PIK3CA Mutations Detected in Patients with Central Nervous System Metastases of Non-small Cell Lung Cancer

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Abstract. *Background: In non-small cell lung cancer (NSCLC) the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) gene mutations have been reported in fewer than 5% of primary tumors. Materials and Methods: We assessed PIK3CA gene mutations in 145 tissue samples from central nervous system (CNS) metastases of NSCLC using three polymerase chain reaction (PCR) techniques: high resolution melting-PCR (HRM-PCR), allele-specific-quantitative PCR (ASP-qPCR) and TaqMan PCR. Results: HRM analysis allowed us to select three PIK3CA-positive specimens (2.1% of the studied group) and ASP-qPCR techniques identified them as one E542K and two H1047R substitutions, which were confirmed by TaqMan probes. The PIK3CA mutations were indicated only in males (3% of all males). One of the patients was reported to be a non-smoker with adenocarcinoma (AC; 2.5% of the AC group), however, the other two patients were smokers with squamous cell carcinoma (SCC; 3.4% of SCC group). Conclusion: This is the first report of the presence of PIK3CA gene mutation in CNS-metastatic lesions of NSCLC worldwide that could broaden therapeutic choices in such patients.*

Phosphatidylinositol 3-kinases (the PI3K protein family) are lipid kinases encoded by the phosphatidylinositol-4,5-*bis*phosphate 3-kinase, catalytic subunit alpha (*PIK3CA*) gene. PI3Ks play a major role in important cellular functions, including growth, adhesion, survival, motility and cell proliferation. The function of PI3Ks is regeneration of phosphorylated forms of phosphatidylinositol, which is a key mediator between growth factor receptors and intracellular downstream signaling pathways (1-4).

PIK3CA gene mutations are somatic missense substitutions that are generally clustered in exon 9 (E542K, E545K, E545Q), which encodes the PI3K helical domain, and in exon 20 (H1047R, H1047L), which encodes the catalytic domain of PI3K (3-6). It has been shown that mutations of the *PIK3CA* gene can induce oncogenic cellular transformation, which is correlated with enhancing of PI3K enzymatic activity that leads to constitutive activation of murine thymoma viral oncogene (AKT) signaling pathway in cancer cells. It has been associated with more aggressive diseases, which correlate with a poor prognosis for patients (3-7).

PIK3CA gene mutations are most frequently reported in colorectal cancer, gliobloastoma, and gastric and breast cancer (30%) (1, 2, 5). In non-small-cell lung cancer (NSCLC), *PIK3CA* gene mutations have been reported in fewer than 5% of primary tumors, especially in adenocarcinoma (AC) and squamous cell carcinoma (SCC) subtypes. Moreover, the coexistence of *PIK3CA* gene mutations and other oncogene mutations (mainly epidermal growth factor receptor (*EGFR*) and vital rapidly accelerated fibrosarcoma homolog B1 (*BRAF*) in lung adenocarcinoma have also been described (6-9).

Reliable and sensitive molecular detection assays are in demand for predicting response or survival benefit during standard or targeted therapy in patients with NSCLC (10). However, only exact reporting of methodology will allow for detectability of mutations by different molecular methods to be evaluated (11). In spite of low sensitivity (especially in poorly cellular biopsies and degraded formalin-fixed paraffin-embedded (FFPE) tissue samples), direct DNA sequencing was considered a gold standard for the detection of driver mutations (11-13). Currently, techniques certified for *in vitro* diagnosis followed by melting-curve analysis or hybridization probes in real-time polymerase chain reaction (PCR) technique are described as an efficient approach to screening of well-known mutations (14). The high detectability of driver

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mutations is also provided by next-generation sequencing (NGS). In the future, the NGS method may play a major role both in the qualification of patients for personalized therapy and in the detection of residual disease (15).

To date analysis of E542K, E545K, E545Q,H1047R, and H1047L substitutions for the *PIK3CA* gene was performed mainly in primary tumors of NSCLC. However, in our study we estimated their incidence in central nervous system (CNS) metastases of NSCLC. Moreover, we compared the results of *PIK3CA* mutation detection using high-resolution melting PCR (HRM-PCR), allele-specific quantitative real-time PCR (ASP-qPCR) and real-time PCR with TaqMan hydrolysis probes. With these approaches, we aimed to determine the screening process for *PIK3CA* gene status by determining the concordance rate between the results obtained for the analyzed samples and control DNA with the presence or absence of *PIK3CA* mutation.

Materials and Methods

Patients and Materials. A total of 145 FFPE tissue samples were obtained from surgically resected CNS metastases of Caucasian patients with advanced NSCLC. Thirty corresponding primary NSCLC tumors were simultaneously available. The patients underwent routine neurosurgical procedures with a palliative intent. The median overall survival (OS) time from cancer diagnosis to death was 13.5 months (range=0.1-78.2 months; information available from 119 patients). All of the studied patients were untreated by chemotherapy, radiotherapy or other molecularly targeted therapies. According to the number of smoked cigarettes, patients were qualified as heavy smokers (≥15 pack-years), light smokers (<15 pack-years) and non-smokers. Detailed characteristics of the studied group are presented in Table I.

The study was approved by the Ethics Committee of the Medical University of Lublin, Poland (no. KE-0254/86/2013).

Mutation analysis. DNA was isolated from FFPE samples of metastatic tissue using QIAamp a DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Estimation of *PIK3CA* gene mutations (substitutions E542K, E545K, H1047R) was conducted using three methods based on a real-time PCR reaction. The HRM-PCR technique was used as a screening method. ASP-qPCR was performed for the identification of *PIK3CA* mutation type. TaqMan (Applied Biosystems, Foster City, CA, USA) hydrolysis probes, as well as real-time PCR technique, were used to confirm the results obtained with the two previously mentioned methods. DNA isolated from *PIK3CA* gene mutation-positive cell lines (SW48 cell-line: substitution E542K; MCF10A cell-line: substitution E545K and H1047R) acted as positive control for the analysis. The control DNA was provided by Horizon Discovery (Horizon Discovery, Cambridge, UK) at a concentration of 20 ng/μl. DNA isolated from peripheral blood leukocytes of healthy individuals acted as the negative control. In addition, in our previous studies, we published the incidence of *EGFR*, human epidermal growth factor receptor 2 (*HER2*), discoidin death receptor 2 (*DDR2*) and Kirsten rat sarcoma virus (*KRAS*) gene mutations in the same analyzed material (16).

Table I. *Study group characteristics*.

Characteristic	Value
Gender	
Male, n (%)	100 (69)
Female, n (%)	45 (31)
Age	
Median±SD years	60±8.8
≥60 Years, n (%)	72 (49.7)
<60 Years, n (%)	73 (50.3)
Histopathology	
Adenocarcinoma, n (%)	80 (55.2)
Squamous-cell carcinoma, n (%)	29 (20)
Large-cell carcinoma, n (%)	22 (15.1)
NSCLC-NOS, n (%)	14 (9.7)
Smoking status	
Current smokers, n (%)	73 (50.4)
Former smokers, n (%)	21 (14.5)
Non-smokers, n (%)	36 (24.8)
Lack of data, n (%)	15 (10.3)
Performance status	
0, n (%)	22 (15.2)
1, n (%)	76 (52.4)
2, n (%)	31 (21.4)
3, n (%)	16 (11)

SD: Standard deviation; NSCLC-NOS: non-small-cell lung cancer not-other specified.

HRM-PCR method. PIK3CA gene substitutions were analyzed using real-time PCR method with DNA intercalating dye and two pairs of primers flanking the mutated site of the gene. One pair of primers flanked the mutations located in exon 9 (substitutions E542K and E545K) and a second pair of primers flanked a mutation located in exon 20 (substitution H1047R). Before HRM-PCR, all DNA samples were diluted to a concentration of 20 ng/μl. The total reaction mixture (15 μl) was prepared with 8 μl of HRM Primer Design Master Mix (PrimerDesign Ltd, Southampton, UK) with DNA-intercalating dye (Chromofy™), 1 μl of purified genomic DNA (20 ng/μl), 1 μl of each forward and reverse primer and 4 μl of nuclease-free water. The amplification of the examined region was performed in 48-well plates using an Eco real-time PCR device (Illumina, San Diego, CA, USA) in the following steps: pre-denaturation at 95˚C for 10 min and 45 cycles of: 95˚C for 15 s and 62˚C for 60 s. In the next step, the HRM procedure was performed for PCR products as follows: 92˚C for 5 s, 72˚C for 20 s, and 92˚C for 5 s. Different genotypes of *PIK3CA* were distinguished according to the raw data plots and difference graph derived from the normalization data. A comparison of amplification and melting curves in the positive and negative controls allowed us to distinguish the mutant and wild-type samples.

ASP-qPCR method. The *PIK3CA* gene mutations were analyzed using the ASP-qPCR method with DNA-intercalating dye and allele-specific primers for mutant and wild-type *PIK3CA* gene. Some of the reagents and DNA, as well as total volume of PCR mixture, were the same as in the HRM procedure. The amplification of the examined region was performed in 48-well plates using an Eco real-time PCR device (Illumina) in the following steps: pre-denaturation at 95˚C for 10 min

Table II. *Characteristics of patients with non-small-cell lung cancer with phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) gene mutation.*

Patient	Age, years	*PIK3CA* mutation	Histopathology	Smoking status	Overall survival, months
1	77	E542K	Adenocarcinoma	Never-smoker	0.1
2	67	H1047R	Squamous cell carcinoma	174 Pack years	5.8
3	54	H1047R	Adenocarcinoma	20 Pack years	18.7

and 35 cycles of: 95˚C for 15 s and 63˚C for 60 s. The mutant and wild-type *PIK3CA* gene were tested in separate reactions with specific forward primers for these variants of the gene. Samples were assessed as positive if amplification in the ASP-qPCR was observed both for mutant and wild-type *PIK3CA* gene. Samples with late amplification (Ct>32-cycle) of wild-type *PIK3CA* gene were excluded from analysis and samples with late amplification (Ct>32-cycle) of mutant region of the *PIK3CA* gene were assessed as negative.

Real-time PCR with TaqMan probes. The TaqManFAM™-labeled hydrolysis probes are able to bind only to mutant regions of DNA (*i.e.* emission of fluorescence is observed only in positive samples). The PCR was performed in a final volume of 15 μl: 10 μl of 2× TaqMan® genotyping master mix (Applied Biosystems), 1 μl of 10× TaqMan hydrolysis probe, 3 μl of nuclease-free water and 1 μl of DNA template (20 ng/μl) in 48-well plates using an Eco real-time PCR device (Illumina). Thermo cycling conditions were recommended by Applied Biosystems: 95˚C for 10 min, five cycles of 92˚C for 15 s and 58˚C for 1 min, then 40 cycles of 92˚C for 15 s and 60˚C for 60 s. Samples were assessed as mutant for *PIK3CA* if we observed amplification between 25-30 cycles. The results were compared with amplification plots for positive and negative controls. Samples with (Ct>40-cycle) or without late amplification were assessed as having wild-type *PIK3CA* gene.

Analysis of results. Results obtained in originally designed HRM- and ASP-qPCR methods were compared to the results produced by TaqMan probes that were dedicated to detection of E542K, E545K and H1047R substitutions in *PIK3CA* gene. In all three analyses, the threshold was established according to amplification plots for positive and negative controls.

Coexistence of different driver mutations with *PIK3CA* mutations has also been described. We published the incidence of EGFR, *HER2, DDR* and *KRAS* gene mutations in NSCLC metastases to the CNS in our previous studies (16).

Results

In the first part of the analysis, the HRM-PCR technique was used for screening of mutations in exons 9 and 20 of the *PIK3CA* gene. Flanking HRM primers allowed amplification of selected PIK3CA gene regions in all analyzed samples and controls (mean Ct=25 cycles). Based on the differences in melting curves of positive and negative controls, we were able to distinguish mutant and wild-type *PIK3CA* gene (Figure 1). We selected three samples with probable *PIK3CA* gene mutations (one in exon 9 and two in exon 20). In the second part of the analysis, we performed ASP-qPCR on all examined samples to define the type of screened mutations and to compare the sensitivity of the methods (Figure 2). Using the ASP-qPCR technique we were able to detect mutations in positive controls and in all three samples previously selected by HRM-PCR. We found one E542K and two H1047R substitutions of the PIK3CA gene. Amplification of mutant *PIK3CA* gene was observed on average in the 25th cycle. In these cases, the amplification of wild-type *PIK3CA* gene was observed on average in the 20th cycle. The presence of amplification of the mutant *PIK3CA* gene in the negative control was not shown. In the third part of the analysis, we used TaqMan assays (dedicated to the distinction of E542K, E545K and H1047R substitutions in the *PIK3CA* gene) as a reference technique (Figure 3). This analysis confirmed our results obtained both in HRM-PCR and ASP-qPCR. We observed amplification curves (mean Ct=35th cycle) for three samples positive by HRM-PCR and ASP-qPCR and in the positive control. In wild-type samples, no amplification was observed.

We detected one E542K and two H1047R substitutions in the *PIK3CA* gene in three CNS metastases of male patients with NSCLC. Incidence of *PIK3CA* gene mutations was 2.1%. In all cases, the *PIK3CA* gene was mutated exclusively (without *EGFR, HER2, DDR* and *KRAS* gene mutations). Two patients with *PIK3CA* gene mutations had lung adenocarcinoma (2.5% of patients with AC) and one patient squamous cell lung cancer (3.4% of patients with SCC). One patients with AC was described as a never smoker (77 years old); however, the other *PIK3CA* gene mutation-positive patients had reported a smoking history (20 pack-year 54-year-old patient with AC; and 174 pack-year 67-year-old patient with SCC). The median OS for patients without *PIK3CA* gene mutation with NSCLC treated with resection of CNS metastases was 13.5 months. A never-smoking patient with AC with *PIK3CA* mutation died in the postoperative period. However, OS for a mutation-positive patient with SCC was 5.8 months and in a second mutation-positive patient with AC was 18.7 months (Table II). Analysis of corresponding primary tumors indicated only wild-type *PIK3CA* gene. Corresponding primary tumors were not available for *PIK3CA* mutation-positive patients with CNS metastases.

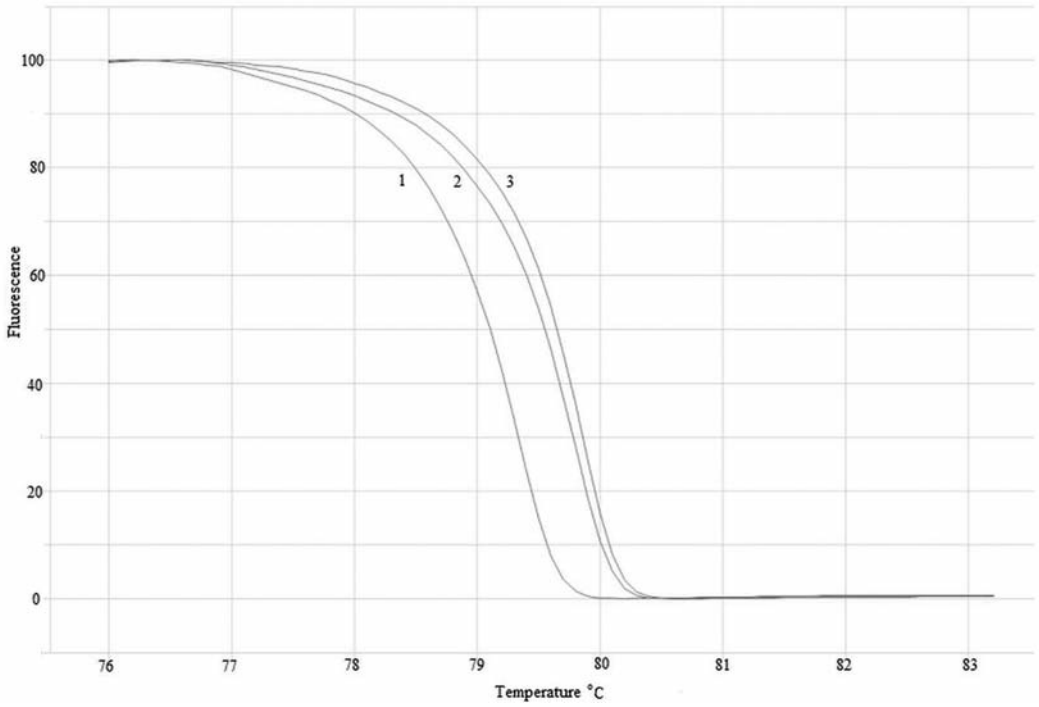

Figure 1. *Melting curves of phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) gene amplification product in high-resolution melting-polymerase chain reaction analysis. Curves represent: 1: Amplification of wild-type PIK3CA region of negative control; 2: amplification of mutant PIK3CA region in a central nervous system metastasis from non-small-cell lung cancer; 3: amplification of mutant PIK3CA region in positive control DNA.*

Discussion

We detected three *PIK3CA* gene mutations in CNS metastases of NSCLC (3/145; 2.1%). This result is in accordance with results of the majority of studies reporting a frequency of *PIK3CA* gene mutations of lower than 5% in primary NSCLC tumors (1, 14, 17, 18). However, unlike in previous studies, *PIK3CA* mutations were detected here in heavily smoking patients and in CNS metastases of NSCLC. Moreover, in our studied group, *PIK3CA* mutations were mutually exclusive. In the primary tumor, coexistence of *PIK3CA* and *EGFR* mutations was previously described. An *et al*. reported 20 cases with *PIK3CA* mutation in 452 patients with NSCLC (4.4%). Mutation presence was insignificantly related with histological type of NSCLC and smoking history (mutations were more frequently detected in adenocarcinoma and in non-smokers). Moreover, they observed overlapping of *PIK3CA* mutations and cellular-mesenchymal to epithelial transition factor (*c-MET*) amplification in smokers, both with SCC and AC subtypes (17). Kawano *et al*. indicated eight cases with *PIK3CA* gene mutation (8/235; 3.4%) in NSCLC samples. The *PIK3CA* mutation incidence was lower in those with AC (2/135; 1.5%) than in SCC (5/77; 6.5%). Moreover, in three cases *PIK3CA* mutations co-existed with *EGFR* gene mutations. Kawano *et al*. also mentioned that the most frequent mutations were E545K (26.7%), H1047R (12.9%) and E542K (8.9%) substitutions, whereas the incidence of E545Q and H1047L substitutions was estimated to be in the range of 0.5-2.5% (14). Schildgen *et al*. detected two cases with *PIK3CA* mutation (4.9%) in 41 *EGFR*-negative NSCLC samples (18). However, there are also data of an extremely low (<1%) or high (7%) frequency of *PIK3CA* gene mutations in patients with NSCLC (7, 19). Kris *et al*. identified only six cases with *PIK3CA* gene mutations in 733 NSCLC primary specimens (0.82%) (7). On the other hand, Bar *et al*. described an exceedingly high incidence of *PIK3CA* gene mutations (7/100; 7%). Moreover, Bar *et al*. suggested that the variability in *PIK3CA* gene mutations may be associated with genetic predisposition to lung cancer (19).

The pre-clinical data have shown that the activation of *PIK3CA* mutations in *EGFR*-mutated lung cancer cell lines provides acquired resistance to EGFR tyrosine kinase inhibitors (TKIs). Therefore, treatment based on combination of EGFR TKIs with orally bioavailable inhibitors of class I PI3K isoforms such as NPV-BKM120 or inhibitor of mammalian target of rapamycin (mTOR) (everolimus) have

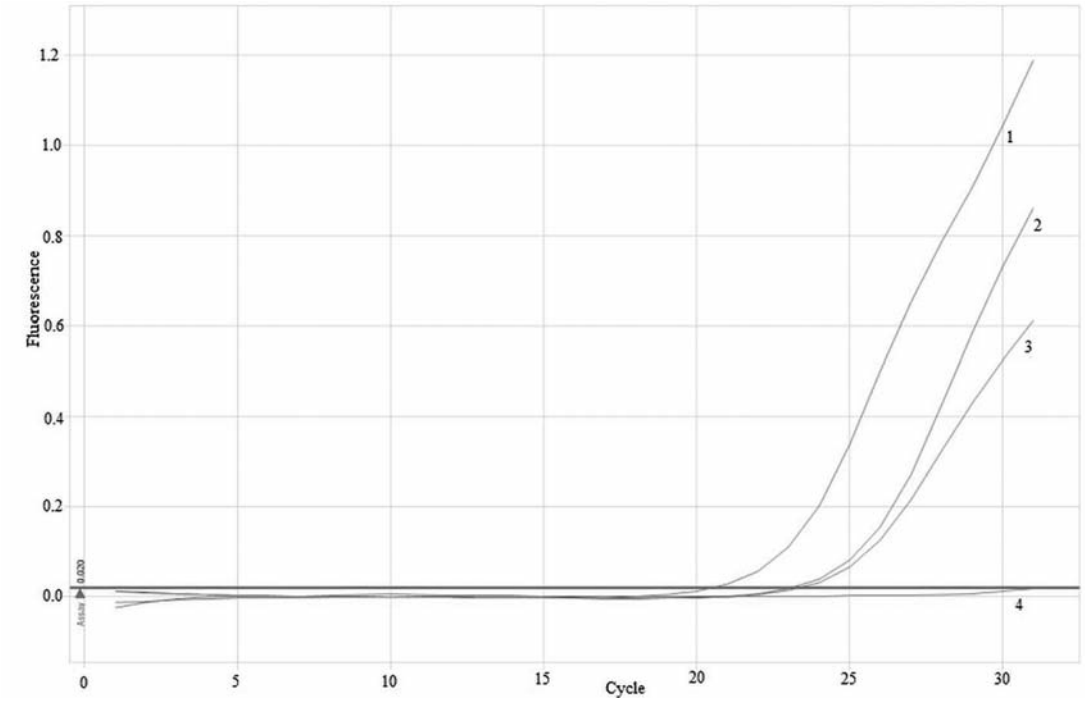

Figure 2. *Amplification curves for the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) gene in allele-specific quantitative polymerase chain reaction. Curves represent: 1: Amplification of wild-type PIK3CA region in a negative control; 2: amplification of mutant PIK3CA region in a central nervous system metastasis from non-small-cell lung cancer; 3: amplification of mutant PIK3CA region in positive control DNA; 4: lack of amplification of mutant PIK3CA region in negative control DNA (pair of primers specific to the mutant PIK3CA region).*

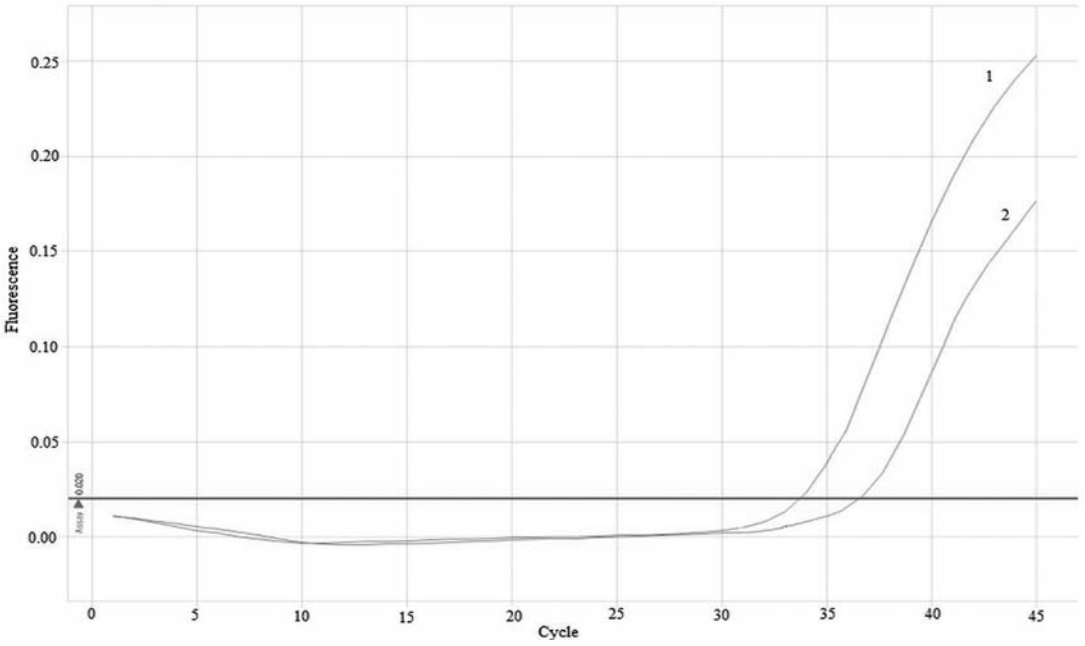

Figure 3. *Amplification curves for the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) gene in real-time polymerase chain reaction with TaqMan probes. Curves represent: 1: Amplification of mutant PIK3CA region in positive control DNA; 2: amplification of mutant PIK3CA region in a central nervous system metastasis from non-small-cell lung cancer.*

shown promising activity in such cases (3, 4, 8, 9, 20, 21). Moreover, the combination of dual PI3K/mTOR inhibitors (PI-103 or NVP-BEZ235) with selumetinib (selective mitogen-activated protein kinase 1/2 (MEK1/2) inhibitor) can enhance anti-angiogenic and antitumor effects in gefitinib-resistant NSCLC cell line models (20-23). The combination of pictilisib, a novel, oral, highly specific, small-molecule, ATP-competitive class I PI3K inhibitor (GDC-0941) with standard chemotherapy (paclitaxel or carboplatin) revealed better antitumor activity against lung cancer cells than did PI3K inhibition alone (3, 20-24). It was also mentioned that the dual PI3K/mTOR inhibitor dactolisib (NVP-BEZ235) has also shown efficacy against both *PIK3CA*- and *KRAS*-mutated NSCLC tumors (25). Therefore, the development of effective methods for *PIK3CA* mutation testing may be important for the future qualification of molecularly targeted therapies in patients with NSCLC.

In the subsequent analysis, we used originally designed techniques (HRM-PCR and ASP-qPCR methods) that allowed us to detect *PIK3CA* gene mutations in CNS metastases of NSCLC and in DNA from mutation-positive cell lines. ASP-qPCR was more useful in the identification of *PIK3CA* mutation types. Moreover, a real-time PCR technique with TaqMan hydrolysis probes confirmed HRM-PCR and ASP-qPCR results. Lindeman *et al*. suggested that all assays with detectability of mutation in specimens with 10% cancer cells can be used in the screening of routine driver mutations (26). Previously, we described that our ASP-qPCR method is able to detect mutations in material with >1% content of mutant DNA (27). However, the melting-curve analysis may be useful as a prescreening method allowing the selection of specimens for sequence analysis and genotype confirmation (10, 28, 29). Heideman *et al*. (10) and Kramer *et al*. (29) described sequencing of HRM-PCR products as a sensitive technique in the identification of mutation types in FFPE tissue samples. However, in the future, the NGS method may play a major role in the screening of mutations (15, 30). Tuononen *et al*. (30) reported a high (96.3-100%) concordance of the results between targeted NGS and certified real-time PCR tests (for *EGFR, KRAS* and *BRAF* screening). Surprisingly, the NGS method showed lower sensitivity than real-time PCR in which four additional mutations were observed. This may be due to FFPE sample purity, DNA degradation and low content of mutant DNA (<20%). A low total number of reads and hybridization-based enrichment NGS method can also favor the wild-type allele. However, NGS allows new and unknown variants of driver mutations to be identified. The implementation of the NGS technique into routine diagnostics will be possible when data for the impact of novel gene variants on cancer pathogenesis, treatment possibilities and prognosis become available (15, 30).

Based on the overall data, we would like to conclude that the designed PCR techniques were sensitive enough to detect *PIK3CA* gene mutations in the CNS metastases of NSCLC. The HRM-PCR technique can be useful in the selection of positive samples, however, ASP-qPCR or real-time PCR with TaqMan probes allow identification of the types of mutation. The incidence of *PIK3CA* gene mutations in CNS metastases of NSCLC does not exceed 5% and mutation frequency is probably similar to that in primary tumors.

Conflicts of Interest

The Authors declare no conflicts of interest.

References

1 Kang S, Bader AG and Vogt PK: Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proc Natl Acad Sci USA *102*: 802-807, 2015.

2 Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, Carey M, Hu Z, Guan Y, Sahin A, Symmans WF, Pusztai L, Nolden LK, Horlings H, Berns K, Hung MC, van de Vijver MJ, Valero V, Gray JW, Bernards R, Mills GB and Hennessy BT: An integrative genomic and proteomic analysis of *PIK3CA, PTEN*, and *AKT* mutations in breast cancer. Cancer Res *68*: 6084-6091, 2008.

3 Cheng H, Shcherba M, Pendurti G, Liang Y, Piperdi B and Perez-Soler R: Targeting the PI3K/AKT/mTOR pathway: potential for lung cancer treatment. Lung Cancer Manag *3*: 67-75, 2014.

4 Martini M, Ciraolo E, Gulluni F and Hirsch E: Targeting PI3K in cancer: Any good news? Front Oncol *3*: 108-106, 2013.

5 Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B and Velculescu VE: High frequency of mutations of the *PIK3CA* gene in human cancers. Science *304*: 554-561, 2004.

6 Pao W and Girard N: New driver mutations in non-small-cell lung cancer. Lancet Oncol *12*: 175-180, 2011.

7 Kris MG, Johnson BE, Berry LD, Kwiatkowski DJ, Iafrate AJ, Wistuba II, Varella-Garcia M, Franklin WA, Aronson SL, Su PF, Shyr Y, Camidge DR, Sequist LV, Glisson BS, Khuri FR, Garon EB, Pao W, Rudin C, Schiller J, Haura EB, Socinski M, Shirai K, Chen H, Giaccone G, Ladanyi M, Kugler K, Minna JD and Bunn PA: Using multiplexed assays of oncogenic drivers in lung cancers to select targeted drugs. JAMA *311*: 1998-2006, 2014.

8 Planchard D: Identification of driver mutations in lung cancer: first step in personalized cancer. Target Oncol *8*: 3-14, 2013.

9 An SJ, Chen ZH, Su J, Zhang XC, Zhong WZ, Yang JJ, Zhou Q, Yang XN, Huang L, Guan JL, Nie Q, Yan HH, Mok TS and Wu YL: Identification of enriched driver gene alterations in subgroups of non-small cell lung cancer patients based on histology and smoking status. PLoS One *7*: e40109, 2012.

10 Kramer D, Thunnissen FB, Gallegos-Ruiz MI, Smit EF, Postmus PE, Meijer CJ, Snijders PJ and Heideman DA: A fast, sensitive and accurate high resolution melting (HRM) technology-based assay to screen for common K-RAS mutations. Cell Oncol *31*: 161-167, 2009.

11 Penzel R, Sers C, Chen Y, Lehmann-Mühlenhoff U, Merkelbach-Bruse S, Jung A, Kirchner T, Büttner R, Kreipe HH, Petersen I, Dietel M and Schirmacher P: EGFR mutation detection in NSCLC-assessment of diagnostic application and recommendations of the German Panel for Mutation Testing in NSCLC. Virchows Arch *458*: 95-98, 2011.
12 Kim HJ, Lee KY, Kim YC, Kim KS, Lee SY, Jang TW, Lee MK, Shin KC, Lee GH, Lee JC, Lee JE and Kim SY. Detection and comparison of peptide nucleic acid-mediated real-time polymerase chain reaction clamping and direct gene sequencing for epidermal growth factor receptor mutations in patients with non-small cell lung cancer. Lung Cancer *75*: 321-325, 2012.
13 Roma C, Esposito C, Rachiglio AM, Pasquale R, Iannaccone A, Chicchinelli N, Franco R, Mancini R, Pisconti S, De Luca A, Botti G, Morabito A and Normanno N: Detection of *EGFR* mutations by TaqMan mutation detection assays powered by competitive allele-specific TaqMan PCR technology. Biomed Res Int 2013:385087. DOI: 10.1155/2013/385087, 2013.
14 Kawano O, Sasaki H, Endo K, Suzuki E, Haneda H, Yukiue H, Kobayashi Y, Yano M and Fujii Y: *PIK3CA* mutation status in Japanese lung cancer patients. Lung Cancer *54*: 209-215, 2006.
15 Lin MT, Mosier SL, Thiess M, Beierl KF, Debeljak M, Tseng LH, Chen G, Yegnasubramanian S, Ho H, Cope L, Wheelan SJ, Gocke CD and Eshleman JR: Clinical validation of *KRAS*, *BRAF*, and *EGFR* mutation detection using next-generation sequencing. Am J Clin Pathol *141*: 856-866, 2014.
16 Nicoś M, Krawczyk P, Jarosz B, Sawicki M, Szumiłło J, Trojanowski T and Milanowski J: Analysis of *KRAS* and *BRAF* genes mutation in the central nervous system metastases of non-small cell lung cancer. Clin Exp Med DOI: 10.1007/s10238-015-0349-2, 2015.
17 Schildgen V, Lüsebrink J, Appel JD, Wübben C, Engel-Riedel W, Ludwig C, Stoelben E, Schildgen O and Brockmann M: Identification of uncommon *PIK3CA* mutations in lung cancer by using pyrosequencing. Diagn Mol Pathol *22*: 22-27, 2013.
18 An SJ, Chen ZH, Su J, Zhang XC, Zhong WZ, Yang JJ, Zhou Q, Yang XN, Huang L, Guan JL, Nie Q, Yan HH, Mok TS and Wu YL: Identification of enriched driver gene alterations in subgroups of non-small cell lung cancer patients based on histology and smoking status. PLoS One *7*: e40109, 2012.
19 Bar J, Damianovich M, Hout Siloni G, Dar E, Cohen Y, Perelman M, Ben Nun A, Simansky D, Yellin A, Urban D and Onn A: Genetic mutation screen in early non-small-cell lung cancer (NSCLC) specimens. Clin Lung Cancer *15*: 159-165, 2014.
20 Reungwetwattana T and Dy GK: Targeted therapies in development for non-small cell lung cancer. J Carcinog *31*: 22-32, 2013.
21 Heavey S, O'Byrne KJ and Gately K: Strategies for co-targeting the PI3K/AKT/mTOR pathway in NSCLC. Cancer Treat Rev *40*: 445-456, 2014.
22 Qu Y, Wu X, Yin Y, Yang Y, Ma D and Li H: Antitumor activity of selective MEK1/2 inhibitor AZD6244 in combination with PI3K/mTOR inhibitor BEZ235 in gefitinib-resistant NSCLC xenograft models. J Exp Clin Cancer Res *33*: 52-61, 2014.
23 Zou ZQ, Zhang XH, Wang F, Shen QJ, Xu J, Zhang LN, Xing WH, Zhuo RJ and Li D: A novel dual PI3K/mTOR inhibitor PI-103 with high antitumor activity in non-small cell lung cancer cells. Int J Mol Med *24*: 97-101, 2009.
24 Spoerke JM, O'Brien C, Huw L, Koeppen H, Fridlyand J, Brachmann RK, Haverty PM, Pandita A, Mohan S, Sampath D, Friedman LS, Ross L, Hampton GM, Amler LC, Shames DS and Lackner MR: Phosphoinositide 3-kinase (PI3K) pathway alterations are associated with histologic subtypes and are predictive of sensitivity to PI3K inhibitors in lung cancer preclinical models. Clin Cancer Res *18*: 6771-6783, 2012.
25 Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, Maira M, McNamara K, Perera SA, Song Y, Chirieac LR, Kaur R, Lightbown A, Simendinger J, Li T, Padera RF, García-Echeverría C, Weissleder R, Mahmood U, Cantley LC and Wong KK: Effective use of PI3K and MEK inhibitors to treat mutant *K-Ras* G12D and *Pik3ca* H1047R murine lung cancers. Nat Med *14*: 1351-1356, 2008.
26 Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, Jenkins RB, Kwiatkowski DJ, Saldivar JS, Squire J, Thunnissen E and Ladanyi M: Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. J Thorac Oncol *8*: 823-859, 2013.
27 Nicoś M, Powrózek T, Krawczyk P, Jarosz B, Pająk B, Sawicki M Kucharczyk K, Trojanowski T and Milanowski J: Sensitive methods for detection of the S768R substitution in exon 18 of the *DDR2* gene in patients with central nervous system metastases of non-small cell lung cancer. Med Oncol *10*: 176-185, 2014.
28 Tsiatis AC, Norris-Kirby A, Rich RG, Hafez MJ, Gocke CD, Eshleman JR and Murphy KM: Comparison of Sanger sequencing, pyrosequencing, and melting curve analysis for the detection of *KRAS* mutations: diagnostic and clinical implications. J Mol Diagn *12*: 425-432, 2010.
29 Heideman DA, Lurkin I, Doeleman M, Smit EF, Verheul HM, Meijer GA Snijders PJ, Thunnissen E and Zwarthoff EC: *KRAS* and *BRAF* mutation analysis in routine molecular diagnostics: comparison of three testing methods on formalin-fixed, paraffin-embedded tumor-derived DNA. J Mol Diagn *14*: 247-255, 2012.
30 Tuononen K, Mäki-Nevala S, Sarhadi VK, Wirtanen A, Rönty M, Salmenkivi K, Andrews JM, Telaranta-Keerie AI, Hannula S, Lagström S, Ellonen P, Knuuttila A and Knuutila S: Comparison of targeted next-generation sequencing (NGS) and real-time PCR in the detection of *EGFR, KRAS*, and *BRAF* mutations on formalin-fixed, paraffin-embedded tumor material of non-small cell lung carcinoma-superiority of NGS. Genes Chromosomes Cancer *52*: 503-511, 2013.

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