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Prevalence of *BRAF* and *NRAS* Mutations and a Comparative Analysis in Primary and Metastatic Melanoma of Korean Patients

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Objectives: The aim of this study is to verify the status and the clinical significance of *BRAF* and *NRAS* mutations in patients of one of the university hospitals in Korea.
Methods: Polymerase chain reaction (PCR) amplification and direct sequencing were performed for the analysis of melanoma samples (n=22) for the detection of mutations in exon 15 of the *BRAF* gene, and exons 2 and 3 of the *NRAS* gene in genomic DNA. Mutations of the *BRAF* gene were correlated with the clinicopathologic features of patients and the *BRAF* mutation status was compared in 18 paired primary and metastatic tumors.
Results: Incidence of somatic mutations within the *BRAF* and *NRAS* genes was 27.3% (6/22) and 0% (0/22), respectively. Age, gender, Breslow thickness, and ulceration did not show correlation with *BRAF* mutations. Among 18 patients with metastasis, *BRAF* mutation was detected in 22.2% of cases (4/18), and all four cases with *BRAF* mutations were identified in metastatic lymph node tissues. *BRAF* mutations were only found in lymph node metastases, which was statistically significant (28.6% vs 0%, P<0.01).
Conclusion: The incidence of *BRAF* mutation is as low as in other Asian reports and the *NRAS* mutation was not found in patients of our institute. **(Ewha Med J 2014;37(1):30-35)**

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Introduction

The incidence of cutaneous melanoma is increasing throughout most of the Western world [1]. Although cure is achieved with surgery only in the majority of patients with early-stage disease, curative treatment for patients with metastatic melanoma remains elusive. The median survival time for melanoma patients with metastatic disease is 8~9 months, and the 3-year overall survival rate is less than 15% [2]. Melanoma is a heterogeneous disease. As a result of intensive molecular analyses, the influence of several important oncogenes has been discovered. These include oncogenic activation by mutation or amplification in *BRAF*, *KIT*, *NRAS*, cyclin D, cyclin dependent kinase 4, and alterations in the *ERBB4* gene [3]. To date, several targeted therapies have been approved for treating melanoma [4].

Mutations in the *BRAF* gene occur in approximately 50% of cases of cutaneous melanoma [5,6] and the most common *BRAF* mutation is a T1799A transversion mutation in exon 15 of the gene, which causes V600E (Val600Glu) amino acid substitution in the protein. This results in constitutive activation of *BRAF* and activation of the MAPK pathway [7]. The presence of mutated *NRAS*, which has been reported in approximately 15% of cases of cutaneous melanoma [5,6], leads to up-regulation of the MAPK pathway, the phosphatidylinositol 3′ kinase (PI3K) pathway, and the RAL pathway, resulting in inhibition of apoptosis, promotion of cell proliferation, invasion, and anchorage-independent growth [8,9]. The most common *NRAS* codon 61 mutations in exon 2 in cutaneous melanoma are the Q61R (CAA/CGA) and Q61K (CAA/AAA) changes, which lead to substitutions from glutamine to arginine or to ly-

sine, respectively. Genetic mutations in *BRAF* and *NRAS* genes have shown correlation with the clinicopathologic features and progression of melanoma; however, the effect of these mutations on the clinical outcome remains uncertain, and previous studies have reported conflicting results [10-15]. Some investigators have suggested that *BRAF* mutation may be acquired during development of metastasis and may show correlation with progression rather than initiation [16-18]; however, other investigators have suggested that *BRAF* mutations are most likely to occur before the development of melanoma metastasis [14,19].

With the advent of BRAF V600E-specific inhibitors, identification of mutations in the *BRAF* and *NRAS* genes may be of importance in clinical trials. However, the majority of these studies included patients with recurrent or advanced stage disease and the observations were conducted in Caucasian populations [20-23]. The incidence of *KIT* gene mutations in Asians (Chinese and Koreans) was lower than that reported in Western patients (7.9~11% *vs*. 29%) [24-26], indicating that the mutation status of the *BRAF* and *NRAS* genes may also differ between Asians and Caucasians. Recently, two large-scale analyses of *BRAF* and *NRAS* mutations in Chinese patients demonstrated a lower incidence of *BRAF* and *NRAS* mutations in Chinese patients [25,27]. Even though screening for *BRAF* and *NRAS* mutations was performed in one Korean study, the incidence of these mutations was extraordinarily low compared to results from Western or other Asian studies [26].

To the best of our knowledge, this is the first study in Korea to investigate the frequency of *BRAF* and *NRAS* mutations and to compare genetic differences between primary melanoma and metastatic melanoma tissues. Despite the fact that this was a small series study, findings of this study may be informative because the incidence of *BRAF* and *NRAS* mutations among Korean melanoma patients has not been well studied to date.

Methods

1. Patients

We conducted a retrospective review of the clinical records of 130 patients diagnosed as having a malignant melanoma at Gachon University Gil Hospital (GUGH) between 1999 and 2010. Among patients with follow-up information, we selected 10 patients with primary cutaneous melanoma without metastasis during a period of five years and 20 patients with metastatic melanoma. Paraffin blocks or clinical data were available in all cases. Because of technical problems, analysis for the *BRAF* mutation was performed at least one site in only 22 cases. Only four primary cutaneous melanoma patients without metastasis and 18 patients with metastatic melanoma were included in this study. Clinical characteristics of all 22 patients are shown in Table 1. The Institutional Review Board of GUGH approved the acquisition, analysis, and reporting of patient data and human tissue (approval number: GIRBA 2487).

2. DNA preparation and screening for mutation

Using the QIAgen Tissue kit (DNeasy Blood & Tissue kit, Cat. No. 69506; Qiagen, Valencia, CA), tumor-rich areas were extracted from five paraffin sections of 10 μm thickness containing a representative portion of each tumor block. For detection of hotspot mutations, we amplified exon 15 of the *BRAF* gene, and exons 2 and 3 of the *NRAS* gene, using PCR. PCR primers for *BRAF* and *NRAS* mutational analyses were as follows: *BRAF* exon 15 (forward) 5'-TGCTTGCTCTGATAGGAAAAT-3' and (reverse) 5'-AGCATCTCAGGGCCAAAAAT-3'; *NRAS* exon 2 (forward) 5'-GAACCAAATGGAAGGTCACA-3' and (reverse) 5'-TGGGTAAAGATGATCCGACA-3', *NRAS* exon 3 (forward) 5'-AGGCAGAAATGGGCTTGGAT-3' and (reverse)

Table 1. BRAF mutation and clinicopathologic factors

Variable	Patients with *BRAF* mutation (%)	P value
Gender (n=22)		
Male (n=14)	4 (28.6)	0.190
Female (n=8)	2 (25.0)	
Age (yr) (n=22)		
≤60 (n=13)	4 (30.8)	0.210
>60 (n=9)	2 (22.2)	
Breslow thickness (mm) of primary cutaneous melanoma (n=10)		
<2 (n=4)	1 (25.0)	0.301
2-5 (n=4)	1 (20.0)	
>5 (n=2)	1 (50.0)	
Ulceration of primary cutaneous melanoma (n=10)		
Present (n=5)	2 (40.0)	0.172
Absent (n=5)	1 (20.0)	
Site of metastatic melanoma (n=18)		
Lymph node (n=14)	4 (28.6)	<0.001
Others (n=4)	0	

5'-CGCCTGTCCTCATGTATTGG-3'. Purified PCR products were sequenced directly on both strands. An ABI PRISM 3100 DNA analyzer (Applied Biosystems, Foster city, CA) was used to perform sequencing reactions. Repeat PCR and sequencing were performed using a different primer for confirmation of the mutations.

3. Statistical Analysis

Fisher exact test was used for the analysis of the categorical data, unless otherwise specified. SAS ver. 9.2 (SAS Institute, Cary, NC, USA) was used in the performance of all statistical calculations. A two-tailed test of $P<0.05$ was considered statistically significant.

Results

Overall, mutations in *BRAF* exon 15 were detected in six of 22 cases (27.3%) and all *BRAF* mutations were represented by the valine to glutamic acid substitution at position 600 (V600E). Mutations in *NRAS* exons 2 and 3 were not detected in any of the cases (Tables 1, 2). *BRAF* mutations were compared with known prognostic factors in patients with primary and metastatic melanoma. Age, gender, Breslow thickness, and ulceration did not show correlation with the *BRAF* mutations (Table 1). Among the cases involving metastasis, *BRAF* mutations were detected in four of 18 cases (22.2%), and in all four cases, *BRAF* mutations were identified in metastatic lymph nodal tissues. The increase in frequency of *BRAF* mutation in lymph node metastases, compared with that of other sites, was statistically significant (28.6% vs. 0%, $P<0.01$).

Table 2 shows the *BRAF* mutation status of 18 paired primary melanomas and metastatic melanomas. Sites of primary melanoma included the skin in nine cases (there were three cases in the toe, two in the sole and one each in the finger, inguinal area, flank and back), the mucosa in four cases (one case each in the lip, vagina, esophagus and nasal cavity), and the viscera

Table 2. *BRAF* mutation status of paired primary and metastatic tumor

Cases	Sex/age	Primary site	Subtype	*BRAF* mutation of primary tumor	Metastatic site	*BRAF* mutation of metastatic tumor	Time for metastasis (mo)
1	M/39	Skin, 3rd finger tip, Rt	Acral	WT	Skin, flank, Rt	WT	10
2	M/67	Skin, sole, Lt	Acral	WT	LN, inguinal, Lt	WT	36
3	M/50	Lip	Mucosal	WT	LN, neck, Lt	WT	5
4	F/75	Vagina	Mucosal	WT	Stomach	WT	1
5	M/70	Small intestine	EC	WT	LN, inguinal, brain	WT (both)	1 (LN), 4 (brain)
6	F/62	Small intestine	EC	WT	LN, inguinal, Rt	WT	0
7	M/46	Esophagus	Mucosal	Fail	LN, neck	WT	1
8	M/72	Nasal cavity	Mucosal	Fail	Small intestine	WT	3
9	M/39	Foot, big toe, Rt	Acral	Fail	LN	WT	0
10	M/72	Skin, ingunal area, Rt	Non-CSD	Fail	LN, inguinal, Rt	WT	0
11	M/70	Foot, 2nd toe, Rt	Acral	Fail	LN, inguinal, Rt	WT	24
12	F/75	Big toe, Rt	Acral	Fail	Liver, multiple	WT	10
13	M/57	Heel, Rt	Acral	Fail	LN, inguinal, Rt	WT	36
14	F/46	Ovary, Lt	EC	Mutant	LN, pelvic, Lt	Mutant	0
15	M/49	Skin, flank, Lt	Non-CSD	Mutant	LN, axilla, Lt	Mutant	2
16	M/73	Skin, back	Non-CSD	Fail	LN, axilla, Lt	Mutant	1
17	M/43	Unknown	-	NA	LN, Rt parotid gland area	Mutant	-
18	M/43	Unknown	-	NA	LN	WT	-

EC, extracutaenous; Non-CSD, melanomas on skin without chronic sun-induced damage; Rt, right; Lt, left; WT, wild type; NA, not available; LN, lymph node.

in three cases (two cases were in the small intestine and one case in the ovary), and two cases were of an unknown primary. Sites of metastatic melanoma included 14 cases in the lymph nodes and one in the skin, stomach, small intestine and liver. In six pairs, wild-type genes were detected in both the primary tumors and metastases (cases 1~6), and wild-type genes were detected in the metastases in seven pairs; however, evaluation of the *BRAF* mutation in the primary tumors failed (cases 7~13). The presence of a *BRAF* mutation was detected in metastatic tumors in three pairs. Among them, *BRAF* mutations were detected in both the primary and the metastasis in two pairs (cases 14, 15); however, evaluation of the *BRAF* mutation in the primary tumor of one pair failed (case 16). Among two cases involving metastatic melanomas of an unknown primary, a *BRAF* mutation was detected in one case (case 17) and no *BRAF* mutation was detected in the other case (case 18).

Discussion

The occurrence of *BRAF* mutations in patients with cutaneous melanoma ranges from 22% to 72% [28]. Approximately 90% of the reported *BRAF* mutations occur at residue 600, located in the activation domain of this kinase [29,30]. In the Catalogue of Somatic Mutations in Cancer (COSMIC) database, the occurrence of a number of less common variant codon 600 mutations has been reported in patients with malignant melanoma; these non-V600E mutations comprise approximately 10% of all codon 600 mutations reported in patients with malignant melanoma (MM) [31]. However, these studies have been conducted mainly in Caucasian populations, and the mutational status of the *BRAF* gene and the clinical significance of this mutation have not been well studied in Asian populations. Recently, findings from two large-scale studies examining *BRAF* V600E mutations in the Chinese population demonstrated the presence of *BRAF* mutations in 15% and 25.5% of cases of MM, respectively [25,27]. In addition, one Japanese study reported the detection of the *BRAF* mutation in only nine of 35 patients (26%) with MM [32]. According to the results of one previous Korean study, the *BRAF* mutation was detected in only one of 49 patients (2%) with MM [26]. However, compared to results from Western or other Asian studies, this incidence was very low. This study has a limitation in that the study samples were taken from acral and mucosal melanomas only. In our study, 27.3% of the patients with MM harbored *BRAF* mutations. This result was similar to the results of other Asian studies and this also suggests that the incidence of *BRAF* mutations in the Asian population is lower than that reported by Western studies. The incidence of *BRAF* mutation varies according to histologic subtype and tumor location [28]. In addition, Caucasians often show superficial spread of MM and nodular MM [33], whereas Asians present with acral lentigenous MM [32]. Findings of one Korean study demonstrated that 65% of the melanomas were located on the hands and feet, with acral lentiginous melanoma being the most common histologic subtype [34]. Therefore, racial, histological, and locational differences may be one explanation for the lower rate of *BRAF* mutations in Koreans and Asian patients with MM.

BRAF mutation was detected in four out of 14 cases (28.6%) of lymph node metastases; however, no mutations in the metastases of other sites were observed, which is statistically significant ($P<0.01$). However, the size of the study sample may be too small to draw an accurate conclusion. One study reported a somewhat higher frequency of *BRAF* mutations in primary melanomas associated with concurrent lymph node metastasis; however, the difference was not statistically significant [18]. The association of *BRAF* mutation with lymph node metastasis remains unclear.

In our study, unfortunately, eight primary tumors failed to demonstrate their *BRAF* mutational status. Traditional direct (Sanger) sequencing has been widely used in clinical laboratories for mutation testing, including *BRAF* mutations [35]; however, this method suffers from limited sensitivity in the detection of mutations that are present in low percentages in a specimen [36]. In one study, the failure rate of Sanger sequencing was reported as 9.2% [37]. These eight cases were punch-biopsied specimens from the skin or mucosa, with a very low tumor content. Efforts to secure specimens with a high tumor content and to enrich the tumor by macro- or microdissection may result in the enhanced accuracy of Sanger sequencing [37]. In addition, new methods, such as pyrosequencing assays, should be applied for accurate and quantitative identification of *BRAF* mutations [38].

Unfortunately, *NRAS* mutation was not identified in this study. The occurrence of *NRAS* mutations in patients with cutaneous melanoma ranges from 0% to 50%, and *NRAS* mutations have been reported to show a significant association with

nodular melanoma [28]. In one large-scale Chinese study, the incidence of *NRAS* mutations was reported as 7.2% [25]. In a Korean study, *NRAS* mutation was detected in only one of 47 patients (2.1%) with mucosal or acral melanoma [26]. Further studies are needed in order to determine the incidence of *BRAF* and *NRAS* mutations in Korean populations according to the histologic subtype and to determine whether *BRAF* or *NRAS* mutations in conjunction with other genetic aberrations found in MMs, such as epigenetic changes of tumor suppressor genes and/or allelic imbalance, play a role in tumor progression or metastasis.

For comparison of the mutational status between primary and metastatic MM, we assessed 18 pairs of primary MM tumors and their nodal or extranodal metastases. In six pairs, the wild-type gene was detected in both the primary tumors and metastases, and among six pairs, the wild-type gene was detected at three sites (primary tumor, lymph node metastasis, and brain metastasis) in one pair (case 5). In two pairs, *BRAF* mutations were detected in both the primary tumors and their respective metastases.

In conclusion, the incidence of *BRAF* mutations in the Korean population is low as in the Japanese and the Chinese reports and *NRAS* mutation is a very rare event. Although the number of tumors included in this study was too small to be conclusive, the results of our study indicated that the *BRAF* mutation may be an early event that occurs before metastasis, and that *BRAF* mutation did not appear to contribute to disease progression or metastasis. Larger studies will be needed in order to determine whether the *BRAF* mutation plays a significant role in lymph node metastasis.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;55:74-108.
2. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* 2009;27:6199-6206.
3. Devitt B, Liu W, Salemi R, Wolfe R, Kelly J, Tzen CY, et al. Clinical outcome and pathological features associated with NRAS mutation in cutaneous melanoma. *Pigment Cell Melanoma Res* 2011;24:666-672.
4. Finn L, Markovic SN, Joseph RW. Therapy for metastatic melanoma: the past, present, and future. *BMC Med* 2012;10:23.
5. Hocker T, Tsao H. Ultraviolet radiation and melanoma: a systematic review and analysis of reported sequence variants. *Hum Mutat* 2007;28:578-588.
6. Tsao H, Zhang X, Fowlkes K, Haluska FG. Relative reciprocity of NRAS and PTEN/MMAC1 alterations in cutaneous melanoma cell lines. *Cancer Res* 2000;60:1800-1804.
7. Peyssonnaux C, Eychene A. The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell* 2001;93:53-62.
8. Mishra PJ, Ha L, Rieker J, Sviderskaya EV, Bennett DC, Oberst MD, et al. Dissection of RAS downstream pathways in melanomagenesis: a role for Ral in transformation. *Oncogene* 2010;29:2449-2456.
9. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* 1994;370:527-532.
10. Akslen LA, Angelini S, Straume O, Bachmann IM, Molven A, Hemminki K, et al. BRAF and NRAS mutations are frequent in nodular melanoma but are not associated with tumor cell proliferation or patient survival. *J Invest Dermatol* 2005;125:312-317.
11. Edlundh-Rose E, Egyhazi S, Omholt K, Mansson-Brahme E, Platz A, Hansson J, et al. NRAS and BRAF mutations in melanoma tumours in relation to clinical characteristics: a study based on mutation screening by pyrosequencing. *Melanoma Res* 2006;16:471-478.
12. Jiveskog S, Ragnarsson-Olding B, Platz A, Ringborg U. N-ras mutations are common in melanomas from sun-exposed skin of humans but rare in mucosal membranes or unexposed skin. *J Invest Dermatol* 1998;111:757-761.
13. Kumar R, Angelini S, Czene K, Sauroja I, Hahka-Kemppinen M, Pyrhonen S, et al. BRAF mutations in metastatic melanoma: a possible association with clinical outcome. *Clin Cancer Res* 2003;9:3362-3368.
14. Omholt K, Platz A, Kanter L, Ringborg U, Hansson J. NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. *Clin Cancer Res* 2003;9:6483-6488.
15. Ugurel S, Thirumaran RK, Bloethner S, Gast A, Sucker A, Mueller-Berghaus J, et al. B-RAF and N-RAS mutations are preserved during short time in vitro propagation and differentially impact prognosis. *PLoS One* 2007;2:e236.
16. Dong J, Phelps RG, Qiao R, Yao S, Benard O, Ronai Z, et al. BRAF oncogenic mutations correlate with progression rather than initiation of human melanoma. *Cancer Res* 2003;63:3883-3885.
17. Gorden A, Osman I, Gai W, He D, Huang W, Davidson A, et al. Analysis of BRAF and N-RAS mutations in metastatic melanoma tissues. *Cancer Res* 2003;63:3955-3957.
18. Shinozaki M, Fujimoto A, Morton DL, Hoon DS. Incidence of BRAF oncogene mutation and clinical relevance for primary cutaneous melanomas. *Clin Cancer Res* 2004;10:1753-1757.
19. Libra M, Malaponte G, Navolanic PM, Gangemi P, Bevelacqua V, Proietti L, et al. Analysis of BRAF mutation in primary and metastatic melanoma. *Cell Cycle* 2005;4:1382-1384.
20. Ascierto PA, Schadendorf D, Berking C, Agarwala SS, van Herpen CM, Queirolo P, et al. MEK162 for patients with advanced

melanoma harbouring NRAS or Val600 BRAF mutations: a non-randomised, open-label phase 2 study. *Lancet Oncol* 2013;14:249-256.
21. Johnson DB, Sosman JA. Update on the targeted therapy of melanoma. *Curr Treat Options Oncol* 2013;14:280-292.
22. Kudchadkar RR, Smalley KS, Glass LF, Trimble JS, Sondak VK. Targeted therapy in melanoma. *Clin Dermatol* 2013;31:200-208.
23. Menzies AM, Long GV. Recent advances in melanoma systemic therapy: BRAF inhibitors, CTLA4 antibodies and beyond. *Eur J Cancer* 2013;49:3229-3241.
24. Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. *J Clin Oncol* 2006;24:4340-4346.
25. Si L, Kong Y, Xu X, Flaherty KT, Sheng X, Cui C, et al. Prevalence of BRAF V600E mutation in Chinese melanoma patients: large scale analysis of BRAF and NRAS mutations in a 432-case cohort. *Eur J Cancer* 2012;48:94-100.
26. Yun J, Lee J, Jang J, Lee EJ, Jang KT, Kim JH, et al. KIT amplification and gene mutations in acral/mucosal melanoma in Korea. *APMIS* 2011;119:330-335.
27. Qi RQ, He L, Zheng S, Hong Y, Ma L, Zhang S, et al. BRAF exon 15 T1799A mutation is common in melanocytic nevi, but less prevalent in cutaneous malignant melanoma, in Chinese Han. *J Invest Dermatol* 2011;131:1129-1138.
28. Lee JH, Choi JW, Kim YS. Frequencies of BRAF and NRAS mutations are different in histological types and sites of origin of cutaneous melanoma: a meta-analysis. *Br J Dermatol* 2011;164:776-784.
29. Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, et al. BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Res* 2002;62:6997-7000.
30. Fecher LA, Cummings SD, Keefe MJ, Alani RM. Toward a molecular classification of melanoma. *J Clin Oncol* 2007;25:1606-1620.
31. Wellcome Trust Sanger Institute. Catalogue of somatic mutations in cancer (COSMIC) [Internet]. 2012 [cited 2013 Nov 15]. Available from: http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/
32. Sasaki Y, Niu C, Makino R, Kudo C, Sun C, Watanabe H, et al. BRAF point mutations in primary melanoma show different prevalences by subtype. *J Invest Dermatol* 2004;123:177-183.
33. Lang J, MacKie RM. Prevalence of exon 15 BRAF mutations in primary melanoma of the superficial spreading, nodular, acral, and lentigo maligna subtypes. *J Invest Dermatol* 2005;125:575-579.
34. Roh MR, Kim J, Chung KY. Treatment and outcomes of melanoma in acral location in Korean patients. *Yonsei Med J* 2010;51:562-568.
35. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1977;74:5463-5467.
36. Tan YH, Liu Y, Eu KW, Ang PW, Li WQ, Salto-Tellez M, et al. Detection of BRAF V600E mutation by pyrosequencing. *Pathology* 2008;40:295-298.
37. Anderson S, Bloom KJ, Vallera DU, Rueschoff J, Meldrum C, Schilling R, et al. Multisite analytic performance studies of a real-time polymerase chain reaction assay for the detection of BRAF V600E mutations in formalin-fixed, paraffin-embedded tissue specimens of malignant melanoma. *Arch Pathol Lab Med* 2012;136:1385-1391.
38. Spittle C, Ward MR, Nathanson KL, Gimotty PA, Rappaport E, Brose MS, et al. Application of a BRAF pyrosequencing assay for mutation detection and copy number analysis in malignant melanoma. *J Mol Diagn* 2007;9:464-471.