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A COST-EFFECTIVENESS METHOD FOR THE ENRICHMENTS OF MINORITY MUTANT DNA IN A LARGE EXCESS OF WILD TYPE DNA. DETECTION OF B-RAF 1799T>A (V600E) MUTATIONS IN THE THYROID FINE NEEDLE ASPIRATE

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ABSTRACT: Background: *Molecular detection of 1799 T>A point mutation in* BRAF *gene is prognostic and diagnostic for papillary thyroid carcinoma (PTC 70%). Furthermore,* BRAF *inhibitor molecules like vemurafenib are proving highly effective to treat patients with* BRAF *mutated tumors, in melanoma; in the next future these approach could be suitable for PTC treatments. This can prove a challenge and underlines the importance of utilizing a detection method that is easy, sensitive, reproducible and cost-effective.*

Materials and methods: *We developed a cost-effective and sensitivity method based on Locked Nucleic Acid (LNA) oligomers for selective detection of* BRAF *1799T>A V600E. LNA have been used to enhance mutant allele of minor background variants in a large excess of wild type allele derived from DNA extracted by of 14 thyroid biopsy; 14 of them obtained from cytological slides and 5 from fresh Fine Needle Aspirate (FNA) of patients with papillary thyroid carcinoma (PTC). In addition, we compared the results with those obtained by direct sequencing*

Results: *TThe assay sensitivity of the method was 0.1% of mutant alleles, assessed by serial dilution of DNA from ARO cell line (carrying heterozygous V600E), mixed to wild type DNA obtained from Healthy donors. Optimized concentration of the primer LNA clamping-wild type (wt) DNA is 12 μM per reaction. Direct sequencing was able to detect mutation in only 26.7% (5/19) while, LNA-based PCR assay 57.9% (11/19) of patients, carrying mutation at codon V600. The estimated reagents costs is about € 20,00 per sample, including controls and pre-analytical steps. This assay could be performed in a simple thermalcycler and results visualized by agarose ethidium bromide stained gels.*

KEY WORDS: *B-RAF mutations, Mutant minor allele detection, Pharmacogenomics, Vemurafenib.*

INTRODUCTION

Oncogenic *B-RAF* mutations occur in up to 15% of all human tumors. The majority (>90%) being nucleotide 1799 T>A substitutions that lead to the replacement of Valine with Aspartic acid (V600E) causing constitutive *BRAF* activation. Molecular detection of 1799 T>A point mutation is prognostic and diagnostic for melanoma (70%) papillary thyroid carcinoma (PTC; 60%), pancreas (40%)

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and colon (20%)[1]. PTC accounts for the majority of thyroid cancers (~85%) and generally carries an excellent prognosis, with a 10-year survival rate >90%. However, a small subset of PTC patients goes on to relapse[2]. Ultrasensitive methods of identifying earlier and treating these patients are very much needed[3]. Improved understanding of the genetic basis underlying the development of thyroid cancer has evolved that will certainly lead to necessary improvements in the management of PTC patients. It has been known that the development of PTC involves activation of the mitogen-activated protein kinase (MAPK) signaling pathway, which mediates cellular response to various growth signals. The *BRAF* V600E alteration leading to constitutive MAPK pathway stimulation. The *BRAF* V600E mutation is the most common genetic alteration in PTC and has been reported to occur in up to 60% of papillary thyroid cancers, although most experts quote a prevalence of about 45% in PTCs[4]. Among the various histologic subtypes of PTC, *BRAF* V600E mutation is most commonly found in the conventional and tall-cell histologic variants (67%-68% and 80%-83%, respectively), and less commonly found in the follicular variant (12%-18%) of PTC[5]. *BRAF* mutations may also occur in thyroid lymphomas and anaplastic and poorly differentiated thyroid cancers, but have not been identified in follicular or medullary carcinomas and have only very rarely been identified in benign hyperplastic nodules[6]. Fine-needle aspiration biopsy (FNAB) is the gold standard for the evaluation of thyroid nodules because it is safe, quick, cost-effective, and accurate. When malignant cells are seen on cytology, the decision to proceed to surgery is simple, and most experts agree on the extent of initial thyroidectomy (i.e., near-total or total thyroidectomy)[7]. However, 10%-15% of thyroid nodule FNABs fall into the indeterminate category, which includes follicular and oncocytic neoplasms, follicular lesions of undetermined significance, and suspicious nodules, according to the most current categorization of thyroid nodules established by the Bethesda Criteria of the National Cancer Institute[8]. To date, diagnostic thyroidectomy is recommended to definitively exclude malignancy in patients with indeterminate lesions. Although the risk is extremely low in specialized centers, thyroid surgery is not minus a risk for complications and carries health care costs.

In this report we describe a new assay method based on 3'-locked nucleic acid (LNA)-modified reverse primer able to clump the amplification of wild type (wt) DNA. This detection method is easy, sensitive, reproducible and cost-effective. Finally, ideal for small laboratory with few samples.

MATERIALS AND METHODS

Patient samples

Since BRAF mutational analysis is part of proper diagnostic protocols, the need for ethic committee's approval was not necessary for this study. Accordingly to these guidelines, a comprehensive written informed consent was signed for the FNA procedures that produced the tissue samples and the related diagnostic assay. All information regarding the human material used in this study was managed using anonymous numerical codes, clinical data were not used and samples were handled in compliance with the Helsinki declaration (http://www.wma.net/en/30publications/10policies/b3/).

Nineteen consecutive FNA thyroid samples from the "Ambulatorio Endocrinologia" of CETAC Research Center (Caserta, Italy), were selected for the study. For cytology preparations (14 samples) the slides with the highest tumor content were selected and material collected after removal of the cover slide. The tumor area selected for the analysis was marked on the control slide to ensure, whenever possible, greater than 70% content of neoplastic cells, in accordance with published guidelines[9]. Tumor material was manually scraped 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 derived 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 sample and all experiments included negative control (water) from all stage of the reaction.

Design primers and Estimation of LNA concentration

Primers and LNA were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/).

Specific forward primers (on intron 14) 5'GTT TTC CTTT TAC TTA CTA CAC CTC AG 3' and reverse (on exon 15) 5' CCA CTC CAT CGA CGA GAT TTC **T**C 3' designed to bind the mutand allele.

LNA primer was designed to bind antiparallel wild-type DNA in the region surrounding nt A1799 of *BRAF* gene (GeneBank data base entry accession number NM_004333) 5' CAT CGA GAT T-68-TC ACT C 3'end-blocker (in bold nucleotide mutated).

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

PCR

All 19 DNA samples were screened for *BRAF* exon 15 by AmpliSet BRAF (Diachem, Naples, Italy). In our hand, PCR reactions were performed using the FastStartTaq DNA polymerase (Roche Applied Science, Mannheim, Germany) following the instructions of the manufacturer.

Starting from 30-50 ng for DNA from cytology slide, from fresh tumor sample and cell line, were amplified using two PCR separate tubes: i) for allele specific reverse mutated primer and ii) LNAwt reverse primer. In both case a reaction mix was prepared containing: 10 X buffer (100 mM Tris-HCl pH 8.3, 0,5 mM KCl), 1,5 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM of Primers, and distilled water for 25 ml of total volume. Reaction conditions for the first round were 10 minutes at 95°C followed by 35 cycles of 30 seconds at 94°C (denaturation), grading of 5 seconds from 71° to 64° (allowing LNA annealing), 30 seconds at 58°C (primers annealing) and 1 minute at 72°C (extension) and final extension of 5 minutes at 64°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'CAT 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 Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase FS (Applied Biosystems). 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 BigDye Terminator Cycle Sequencing Ready Reaction Kit, and 10 μL of distilled water. Thermal cycling was conducted in a PerkinElmer Model 9700 thermocycler and started with 1 min at 96°C. The reaction then underwent 25 cycles of 96°C for 15 sec (denaturation), 50°C for 5 sec (annealing), and 60°C for 2 min (extension). The DNA product was purified using Edge Gel Filtration Cartridges (Edge Biosystems). The samples were dried on a RC10.10 Centrifugal Evaporator (Jouan) and reconstituted in 20 μL of Template Suppression Reagent (Life Technologies, Milan, Italy).

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 different amount of mutant DNA from ARO cell Line mixed at various ratio (100% through 0.01%) with genomic wtDNA from healthy donor as templates (50 ng). Each assay was performed in triplicate for confirmation the lowest standard point that produce the most consistent and unambiguous positive results.

Statistical measures of performance

True positive (TP), false positive (FP), 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

Estimation of the LNA concentration allowing wild type Clamping PCR

Through a PCR clamping approach, primer forward can be replaced by LNA oligonucleotides due to high binding affininty to DNA wild type strandt. The increased DNA/LNA duplex affinity is allowed by an additional annelaing step at 65°C (I) performed before primers annealing 59°C (II) in each 35 cycles of amplifications. The extension phase was performed at 64° and LNA strongly annealed to wild type DNA, allow the amplification of the eventually present Mutant DNA (Figure 1).

The concentration effect of LNAwt (which was designed to inhibit the wild-type nucleotide) is clearly indicated and shows a maximum inhibition of the PCR at 12 μmol/L (Figure 2). 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 (93. bp).

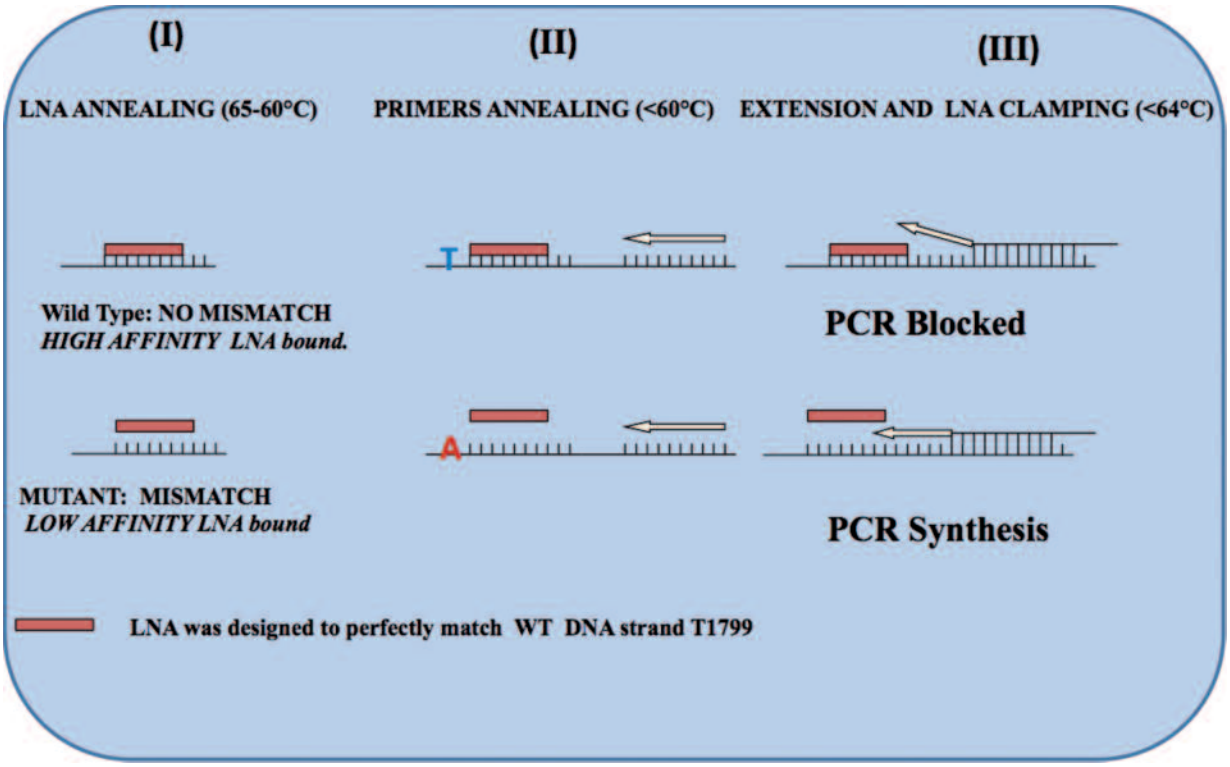

Figure 1. Annealing of LNA affinity binding to wild type and mutant DNA.

Analytical sensitivity assay

Considering that all ARO cells carry 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 3).

BRAF Detection assay on samples

Nineteen patients with conventional PTC cytology underwent thyroidectomy. The molecular assay reported that *BRAF* mutational analysis was present in 5/19 FNA of cytology by sequencing and 11/19 by our LNA clamping wtDNA based PCR method.

Early Outline of Genotyping Costs

We have evaluated the cost-analysis of custom genotyping service by a random selection of 20 interrogated certified laboratories on 95, present on the site of GeneTests™ http://www.ncbi.nlm.nih.gov /sites/GeneTests/lab?db=GeneTests (as to July 2014); it is

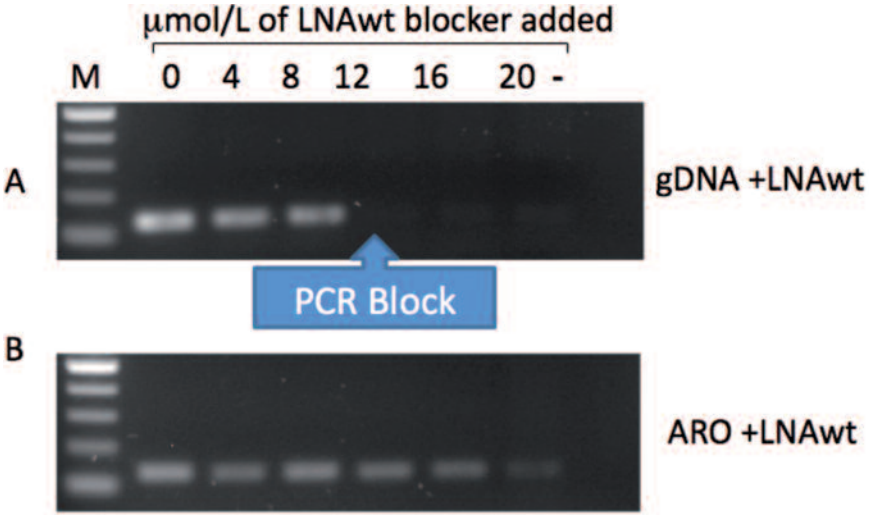

Figure 2. Estimation of the LNA concentration allowing wild type Clamping PCR. The concentration effect of LNAwt (which was designed to inhibit the wild-type nucleotide) is clearly indicated by arrow and shows a maximum inhibition of the PCR at 12 µmol/L on wtDNA **(A)**. Same amplification condition was applied to mutant DNA from ARO Cell lines. No block of PCR was noticed **(B)**.

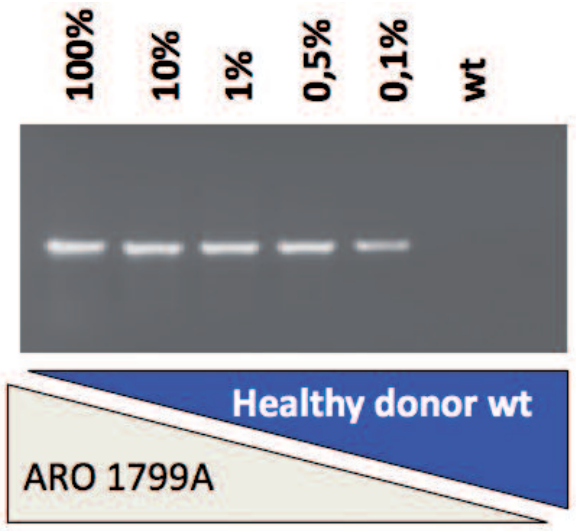

Figure 3. Sensitivity curve. Low detection method was inferred to 0,1% of mutant DNA from ARO mixed to wtDNA from healthy donors.

approximately € 120,00 per mutation detection, acid nucleic isolation and shipping included. Specialized laboratories can perform V600E detection panel "in house", which may further reduce the costs. The price of instrumentation is only an approximate estimation, since the prices can vary, depending on the manufacturer, model and institutional discount. The cost of the reagents also varies widely in different genotyping methods. Evaluation costs per Single point Mutation were scored as very low (<10€), low (<20€), middle (<30€), high (<50€), very high (>50€), including positive and negative controls (Table 1). In addition, appreciative time-labour is referred to single test assay within one work session. In conclusion, estimated cost of a patient's genotyping profile performed with LNA clamping wt DNA is about € 20,00/sample, in according to other genotyping estimation costs[11].

DISCUSSION

Today several procedures currently evaluate *BRAF* mutation analysis in the preoperative setting. In our report, we prospectively evaluated a cohort of patients with nodules deemed suspicious sonographically, for PTC.

TABLE 1. MOST COMMON DESCRIBED METHODS FOR DETECTION OF BRAF V600 IN CLINICAL SETTING.

METHODS	Genotyping costs#	Samples	Disease	Reference
PNA-mediated PCR and direct sequencing	++	92	Colon-rectum cancer (CRC)	[14]Kwon MJ et al 2011
HRM high resolution melting	++	120	CRC and lung cancer	[15]Borràs E et al 2011
Real time PCR	+	28	CRC	[16]Hamfjord J et al 2011
Multiplex snapshot assay	+++	110	CRC	[17]Magnin S et al 2011
Selected cleavage of Wild Type DNA by TspR1 Restriction assay	+	21	Circulant Melanoma cancer cells	[18]Fusi A et al. 2011
Pyrosquencing and direct DNA sequencing	+++	17+168	Papillary thyroid Carcinoma (PTC)	[19]Guerra A et Al 2012
PCR allele-specific TaqMan	++	125	CRC	[20]Lang AH et al 2011 Jan
PCR multiplex HTP	++		Tessuti tumorali	[21]Li G et al 2011 Feb
Cold PCR + HRM	++	117	CRC	[22]Pinzani P et Al 2011
PCR allele specifica + direct Dna sequencing + pyrosequencing		85	Thyroid carcinomas	[23]Lee HJ et al 2010
Maldi-tof mass spectrometry +	++++ (Oncocarta® panel)	239 + 39	Metastatic CRC	[24]Fumagalli D et al 2010
\				
HRM and Snapshot	+++	195	Generic cancer	[25]Heideman DA et al 2012
HRM	++	51	Hairy cell leucemia (HCL)	[26]Blombery PA et al 2011
MEMO PCR	+	47	Thyroid carcinomas	[27].Bernacki KD et al 2012
Allele specific LNA qPCR	++	300	Generic cancer	[28]Morandi et Al 2012

#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. #.

We evaluated 19 samples from PTC patients. In 5 cases the DNA isolation was from fresh FNAB specimens, whereas in other cases it was retrospectively obtained 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 was performed for optimization high sensitivity BRAF V600E detection assay (Table 1) without defining the best standard method. To date, relative costs of PG tests, here, were evaluated by "manually cured criteria" due to lack of specific guidelines[12].

In terms of assay costs, our LNA PCR-based methods could be like either Allele Specific-PCR (AS-PCR) and/or Restriction Fragment Length Polymorphism (RFLP). They do not need specialized equipment (scored as very low) or reagents, but these technologies suffer from a concomitant significant decrease in throughput and time-labour. Nearly all methods require at least one fluorescent reagent, which can dramatically increase the cost of the reaction (scored as middle). However, this type of reagent also increases 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 each simultaneously labelled 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-labelled reagent, but do have other specific requirements, which can be rather expensive. For example, Pyrosequencing, and MALDI TOF systems require 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 available only on custom service (scored as very high). These methods are useful only when a large number of the samples is assayed. A review of major applied methods to detect BRAF mutation in clinical setting are summarized in Table 1.

CONCLUSION AND FUTURE OUTLOOK

LNA mediated clamping PCR can be performed in any laboratory with basic PCR equipment. It is very cost-effective and can easily be adapted to detect hot spot mutations in other genes. Our results, needs to be confirmed on large scale PTC samples. In the next future the oncologists will have new means based on the genetic profile of the individual, to make treatment decisions for their patients in order to maximize benefits and minimize toxicity[32]. Therefore, it is fundamental that pharmaceutical and biotechnology companies join, in order to develop an extensive study on the standardization method to validated tests suitable for routine diagnostics in pharmacogenomics,

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Conflict of Interests:
The Authors declare that they have no conflict of interests.

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