

Original Article

Comparison between two widely used laboratory methods in BRAF V600 mutation detection in a large cohort of clinical samples of cutaneous melanoma metastases to the lymph nodes

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Abstract: Aims: The study compares detection rates of oncogenic *BRAF* mutations in a homogenous group of 236 FFPE cutaneous melanoma lymph node metastases, collected in one cancer center. *BRAF* mutational status was verified by two independent in-house PCR/Sanger sequencing tests, and the Cobas® 4800 BRAF V600 Mutation Test. Results: The best of two sequencing approaches returned results for 230/236 samples. In 140 (60.9%), the mutation in codon 600 of *BRAF* was found. 91.4% of all mutated cases (128 samples) represented p.V600E. Both Sanger-based tests gave reproducible results although they differed significantly in the percentage of amplifiable samples: 230/236 to 109/143. Cobas generated results in all 236 cases, mutations changing codon V600 were detected in 144 of them (61.0%), including 5 not amplifiable and 5 negative in the standard sequencing. However, 6 cases positive in sequencing turned out to be negative in Cobas. Both tests provided us with the same *BRAF* V600 mutational status in 219 out of 230 cases with valid results (95.2%). Conclusions: The total *BRAF* V600 mutation detection rate didn't differ significantly between the two methodological approaches (60.9% vs. 61.0%). Sequencing was a reproducible method of V600 mutation detection and more powerful to detect mutations other than p.V600E, while Cobas test proved to be less susceptible to the poor DNA quality or investigator's bias. The study underlined an important role of pathologists in quality assurance of molecular diagnostics.

Keywords: Melanoma, BRAF, mutation, detection, analytical sensitivity, sequencing

Introduction

The recently introduced personalized therapy has provided the much awaited breakthrough in metastatic melanoma management. As the therapy is dedicated to the specific, often quite narrow, molecular profile of the disease, molecular diagnostics of specimens is starting to gain a crucial role in providing the best selection for the tailored treatment [1]. This issue is important from the point of view of every involved party: 1) a patient, as wrongly selected treatment option will bring no clinical benefit but possibility of extensive adverse events, 2) a

funding institution as the amount of funds is restricted, 3) a drug provider, since an efficient drug is likely to be recognized and widely accepted even at a high price. Vemurafenib (Zelboraf®; Roche Molecular Systems Inc., Pleasanton, CA, U.S.A.) was the first BRAF inhibitor that got FDA approval, became available in the US and Europe for melanoma treatment and provided dramatic responses in disseminated melanoma cases, based on the results of a pivotal randomized phase 3 trial [2]. Then the second BRAF inhibitor-dabrafenib was approved, demonstrating similar activity to vemurafenib [3]. Together with vemurafenib, the ded-

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icated diagnostic Cobas® 4800 BRAF V600 Mutation Test was developed with the aim to select patients that were the most likely to benefit from the treatment. Cobas Test received CE-IVD certificate right before vemurafenib approval [4].

BRAF activating mutation is the most common molecular alternation in cutaneous melanoma, with total incidence of up to 60% cases depending on the study and examined population [5, 6]. Moreover, the majority of alternations are caused by a single nucleotide substitution resulting in p.V600E, which leads to a 5000 fold increase in its kinase activity, making it a unique target for personalized therapy. A two-nucleotide substitution resulting in p.V600K, accounts for the second most common mutation in *BRAF* gene and is reported in up to one third of all BRAF mutated cases, depending on the study group [7-9]. Other mutation variants located inside and outside codon 600 (missense and deletions) are present, but much less common. Vemurafenib therapy is predominantly dedicated to cases harboring p.V600E substitution, however, the efficacy was also observed in p.V600K cases [7, 10] and other V600 mutations [11, 12].

Since proper testing is essential for the delivery of optimal treatment option, various approaches were applied and subsequently validated for *BRAF* V600 mutation detection: Sanger sequencing, mismatch ligation assay, ligase detection reaction, denaturing high-performance liquid chromatography, SNAPshot, high-resolution melting, mutation-specific real-time PCR, pyrosequencing, immunohistochemistry, next-generation sequencing and mass spectrometry [13-15]. Each method has its own sensitivity, specificity, cost and response delay [16, 17]. Some data indicate that Sanger sequencing fails to efficiently detect *BRAF* mutations in many melanomas and therefore should no longer be considered as the reference test [18, 19]. Recent development and discussion reflect rapid increase in the demand for reliable mutation testing in both clinical setting and research.

The aim of the study was to compare feasibility, mutation detection rates and agreement between Cobas® 4800 BRAF V600 Mutation Test and direct Sanger sequencing. By conducting two independent Sanger sequencing-based tests intra-method reproducibility was also veri-

fied. A homogenous group of cutaneous melanoma cases, with clinical lymph nodes metastases only, surgically treated in Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology in Warsaw (CCIO), was subject to examination. None of the patients was treated with BRAF inhibitors prior to or after the testing.

Materials and methods

All patients eligible for the study were diagnosed with clinical stage III B, C cutaneous melanoma according to the American Joint Committee on Cancer (AJCC) classification [20] and had undergone radical lymph node dissection (LND) at the Department of Soft Tissue/Bone Sarcoma and Melanoma at CCIO between May 1995 and November 2010. Each patient provided written informed consent for using their biological material in molecular studies. The study was approved by the local Bio-Ethics Committee according to Best Clinical Practice Guidelines. All cases were reviewed by a pathologist for the purpose of this study and 236 paraffin blocks (one per patient) with the highest tumor load and the best possible material quality were chosen as described [21]. Only metastatic lymph node material was subject to the study. The majority of FFPE samples had a tumor content of >90%, and none <10%. The samples were cut from the whole block surface. Genomic DNA for the first round Sanger test and Cobas was isolated with the Sherlock AX DNA kit (A & A Biotechnology, Gdynia, Poland) and re-isolated with Cobas DNA Isolation Kit (Roche Molecular Diagnostics, Pleasanton, CA, USA) if DNA didn't amplify after Sherlock. The second-round Sanger testing followed isolation by QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany).

Mutation analysis

An in-house PCR/Sanger sequencing test-the first round: After successful PCR amplification, analysis of exon 15 sequence of the *BRAF* gene with flanking intronic sequences was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit and a ABI Prism 3130 xL Genetic Analyzer (both Applied Biosystems, Carlsbad, CA, USA) according to the previously described protocol [21]. Sequences were then compared to the *BRAF* GenBank reference (NM_004333.4). Test sensitivity cut-off has

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Table 1. Comparison of Sanger sequencing vs. Cobas test results for *BRAF* mutations detection

Mutations as by: Sanger sequencing		Cobas test		
Mutation type	Total	Mutation detected	Mutation not detected	<i>P</i>
p.V600E	128	126	2	n.s.
p.V600K	9	5	4	n.s.
p.V600E (2)	2	2	0	n.s.
p.V600D	1	1	0	n.s.
WT	86	5	81	n.s.
no result	6	5	1	n.a.
Outside V600	4	n.a.	4	n.a.

n.s. -not significant, n.a. -not applicable.

been estimated (in serial dilution test) as >10% of the mutated tumor sample.

An in-house PCR/Sanger sequencing test-the second round: 149 consecutive project FFPE blocks, after cutting for the first round DNA extraction, had HE slides fresh-made and analyzed by an independent (second) pathologist. Among them, 143 blocs still fulfilled the study criteria. These samples were cut and processed as for the first round except extraction kit used, by independent staff and with another primer set. 109 DNAs (76.2%) were amplifiable and returned sequencing results.

The Cobas® 4800 BRAF V600 mutation test: (Cobas test) with respective equipment (Roche Molecular Diagnostics, Pleasanton, CA, USA). Cobas is a real-time-PCR based test dedicated to p.V600E (c.1799T>A) mutation detection in DNA isolated from FFPE samples [18]. Inconsistent cross reactivity with other V600 point mutations such as p.V600K, p.V600E (2) (c.1799_1800delinsAA), p.V600D (but not with p.V600R) was observed [19, 22]. No information about the type of mutation detected is available with this test. Tests were performed according to the manufacturer's protocol, except for the DNA extraction step, that was performed by recommended protocol only when routine laboratory method failed (no DNA amplification by PCR or Cobas).

Retesting was performed for samples with tumor load <90%, in case of invalid result in any of the tests, or when mutation other than p.V600E was identified by sequencing.

All statistical analyses were performed using Statistica version 7.0 statistical software

(Tulsa, OK, USA). Contingency tables were analyzed using the chi-square test. Statistical significance was accepted for *P*-values <0.05.

Results

Sanger sequencing

Considered a gold standard in DNA mutation analysis, the sequencing approach enabled us to obtain results for 230/236 samples (97.5%). In 140 samples (60.9%), the mutation in codon 600 of *BRAF* was found. Four mutations were identified outside V600 codon. The 86 remaining samples were of wild type (WT). The p.V600E substitution accounted for 91.4% of all mutated cases (128 samples) followed by p.V600K (9 samples, 6.4%), p.V600E (2) in two cases and one p.V600D (**Table 1**).

The results of the second round sequencing, when possible to obtain, were in full accordance with the results of the first round with the exception of a high rate of unsuccessful amplification (lack of PCR product for 34/143, all samples tested once only).

Cobas® 4800 BRAF V600 mutation test

Cobas test generated results in all 236 cases, V600 mutations were detected in 144 of them (61.0%). Among 6 cases that were not amplifiable by the standard PCR/sequencing approach, 5 turned out to be positive and 1 was negative for the V600 mutation (**Table 1**). Additionally the Cobas test detected mutations in 5 samples that were negative by the other method.

Sequencing allowed us to detect codon 600 mutations in 6 cases negative in Cobas test - 2 p.V600E samples and 4 that were p.V600K. Considering mutations affecting V600, other than p.V600E, the Cobas test detected 5/9 p.V600K mutations (55%); rare mutation cases (p.V600E (2) and p.V600D) were also indicated as mutated.

In total, both testing methodologies provided us with the same *BRAF* V600 mutational status in 219 out of 230 cases (95.2%) (**Table 2**).

Discussion

Since the success of vemurafenib treatment in melanoma patients depends on accurate selection process, a need for a proper diagnos-

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Table 2. Comparison of Sanger Sequencing and Cobas test in terms of V600 codon status

TEST	output	Cobas test	
		Mutation detected	Mutation not detected
	Total (230)	139	91
Sanger sequencing	Mutation detected	140	6
	Mutation not detected	90	85

tic test has emerged. The main parameters to be considered when choosing a suitable diagnostic test should be sensitivity, specificity and failure rates [4]. Turnaround time, lab resources and test price are not to be omitted in routine testing.

In practice sensitivity and reproducibility vary between different testing strategies and laboratories as a result of the methodology used, the experience of the testing laboratory and the sample selection and preparation.

It is mainly the decision on methodology (excluding lab errors) that influences both the testing process (i.e. a need for proper sample when the test sensitivity is low) and patient selection (mutation positive as a result of only a small fraction of tumor cells carrying the mutation when a highly sensitive test is applied; such tumors may not respond to targeted therapy as expected). Similarly to previous results with *KRAS* mutation testing, the present study demonstrated a high intra- and inter-method accordance in *BRAF* mutation detection when screening was carried out by an experienced laboratory working in a clinical setting [23]. The overall agreement between the Cobas and Sanger sequencing reached 95.2% and the majority of discrepancies resulted logically from either sensitivity or specificity of the approaches.

Cobas V600 mutation test was developed for p.V600E *BRAF* mutation detection during the selection process for vemurafenib treatment. Although still treated with caution by some authors [22], Cobas has already proven in many studies to be fast and sensitive, allowing for the detection of mutation in material containing as little as 5% of tumor load and providing valid results in a highly reproducible manner for >99% of samples tested [4, 16, 19, 24]. There was no test failure with Cobas observed in the present study and samples both not amplifi-

able (6 cases) and negative (5 cases) in sequencing were returned with valid results.

By conducting the comparison presented here, we challenged the criticism of application of Sanger sequencing in targeted therapy testing. Direct sequencing is claimed to be insufficiently sensitive and specific to cope with heterogeneous and generally degraded FFPE tumor-derived DNA and our results argue both for and against that statement. Performed by experienced laboratory, Sanger sequencing turned out to be highly specific and reproducible in two independent analyses. Finally the first round Sanger test might be considered false-negative (in comparison to Cobas) in 2.2% and failing to provide results in only 2.5% (superior to the previously reported Sanger failure rate of 6.8% [22] or even 9.2%, despite being retested [19]). In contrast, the second round lost almost 24% for testing-number left without an attempt of retesting this time and reflecting difficulties in diagnostic utilization of FFPE-derived DNA.

If a laboratory uses sequencing, the detection rate would highly depend on sufficient quality and quantity of diagnostic material. It is worth underlining that we have examined a homogeneous group of macrometastases in lymph nodes that were managed in one center, with the highest quality material selected for the study. Moreover, the same blocks have been used as a source of DNA for all approaches. Thus, isolation kit dedicated to Cobas test, that returned results in all cases tested, must be acknowledged as enabling to generate DNA of a superb quality, not only sufficient for successful testing in all cases, but also suitable for direct sequencing assay (finally in the first round sequencing only 2.5% of samples didn't amplify in PCR, comparing to 24% of unsuccessful second-round testing, where routine extraction method was applied and no attempt to retest was made). In the first round set, Sanger sequencing failed to provide results in only 6 out of 236 samples. As our archival

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material dates back to even 1995, it is to be considered a good result, superior to the previously published data [4, 19, 22]. Two of our not amplifiable samples had high melanin content-possible reason for test failure. First run of Cobas test was also invalid for them and mutational status was resolved only after re-testing.

Sanger Sequencing revealed p.V600E mutation in two cases negative by Cobas. In both cases mutation peaks were weak and sample contamination due to poor DNA quality cannot be excluded - although Sanger-only mutation positive results were reported before [4, 19, 22].

Discordance in results between Cobas test and sequencing should not be automatically interpreted in favor of Cobas. When next generation sequencing (NGS) was engaged as reference methodology, among the 8 Cobas mutation-not-detected/Sanger mutation-detected discordant samples, three were p.V600E (c.1799T>A). Two of them had percentage of mutant alleles at the 5% [19]. According to producer information, Cobas test should be sensitive down to as little as 5% of tumor load in the specimen; in practice it often requires more [24]. Whatever are the precise numbers they are expected always lower than Sanger sequencing safe threshold (10% of tumor cells in the current analysis). Thus recurrent lack of detection of p.V600E by Cobas would be difficult to explain.

In the study by Qu et al. [22] the Cobas assay missed almost 20% of V600 mutations in comparison to sequencing, but only one of p.V600E. Thus the addition of Sanger sequencing for samples with negative Cobas resulted in significantly increased detection rate due to sequencing specificity towards less frequent and mainly dinucleotide substitutions. We did not entirely share the same experience. Four out of 9 p.V600K in our series were indeed negative by Cobas but all 3 rare variants were properly identified (two p.V600E (2) and one p.V600D). Decreased specificity of Cobas resulting in low discovery rate (at least 30% not detected) for p.V600K was underlined by others [22] and might be an important issue as those mutants was reported to respond to vemurafenib or dabrafenib treatment. Other rare BRAF mutants definitely need more studies, first-their sensitivity to BRAF inhibitors, second-the optimal mode of detection since targeted genotyping would not cover all possibilities.

By definition Cobas cannot detect activating BRAF mutation located outside codon V600 (four cases in our study). For those variants, detection based on sequence analysis (Sanger/NGS) seems to be method of choice at current state, until collecting sufficient knowledge about their significance for melanoma treatment decisions.

The present study has several limitations. Since none of patients was treated with BRAF inhibitors, we cannot verify whenever detection of mutation other than p.V600E would result in any clinical benefit that may justify testing. Since archival FFPE material was tested, the turnover time was not an issue and less experienced sequencing team might have miss some of weakly BRAF-positive cases reported here, further decreasing Sanger detection rate. Neither micro nor macrodissection was performed to reflect routine/rapid testing-selecting for sufficient tumor content was performed instead, which in practice might be discriminating for patients with micro-metastases only. In clinical practice, specimens with high tumor content may not always be available, but neither macrodissection may be feasible in every case [19].

Nevertheless, we consider our results highly both intra- and inter-approach repeatable and postulate the selection of the diagnostic material as the key for diagnostic accuracy [25]. Despite publications that, in the molecular era, question pathological estimation as inherently imprecise [18, 26] we postulate that careful examination of specimen by pathologist in order to select block surface with sufficient tumor cellularity (>5-20% depending of the analytical sensitivity of the applied method and feasibility of subsequent dissection) but also without extensive necrosis, blood or melanin pigments content compromises a key step for reliable testing, regardless testing method.

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Disclosure of conflict of interest

None.

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References

- [1] Eggermont AM, Robert C. New drugs in melanoma: It's a whole new world. *Eur J Cancer* 2011; 47: 2150-2157.
- [2] Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 2011; 364: 2507-2516.
- [3] van Akkooi AC, Nijsten T. A costly revolution for a subgroup of patients with metastatic melanoma. *Br J Dermatol* 2013; 168: 467-470.
- [4] Lopez-Rios F, Angulo B, Gomez B, Mair D, Martinez R, Conde E, Shieh F, Vaks J, Langland R, Lawrence HJ, de Castro DG. Comparison of Testing Methods for the Detection of BRAF V600E Mutations in Malignant Melanoma: Pre-Approval Validation Study of the Companion Diagnostic Test for Vemurafenib. *PLoS One* 2013; 8: e53733.
- [5] Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. *Nature* 2002; 417: 949-954.
- [6] 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.
- [7] Rubinstein JC, Sznol M, Pavlick AC, Ariyan S, Cheng E, Bacchicocchi A, Kluger HM, Narayan D, Halaban R. Incidence of the V600K mutation among melanoma patients with BRAF mutations, and potential therapeutic response to the specific BRAF inhibitor PLX4032. *J Transl Med* 2010; 8: 67.
- [8] Menzies AM, Haydu LE, Visintin L, Carlino MS, Howle JR, Thompson JF, Kefford RF, Scolyer RA, Long GV. Distinguishing clinicopathologic features of patients with V600E and V600K BRAF-mutant metastatic melanoma. *Clin Cancer Res* 2012; 18: 3242-3249.
- [9] Wellcome Trust Sanger Institute: Catalogue of somatic mutations in cancer (COSMIC) database. www.sanger.ac.uk/genetics/CGP/cosmic.
- [10] Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, McArthur GA, Hutson TE, Moschos SJ, Flaherty KT, Hersey P, Kefford R, Lawrence D, Puzanov I, Lewis KD, Amaravadi RK, Chmielowski B, Lawrence HJ, Shyr Y, Ye F, Li J, Nolop KB, Lee RJ, Joe AK, Ribas A. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med* 2012; 366: 707-14.
- [11] Klein O, Clements A, Menzies AM, O'Toole S, Kefford RF, Long GV. BRAF inhibitor activity in V600R metastatic melanoma. *Eur J Cancer* 2013; 49: 1073-1079.
- [12] Bahadoran P, Allegra M, Le Duff F, Long-Mira E, Hofman P, Giaccherio D, Passeron T, Lacour JP, Ballotti R. Major clinical response to a BRAF inhibitor in a patient with a BRAF L597R-mutated melanoma. *J Clin Oncol* 2013; 31: e324-326.
- [13] Heideman DAM, Lurkin I, Doleman M, Smit EF, Verheul HM, Meijer GA, Snijders PJ, Thunnissen E, 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* 2012; 14: 247-255.
- [14] Carbonell P, Turpin MC, Torres-Moreno D, Molina-Martinez I, Garcia-Solano J, Perez-Guillermo M, Conesa-Zamora P. Comparison of allelic discrimination by dHPLC, HRM, and TaqMan in the detection of BRAF mutation V600E. *J Mol Diagn* 2011; 13: 467-473.
- [15] Capper D, Berghoff AS, Magerle M, Ilhan A, Wöhrer A, Hackl M, Pichler J, Pusch S, Meyer J, Habel A, Petzelbauer P, Birner P, von Deimling A, Preusser M. Immunohistochemical testing of BRAF V600E status in 1,120 tumor tissue samples of patients with brain metastases. *Acta Neuropathol* 2012; 123: 223-233.
- [16] Curry JL, Torres-Cabala CA, Tetzlaff MT, Bowman C, Prieto VG. Molecular platforms utilized to detect BRAF V600E mutation in melanoma. *Semin Cutan Med Surg* 2012; 31: 267-273.
- [17] Wilson MA, Nathanson LN. Molecular testing in Melanoma. *Cancer J* 2012; 18: 117-123.
- [18] Halait H, Demartin K, Shah S, Soviero S, Langland R, Cheng S, Hillman G, Wu L, Lawrence HJ. Analytical performance of a real-time PCR-based assay for V600 Mutations in the BRAF gene, used as the companion diagnostic test for the novel BRAF inhibitor vemu-

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- rafenib in metastatic melanoma. *Diagn Mol Pathol* 2012; 21: 1-8.
- [19] Anderson S, Bloom KJ, Vallera DU, Rueschoff J, Meldrum C, Schilling R, Kovach B, Lee JR, Ochoa P, Langland R, Halait H, Lawrence HJ, Dugan MC. 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.
- [20] Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Ding S, Byrd DR, Cascinelli N, Cochran AJ, Coit DG, Eggermont AM, Johnson T, Kirkwood JM, Leong SP, McMasters KM, Mihm MC Jr, Morton DL, Ross MI, Sondak VK. Multivariate analysis of prognostic factors among 2,313 patients with stage III melanoma: comparison of nodal micrometastases versus macrometastases. *J Clin Oncol* 2010; 28: 2452-2459.
- [21] Rutkowski P, Gos A, Jurkowska M, Switaj T, Dziewirski W, Zdzienicki M, Ptaszyński K, Michej W, Tysarowski A, Siedlecki JA. Molecular alterations in clinical stage III cutaneous melanoma correlation with clinical-pathological features and patient outcome. *Oncol Lett* 2014; 8: 47-54.
- [22] Qu K, Pan Q, Zhang X, Rodriguez L, Zhang K, Li H, Ho A, Sanders H, Sferruzza A, Cheng SM, Nguyen D, Jones D, Waldman F. Detection of BRAF V600 mutations in metastatic melanoma: comparison of the Cobas 4800 and Sanger sequencing assays. *J Mol Diagn* 2013; 15: 790-795.
- [23] Richter A, Grieu F, Carrello A, Amanuel B, Namdarian K, Rynska A, Lucas A, Michael V, Bell A, Fox SB, Hewitt CA, Do H, McArthur GA, Wong SQ, Dobrovic A, Iacopetta B. A multisite blinded study for the detection of BRAF mutations in formalin-fixed, paraffin-embedded malignant melanoma. *Sci Rep* 2013; 3: 1659.
- [24] Lade-Keller J, Rømer KM, Guldberg P, Ribber-Hansen R, Hansen LL, Steiniche T, Hager H, Kristensen LS. Evaluation of BRAF mutation testing methodologies in formalin-fixed, paraffin-embedded cutaneous melanomas. *J Mol Diagn* 2013; 15: 70-80.
- [25] Dudley JC, Gurda GT, Tseng LH, Anderson DA, Chen G, Taube JM, Gocke CD, Eshleman JR, Lin MT. Tumor cellularity as a quality assurance measure for accurate clinical detection of BRAF mutations in melanoma. *Mol Diagn Ther* 2014; 184: 409-18.
- [26] Bellon E, Ligtenberg MJ, Tejpar S, Cox K, de Hertogh G, de Stricker K, Edsjö A, Gorgoulis V, Höfler G, Jung A, Kotsinas A, Laurent-Puig P, López-Ríos F, Hansen TP, Rouleau E, Vandenberghe P, van Krieken JJ, Dequeker E. External quality assessment for KRAS testing is needed: setup of a European program and report of the first joined regional quality assessment rounds. *Oncologist* 2011; 16: 467-478.