World Cancer Research Journal WCRJ 2019; 6: e1291

A SIMPLE AND HIGH SENSITIVE METHOD FOR DETECTION OF B-RAF 1799T>A (V600E) MUTATION IN THE THYROID FINE NEEDLE ASPIRATE

C. SANTONASTASO[1], M. DI PAOLO[2], A. TOMMASELLI[2], L. MORELLI[3], S. PUGLIESE[2], A. TROISI[2], T. MUTO[4], S. DI MARTINO[2,5,6]

[1]Pathological Anatomy, National Cancer Institute "G. Pascale", Naples, Italy
[2]CETAC Research Centre, Caserta, Italy
[3]Cytopatology Service ASL CE, Caserta, Italy
[4]Hematology and Cellular Immunology (Clinical Biochemistry), A.O. dei Colli Monaldi Hospital, Naples, Italy
[5]Pathology Unit, Cav. Apicella Hospital, Pollena Trocchia (ASL NA 3 SUD), Naples, Italy
[6]Pathology Unit, San Leonardo Hospital (ASL NA 3 SUD), Naples, Italy

Abstract – *Objective:* *In our report, we prospectively estimated a cohort of patients with nodules considered suspicious sonographically, for PTC. We evaluated 24 samples from PTC patients. The sensitivity, specificity, and negative predictive values (NPV) of FNAB BRAF testing in this study were 0.1% (analytical sensitivity), 100%, and 78%, respectively. In terms of assay costs, our PNA PCR-based method could be like either Allele Specific-PCR (AS-PCR) and/or Restriction Fragment Length Polymorphism (RFLP).*

Patients and Methods: *PNA chemistry has been performed to amplify selectively minor mutant allele background variants in a large amount of wild type (wt) allele derived from DNA extracted by 24 thyroid biopsies; 14 of them were obtained from cytological slides and 10 from fresh Fine Needle Aspirate (FNA) of patients with papillary thyroid carcinoma (PTC). Finally, we compared the results with those gained by direct sequencing.*

Results: *The assay sensitivity of the method was 0.1% of mutant alleles, evaluated by serial dilution of DNA from ARO cell line (carrying heterozygous V600E), mixed to wild type DNA gained from healthy donors. Optimized concentration of the primer PNA clamping-wt DNA is 12 μM per reaction. Direct sequencing was able to detect mutation in only 33.3% (8/24), while PNA-based PCR assay 45.8% (11/24) of patients, carrying mutation at codon V600. The estimated reagents costs are about €20.00 per sample, including controls and pre-analytical steps. This assay could be performed in a simple thermal cycler and results visualized by agarose ethidium bromide stained gels.*

Conclusions: *PNA clamping wt-DNA could be performed in any laboratory with very cheap PCR equipment. It is very cost-effective and could easily be adapted to detect hot spot mutations in any other genes.*

KEYWORDS: *NB-RAF mutations, Mutant minor allele detection, Pharmacogenomics, PNA-clamping PCR.*

Corresponding Author: Salvo Di Martino, MD; e-mail: salvo-dimartino@libero.it

INTRODUCTION

Oncogenic B-RAF mutations occur in up to 19% of all human cancers. Most of them (>90%) are nucleotide 1799 T>A substitutions that lead to the replacement of valine with aspartic acid (V600E) generating constitutive BRAF activation. Molecular detection of 1799 T>A point mutation is prognostic and diagnostic for melanoma (70%), papillary thyroid carcinoma (PTC; 55%), pancreas (40%) and colon (20%)[1]. PTC regards for the majority of thyroid cancers (around 80%) and generally brings a good prognosis, with a 10-year survival rate > 90%. Unlikely, a minor subgroup of PTC patients goes on to relapse[2]. Highly sensitive methods allowing early identification of PTC patients are very much-needed[3]. The necessary improvements in the management of PTC patients will certainly be obtained thanks to a better understanding of the genetic basis underlying the development of thyroid cancer. It has been known that the development of PTC implicates activation of the mitogen-activated protein kinase (MAPK) signaling pathway, which moderates cellular response to various growth signals. The most common reported genetic alteration in PTC is BRAF V600E mutation, occurring in up to 55% of papillary thyroid cancers, although most experts quote a prevalence of about 45% in PTC[4]. In the various histologic subtypes of PTC, BRAF V600E mutation is most commonly detected in the tall-cell and conventional histologic variants (80-83% and 67-68%, respectively) and less generally detected in the follicular variant (12-18%) of PTC[5]. BRAF mutations may also regard thyroid lymphomas and anaplastic and poorly differentiated thyroid cancers, but have not been recognized in medullary or follicular carcinomas and have only rarely been recollected in benign hyperplastic nodules[6]. Being safe, quick, cost-effective and accurate, fine-needle aspiration biopsy (FNAB) is the gold standard for the evaluation of thyroid nodules. When malignant cells are citologically detected, surgery is preferred and many experts accord with the extention of initial thyroidectomy (i.e., near-total or total thyroidectomy)[7]. According to the most current categorization of thyroid nodules, accepted by the Bethesda Criteria of the National Cancer Institute[8], 10-15% of thyroid nodule FNABs, comprising oncocytic and follicular neoplasms, follicular lesions of undetermined significance and suspicious nodules, fall into the indeterminate category. Currently, in patients with indeterminate lesions, diagnostic thyroidectomy is recommended to clearly exclude malignancy. Although the risk is very low in specialized centers, thyroid surgery doesn't reduce the risk for complications and brings health care costs. The new assay method we describe in this report is based on Peptide Nucleic Acid (PNA)-modified reverse primer able to assemble the amplification of wt-DNA. This method is easy, sensitive, reproducible and cost-effective. Finally, ideal for a small laboratory with few samples.

PATIENTS AND METHODS

Patient samples

Since BRAF mutational analysis is part of diagnostic protocols, the need for Ethic Committee's endorsement was not required for this study. Accordingly to these guidelines, an understanding written informed consent was granted for the FNA procedures that generated the tissue samples and the related diagnostic assay. Information regarding the human material used in this study was administered using anonymous numerical codes, clinical data were not used and samples were handled according to the Helsinki declaration (http://www.wma.net/en/30publications/10policies/b3/).

Twenty-four serial FNA thyroid samples from the "ambulatorio endocrinologia" of CETAC Research Center (Caserta, Italy) were selected for the study. For cytology preparations, the slides with the highest tumor content were selected and material collected after removal of the coverslide. The tumor area elected for the analysis was marked on the control slide to confirm, if possible, greater than 70% content of neoplastic cells, in accordance with published guidelines[9]. Tumor material was manually scoured under microscopic guidance from the corresponding sections using a sterile blade. Dissected tumor areas ranged from 0.25-1.0 cm^2. The last five samples resulted from cells obtained by fresh FNA harvested in Phosphate Buffered Solution (PBS).

DNA isolation

DNA was extracted from cytological slides using the Ampli DNA extraction kit (Dia-Chem, Naples, Italy), according to the manufacturer's recommendation. DNA from ARO cell line (carrying heterozygous V600E) was used as positive control in all experiments. DNA concentration was measured using by 260/280 nm UV detection. Rigorous precaution was taken to prevent cross-contamination of the sample and all experiments included a negative control (water) from all stage of the reaction.

Design primers and estimation of PNA concentration

Primers and PNA were designed using Primers software (http://frodo.wi.mit.edu/primer3/).

Specific forward primers (on intron 14) 5'GTT TTC CTTT TAC TTA CTA CAC CTC AG3' and reverse (on exon 15) 5' CCA CTC CAT CGA CGA GAT TTC TC 3' were designed to bind the mutant allele.

PNA primers were designed to bind anti-parallel wild-type DNA in the region nearby nt A1799 of BRAF gene (GeneBank data base entry accession number NM_004333) 5'CAT CGA GAT T-68-TC ACT C3'end-blocker (in bold nucleotide mutated).

The melting temperature (T_m) of these 16-oligomer segment is 67°C for DNA/PNA duplex. Determination of the thermal T_m for the PNA/wt-DNA and PNA/mut-DNA as well as the optimal conditions for amplification and sequencing was necessary for success. Estimation of required amount of PNA wt for knock out wt-DNA amplification was performed by PCR in the presence of several magnitude concentration (0, 4, 8 , 12 and 20 µmol/L) of PNA wt primer.

PCR

All 24 DNA samples were screened for BRAF exon 15 by AmpliSet BRAF (Diachem, Naples, Italy). PCR reactions were executed using the AccuraTaq DNA polymerase (Lucigen, Castenaso, BO, Italy) following the instructions of the manufacturer. Starting of 50 ng for DNA either from cytology slide and/or from the fresh tumor sample and cell line, were amplified using two PCR separate tubes: i) for allele-specific reverse mutated primer and ii) PNA wt reverse primer. In both cases, a reaction mix was prepared by: 10 X buffer (100 mM Tris-HClpH 8.3, 0.5 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM of Primers, and distilled water for 25 µL of total volume. Reaction conditions for the first round were 10 min at 95°C followed by 35 cycles of 30 s at 94°C (denaturation), grading of 5 s from 70°C to 65°C (allowing PNA annealing), 30 s at 59°C (primers annealing), 1 min at 72°C (extension) and final extension of 5 min at 65°C. In both assays, 10 µl of amplified products were run on a 2% agarose ethidium bromide-stained gel.

Sequencing

PCR products were sequenced in the forward directions with the following primers 5'AT GAA GAC CTC ACA GTA AAA AAT TAG3' by using the PCR amplicon. Cycle sequencing with fluorescent dye-labeled terminators was performed using an ABI PRISM® BigDye Terminator Cycle SequencingReadyReaction Kit with AmpliTaq® DNA Polymerase FS (Applied Biosystems, Foster City, CA, USA). Samples were prepared using 1 µL of primer (initial concentration, 2.5 µmol/L), 1 µL of DNA, 8 µL of the mixture from the Big Dye Terminator Cycle Sequencing Ready Reaction Kit, and 10 µL of distilled water.

Determination of lowest detectable mutant allele

To determine the assay's sensitivity to detect mutant allele in a large excess of wt allele, we used a different amount of mutant DNA from ARO cell Line mixed at a various ratio (100% through 0.01%) with genomic wt-DNA from an healthy donor as templates (50 ng). Each assay was executed in triplicate to confirm the lowest standard point producing the most coherent and unambiguous positive results.

Statistical measures of performance

False positive (FP), true positive (TP), true negative (TN), false negative (FN), test sensitivity (SEN), specificity (SPEC), negative predictive value (NPV), positive predictive value (PPV), accuracy (ACC), false discovery rate (FDR)[10].

RESULTS

Assessment of the PNA concentration allowing wild type Clamping PCR

Through a PCR clamping approach, primer forward can be replaced by PNA oligonucleotides due to high binding affinity to wt-DNA strands. The increased DNA/PNA duplex affinity is allowed by an additional annealing step at 65°C (I) executed before primers annealing 59°C (II) in every 35 cycles of amplifications. The extension phase was performed at 65°and PNA strongly annealed to wt-DNA, allowing the amplification of the eventually present Mutant DNA (Figure 1).

The concentration effect of PNA wt (which was designed to link the wt nucleotide) is clearly indicated and shows a maximum inhibition of the PCR at 10 µmol/L. Two percent agarose gels of the PCR products with the PNAs in the PCR reaction mixture showed single strong bands of the expected amplicon size (98 bp).

Analytical sensitivity assay

Considering that all ARO cells bring the heterozygous V600E that it is possible to have one rearranged gene/tumor cell, we are able to detect 1 mutant cell over 1000 wt cells (Figure 2).

BRAF Detection assay on samples

Twenty-four patients positive for PTC diagnosy (TIR 3b) by cytology assay, underwent the molecular assay. The results reported BRAF V600 positive in 8/24 FNA of cytology by sequencing and 11/24 by our PNA clamping wtDNA based PCR method.

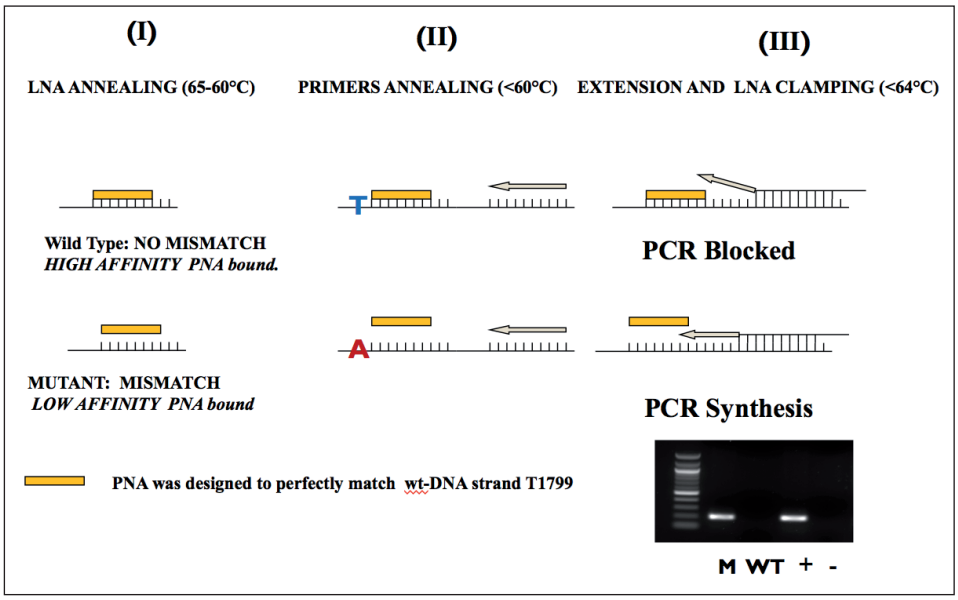

Fig. 1. Annealing of PNA affinity binding to wild type and mutant DNA.

Early Outline of Genotyping Costs

We have evaluated the cost-analysis of custom genotyping service by a random selection of 20 examined certified laboratories on 95, present on the site of GeneTests™http://www.ncbi.nlm.nih.gov/sites/GeneTests/lab?db=GeneTests (as to November 2018); it is approximately € 120.00/sample detection, acid nucleic isolation included. Specialized laboratories may perform V600E detection panel "in-house", which may further decrease the costs. As the prices can vary, depending on the model, manufacturer and institutional discount, price of instrumentation is only an approximate estimation. The cost of the reagents also differs widely in various genotyping methods. Evaluation costs per Single Point Mutation were gained as very low (<10€), low (<20€), middle (<30€), high (<50€), very high (>50€), including positive and negative controls (Table I). Moreover, appreciative time-labor is referred to a single test assay within one work session. In conclusion, the estimated cost of a patient's genotyping profile performed with PNA clamping wt-DNA is about € 20.00/sample, according to other genotyping estimation costs[11].

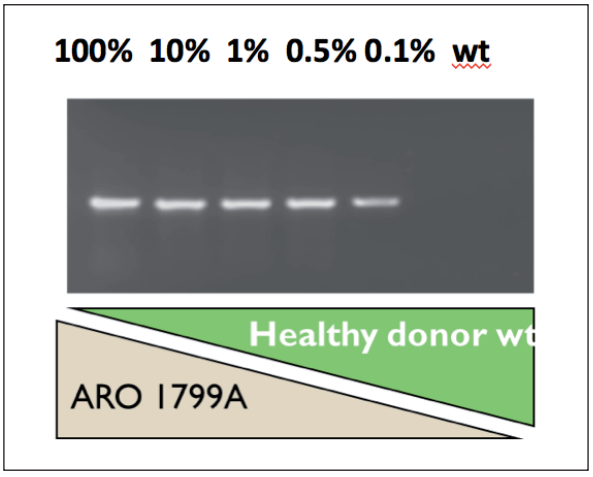

Fig. 2. Sensitivity curve. Low detection method was inferred to 0.1% of mutant DNA from ARO mixed to wtDNA from healthy donors.

DISCUSSION

Several procedures currently assess BRAF mutation analysis in the pre-operative setting. In our report, we prospectively estimated a cohort of patients with nodules considered suspicious sonographically, for PTC.

We evaluated 24 samples from PTC patients. In 5 cases, isolation of DNA was from fresh FNAB specimens whereas in other cases it was retrospectively achieved from archived cytological slide samples. The sensitivity, specificity and negative predictive value (NPV) of FNAB BRAF testing in this study were 0.1% (analytical sensitivity), 100%, and 78%, respectively.

Several studies were executed for optimization of high sensitivity BRAF V600E detection assay (I) without defining the best standard method. To date, relative costs of PG tests were evaluated by "manually cured criteria" due to lack of specific guidelines[12].

In terms of assay costs, our PNA PCR-based method could be like either Allele Specific-PCR (AS-PCR) and/or Restriction Fragment Length Polymorphism (RFLP). They do not need dedicated equipment (scored as very low) or reagents, but these technologies undergo a concomitant signifi-

TABLE 1. Most common described methods for detection of BRAF V600 in clinical setting.

Methods	*Genotyping costs#*	*Samples*	*Disease*	*References*
PNA-mediated PCR and direct sequencing	++	XCII	Colon-rectum cancer (CRC)	Kwon et al[13]
HRM high resolution melting	++	CXX	CRC and lung cancer	Borràs et al[14]
Real-time PCR	+	XXVIII	CRC	Hamfjord et al[15]
Multiplex snapshot assay	+++	CX	CRC	Magnin et al[16]
Selected cleavage of wild type DNA by TspR1 restriction assay	+	XXI	Circulant Melanoma cancer cells	Fusi et al[17]
Pyrosequencing and direct DNA sequencing	+++	XVII+CLXVIII	Papillary thyroid Carcinoma (PTC)	Guerra et al[18]
PCR allele-specific TaqMan	++	CXXV	CRC	Lang et al[19]
PCR multiplex HTP	++		Tumoral tissues	Li et al[20]
Cold PCR + HRM	++	CXVII	CRC	Pinzani et al[21]
PCR allele specifica + direct Dna sequencing + pyrosequencing		LXXXV	Thyroid carcinomas	Lee et al[22]
Maldi-tof mass spectrometry + (Oncocarta® panel)	++++	CCXXXIX + 39	Metastatic CRC	Fumagalli et al[23]
HRM and Snapshot	+++	CICV	Generic cancer	Heideman et al[24]
HRM	++	LI	Hairycell leucemia (HCL)	Blombery et al[25]
MEMO PCR	+	ILVII	Thyroid carcinomas	Bernacki et al[26]
Allele specific LNA qPCR	++	CCC	Generic cancer	Morandi et al[27]

#Reagent costs were scored as [+] very low (< 10€), [++] low (< 20€), [+++] moderate (< 30€), [++++]high (< 50€), [++++++] very high (> 50€) per sample, including DNA isolation step, positive and negative controls #.

cant decrease in throughput and time-labor. Almost all methods require at least one fluorescent reagent, which can dramatically increase the cost of the reaction (scored as a middle). However, this type of reagent also rises the sensitivity of the assay, thereby reducing the amount of template and reaction volume required. For example, all FRET-based methods require two fluorescent probes simultaneously labeled for allelic discrimination. The assay-specific probes are not required in the HRM platform, since the double stranded-DNA binding dye SYBR green can be used instead (scored as low cost). Other genotyping methods do not require any fluorescently-labeled reagent, but do have other specific requirements, which can be rather expensive. For example, pyrosequencing and MALDI TOF systems request tagged primers (e.g., biotin) for pre-detection purification steps (scored as high cost). Finally, the chip-based assays have physical matrix requirements (synthesized DNA chip arrays tailored to SNPs) that are disposable only on custom service (scored as very high). These methods are helpful only when a large number of samples is assayed. A review of major applied methods to reveal BRAF mutation in the clinical setting is summarized in Table I.

CONCLUSIONS

PNA mediated clamping PCR can be executed in any laboratory with basic PCR equipment. It is very cost-effective and can simply be adapted to detect hot spot mutations in other genes. Our results need to be confirmed on large-scale PTC samples. In the future the oncologists will have new instruments based on the individual genetic profile, to give treatment decisions for their patients in order to minimize toxicity and maximize benefits. Therefore, it is very important that biotechnology and pharmaceutical companies work closer, in order to generate a large study on the standardization method to validated tests suitable for routine diagnostics in pharmacogenomics.

Acknowledgments:
We are grateful to “Fondazione Muto ONLUS” for bibliografy support.

Conflict of Interest:

The Authors declare that they have no conflict of interests.

REFERENCES

1. De Monaco A, Faioli D, Di Paolo M, Catapano O, D'Orta A, Del Buono M, Del Buono R, Di Francia, R. Pharmacogenomics markers for prediction response and toxicity in cancer therapy. WCRJ 2014; 1: e276.
2. Yarchoan M, LiVolsi VA, Brose MS. Braf mutation and thyroid cancer recurrence. J Clin Oncol 2014; 59: 3657-3665.
3. Tirelli U, Berretta M, Bearz A, Carbone A. Grouping of molecularly targeted anti-cancer agents based on cost-effectiveness analysis. Eur Rev Med Pharmaco 2011; 15: 1355-1356.
4. Albores-Saavedra J, Henson DE, Glazer E, Schwartz AM. Changing patterns in the incidence and survival of thyroid cancer with follicular phenotype – papillary, follicular, and anaplastic: a morphological and epidemiological study. Endocr Pathol 2007; 18: 1-7.
5. Lee JH, Lee ES, Kim YS. Clinicopathologic significance of BRAF V600E mutation in papillary carcinomas of the thyroid: a meta-analysis. Cancer 2007; 110: 38-46.
6. Nikiforova MN, Nikiforov YE. Molecular diagnostics and predictors in thyroid cancer. Thyroid 2009; 19: 1351-1361.
7. Nikiforov YE. Thyroid carcinoma: molecular pathways and therapeutic targets. Modern Pathol 2008; 21: S37-S43.
8. Fisher KE, Cohen C, Siddiqui MT, Palma JF, Lipford EH. 3rd, Longshore JW. Accurate detection of BRAF p.V600E mutations in challenging melanoma specimens requires stringent immunohistochemistry scoring criteria or sensitive molecular assays. Hum Pathol 2014; 45: 2281-2293.
9. Seo JY, Choi JR, Moon HJ, Kim EK, Han KH, Kim H, Kwak JY. Clinical implication of highly sensitive detection of the brafv600e mutation in fine-needle aspirations according to the thyroid bethesda system in patients with conventional papillary thyroid carcinoma. Ann Otol Rhinol Laryngol 2014; 124: 392-399.
10. Van Krieken JH, Jung A, Kirchner T, Carneiro F, Seruca R, Bosman FT, Quirke P, Fléjou JF, Plato Hansen T, De Hertogh G, Jares P, Langner C, Hoefler G, Ligtenberg M, Tiniakos D, Tejpar S, Bevilacqua G, Ensari A. KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. Virchows Arch 2008; 453: 417-431.
11. De Monaco A, Berretta M, Pugliese S, Valente D, Ciaffarafa S, Di Francia R. Evaluation of genotyping costs. Eur Rev Med Pharmacol Sci 2014; 18: 2084-2087.
12. Di Francia R, Berretta M, Catapano O, Canzoniero LM, Formisano L. Molecular diagnostics for pharmacogenomic testing of fluoropyrimidine based-therapy: costs, methods and applications. Clin Chem Lab Med 2011; 49: 1105-1111.
13. Kwon MJ, Ki CS, Kim JY, Lee ST, Kim JW, Kang SY. Multiplex ligation-dependent probe amplification (MLPA) assay for the detection of mitochondrial DNA deletion in chronic progressive external ophthalmoplegia (CPEO). Ann Clin Lab Sci 2011; 41: 385-389.
14. Borràs E, Jurado I, Hernan I, Gamundi MJ, Dias M, Martí I, Mañé B, Arcusa A, Agúndez J. AG, Blanca M, Carballo M. Clinical pharmacogenomic testing of KRAS, BRAF and EGFR mutations by high resolution melting analysis and ultra-deep pyrosequencing. BMC Cancer 2011; 11: 406.
15. Hamfjord J, Stangeland AM, Skrede ML, Tveit KM, Ikdahl T, Kure EH. Wobble-enhanced ARMS method for detection of KRAS and BRAF mutations. Diagn Mol Pathol 2011; 20: 158-165.
16. Magnin S, Viel E, Baraquin A, Valmary-Degano S, Kantelip B, Pretet JL, Mougin C, Bigand M, Girardo B, Borg C, Ferrand C. A Multiplex SNaPshot assay as a rapid method for detecting KRAS and BRAF mutations in advanced colorectal cancers. J Mol Diagn 2011; 13: 485-492.
17. Fusi A, Berdel R, Havemann S, Nonnenmacher A, Keilholz U. Enhanced detection of BRAF-mutants by pre-PCR cleavage of wild-type sequences revealed circulating melanoma cells heterogeneity. Eur J Cancer 2011; 47: 1971-1976.
18. Guerra A, Sapio MR, Marotta V, Campanile E, Rossi S, Forno I, Fugazzola L, Budillon A, Moccia T, Fenzi G, Vitale M. The primary occurrence of BRAF(V600E) is a rare clonal event in papillary thyroid carcinoma. J Clin Endocr Metab 2012; 97: 517-524.
19. Lang AH, Drexel H, Geller-Rhomberg S, Stark N, Winder T, Geiger K, Muendlein A. Optimized allele-specific real-time PCR assays for the detection of common mutations in KRAS and BRAF. J Mol Diagn 2011; 13: 23-28.
20. Li G, Luo X, He J, Zhu Z, Yu G, Qin H, Zeng T, Liu Z, Wu S, Xu J, Ren-Heidenreich L. A novel liquidchip platform for simultaneous detection of 70 alleles of DNA somatic mutations on EGFR, KRAS, BRAF and PIK3CA from formalin-fixed and paraffin-embedded slides containing tumor tissue. Clin Chem Lab Med 2011; 49: 191-195.
21. Pinzani P, Santucci C, Mancini I, Simi L, Salvianti F, Pratesi N, Massi D, De Giorgi V, Pazzagli M, Orlando C. BRAFV600E detection in melanoma is highly improved by COLD-PCR. Clin Chim Acta 2011; 412: 901-905.
22. Lee HJ, Choi J, Hwang TS, Shong YK, Hong SJ, Gong G. Detection of BRAF mutations in thyroid nodules by allele-specific PCR using a dual priming oligonucleotide system. Am J Clin Pathol 2010; 133: 802-808.
23. Fumagalli D, Gavin PG, Taniyama Y, Kim SI, Choi HJ, Paik S, Pogue-Geile KL. A rapid, sensitive, reproducible and cost-effective method for mutation profiling of colon cancer and metastatic lymph nodes. BMC Cancer 2010; 10: 101.
24. Heideman DA, Lurkin I, Doeleman 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.
25. Blombery PA, Wong SQ, Hewitt CA, Dobrovic A, Maxwell EL, Juneja S, Grigoriadis G, Westerman DA. Detection of BRAF mutations in patients with hairy cell leukemia and related lymphoproliferative disorders. Haematologica 2012; 97: 780-783.
26. Bernacki KD, Betz BL, Weigelin HC, Lao CD, Redman BG, Knoepp SM, Roh MH. Molecular diagnostics of melanoma fine-needle aspirates: a cytology-histology correlation study. Am J Clin Pathol 2012; 138: 670-677.
27. Morandi L, De Biase D, Visani M, Cesari V, De Maglio G, Pizzolitto S, Pession A, Tallini G. Allele specific locked nucleic acid quantitative PCR (ASLNAqPCR): an accurate and cost-effective assay to diagnose and quantify KRAS and BRAF mutation. PLoS One 2012; 7: e36084.