

Increased *BRAF* copy number in lung adenocarcinoma

HIDEFUMI SASAKI¹, MASAHIKO MAEKAWA², TSUTOMU TATEMATSU¹, KATSUHIRO OKUDA¹,
SATORU MORIYAMA¹, MOTOKI YANO¹ and YOSHITAKA FUJII¹

¹Department of Oncology, Immunology and Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi 467-8601; ²GSP Lab Inc., Kawasaki, Kanagawa 212-0032, Japan

Received April 24, 2014; Accepted October 29, 2014

DOI: 10.3892/ol.2014.2719

Abstract. Point mutation of the *BRAF* gene is a genetic event that occurs in a subset of lung adenocarcinoma cases. For example, *BRAF* V600E is a driver mutation that can be effectively targeted using selective BRAF and/or MEK inhibitors. The present study hypothesized that an increase in *BRAF* copy number may be correlated with certain clinicopathological features of lung adenocarcinoma in Japanese patients. The *BRAF* gene copy number was analyzed using quantitative polymerase chain reaction amplifications in 29 surgically treated lung adenocarcinoma cases without *EGFR* or *Kras* mutations from Nagoya City University Hospital (Nagoya, Japan). Seven *BRAF*-mutant cases were included. Increased *BRAF* gene copy number was identified in three lung adenocarcinoma patients (10.3%), all of which exhibited the V600E mutation. Using fluorescence *in situ* hybridization with *BRAF*-specific and chromosome 7 centromeric probes, increased copy number status was associated with gene amplification or gain of chromosome 7. Although increased *BRAF* copy number was correlated with *BRAF* V600E mutations, numerical changes in BRAF copy number were rare and mild in lung adenocarcinoma, resulting in no significant difference in pathological tumor status or tumor stage.

Introduction

Despite recent improvements in its diagnosis, lung cancer remains a significant cause of mortality among malignant diseases due to its high incidence rate, malignant behavior and a lack of major advancements in treatment strategies (1). In Japan in 2011, the majority of respiratory surgeries performed were a result of lung cancer (48.9%) and >33,000 patients underwent surgery for lung cancer (2). The clinical behavior

of lung cancer is predominantly associated with its stage; thus, the treatment of lung cancer by surgery is only achieved in cases presenting in an early stage (3).

In addition to epidermal growth factor receptor (*EGFR*) and anaplastic lymphoma kinase gene alternations, genomic studies in lung adenocarcinoma have identified other potential therapeutic targets, including activating mutations in *Kras*, *BRAF*, *HER2* and *PIK3CA*, in frequencies >1% (4-6). *BRAF* mutations in lung adenocarcinoma would be of interest as these mutations may be associated with increased sensitivity to agents directly targeting BRAF or BRAF-mediated downstream signaling pathways (7,8). For example, *BRAF* V600E is a driver mutation that can be effectively targeted with selective BRAF and/or MEK inhibitors (9-11). Previous reports identified *BRAF* mutations in 1-4% of cases of lung adenocarcinoma (12-15), and 40-50% of lung cancer cases have been demonstrated to harbor non-V600E mutations distributed in exons 11 and 15 (12-17). A number of these non-V600E mutations exhibit only intermediate or low kinase activity, and the analysis of preclinical data indicates that non-V600E-mutant BRAF kinases may be resistant to BRAF-targeted therapy (17,18).

Although *BRAF* copy number gain has been investigated in thyroid tumors (19), to the best of our knowledge, the association between *BRAF* gene mutation and copy number gain in Japanese lung adenocarcinoma patients has not previously been reported. In the present study, the possibility that *BRAF* copy number gain represents a novel mechanism for *BRAF* gene mutation is investigated. To determine the *BRAF* copy number status in Japanese lung adenocarcinoma patients, quantitative polymerase chain reaction (qPCR) amplification was performed. The findings were compared with the clinicopathological features of the lung cancer patients and data from fluorescence *in situ* hybridization (FISH) performed using *BRAF*-specific and chromosome 7 centromeric probes. Typically, increases in *BRAF* copy number are moderate; however, in V600E lung adenocarcinomas, *BRAF* copy number increases occur with significant prevalence.

Patients and methods

Patients. The study group included 29 lung adenocarcinoma patients who had undergone surgery at the Department of Oncology, Immunology and Surgery, Nagoya City University Hospital (Nagoya, Japan) between 2002 and 2011. All tumor

Correspondence to: Dr Hidefumi Sasaki, Department of Oncology, Immunology and Surgery, Nagoya City University Graduate School of Medical Sciences, 1 Aza Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8601, Japan
E-mail: hisasaki@hotmail.com

Key words: BRAF, lung cancer, adenocarcinoma, copy number, V600E

samples were immediately frozen and stored at -80°C until assaying.

The clinical and pathological characteristics of the 29 lung adenocarcinoma patients were as follows: Stage I, 16 cases; stage II, six cases; and stage III, seven cases. The mean age of the patients was 67.5 years (range, 47-84 years). Among the 29 lung adenocarcinoma patients, eight were female and 10 were non-smokers. The samples from these patients had previously been analyzed for *EGFR* or *Kras* gene status (20,21) and were considered to be wild-type. This study was approved by the ethics committee of Nagoya City University (Nagoya, Japan) and written informed consent was obtained from all patients.

PCR assays for *BRAF*. Genomic DNA was extracted from the lung cancer tissues using the Wizard[®] SV Genomic DNA Purification system (Promega Corporation, Madison, WI, USA), according to the manufacturer's instruction. The DNA concentration was determined using a NanoDrop spectrophotometer (ND-1000, version 3.0; Thermo Fisher Scientific, Wilmington, DE, USA) and adjusted to a concentration of 2.5 ng/ml. *BRAF* copy number was analyzed by performing qPCR assays on a 7500 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA) using a QuantiTect SYBR Green[®] PCR kit (Qiagen, Valencia, CA, USA), with 5 μl DNA from each tumor sample (20,21). The DNA of each tumor sample was quantified by comparing the target locus (*BRAF*) to the reference long interspersed nucleotide element (*Line-1*), a repetitive element for which the copy number per haploid genome is similar in all healthy and neoplastic human cells (22). The quantification was based on a standard curve previously determined from a serial dilution of healthy human genomic DNA (Roche Diagnostics, Indianapolis, IN, USA) and the relative *BRAF* copy number was normalized to the healthy human genomic DNA (calibrator). Furthermore, the change in *BRAF* gene copy number relative to *Line-1* and the calibrator was determined using the following formula: $(T \text{ BRAF} / T \text{ Line-1}) / (C \text{ BRAF} / C \text{ Line-1})$, where T and C represent the quantity present in the tumor DNA and the calibrator, respectively. *BRAF* copy number was determined by assaying *BRAF* for each sample using the following primers: Forward, 5'-TCATAATGCTTGCTCTGATAGGA-3' and reverse, 5'-GGCCAAAATTTAATCAGTGGA-3'. In addition, the total DNA content was estimated by assaying *Line-1* elements for each sample using the following primers: Forward, 5'-AAAGCCGCTCAACTACATGG-3' and reverse, 5'-TGCTTTGAATGCGTCCCAGAG-3'. PCR was performed in triplicate for each primer set and the cycling conditions were as follows: Initial denaturation at 95°C for 15 min followed by 40 cycles at 94°C for 15 sec, 56°C for 30 sec and 72°C for 34 sec.

***BRAF* FISH analysis.** Unstained 5- μm sections of formalin-fixed and paraffin-embedded tumor tissue were submitted to dual-color FISH analysis using four probe sets. The *BRAF/CEN 7q* probe sets were developed at GSP Research, Inc. (Kawasaki, Japan) and were labeled with Texas Red[®] (TexRed) and fluorescein isothiocyanate (FITC). The probe sets were as follows: *BRAF1* (390 kb; 140.3-140.7 MB) at chromosome 7p12-TexRed; and *CEN 7q* (820 kb; 64.2-65.1 MB)-FITC at chromosome 7q11.21. The

lung adenocarcinoma slides were deparaffinized and pre-incubated with Pretreatment Solution (GSP Research, Inc.) at $95-99^{\circ}\text{C}$ for 30 min, followed by protease digestion buffer at 37°C for 10-20 min. The slides were subsequently washed and dried. In addition, labeled probe sets (10 μl) were cohybridized at 37°C for 72 h following denaturation at 75°C for 5 min. A stringency wash was conducted at 72°C with 2X saline-sodium citrate/0.3% Nonidet P-40 (Sigma-Aldrich, St. Louis, MO, USA) for 1-2 min and the slides were counterstained with DAPI. The slides were then visualized using the Leica MM AF imaging system (Leica Microsystems, Wetzlar, Germany).

Statistical analysis. Statistical analyses of unpaired samples were performed using the Mann-Whitney U test, and correlation coefficients were determined by rank correlation using Spearman's rank correlation analysis and the χ^2 test. All analyses were performed using StatView software (Abacus Concepts, Inc., Berkeley, CA, USA) and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

***BRAF* gene status in Japanese lung adenocarcinoma patients.** The clinicopathological data of the 29 lung cancer patients is indicated in Table I. Using primers sets for *BRAF*, 3/29 patients were identified to express >3 copies of the *BRAF* gene. *BRAF* gene copy status was not significantly correlated with gender (male, 9.5% vs. female, 12.5%; $P > 0.9999$), tobacco-smoking (non-smoker, 0% vs. smoker, 15.8%; $P = 0.5320$), pathological tumor (pT) status (pT1, 18.2% vs. pT2-4, 5.6%; $P = 0.5394$), tumor stage (stage I vs. stage II-IV, $P = 0.9999$) or age (<65 vs. ≥ 65 , $P = 0.5320$). No non-V600E *BRAF*-mutant cases exhibited an increased *BRAF* copy number; however, *BRAF* V600E status was correlated with an *BRAF* increased copy number.

FISH. The screening of seven *BRAF*-mutant tumors by FISH using a *BRAF*-specific probe revealed two cases (28.6%) with *BRAF* gene amplification (Fig 1). The two cases were V600E mutants and demonstrated an association between the *BRAF* copy number and chromosome 7 centromeric signals, indicating an association between numerical changes of the *BRAF* locus and whole chromosome 7 amplification. The *BRAF* copy number in the FISH-positive cases (whole chromosome 7 amplification) was three, 4/5 stage I cases were FISH-negative and 1/2 stage II cases were FISH-positive.

Discussion

In the present study, increased *BRAF* gene copy number was identified in 10.3% of Japanese lung adenocarcinoma patients without *EGFR* or *Kras* mutations. The *BRAF* gene status was correlated with *BRAF* V600E mutation and whole chromosome 7 amplification.

A previous report demonstrated that the clinical outcomes of *BRAF* mutation-positive patients to platinum-based combination chemotherapy resembled those of wild-type lung cancer patients (23). Within the *BRAF*-mutant cohort, patients with V600E mutations exhibited lower response rates to platinum-based chemotherapy and shorter progression-free survival compared with non-V600E mutation patients (23,24).

Table I. Clinicopathological data of 29 lung cancer patients.

Factor	<i>BRAF</i> gene status		P-value
	Increased (n=3)	Normal (n=26)	
Mean age, years ^a (mean±SD)	75.0±7.0	66.7±9.8	0.1670
Age, years [n (%)]			
<65	0 (0.0)	9 (36.6)	0.5320
≥65	3 (100.0)	17 (65.4)	
Gender, n (%)			
Male	2 (66.7)	19 (73.1)	0.9999
Female	1 (33.3)	7 (26.9)	
Tumor stage, n (%)			
I	2 (66.7)	14 (53.8)	0.9999
II-IV	1 (33.3)	12 (46.2)	
Lymph node metastasis, n (%)			
N0	2 (66.7)	17 (65.4)	0.9999
N ⁺	1 (33.3)	9 (36.6)	
Smoking status, n (%)			
Never-smoker	0 (0.0)	10 (38.5)	0.5320
Smoker	3 (100.0)	16 (61.5)	
<i>BRAF</i> mutation, n (%)			
V600E	3 (100.0)	2 (7.7)	0.0027
Non-V600E or wild-type	0 (0.0)	24 (92.3)	
Pathological T status, n (%)			
T1	2 (66.7)	9 (34.6)	0.5394
T2-4	1 (33.3)	17 (65.4)	

^aMean age of total patients, 67.5±9.8 years. SD, standard deviation; T, tumor.

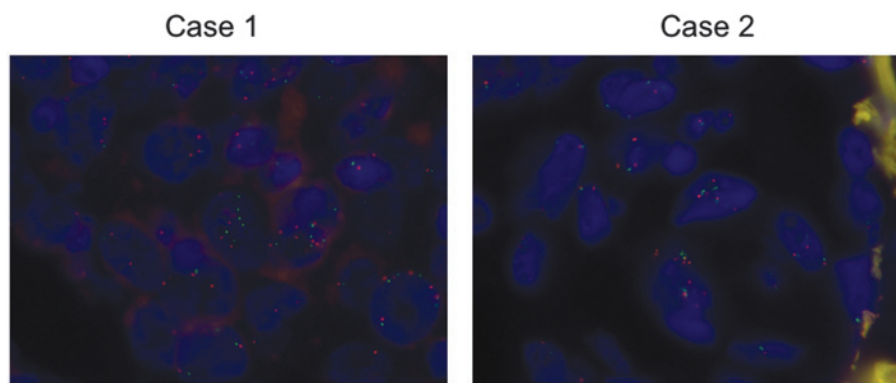


Figure 1. Dual-color fluorescence *in situ* hybridization analysis using the *BRAF*-specific (red) and chromosome 7 centromeric (green) probes, demonstrating the tumor cells exhibiting amplification (magnification, x1,000).

Previous studies have identified that V600E-mutated tumors are frequently associated with a more aggressive histotype (24,25). Furthermore, current second-generation *BRAF* inhibitors, such as vemurafenib and dabrafenib, have potent, selective activity against the V600-mutant *BRAF* kinases. One study in the literature described a *BRAF* V600E-mutant lung cancer patient responding to vemurafenib (7) and two studies described a response to dabrafenib (8,26).

Polysomy of chromosome 7 has been identified in the majority of solid tumors (27) and it is well-established that clonal numerical changes of chromosome 7 are common in lung cancer (28,29). Comparative genomic hybridization analysis demonstrated that 65% of lung cancer cases exhibit overrepresentation of chromosome 7p (28). This chromosome 7p gain has been associated with lymph node metastasis in lung cancer (29) and a detailed analysis of chromosome 7

identified various regions of alteration (30), including *EGFR*. Although gains of chromosome 7 result in an increase in the copy number of various genes located on this chromosome, data from the present study indicate that *BRAF* may also represent a target for its selection and clonal progression (19). The present study supports this role of *BRAF* due to the identification of chromosome 7 amplification in the *EGFR/Kras* wild-type, *BRAF* V600E-mutant cases screened. In a previous study, no overlap was identified between *BRAF* copy number changes and *RAS* mutations that are known to activate MAPK (19).

The numerical changes in *BRAF* determined in the present study included gains of three copies of the gene, which would be expected to result in its modest overexpression. However, one of the lymph node-positive V600E cases demonstrated increased copy number. Furthermore, one patient with an increased *BRAF* copy number had experienced cancer recurrence. Thus, *BRAF* copy number gain may serve as a marker of the more aggressive behavior of V600E lung adenocarcinoma (19).

In conclusion, the present study determined *BRAF* amplification in lung cancer for the first time and demonstrated that *BRAF* copy number gain may be present in *BRAF* V600E cases. *BRAF* copy number gain is rare in lung adenocarcinomas, however, it does occur in the aggressive V600E subtype.

Acknowledgements

The authors would like to thank Miss Ito Yamamoto for her technical assistance. The present study was supported by Grants-in-Aid for Scientific Research, Japan Society for the Promotion of Science (grant nos. 23659674, 24592097 and 25293303).

References

- Ginsberg RJ, Kris MK and Armstrong JG: Cancer of the lung. In: Principles and Practice of Oncology. 4th edition. JB Lippincott, Philadelphia, PA, pp673-682, 1993.
- Amano J, Kuwano H and Yokomise H: Thoracic and cardiovascular surgery in Japan during 2011: Annual report by the Japanese Association for Thoracic Surgery. *Gen Thorac Cardiovasc Surg* 61: 578-607, 2013.
- Postus PE: Chemotherapy for non-small cell lung cancer: the experience of the Lung Cancer Cooperative Group of the European Organization for Research and Treatment of Cancer. *Chest* 113 (Suppl 1): 28S-31S, 1997.
- Ding L, Getz G, Wheeler DA, *et al*: Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 455: 1069-1075, 2008.
- Sun Y, Ren Y, Fang Z, *et al*: Lung adenocarcinoma from East Asian never-smokers is a disease largely defined by targetable oncogenic mutant kinases. *J Clin Oncol* 28: 4616-4620, 2010.
- Weir BA, Woo MS, Getz G, *et al*: Characterizing the cancer genome in lung adenocarcinoma. *Nature* 450: 893-898, 2007.
- Gautschi O, Pauli C, Srobel K, *et al*: A patient with *BRAF* V600E lung adenocarcinoma responding to vemurafenib. *J Thorac Oncol* 7: e23-e24, 2012.
- Falchook GS, Long GV, Kurzrock R, *et al*: Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumors: a phase I dose-escalation trial. *Lancet* 379: 1893-1901, 2012.
- Chapman PB, Hauschild A, Robert C, *et al*: BRIM-3 Study Group: Improved survival with vemurafenib in melanoma with *BRAF* V600E mutation. *N Engl J Med* 364: 2507-2516, 2011.
- Flaherty KT, Infante JR, Daud A, *et al*: Combined *BRAF* and MEK inhibition in melanoma with *BRAF* V600E mutations. *N Engl J Med* 367: 1694-1703, 2012.
- Flaherty KT, Robert C, Hersey P, *et al*: METRIC Study Group: Improved survival with MEK inhibition in *BRAF*-mutated melanoma. *N Engl J Med* 367: 107-114, 2012.
- Marchetti A, Felicioni L, Malatesta S, *et al*: Clinical features and outcome of patients with non-small-cell lung cancer harboring *BRAF* mutations. *J Clin Oncol* 29: 3574-3579, 2011.
- Cardarella S, Ogino A, Nishio M, *et al*: Clinical, pathologic, and biologic features associated with *BRAF* mutations in non-small cell lung cancer. *Clin Cancer Res* 19: 4532-4540, 2013.
- Sasaki H, Shitara M, Yokota K, *et al*: *Braf* and *erbB2* mutations correlate with smoking status in lung cancer patients. *Exp Ther Med* 3: 771-775, 2012.
- Paik PK, Arcila ME, Fara M, *et al*: Clinical characteristics of patients with lung adenocarcinomas harboring *BRAF* mutations. *J Clin Oncol* 29: 2046-2051, 2011.
- Sasaki H, Shimizu S, Tani Y, *et al*: Usefulness of immunohistochemistry for the detection of the *BRAF* V600E mutation in Japanese lung adenocarcinoma. *Lung Cancer* 82: 51-54, 2013.
- Pratilas CA, Hanrahan AJ, Halilovic E, *et al*: Genetic predictors of MEK dependence in non-small cell lung cancer. *Cancer Res* 68: 9375-9383, 2008.
- Wan PT, Garnett MJ, Roe SM, *et al*: Cancer Genome Project: Mechanism of activation of the *RAF*-*ERK* signaling pathway by oncogenic mutations of B-*RAF*. *Cell* 116: 855-867, 2004.
- Ciampi R, Zhu Z and Nikiforov YE: *BRAF* copy number gains in thyroid tumors detected by fluorescence in situ hybridization. *Endocr Pathol* 16: 99-105, 2005.
- Endo K, Sasaki H, Yano M, *et al*: Evaluation of the epidermal growth factor receptor gene mutation and copy number in non-small cell lung cancer with gefitinib therapy. *Oncol Rep* 16: 533-541, 2006.
- Sasaki H, Okuda K, Kawano O, *et al*: *Nras* and *Kras* mutation in Japanese lung cancer patients: Genotyping analysis using LightCycler. *Oncol Rep* 18: 623-628, 2007.
- Wang TL, Maierhofer C, Speicher MR, *et al*: Digital karyotyping. *Proc Natl Acad Sci USA* 99: 16156-16161, 2002.
- Cardarella S, Ogino A, Nishino M, *et al*: Clinical, pathologic, and biologic features associated with *BRAF* mutations in non-small cell lung cancer. *Clin Cancer Res* 19: 4532-4540, 2013.
- Marchetti A, Felicioni L, Malatesta S, *et al*: Clinical features and outcome of patients with non-small-cell lung cancer harboring *BRAF* mutations. *J Clin Oncol* 29: 3574-3579, 2011.
- De Oliveira Duarte Achcar R, Nikiforova MN and Yousem SA: Micropapillary lung adenocarcinoma: *EGFR*, *K-ras*, and *BRAF* mutational profile. *Am J Clin Pathol* 131: 694-700, 2009.
- Rudin CM, Hong K and Streit M: Molecular characterization of acquired resistance to the *BRAF* inhibitor dabrafenib in patient with *BRAF*-mutant non-small-cell lung cancer. *J Thorac Oncol* 8: e41-e42, 2013.
- El-Naggar AK, Dinh M, Tucker SL, *et al*: Numerical chromosomal changes in DNA hypodiploid solid tumors; restricted loss and gain of certain chromosomes. *Cytometry* 37: 107-112, 1999.
- Balsara BR, Sonoda G, du Manoir S, *et al*: Comparative genomic hybridization analysis detects frequent, often high-level, overrepresentation of DNA sequences at 3q, 5p, 7p, and 8q in human non-small cell lung carcinomas. *Cancer Res* 57: 2116-2120, 1997.
- Ubagai T, Matsuura S, Tauchi H, *et al*: Comparative genomic hybridization analysis suggests a gain of chromosome 7p associated with lymph node metastasis in non-small cell lung cancer. *Oncol Rep* 8: 83-88, 2001.
- Garnis C, Lockwood WW, Vucic E, *et al*: High resolution analysis of non-small cell lung cancer cell lines by whole genome tiling path array CGH. *Int J Cancer* 118: 1556-1564, 2006.