Intra-tumor heterogeneity of *BRAF V600E* mutation in lung adenocarcinomas

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Abstract. *BRAF* mutations exist in numerous types of cancer, including melanomas, colorectal cancers and lung cancers. The *V600E*-specific inhibitor vemurafenib has marked clinical activity in patients with *BRAF V600E*-mutated melanoma. However, there are many cases of resistance to vemurafenib. This may be due to the reported intra-tumor heterogeneity of the *BRAF V600E* mutation in primary melanomas. *BRAF* mutations are found in 1-5% of non-small cell carcinomas (NSCLCs), almost exclusively in adenocarcinoma. A few cases have been reported in which vemurafenib was effective against *BRAF V600E*-mutated lung cancers. In a previous study, five lung adenocarcinomas with *BRAF V600E* mutation were detected by direct sequencing. The present study analyzed these tumors for the percentage of mutation (%mutation) by competitive allele-specific polymerase chain reaction (CAST-PCR) assay. In addition, sections of all components of the adenocarcinomas were obtained by laser microdissection and analyzed. The %mutations of *BRAF V600E* within the macrodissected tumors (cases 1-5) were: Case 1, 10.0%; case 2, 8.0%; case 3, 8.9%; case 4, 21.5%; and case 5, 14.9%. In four cases (cases 2-5), the %mutations of each adenocarcinoma component were as follows: Case 2, lepidic growth 6.5-24.5%, papillary 1.3-11.2% and acinar 9.8%; case 3, solid 2.5-69.9%, acinar 12.4-27.1% and papillary 3.7-17.4%; case 4, acinar 10.0-45.0% and papillary 44.0%; and case 5, papillary 3.7-93.4%. Sensitive *BRAF* mutation detection methods were used and evidence for heterogeneity of the *BRAF V600E* mutation in these lung adenocarcinoma cases was observed. Targeted therapy with a *BRAF V600E* inhibitor such as vemurafenib may have potential in the treatment of lung cancer with this mutation; however, it is necessary to consider how the treatment effect of and drug resistance to *BRAF V600E* inhibitors are affected by the presence of heterogeneity in future studies.

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Introduction

Lung cancer is a major cause of mortality due to its high incidence rate and malignant behavior, and a lack of major advancements in treatment strategy (1). Adenocarcinoma is the most common histological class of lung cancer, and its relative incidence is increasing (2). A great deal of progress has been made in the targeted therapy of non-small cell lung cancer (NSCLC), largely owing to the development of small-molecular inhibitors, such as epidermal growth factor receptor (EGFR) (3-5) and anaplastic lymphoma kinase (ALK) inhibitors (6) for lung adenocarcinomas.

The pathway successively linking receptor tyrosine kinases to Ras family proteins, Raf serine-threonine kinase and mitogen-activated protein (MAP) kinase is critical for cell proliferation and is frequently activated in human cancers (7). MAP kinase, also known as extracellular signal-regulated protein kinase (ERK), is crucial for the transduction of growth signals from several key growth factors, such as epidermal growth factor (EGF). Mutations of *BRAF* have been reported in melanomas (>60%) and colorectal cancers (8-11). The *V600E* mutant of *BRAF* activates the RAF/MEK/ERK pathway in human melanoma cells *in vitro*, and the transformation of a melanocyte cell line with *BRAF V600E* activates the MAP kinase pathway (8). In patients with *BRAF V600E*-mutated metastasizing melanoma, the V600E specific inhibitor vemurafenib has evident clinical activity (9). However, there are numerous cases of resistance to vemurafenib, which usually develops within 8 months (9-11). Yancovitz *et al* reported that there is a possibility that intra-tumor heterogeneity is involved in the resistance (11). *BRAF* mutations are found in ~1-5% of NSCLCs, almost exclusively in adenocarcinoma (12-14). There have been only a few case reports indicating that vemurafenib is effective against *BRAF V600E*-mutated lung adenocarcinomas (14,15), and the clinical therapeutic effects of vemurafenib are not yet clear. To the best of our knowledge, there have been no reports concerning the intra-tumor heterogeneity of *BRAF* mutations in lung cancer.

Previously, we identified seven (3.95%) patients with *BRAF* mutations (*V600E,* five cases; *N581I*, one case; and *599 insertion T*, one case) in a Japanese adenocarcinoma cohort (16,17). In the present study, these seven *BRAF*-mutated lung adenocarcinomas were investigated. The percentage of *V600E* mutation (%mutation) of these tumors was analyzed by competitive allele-specific polymerase chain reaction (CAST-PCR) technology (18). Furthermore, the intra-tumoral components of the adenocarcinomas with *BRAF V600E* mutations were dissected by laser microdissection and were analyzed for %mutation by CAST-PCR mutation detection.

Materials and methods

Patients. The study group included lung adenocarcinoma patients who had undergone surgery at the Department of Surgery, Nagoya City University Hospital (Nagoya, Japan). All tumor samples were immediately frozen and stored at -80˚C until assayed. Informed consent was obtained from all of the patients. The present study was approved by the Ethics Committee of Nagoya City University Hospital. Previously, seven adenocarcinoma cases with *BRAF* mutations, including five *V600E* cases, a *N581I* case and a *599 insertion T* mutation case were identified (16,17), and these cases were included. A total of 35 'oncogene-negative' adenocarcinoma cases without *EGFR* (16,19), *Kras* codon12-13 (20), *erbB2* (4,16), *BRAF* (16,17) or *KIF5B*/*RET* (21) mutations from previous studies (16,17) were also included. In addition, 16 adenocarcinoma cases with unknown *BRAF* status and without *EGFR* mutations or ALK immunohistochemistry (IHC) positivity were included. In total, 58 adenocarcinoma cases were evaluated by *BRAF V600E* CAST-PCR mutation detection assay.

CAST-PCR mutation detection assay for BRAF V600E. Genomic DNA was extracted from lung cancer tissues using the Wizard SV Genomic DNA Purification system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The DNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Thermo Fisher Scientific, Wilmington, DE, USA) and adjusted to a concentration of 10 ng/μl. PCR mutation detection assays were then conducted using 4 μl of each DNA. The CAST-PCRs were run in a final volume of 20 μl in a 96 well plate including 10 μl 2X TaqMan Genotyping Master mix (Life Technologies, Foster City, CA, USA), 2 μl 10X assay mix, 5 μl deionized water and 4 μl each DNA template. PCR was performed using a 7500 Fast Real-Time PCR System (Life Technologies). The CAST-PCR mutation detection assays were executed according to the manufacturers' instructions (18). The cycling conditions were initial denaturation at 95˚C for 10 min, followed by 5 cycles at 92˚C for 15 sec and 58˚C for 1 min, 40 cycles at 92˚C for 15 sec and 60˚C for 1 min. The data from the mutation detection assays were analyzed using Mutation Detector™ software version 2.0 (Life Technologies) and the %mutation was calculated with the following formula: $\%\text{mutation} = [1/2^{\text{normalized}\Delta Ct}/(1/2^{\text{normalized}\Delta Ct} + 1)] \times 100$ where normalizedΔCt = [Ct(mutant allele assay) - Ct(wild-type allele assay)] - calibrationΔCt; and calibrationΔCt =Ct(mutant allele assay positive control) - Ct(wild-type allele).

Laser microdissection to analyze intra-tumor heterogeneity. Freshly cut 10 μm paraffin-embedded sections from the five lung adenocarcinomas with the *BRAF V600E* mutation were mounted onto glass slides. Estimation of the tumor content of the lung adenocarcinoma samples was carried out using a light microscope (DM4000B; Leica Microsystems GmbH, Wetzlar, Germany) at a x400 magnification. Following deparaffinization with xylene, sections were stained with hematoxylin as required for laser microdissection. Laser microdissection of component parts from the lung adenocarcinomas was performed. The dissected area measured ~40,000 μm^2, corresponding to ~30 cells in each dissected component section. One case could be not dissected due to a lack of tumor volume. A minimum of four areas and a maximum of eight areas were dissected from each case. The dissected tissue was digested in 50 μl buffer consisting of Tris-HCl (pH 8.0), 20 mM/l EDTA (pH 8.0), 1 mM/l 0.5% Tween 20 and 200 mg/μl protein K for 24 h at 37˚C, followed by incubation for 15 min at 95˚C to inactivate the proteinase K (22). Aliquots of 4 μl volume were used for each experiment.

Results

BRAF V600E mutation detection assay. The CAST-PCR mutation detection assay for *BRAF V600E* revealed that the 35 oncogene-negative cases and 16 cases of unknown *BRAF* status did not have the mutation (0%). Furthermore, the *N581I* case and the *599 insertion T* mutation case did not show any *V600E* mutation (0%). The %mutations of the five *BRAF V600E* samples were 10.0% (case 1), 8.0% (case 2), 8.9% (case 3), 21.5% (case 4), and 14.9% (case 5; Table I). Although the *BRAF V600E* mutations were detected by direct sequencing for the five samples, there was a maximum mutation rate of only ~20% in the CAST-PCR mutation detection assay if microdissection was not performed. A previous study demonstrated that the CAST-PCR assay had a greater sensitivity in the detection of the *BRAF V600E* mutation, as compared with direct sequencing (18); therefore, one explanation for the low mutation rates is that the presence of normal tissue contributed the low percentage of mutation detection. This possibility was investigated by estimating the tumor content of each lung adenocarcinoma sample using light microscopy. Four of the five *V600E* cases, with the exception of case 3, were evaluated. The tumor contents were as follows: Case 1, <10%; case 2, 30%; case 4, 60%; and case 5, 50%.

Analysis of intra-tumor heterogeneity of the BRAF V600E mutation. Since the low mutation rates from these CAST-PCR assays could not only be accounted for by the presence of normal tissue, it was speculated that individual tumors might be heterogeneous with respect to the *BRAF V600E* mutation. In order to investigate the %mutations in each component of the *BRAF V600E*-mutated lung adenocarcinomas, laser microdissection was used to separate the adenocarcinomas into their component parts (Table II). In case 1, further CAST-PCR assays could not be performed as the tumor volume was insufficient. In case 2, a total six sections (three of lepidic growth, two papillary and one acinar) were dissected. The %mutation of *BRAF V600E* was a minimum of 1.3% and a maximum of 24.5%. In case 3, a total of eight sections (four solid, two acinar

Table I. Percentage (%) mutation of five whole tumors with *BRAF V600E* mutation.

Case number	%mutation
1	10.0
2	8.0
3	8.9
4	21.5
5	14.9

Table II. Percentage (%) mutations in each intra-tumor component of five cases of *BRAF V600E*-mutated lung adenocarcinoma.

Case and component numbers	Component type	%mutation
Case 1	No data	
Case 2		
1	Papillary	11.2
2	Acinar	9.8
3	Lepidic	16.7
4	Papillary	1.3
5	Lepidic	6.5
6	Lepidic	24.5
Case 3		
1	Solid	2.5
2	Solid	69.9
3	Acinar	27.1
4	Acinar	12.4
5	Papillary	17.4
6	Papillary	3.7
7	Solid	36.5
8	Solid	5.3
Case 4		
1	Papillary	44.0
2	Acinar	33.4
3	Acinar	45.0
4	Acinar	10.0
Case 5		
1	Papillary	48.1
2	Papillary	3.7
3	Papillary	93.4
4	Papillary	4.8

and two papillary) were dissected. The %mutation of *BRAF V600E* was a minimum of 2.5% and a maximum of 69.9%. In case 4, a total of four sections (three acinar and one papillary) were dissected. The %mutation of *BRAF V600E* was a minimum of 10.0% and a maximum of 45.0%. In case 5, a total of four sections (all papillary) were dissected. The %mutation of *BRAF V600E* was a minimum of 3.7% and a maximum of 93.4%. In all cases examined, there was significant difference in the %mutation of *BRAF V600E* for each component.

Discussion

In this study, the %mutation of *BRAF V600E* mutated lung adenocarcinomas and the heterogeneity of each intra-tumor component were analyzed by CAST-PCR mutation detection assay. Although the *BRAF V600E* mutation in lung adenocarcinomas could be detected by direct sequencing, the %mutations were only ~20% at most (minimum, 8.0%; maximum, 21.5%). From these results, it was hypothesized that lung adenocarcinomas with the *BRAF V600E* mutation might be heterogeneous. In order to verify this hypothesis, the %mutations for each component within adenocarcinomas were investigated following laser microdissection. There were significant differences among the %mutations of *BRAF V600E* for each component. The results indicate that *BRAF V600E* mutations in lung adenocarcinomas had intra-tumor heterogeneity. Alternatively, *BRAF V600E* mutations may not be an initiating event for all cancer cells in lung adenocarcinoma, even in such cancer in which the mutation is detected, since it may be present in only a subset of the cancer cells.

Human cancers are considered to develop from a single mutated cell, followed by malignant clonal expansion secondary to further genetic and genomic alterations. The continuous acquisition of these changes may cause tumor subclones to emerge with varying phenotypic advantages, including invasion, proliferation and metastasis (23). An analysis of three breast cancer tumors by single nucleus sequencing has clearly demonstrated the polyclonal nature of cancer (24). Intra-tumor heterogeneity, where more than one cancer cell clone is present within a single tumor, has been identified in a number of cancers (25-27). The development of therapies targeting specific oncogenes has enabled the use of mutation-detection strategies aimed at these oncogenes for the assessment of intra-tumor heterogeneity (11,18,28). Such heterogeneity is of significance, as it has been shown to affect the response to molecularly targeted treatments in cancers such as gastrointestinal stromal tumors and lung adenocarcinomas (26,27). In a study of the intra-tumor heterogeneity of *EGFR* mutations in NSCLC, it was observed that tumors containing both mutation-positive and mutation-negative tumor cells were less responsive to gefitinib than tumors that did not display such heterogeneity (27).

BRAF mutations were first reported in melanoma. In addition, *BRAF* mutations are most frequently identified in melanoma (29). The most common is a valine to glutamate substitution at codon 600 (*V600E*), which accounts for >90% of the *BRAF* mutations in melanoma (8). In patients with *BRAF V600E*-mutated metastasizing melanoma, the *V600E*-specific inhibitor vemurafenib has evident clinical activity (9). However, there are many cases of resistance to vemurafenib (9-11), and complete responses are rare. Yancovitz *et al* raised the hypothesis that this resistance may be associated with intra-tumor heterogeneity (11). Reports concerning the *BRAF V600E* mutation in lung cancer are less frequent than those in melanoma. To the best of our knowledge, the presence of heterogeneity of *BRAF V600E*-mutated lung adenocarcinoma has not been reported prior to its investigation in the

present study. There may be cases in which *BRAF V600E* mutations are not detected by direct sequencing because the %mutation is low. If so, the incidence of *BRAF V600E* in lung cancer might be underestimated. In a few case reports from other institutions, the *V600E* inhibitor vemurafenib was shown to be effective against *V600E*-mutated lung cancers (14,15). Due to these factors, it is recommended that *V600E* mutation status is evaluated by sensitive methods such as IHC (17) or CAST-PCR (18) in addition to the conventional sequencing. The analytical sensitivity of CAST-PCR is <1% in optimal conditions (18), and the procedure is suitable for the analysis of low quantity DNA templates (1-30 ng per reaction).

In conclusion, in five *BRAF V600E*-mutated lung cancers detected by direct sequencing and IHC, it was found that these tumors had %mutations of ~20% at most and had intra-tumor heterogeneity in all cases of *V600E* mutation. Targeted therapy with a *BRAF V600E* inhibitor such as vemurafenib may have potential in the treatment of lung cancer with this mutation. It is necessary to consider how the treatment effect of and drug resistance to *BRAF V600E* inhibitors are affected by the presence of heterogeneity in future studies.

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