596R, A598T, and G606R) had not been previously reported in lung cancer. The overall survival rate was not significantly different between patients with wild-type BRAF and those with V600E or non-V600E BRAF mutations (P = 0.49 and P = 0.15, respectively). Histomorphological analysis revealed that focal clear cell changes were present in 75% of V600E-mutated tumors. All V600E BRAF-mutated tumors were negative for other driver gene alterations including epidermal growth factor receptor (EGFR) and KRAS mutations and the anaplastic lymphoma kinase gene translocation, whereas five tumors with non-V600E BRAF mutations (four G469A and one G464E/G466R) showed concomitant EGFR mutations.

Conclusion: The frequency of BRAF mutations in lung cancer was low in an Asian cohort. Furthermore, BRAF mutation status lacked prognostic significance in this patient population.

Key words: Asian, BRAF gene mutation, lung carcinoma

Introduction

BRAF, one of three RAF isoforms, is a serine–threonine-specific protein kinase that is activated downstream of RAS. RAF activates mitogen-activated protein kinase, which in turn activates extracellular signal-regulated kinase (ERK) [1, 2]. Mutations in BRAF constitutively activate ERK signaling through hyperactivation of the RAS–ERK pathway, resulting in enhanced cell proliferation and survival [2].

BRAF mutations have been identified in a variety of tumors [3–6], including lung carcinoma [7–11]. BRAF mutations in...
Lung carcinomas are found in 0.8%-8% [7–13] and have some distinct histomorphological features [8, 11, 14, 15]. V600E represents over 50% of BRAF mutations in lung carcinoma, whereas fewer mutations are found at the G469A and G594G sites [8–10]. PLX4032 is the first BRAF-specific inhibitor to show promising clinical activity in melanoma patients [16]. Interestingly, a lung adenocarcinoma case with BRAF-V600E mutation showed a response to this drug [17].

Ethnic-specific differences in the frequency of BRAF mutations have also been reported in various cancers, as with EGFR mutations in nonsmall-cell lung carcinoma (NSCLC). The frequency of BRAF mutations in colorectal cancer is markedly higher in patients of Anglo-Saxon descent than in those of southern European descent [18], whereas the frequency of BRAF mutations in prostate cancer is higher in Asians than in Americans [3]. However, there have been few reports [7, 12] on the prevalence of BRAF mutations in lung carcinoma patients of Asian ethnicity.

In this article, we determined BRAF mutation status and its correlation with clinicopathological features in large number of Asian populations, by high-resolution melting analysis (HRMA) followed by Sanger sequencing and/or deep sequencing using next generation sequencer.

Materials and Methods

Case Selection

This study was approved by the institutional review board of the National Cancer Center Hospital (Tokyo, Japan). Specimens were obtained from 2043 patients who underwent surgical resection for primary lung cancer at the National Cancer Center Hospital between 1993 and 2009. Forty-two patients who received neoadjuvant chemotherapy were excluded. Histological diagnosis by Sanger sequencing or deep sequencing using a high-speed sequencer.

Analysis of BRAF, EGFR, KRAS Mutation Status, and ALK Rearrangement

Molecular-based mutation status was analyzed in fresh frozen (in liquid nitrogen; 989 of 2001 [49.4%]) and formalin or methanol-fixed, paraffin-embedded (1012 of 2001 [50.6%]) tissues of surgically resected lung cancer specimens. Methods of mutation detection by HRMA followed by verification by Sanger sequencing or deep sequencing using a high-speed sequencer are summarized in supplementary Tables S1 and S2 and Note available at Annals of Oncology online.

Echinoderm microtubule-associated protein-like 4 gene and ALK fusions were analyzed by immunohistochemistry, reverse transcriptase (RT)-PCR, and/or fluorescence in situ hybridization assay [20].

Immunohistochemistry

For immunohistochemical staining, 4-μm-thick sections of the paraffinized tumor samples were routinely deparaffinized. Detailed antigen retrieval methods and antibody dilution for each primary antibody are listed in supplementary Table S3, available at Annals of Oncology online.

All sections were stained with hematoxylin and eosin for morphological analysis by an automated strainer. Only BRAF-mutated tumors were stained with Alcian blue–periodic acid-Schiff (AB-PAS) for detection of mucin-containing tumor cells.

Results

Patient Characteristics

A total of 2001 patients with surgically resected NSCLC were included in the present analysis (supplementary Table S4, available at Annals of Oncology online). All patients were Asian. The NSCLC cases consisted of 1835 adenocarcinomas, 160 squamous cell carcinomas, 5 large cell neuroendocrine carcinomas, and 1 adenosquamous carcinoma. The median follow-up time was 61 months after surgical treatment. Among the 1835 adenocarcinomas, the most common histological subtype was papillary predominant (34.8%), followed by lepidic predominant (19.7%), solid predominant (12.7%), acinar predominant (9.3%), minimally invasive adenocarcinoma (MIA; 8.5%), micropapillary predominant (6.3%), and adenocarcinoma in situ (AIS; 4.7%). Other invasive types of adenocarcinoma included invasive mucinous adenocarcinoma (IMA; 3.9%) and colloid adenocarcinoma (0.05%).

BRAF Mutations

Of the 2001 surgically resected NSCLC cases, the first HRMA screening detected 33 cases (1.6%) with abnormal BRAF band patterns compared with wild-type BRAF. The presence of BRAF mutations was verified by sequencing of PCR products in 26 (78.8%) of the 33 suspicious BRAF mutation cases, whereas mutations were not found in the remaining 7 cases (21.2%). Four cases with BRAF-mutated tumor were detected by Sanger sequencing and 24 cases with BRAF mutated tumor were detected by deep sequencing, respectively (supplementary Figures S1 and S2, available at Annals of Oncology online). We confirmed that two cases with BRAF-mutated tumor were detected by both Sanger sequencing and deep sequencing. On the other hand, seven cases with suspicious BRAF mutation were not detected by deep sequencing (supplementary Table S5, available at Annals of Oncology online); therefore, they were judged as being negative. Thirteen kinds of mutation genotypes were identified in the 26 BRAF mutation cases (supplementary Figure S3, available at Annals of Oncology online). V600E, G469A, and K601E were present in 8 (30.8%), 6 (23.1%), and 4 (15.4%) cases, respectively. Other residual mutations (G464E, G466R, G466V, S467L, D594N, D594G, G596R, A598T, T399V, V600insT, G606R) were present in only one case each (0.04%), and two cases (V600E/G606R or G464E/G466R) had two genotypes. Four cases had genotypes (G464E, G596R, A598T, and G606R) that have not been previously reported in lung cancer. However, all the BRAF mutation types detected in the present study were already registered the Catalogue of Somatic Mutations in Cancer (COSMIC) database (supplementary Table S5, available at Annals of Oncology online); therefore, they were believed to be oncogenic mutations.

Statistical Analysis

Statistical analysis was carried out using the SPSS Statistics 21 program (IBM Corporation, Somers, NY). Survival curves were calculated by the Kaplan–Meier method. A P value of <0.05 was considered statistically significant.
BRAF-mutated tumors were present in 13 women and 13 men with an average age of 64 years (range, 44–81 years). Fourteen patients (54%) were current or former smokers. Six tumors originated in the right upper lobe, five in the right middle lobe, six in the right lower lobe, four in the left upper lobe, two in the left lower lobe, and three in other locations. The average tumor size was 2.2 cm (range, 0.8–4.5 cm). Pathological stage varied and included IA (n = 13), IB (n = 4), IIA (n = 3), IIB (n = 1), IIIA (n = 4), and IIIB (n = 1). Regarding tumor histology, 25 tumors were adenocarcinomas and 1 tumor was squamous cell carcinoma.

BRAF-mutated adenocarcinomas showed varied histomorphology. Papillary or micropapillary growth was present in 50% of BRAF-mutated adenocarcinomas (Figure 1A and B). In V600E-mutated cases, the predominant growth pattern was papillary in two cases, micropapillary in two cases, solid in three cases, and AIS in one case. Focal micropapillary patterns were observed in 50% of V600E-mutated cases. In non-V600E-mutated cases, the predominant growth pattern was papillary in eight cases, solid in two cases, and nonmucinous AIS in three cases. Lepidic, micropapillary, MIA, and IMA were each present in one case. Because of the limited numbers available, histopathological features were not evaluated in the other genotypes. Clear-cell changes were detected at least focally in the majority (75%) of V600E-positive cases (Figure 1C and D). Some, but not all, clear cells were positive for surfactant apoprotein A (SP-A) but negative for AB-PAS. In addition, 86% of V600E-mutated cases were positive for thyroid transcription factor-1 (TTF-1).

correlation between BRAF mutations and other driver gene alterations in NSCLC

EGFR and KRAS mutations were detected in 849 (42.4%) and 171 (8.5%) cases, respectively, by HRMA. ALK rearrangement was present in 41 (3.3%) of the 1247 investigated cases. Tumors with V600E BRAF mutations did not have concomitant mutations in EGFR or KRAS or ALK rearrangement. In contrast, five tumors with non-V600E BRAF mutations (four G469A and one G464E/G466R) showed concomitant EGFR mutations (four L858R and one DEL), and one tumor with S467L BRAF mutation showed a concomitant KRAS mutations.

Clinicopathological features associated with BRAF mutations in NSCLC

BRAF-mutated tumors were present in 13 women and 13 men with an average age of 64 years (range, 44–81 years). Fourteen patients (54%) were current or former smokers. Six tumors originated in the right upper lobe, five in the right middle lobe, six in the right lower lobe, four in the left upper lobe, two in the left lower lobe, and three in other locations. The average tumor size was 2.2 cm (range, 0.8–4.5 cm). Pathological stage varied and included IA (n = 13), IB (n = 4), IIA (n = 3), IIB (n = 1), IIIA (n = 4), and IIIB (n = 1). Regarding tumor histology, 25 tumors were adenocarcinomas and 1 tumor was squamous cell carcinoma.

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**Figure 1.** Morphological features of the BRAF-mutated tumors. (A) Papillary and (B) micropapillary growth were most common in BRAF-mutated adenocarcinomas (×10 magnification). (C and D) In most V600E-positive tumors, clear cell changes were identified at least focally (×10 magnification).

**Figure 2.** Overall survival curves in 2001 patients with NSCLC based on BRAF mutation status.
survival analysis of \textit{BRAF} mutations in NSCLC

Among the 2001 patients, the median follow-up time was 61 months after surgical treatment. Of the 26 patients with \textit{BRAF} mutations, 18 (69.2%) were still alive at the time of the most recent follow-up (mean follow-up, 71 months). Seven patients (26.9%) had evidence of intrapulmonary metastasis and 1 (3.8%) died of their disease at the time of last contact. The seven patients with intrapulmonary metastasis had the following genotypes: V600E/G606R-1, G469A-2, K601E-1, G466V-1, T599V600insT-1, and S467L-1.

Overall survival (Figure 2) was not significantly different between patients with wild-type \textit{BRAF} and those with mutated \textit{BRAF} ($P = 0.42$). In addition, overall survival was not significantly different between patients with wild-type \textit{BRAF} and those with V600E or non-V600E-mutated \textit{BRAF} ($P = 0.49$ and $P = 0.15$, respectively). Overall survival was not significantly different between patients with stage I and II disease who had wild-type \textit{BRAF} and those who had mutated \textit{BRAF} ($P = 0.27$ and $P = 0.80$, respectively). Multivariate analysis was not feasible because of the relatively small number of deaths in each \textit{BRAF} mutation genotype group.

discussion

In the present study, we investigated the incidence of \textit{BRAF} mutations and their association with clinicopathological features and prognosis in a predominantly Asian population with surgically resected NSCLC. We found that the incidence of \textit{BRAF} mutations in NSCLC was 1.3%. Thirteen kinds of \textit{BRAF} mutation genotypes were detected, and 4 of the 13 genotypes had not been previously detected in lung cancer. \textit{BRAF} mutation status did not have any prognostic value in NSCLC.

Previous studies have reported that the frequency of \textit{BRAF} mutations in lung adenocarcinoma in Asian populations is low (0.8%–1.1%) [7, 12]. Our incidence rate of 1.3% is consistent with these reports. These incidence rates were lower than those reported in Caucasian populations (~3%) [8, 9, 11]. The relative paucity of \textit{BRAF} mutations in the Asian population may be related to ethnic differences and the high frequency of \textit{EGFR} mutations in Asian women with lung adenocarcinoma. In the present study, V600E and non-V600E mutation genotypes had an equal sex and smoking history distribution. These results are in agreement with those of previous studies [8, 9, 11].

In lung cancer, several \textit{BRAF} mutation genotypes in NSCLC have been reported. Paik et al. [9] reported the presence of three genotypes in lung adenocarcinoma, including V600E, G469A, and D594G. Marchetti et al. [8] reported the presence of 14 genotypes in NSCLC (13 adenocarcinomas and 1 squamous cell carcinoma). We also identified 13 genotypes, including the predominant V600E and G469A mutations. The frequency of V600E (30.8%) in the present study was lower than that reported in the abovementioned studies, whereas the frequency of G469A (23.1%) was similar to that of Paik et al. [9]. In addition, we detected \textit{BRAF} mutations not previously reported in lung carcinoma, including G464E, G596R, A598T, and G606R. These individual mutations occur at a low frequency in a wide variety of other cancers [1, 2, 6, 8–10, 12]. Although, the lung is the most common site of metastasis in the natural history of malignant tumors, our four cases with G464E, G596R, A598T, or G606R mutation were confirmed to be of pulmonary, as evidenced by the expression of TTF-1 [21].

Our data indicated that postoperative overall survival was similar between NSCLC patients with wild-type \textit{BRAF} and those with mutated \textit{BRAF}. Furthermore, patients with \textit{BRAF} mutations had relatively low-stage disease. In contrast to our study, Marchetti et al. [8] reported that V600E mutations in radically resected primary lung adenocarcinoma had relatively high-stage disease and poor disease-free survival and overall survival. The small number of \textit{BRAF} mutation cases in our study precluded the drawing of definitive conclusions concerning the prognostic significance of \textit{BRAF} mutations in NSCLC. Further multicenter studies with larger patient populations are necessary to establish the role of \textit{BRAF} mutations in NSCLC prognosis. V600E mutations have been associated with poor prognosis in patients with colorectal cancer [5] and papillary thyroid cancer [4]. In contrast, \textit{BRAF} mutations did not affect the disease-free interval or overall survival in primary melanoma [22]. Together, these findings indicate that the prognostic significance of \textit{BRAF} mutations is influenced by disease site.

It has been previously reported that \textit{BRAF}, \textit{EGFR}, and \textit{KRAS} mutations are mutually exclusive events [2, 7, 9, 11, 23]. In our study, V600E \textit{BRAF} mutations were present exclusively; however, five non-V600E \textit{BRAF}-mutated tumors (four G469A and one G464E/G466R) exhibited concomitant \textit{EGFR} mutations (four L858R and one DEL). In agreement with our observations, Marchetti et al. [8] also reported the presence of concomitant V600E \textit{BRAF} and \textit{EGFR} mutations (two DEL) in two lung tumors. Recently, Ohashi et al. [24] reported that lung cancer samples with acquired resistance to EGFR-TKIs occasionally harbor \textit{BRAF} mutations. Of our five non-V600E \textit{BRAF}-mutated and EGFR-mutated NSCLC cases, only one case showed intrapulmonary metastasis after surgery but EGFR-TKIs were not effective. This finding supports a potential mechanistic role of \textit{BRAF} mutations in acquired resistance to EGFR-TKIs in lung cancer. We should pursue the correlation among the clinical implications in the presence or absence of molecularly targeted agent in the future.

\textit{BRAF}-mutated lung carcinomas showed varied histomorphology. According to recent reports, adenocarcinoma is the predominant histological type in \textit{BRAF}-mutated lung carcinoma [7, 8, 13], whereas squamous cell carcinoma is rare [1, 7, 8, 13]. Furthermore, V600E-positive cases have been shown to exhibit a more aggressive tumor histotype, which is characterized by micropapillary features [8, 15] and acinar and solid growth [11, 14]. Intriguingly, we detected a \textit{BRAF} mutation (K601E) in one case of squamous cell carcinoma. In addition, papillary (micropapillary) predominant, solid predominant, and focal micropapillary patterns were present in 50%, 38%, and 50% of V600E-positive adenocarcinomas, respectively. Of note, clear-cell changes were identified at least focally in 75% of V600E-positive cases. The nature of these clear-cell changes was not entirely clear; however, a few clear cells were positive for SP-A and negative for mucin and adipophilin. It is possible that clear-cell changes are induced, at least partially, by excessive accumulation of surfactant proteins in the tumor cell cytoplasm. In contrast to the V600E genotype, there are few reports on the histomorphology of other \textit{BRAF} mutation genotypes. Because of the small number of the other mutation genotypes in our study, histomorphological features could not
be evaluated. Specific histological features, such as the correlation between ALK translocations and the presence of signet-ring cells, may help identify specific genetic alterations in tumors [20]; however, it is insufficient to identify BRAF-mutated patients harboring histomorphological features, such as papillary (mircropapillary) predominant and clear cell changes. Additional large studies are needed to extend and confirm our results.

Recently, indirect methods were developed to detect gene mutations, including Scorpion ARMS, the peptide nucleic acid–locked nucleic acid PCR clamp, mutant-enriched PCR, the smart amplification process, and HRMA. In the present study, 33 (1.6%) cases with abnormal BRAF band patterns were detected by HRMA, and 26 (78.8%) of these 33 cases were confirmed by sequencing of PCR products. The targets of recent developed BRAF inhibitor were V600E or V600K mutations [25]. Our results indicated that sequencing is the gold standard method for detecting specific genotypes; however, indirect methods may serve as screening tools to detect the presence of any BRAF mutation. Next-generation sequencing platforms, such as the Ion Torrent, can introduce some errors. However, BRAF mutations were detected using both the HRMA and Sanger sequencing and/or deep sequencing. All these mutations are deposited in the COSMIC database. Therefore, these BRAF mutations were considered to be oncogenic ones. To obtain precise data, high-quality DNA extracted from an adequate amount of pure tumor cells is required and this is expensive and time-consuming. A novel BRAF antibody offers promise for the immunohistochemical detection of BRAF mutations [15] and needs to be validated in V600E-mutated lung tumors.

In conclusion, BRAF mutations in lung cancer were rare in an exclusively Asian population. BRAF mutation status lacked prognostic significance in this patient population.

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disclosure

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