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Implementation of a companion diagnostic in the clinical laboratory: The *BRAF* example in melanoma

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ABSTRACT

A companion diagnostic test provides information that is essential for the safe and effective use of a corresponding therapeutic product as indicated in the drug instructions. The implementation of a companion diagnostic follows the rules of a molecular test for somatic mutations in a routine clinical laboratory environment and needs guidance on practical aspects, including the choice of the proper analytical method and the procedures for internal and external quality controls. Selection of the appropriate assay for detection of genetic alterations depends on several factors: the type of mutation under study, the sample to be assayed and its preparation procedure. In addition, the results of a molecular assay require a complex interpretation process of the analytical data as the patient's genotype, the translation of the identified variant into a predicted phenotype and knowledge on restrictions of the method used. In relation to these aspects herein we report an opinion paper of the Working Group Personalized Laboratory Medicine jointly constituted by the European Federation of Laboratory Medicine (EFLM) and by the European Society of Pharmacogenomics and Theranostics (ESPT) using, as an example, the BRAF genotype analysis in tumor tissue samples for identification of melanoma patients that can benefit treatment with BRAF inhibitors. The manuscript is focused on the following aspects: i) medical rationale, ii) methodologies of analysis, iii) laboratory performance evaluation and iv) the laboratory specific report for the clinicians. The critical evaluation of these aspects would be useful for the implementation of a companion diagnostic in the clinical laboratory.

1. Introduction

Over the past decade, nucleic acid testing for infectious diseases, human genetics and molecular oncology has grown rapidly. Although molecular testing was recently offered exclusively by specialized reference laboratories possessing appropriate resources and technical expertise, new instruments for sequencing and IVD-labeled assays allow routine clinical laboratories to offer molecular analysis without large investments in research and development. As these technologies will further develop in the future, it can be expected that molecular diagnostic techniques will become easier and more applicable in a routine clinical laboratory.

Abbreviations: IVD, in vitro diagnostics; BRAF, V-RAF murine sarcoma viral oncogene homolog B1; SSCP, single-stranded conformation polymorphism analysis; dHPLC, denaturing high performance liquid chromatography; HRMA, high resolution melting analysis; LDTs, Laboratory Developed Tests; AS real-time PCR, allele-specific real-time polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded; NGS, next-generation sequencing; MALDI TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NRAS, neuroblastoma RAS viral oncogene homolog; KIT, V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; EQA, external quality assessment.

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However, the implementation of molecular diagnostic tests in a routine clinical laboratory needs guidance on several practical aspects during set-up including the choice of the proper analytical method and the procedures for internal and external quality controls. The results of molecular assay require a complex interpretation process of the analytical data based on several factors as the patient's genotype, the translation of the identified variant into a predicted phenotype and knowledge on limitations of the assay method used in addition to specific skills on the disease area (infectious diseases, molecular genetics and molecular oncology).

The approach to new molecular assays in a routine clinical laboratory environment requires expert laboratory specialists able to advise clinicians to select the appropriate biological specimen, to request a suitable test, to evaluate the performance of the pre-analytical and analytical phases and to generate clinically useful and patient-specific reports including the availability of consulting.

Several publications and reviews on molecular genetic and genomic tests produced by international organizations, such as the Agency for Health Quality Research (AHRQ) (www.ahrq.gov), the Cochrane Collaboration (www.cochrane.org/reviews), the Clinical Laboratory Standards Institute (www.clsi.org) and the Organisation for Economic Co-operation and Development (www.oecd.org/science/biotech/) or developed from activities of European projects such as EuroGentest (www.eurogentest.org/) are available. In particular the recent CLSI MM19 document *Establishing Molecular Testing in Clinical Laboratory Environments; Approved Guideline* [1] details all these aspects.

Whereas we suggest referring to the above-mentioned documents in the case of planning the incorporation of molecular diagnostics in a clinical laboratory, the implementation itself poses a number of challenges to which no readily available answer can be found in these documents including the choice of analytical method and the procedure for internal and external quality controls. Here we report the experience of the Clinical Biochemistry Unit of the University of Florence in performing molecular testing for *BRAF* somatic mutations in melanoma patients. This test belongs to the area of the so called "companion diagnostics" whereby molecular tests that identify specific mutations are used to provide a specific therapy for the condition of an individual. An up-to-date list of companion diagnostic devices linked to a specific drug and approved by Food and Drug Administration (FDA) can be consulted at the following link:http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm.

The approach used at the University of Florence has been discussed within the joint Working Group Personalized Laboratory Medicine (WG-PLM) nominated by the European Federation of Laboratory Medicine (EFLM, http://www.efcclm.eu/) and by the European Society of Pharmacogenomics and Theranostics (ESPT, http://www.esptnet.eu/) to produce an opinion paper focused on the following aspects: i) medical rationale, ii) methodologies of analysis, iii) laboratory performance evaluation and iv) the laboratory's specific report for the clinicians.

2. BRAF: medical rationale

BRAF mutations and BRAF-inhibitors are an emblematic example of companion diagnostics with improved clinical response and survival in metastatic melanoma (MM) patients.

Belonging to RAF family, the *BRAF* gene encodes for a serine–threonine protein kinase [2] and its mutations account for approximately 50% of all the genetic alterations in primary cutaneous melanoma [3–5]. About 90% of all the clinically relevant mutations affect exon 15 and they arise as a single-point mutation at position 1799 (thymine to adenine) that converts valine to glutamic acid at 600 position of the amino acid sequence [6–8].

Among the remaining *BRAF* mutations, the most common one involves the variations of two adjacent nucleotides and it is identified as c.1798_1799delinsAA (p.Val600Lys).

Other mutations affecting the same region, such as c.1798_1799delinsAG p.Val600Arg, c.1801A>G p.Lys601Glu and c.1799_1800delinsAA p.Val600Glu are extremely rare (COSMIC, *Catalogue of Somatic Mutations in Cancer Database*, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).

The identification of such a frequent mutation in kinase has provided a hub for the development of a new inhibitor molecule that targets toward the mutated *BRAF* gene product and thus affecting only cancer cells by suppression of essential tumor-growth pathways.

Vemurafenib (RG7204/PLX4032), identified by Plexxikon Inc. (Berkeley, CA, USA) is a first-in-class selective inhibitor of BRAF. The effectiveness of Vemurafenib in comparison to the traditional treatment with dacarbazine has been established by the results of several clinical trials, in particular by the BRIM-3 (BRAF inhibitor in melanoma-3) trial performed on 675 MM patients affected by a BRAF-mutated tumor [9].

On August 2011, the drug Zelboraf (Vemurafenib) was approved by the Food and Drug Administration as "*a drug to treat patients with late-stage (metastatic) or unresectable (cannot be removed by surgery) melanoma*". In the label, it is also clearly specified that the drug is "*for the treatment of patients with melanoma whose tumors express a gene mutation called BRAF V600E*".

On 15 December 2011 European Medicines Agency and the Committee for Medicinal Products for Human Use (CHMP) adopted a positive opinion, recommending the granting of a marketing authorization for the medicinal product Zelboraf for the treatment of adult patients with BRAF V600 mutation-positive unresectable or metastatic melanoma.

However, the recurrence of side effects and relapse using BRAF inhibitors is an important aspect to be considered [10] and the mechanisms surrounding the resistance should be better defined and investigated especially in the context of the targeted therapy [11].

3. *BRAF*: methods for mutation analysis

Selection of the appropriate assay for the detection of genetic alterations depends on several factors: firstly the type of mutation under study, the kind of sample to be assayed and then the sample preparation procedure.

In somatic mutations molecular analysis procedures, the contribution of the pathologist is fundamental to evaluate the fraction of neoplastic cells into the sample as this can influence the choice of the analytical method. As a matter of fact, the most important aspect to be considered is the large excess of wild type DNA, when dealing with the detection of rare mutated DNA molecules in tumor samples. Therefore, techniques with a defined specificity and sensitivity are required to detect "mutant genomes" in a background of wild-type DNA.

Each method has its own characteristics in regard to sensitivity, specificity, coverage (i.e. spectrum of identifiable variants), cost and turnaround time. An overview of the main methods used to date in a routine clinical laboratory to assess mutation status of cancer samples is briefly reported in the following paragraphs (see also Table 1).

3.1. Methods of pre-screening analysis

Pre-screening analysis is a heterogeneous group of methods based on different intrinsic features of the target sequence. The most currently used techniques are the single-stranded conformation polymorphism analysis (SSCP) [12], the denaturing high performance liquid chromatography (dHPLC) [13] and the high resolution melting analysis (HRMA) [14].

Since these techniques are Laboratory Developed Tests (LDTs), they should be used only by expert personnel and after a validation procedure that has demonstrated reliability of the analytical performances. An extensive optimization may be required. Methodological aspects that should be taken into account are listed in Table 1.

Table 1
Major classes of methods suitable for *BRAF* analysis.

Method	Mutation detected	Sensitivity (% mutated allele)	Turnaround time (from PCR amplification)	Validation
Pre-screening analysis methods				
SSCP	Theoretically all nucleotide substitutions inside the sequence amplified by PCR primers.	Approximately 5%, strongly dependent by the optimization of the method	1 day (approximately 24 samples/run)	Home-brew test. Serial dilutions of mutated DNA in wild-type should be used to validate the method. Several nucleotide substitutions could be tested. Control positive and negative samples could be analyzed every time to monitor all the phases of the procedure.
dHPLC	Major aspects to be considered: – Type of substitution – Length of the amplicon – Relative position of the substitution inside the amplicon These methods are not able to identify the specific mutation: a direct method should be applied subsequently.		Few hours (one sample/run)	
HRM			Few hours (up to 96 samples/run)	
Sequencing methods				
Sanger method	Specific characterization of all nucleotide substitutions inside the sequence amplified by PCR primers	Approximately 10–20%, strongly dependent by the optimization of the method and the instrument's maintenance.	2 days (one or more sample/run, depending on the available instrumentation)	Home-brew test. Serial dilutions of mutated DNA in wild-type should be used to validate the method. Several nucleotide substitutions could be tested. Control positive and negative samples could be analyzed every time to monitor all the phases of the procedure.
Pyrosequencing	Variations in the first 30–40 bases from sequencing primer	Approximately 5–10%, strongly dependent by the optimization of the method and the instrument's maintenance.	1 day (up to 96 samples/run)	
therascreen BRAF Pyro Kit (QIAGEN)	Two assays: – Codon 600: V600A, V600E, V600G, and V600M – Codons 464–469: G464E, G464V, G466E, G466V, G469A, G469E, and G469V	5%	1 day (up to 24 samples/assay)	CE-IVD marked on PyroMark Q24 MDx platform
AS real-time PCR methods				
Laboratory Developed Test	Theoretically all known nucleotide substitutions could be recognized by this method. Every variant is detected by specific set of primers and probes. Detection system: Customized.	<1%, strongly dependent by the optimization of the method	3 h	Home-brew test. Serial dilutions of mutated DNA in wild-type should be used to validate the method. Several nucleotide substitutions could be tested. Control positive and negative samples could be analyzed in every assay. Linearity, dynamic range and limit of quantification of the assay should be evaluated to use it as a quantitative method.
cobas® 4800 BRAF V600 Mutation Test (Roche)	Only for *BRAF* p.Val600Glu, it is important to pay attention to the declared cross-reactivity with p.Val600Lys. Detection system: Two different fluorescent dye-labeled TaqMan probes are used to detect the mutated and the wild-type allele.	p.Val600Glu mutation at >5%	<8 h, from the DNA purification	CE-IVD marked on cobas® 4800 System, v2.0
therascreen BRAF RGQ PCR Kit (24) CE (QIAGEN)	Four assays: – p.Val600Glu (GAG) and complex (GAA) – Val600Asp – Val600Lys – Val600Arg Detection system: Allele-specific amplification is achieved by Amplification Refractory Mutation Specific (ARMS) primer-design. The detection of amplification is performed by using Scorpions primers and probe.	– p.Val600Glu 1.82% – p.Val600Glu complex 4.31% – p.Val600Asp 3.19% – p.Val600Lys 4.34% – p.Val600Arg 4.85%	<8 h	CE-IVD marked on Rotor-Gene Q 5plex HRM instrument.
THxIDTM-BRAF bioMérieux	p.Val600Glu, p.Val600Lys Detection system: Two different ARMS primers and sets – For p.Val600Glu detection (documented cross-reactivity with p.Val600Glu complex, p.Val600Glu(+)Lys601Glu and p.Val600Asp) – For p.Val600Lys detection	5%	3 h	CE-IVD marked on the ABI 7500 Fast
Competitive Allele-Specific TaqMan® PCR (castPCR) Life Technologies Corporation	Available single assay for 54 *BRAF* variants (also rarer). Cross-reactivity should be tested. Detection system: Allele-specific FAM TaqMan® MGB probe combined with allele-specific MGB blockers.	<1%	3 h	For research use only

Pre-screening methods do not define the specific nucleotide substitution but are cost effective, and can guarantee a primary evaluation of the amplified product without manipulation and risk of contamination. Furthermore, they can represent a confirmatory test for the presence/absence of a mutation in case of ambiguous results obtained by sequencing analysis. Among them, HRMA allows the submission of the amplified samples directly to further confirmatory techniques such as sequencing or pyrosequencing.

3.2. Sequencing methods

Sanger sequencing analysis, a LDT methodology, should be available in the laboratory to develop, validate and confirm the results obtained by all the other assays and it offers the advantage to set up rapidly new assays in other regions of interest, when requested by clinicians. The principal advantage of sequencing-based methods is the possibility to identify all types of nucleotide substitutions, including rare mutations, within the target sequence. However, several limitations of Sanger sequencing have been underlined: 1) at least 20% of mutated allele is required for detection [15] (interpretation is difficult when the signal related to the mutation is low) and 2) it is time consuming and only some phases of the entire procedure are suited for automation in clinical laboratory setting.

An alternative to direct sequencing method is represented by pyrosequencing [16]. It is based on the detection of the pyrophosphate group released during nucleotide incorporation (sequencing by synthesis) by a chemiluminescent reaction. The analyzed sequence is shorter, but detection limits are lower in comparison to Sanger sequencing (around 5–10% of mutated allele) [17,18].

Commercial kits validated for the screening of the most frequent mutations in cancer, including *BRAF* mutations, are available based on pyrosequencing. This technique is mostly indicated to investigate known nucleotide substitutions: the pyrogram alteration due to a rare mutation is not easily recognizable and it often requires a secondary level of investigation. In addition, assay reliability can be more easily affected by the quality and quantity of the amplification product.

3.3. Allele-specific (AS) real-time PCR methods

AS real-time PCR is a molecular method used to detect known mutations in clinical samples using an amplification system selective for mutated allele and a fluorescently-labeled-probe based detection to increase the assay sensitivity. This technique is considered significantly more sensitive than sequencing (it may detect $<1\%$ of mutated DNA) [19,20]. It is particularly indicated to analyze hot spot mutations in FFPE samples with low tumor cells content. As reported in Table 1, several PCR primer and probe chemistries are now available as IVD (In Vitro Diagnostic devices) methods for *BRAF* mutations detection. A disadvantage of the IVD AS real-time PCR assays is that they have been developed only for the most frequent mutations. To avoid the risk of false negative results occurring with rarer mutations, laboratories with long-standing experience in molecular tests use LTD assays for a customized panel of parameters. In allele-specific (AS) real-time PCR methods the absence of cross-reactivity of the probes among different nucleotide substitutions affecting the same codon should be always evaluated to guarantee the specificity of the assay for the characterization of the genetic variant tested.

3.4. Other allele-specific methods

In clinical laboratories several other methods are currently in use to perform the direct characterization of BRAF mutations. In particular, two different allele-specific methods can be mentioned in this category: SNaPshot [21] and Strip assays. Both have been developed for the easily recognition of known sequencing variations (e.g. single nucleotide polymorphisms) and subsequently have been adapted in terms of sensitivity for the detection of somatic mutations in cancer samples.

SNaPshot test allows the rapid and simultaneous identification of the most common hot spot mutations in cancer genes. The chemistry is based on the dideoxy single-base extension of an unlabeled oligonucleotide primer. The ddNTPs are fluorescently labeled and the fluorescence color readout reports which base was added after the analysis of the generated fragments by a capillary electrophoresis. SNaPshot can analyze up to ten nucleotide substitutions in a single reaction but it is not a closed tube method. The technique gives the specific characterization of the substitution but rarer mutations generally are not investigated.

Similarly, Strip assays are generally pre-designed by manufacturer to test the most prevalent mutations identified in oncogenes. Several kits are now commercially available and require standard laboratory equipment only. For example, ViennaLab KRAS — BRAF StripAssay (ViennaLab Diagnostics GmbH, Vienna, Austria) method is based on reverse-hybridization of biotinylated PCR products and the presence of a mutation is shown by an enzymatic reaction, which happen directly on the strip of the assay, already visible by naked eye or by using a scanner [22].

3.5. Emerging technologies in somatic mutation detection

Recent technological advancements have radically changed the landscape of medical sequencing providing fast, inexpensive and accurate DNA sequencing data. The high demand for low-cost sequencing has driven the development of high-throughput sequencing (or next-generation sequencing, NGS) technologies that utilize clonally amplified or single-molecule templates to parallelize the sequencing process, producing thousands or millions of sequences concurrently [23].

NGS technologies are now being adopted in clinical settings mainly focused on cancer-targeted gene panels often including *BRAF* analysis. The American College of Medical Genetics and Genomics has recently developed specific professional standards and guidelines [24] to assist clinical laboratories with the validation of NGS methods and platforms.

In addition, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) represents a different technological approach that has been proven as a suitable method for the screening of somatic mutations in several cancer types. The application of the MALDI TOF MS to the analysis of somatic mutations has led to the development of a dedicated platform for profiling hundreds of hot spot oncogene mutations in parallel. Sequenom's MassARRAY® system (Sequenom, San Diego, CA) is based on the detection of the extension products obtained by a PCR amplification of the sequence of interest followed by a locus-specific single-base primer-extension reaction [25]. Commercially available specific panels comprise more than 50 oncogenes and tumor suppressor genes previously identified and associated to several neoplastic diseases [26].

An example is represented by the MelaCarta™ v1.0 Panel (Sequenom) composed of a set of pre-validated assays for a cost-effective and efficient mutation screening focused on the identification of the mutational status of *BRAF* and other common genes involved in melanoma.

Despite these high-throughput technologies represent the imminent future in the studies of genetic alterations in cancer, the routine use of these wide analysis is not yet effective in clinical laboratories. Indeed, in the context of targeted therapy it is necessary to follow the specific request outlined by clinicians, i.e. the assessment of the mutational status of one or few hot spots in a single gene, while how it can be used and which is the potential significance of the incidental information, provided by the analysis of multiple genetic loci, remain to be determined.

However, it is important to highlight that in the recent years, the applications of these new technologies as research tools are providing genetic knowledge strictly connected to mechanisms of therapy's resistance and/or disease's recurrence and moreover useful targets for the discovering of novel therapeutic agents. Therefore, the initiation of clinical laboratories toward systems employed for the parallel analysis of multiple genetic markers should be considered in order to meet the need of detecting a growing number of genetic markers with clinical significance.

4. Laboratory performance evaluation

Several issues, deriving from the ISO 15189:2012, should be taken into account in a molecular laboratory dealing with somatic mutation

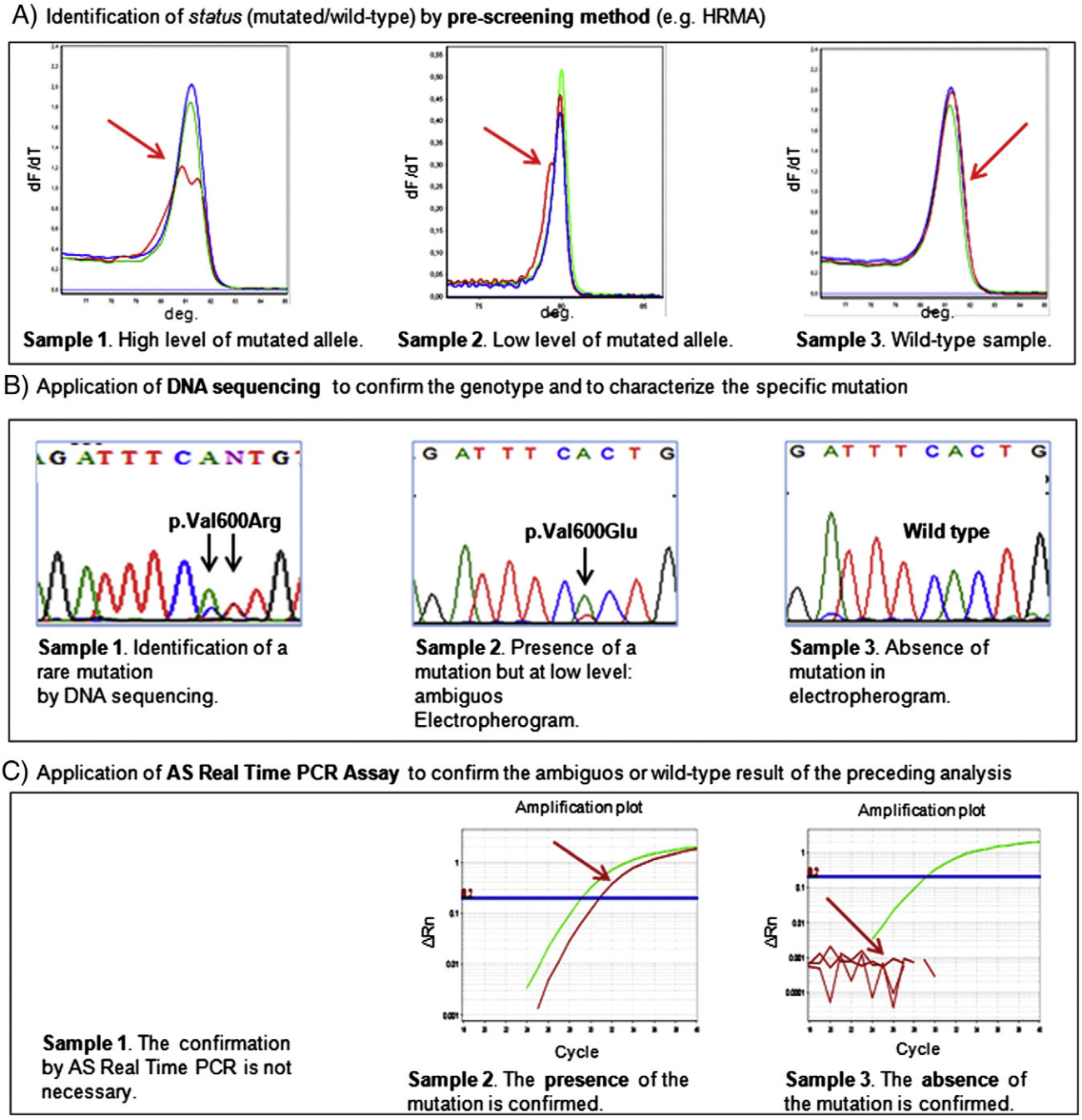

Fig. 1. Methods for *BRAF* analysis used in the Clinical Laboratory of the University of Florence. Description of the multimodal and sequential analysis of *BRAF* mutations in three characteristic cases of FFPE samples by using a pre-screening method (HRMA), the Sanger sequencing and an allele specific real-time PCR method. Panel A) HRM analysis is performed on unknown samples (red line) together with control samples (wild-type references are represented by green and blue lines). The detection of double peaks in the melting graph suggests the presence of a sequence variant in the sample. 1: Mutated sample; 2: sample with a small fraction of mutated DNA; and 3: wild-type sample. Panel B) Sanger sequencing analysis is applied to confirm and characterize the specific nucleotide variants. 1: The presence of a rare *BRAF* mutation (p.Val600Arg) is clearly detectable in the electropherogram. 2: The peak relative to *BRAF* p.Val600Glu mutation is present but at low level, it could be misinterpreted. 3: Sequence variations are not displayed in the wild type sample. Panel C) AS real-time PCR assay specific for p.Val600Glu (cast-PCR, Life Technologies Corporation) is used to confirm the presence or the absence of a low-level mutation. 1: *BRAF* testing gives a conclusive result with the identification of a mutation after the first screening so the confirmatory test is unnecessary. 2: The ambiguous outcome obtained by the previous screening is supported by the result of the AS real-time PCR and the presence of the p.Val600Glu is confirmed. 3: The absence of mutation in the sample is confirmed by a more sensitive assay.

analysis to ensure a high degree of quality. They include: 1) the availability of a suitable biological starting material (pre-analytical phase requirements); 2) the best strategy to use to fit the purpose; 3) the choice of internal quality control materials; and 4) the participation to international external quality control programs.

4.1. Pre-analytical phase

The reference pathologist plays a key role in the selection of tumor samples to be submitted to the assessment of somatic mutation test. To date, the most commonly used sample for *BRAF* testing is formalin-fixed paraffin-embedded (FFPE) tissue. The pathologist is involved in the evaluation of the FFPE block in order to guarantee: i) an adequate amount of tumor sample containing at least 50% of tumor cells and ii) detailed information about the presence of pigmented areas which could compromise the analytical phase (particularly relevant in melanoma specimens).

When necessary, the pathologist will perform the enrichment of the tissue sample, by using e.g. manual macrodissection or laser-assisted microdissection, to provide a suitable sample for the analysis (to reach at least 50% of tumor cells in the specimen).

The tissue sample should be sent to the laboratory of molecular biology, accompanied by an explanatory document, prepared by the pathologist, which contains all the information necessary for its identification and useful for the preparation of the genetic test.

4.2. Analytical performance evaluation

In light of the relevant impact of these tests on the choice of patients' therapeutic treatment and due to the limitations of each of the above reported methodologies, the selection of a unique method for sample analysis is often inappropriate. To guarantee a qualified response, the final result should derive from the use of more than one technique, as a matter of fact the application of several methods on the same sample can be complementary or, at least, confirmatory. In Fig. 1, we report the workflow followed in our laboratory for *BRAF* mutation detection in melanoma tissue samples.

A multi-technique approach can overcome the limits, in terms of specificity and sensitivity, of a single-assay analysis. The use of different technologies and the application of a multi-methodological approach to test the presence of *BRAF* mutation turn out common within many laboratories that perform this assay. The Final Report edited by the European Molecular Genetics Quality Network (EMQN), following the results obtained in the *2012 Pilot EQA scheme for BRAF testing in melanoma*, shows that the methods more frequently used by participating laboratories were sequencing and cobas® 4800 BRAF V600 Mutation Test (Roche) and that 15 laboratories out of 27 have chosen to use a combination of different methodologies, to confirm principally the first result obtained by a pre-screening method or an allele-specific assay (Fig. 2).

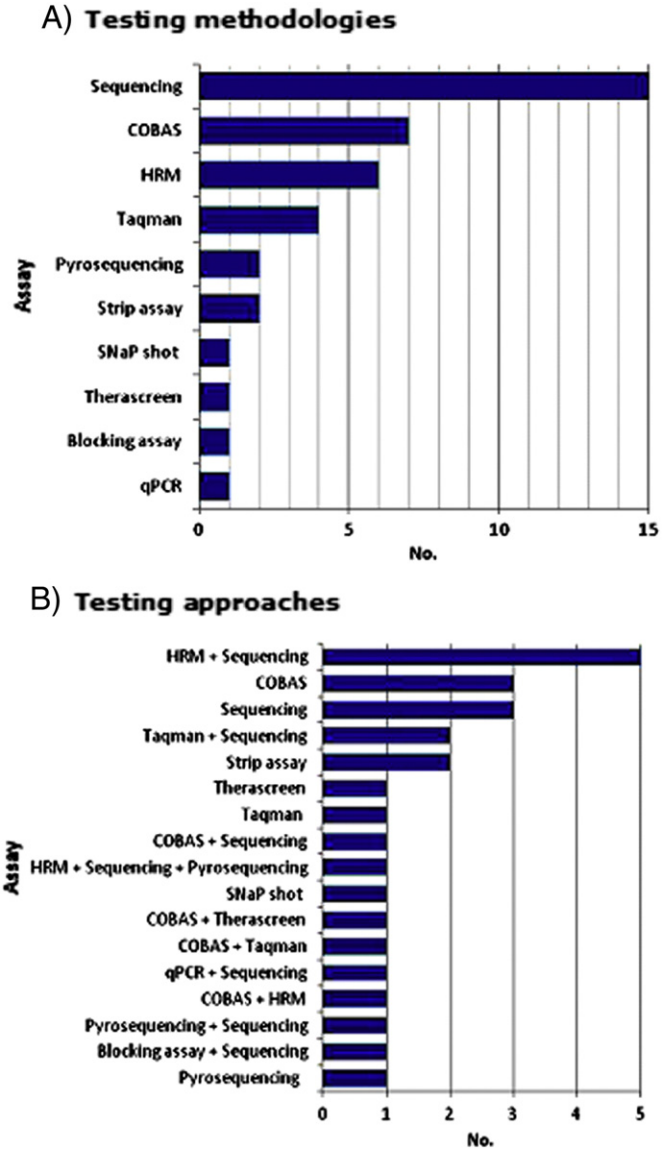

Fig. 2. Distribution of testing methodologies (panel A) and testing approaches (panel B) used by participating laboratories in EMQN *2012 Pilot EQA scheme for BRAF testing in melanoma* (figures extracted from the Final Report and shown by the permission of the EMQN).

4.3. Internal quality control materials

Commercially available controls, cell lines or plasmids (see MM14 CLSI document, reference [27]) can be used as internal quality control. In case of FFPE samples, internal quality control should include pre-characterized FFPE tissues or fixed cell pellets in the matrix used for histological preparation of samples that can mimic the biological characteristics of patients' specimens. In particular, in the context of somatic mutation analysis, the use of reconstituted samples harboring a different percentage of mutant allele up to the detection limit of the technique used is also strongly recommended for monitoring the performance of each experimental session. In our laboratory SK-MEL-28 or A-375 (human melanoma cell lines, homozygous for *BRAF* p.Val600Glu) and HT1197 (human bladder carcinoma cell lines, wild type for *BRAF*) cell lines are routinely used as the positive and negative controls in BRAF V600E testing.

4.4. External quality control programs

For routine clinical chemistry tests, well-developed systems for peer inspection and benchmarking (such as external QA schemes) exist in all countries; on the contrary, EQA programs of molecular diagnostics are less common and, in some cases, they are not target-specific, but more generically addressing each single step of the test procedure (pre-analytical, analytical or post analytical phase: see reference [28]).

Last year EMQN launched an EQA program to evaluate the performance of laboratories in the detection of somatic mutations in genes involved in specific tumor types as lung cancer, colorectal cancer and melanoma.

The determination of mutational status of *BRAF*, *NRAS* and *KIT* genes by PCR-based analysis techniques represents the target of the EQA intended for melanoma. The scheme calls for the assessment of the genotyping and the biological and clinical interpretation on ten mock

clinical samples according to the procedures adopted routinely in the participating laboratory. The samples employed are represented by rolled sections of paraffin embedded matrix samples obtained by a serial dilution of mutated cell lines into wild type cell lines. They represent a good standardized starting material to evaluate the performance in the purification and analysis steps of tumor DNA deriving from FFPE samples. Alternatively, an EQA scheme organized by the RfB Institute (Referenzinstitut für BioanalytiK) has adopted lyophilized DNA samples ready to use for the amplification. The choice of the most appropriate EQA scheme by a laboratory should be dictated by the interest for the evaluation of a specific step or all phases of an assay (pre-examination, examination and post-examination). The results of the external quality assessment of BRAF molecular analysis in melanoma provided by United Kingdom National External Quality Assessment Service (UK NEQAS) have been published recently showing a high standard of testing by laboratories over a period of 12 months and confirming EQA schemes as a mechanism by which educational advice and reference material can be supplied [29].

5. *BRAF*: laboratory specific report for clinicians

Reporting of mutational testing should be carried out by the clinical laboratory in accordance with the standard procedure of the membership organization. The *Guidelines of Quality Assurance in Molecular Genetic Testing* (http://www.oecd.org/science/biotech/geneticsandgenomics.htm) provides guidelines on reporting, although not specifically referred to somatic mutation detection.

If the result entry into the laboratory information system is carried out manually, a system to prevent transcription errors should be in place, e.g. by double-checking by one or more molecular biologists and moreover by the supervisor of the laboratory before validation signature.

The report should include data for patient identification and a detailed description of the sample, i.e. the type of biological material, specific identification data and percentage of tumor cells (information provided with the tissue sample in the accompanying form compiled by the pathologist) along with the reason for the medical prescription.

Molecular Laboratory letterhead
[LOGO, contact details]
[Accreditation or other equivalent recognition]

Patient identification data:
_Name:_____
_Surname: _____
_Sex: _____
_Date of Birth: *dd/mm/yyyy*

Date of arrival: *dd/mm/yyyy*

Exam No: *progressive internal reference number/year of the exam*

Test required by: *referring doctor and provenance of biological sample*

Examined material: DNA extracted from_____ [e.g. *FFPE sections* (10µm thick), *H&E slide*,...] related to the inclusion number: *external reference number*.

Pathologist's evaluation: Tumoral cells content of the provided material: > %.

Pre-analytical procedure:_____ [e.g.*YES/NO micro/macro-dissection performed*].

Reason for the request: suitability for BRAF inhibition therapy in a patient with malignant melanoma. Informed consent for this test has been obtained from patient.

Test required: analysis of the coding region of *BRAF* exon 15 to detect the presence of somatic mutations.

Methods used: [e.g. *DNA amplification by PCR, High Resolution Melting analysis to detect heterozygous samples followed by direct sequencing (Sanger). Further data confirmation by Real-Time PCR assay, specific for p.Val600Glu variant detection*].
Sensitivity of the methodological approach: _____ [*% mutated allele detected respect to wild-type DNA*].

Results:
***BRAF*, exon 15 (NM_004333.4)**: detection of the p.Val600Glu (c.1799T>A) mutation.

Conclusion:
The genetic variant p.Val600Glu (c.1799T>A) has been previously described and is included in the database of somatic mutations in cancer (e.g. COSMIC). The clinical relevance of this variant is supported by several international studies: ***REFERENCES***.

Suitability for BRAF inhibition therapy is confirmed.

Molecular biologist signature ***Supervisor signature***

Date of report: *dd/mm/yyyy* *Page 1 of 1*

Fig. 3. Example of a Clinical Report for *BRAF* analysis.

If different analytical procedures are adopted in the laboratory, they should be reported and their sensitivity should be specified. In addition, it is advisable to indicate the participation to proficiency testing scheme as evidence of the quality assurance guaranteed by the laboratory. This is particularly important when dealing with LTD tests.

The use of internationally accepted nomenclature is mandatory: the description of sequence variants at DNA and protein level and the target reference sequence should be expressed in accordance with the guidelines of Human Genome Variation Society (http://www.hgvs.org/mutnomen/). Moreover, to support the clinicians in the interpretation of the molecular findings, references from the international literature, concerning the result of the molecular test, should be included in the report conclusions.

An example of a Clinical Report for BRAF analysis performed in the Clinical Biochemistry laboratory at the University of Florence is reported in Fig. 3.

6. Discussion

Some significant aspects needed to implement a companion diagnostic in routine clinical practice have been discussed by the WG-PLM based on the procedures used and results obtained in the Clinical Laboratory of the University of Florence, Italy. Table 2 summarizes the main issues discussed in this paper, which are considered essential for the implementation of a new molecular test in a clinical laboratory.

BRAF testing represents just an example of all molecular assays in cancer diagnostics required to assist in estimating prognosis and predicting responsiveness of a targeted therapy. Analogous molecular analysis in other tumor types is expected to grow in the future with the increment of knowledge about these biomarkers. As *BRAF* mutational status test represents a companion diagnostic for Vemurafenib treatment in metastatic melanoma patients, it is crucial to highlight the issue of analytic specificity and to emphasize the importance of analytic sensitivity with respect to mutational analysis. Moreover, somatic genetic testing is characterized by a number of unique features that differentiate it from other types of molecular diagnostic assays, adding complexity to test performance and clinical interpretation. The cooperative work involving pathologists, molecular biologists and technicians is a key step for an accurate and sensitive analysis of somatic mutations aimed to the identification of a personalized therapeutic regimen.

The importance of the implementation of a companion diagnostic in the clinical laboratory for the selection of patients in relation to a treatment is an emerging concept of the personalized medicine. The monitoring and standardization of the entire workflow process of companion diagnostics (the sample handling, the analytical validity and the management of the biological sample and personal health information after the processing) need further discussion and improvements taking into consideration the rapid development of knowledge in this field and the availability of new diagnostic tools which could improve the future individual patient's treatment quality.

Furthermore, when a companion diagnostic test is provided as part of routine clinical practice it is desirable to monitor its utility in collaboration with clinicians.

A central phase in the evaluation of a biomarker is the assessment of its clinical validity. The clinical validity of a biomarker can be defined as its ability to discern in the patient population those patients with the target conditions, i.e. the patients that will benefit from a specific treatment. The increment in the therapeutic benefit should be also measured

Table 2
Relevant aspects to be fulfilled before implementation of a companion diagnostic test in the clinical laboratory.

I. Aims.
- Definition of the medical rationale (diagnostic, therapeutic, prognostic)
- Identification of the patient population
- Determination of expected clinical and technical performances

II. Choice of method for mutation analysis.
- Selection of the appropriate assay concerning technical aspects (molecular target and specimen):
  i. Sample preparation procedure (pathologist evaluation; micro- or macrodissection; nucleic acid extraction methods)
  ii. Source of sample to be assayed (blood; tissue frozen or FFPE; cytological biopsied samples; …)
  iii. Type of mutation under study (hot spot or unknown; single nucleotide substitution or complex mutation; reported frequency in samples; germinal or somatic mutation)
  iv. Sensitivity, specificity, coverage (i.e. spectrum of identifiable variants) of the method
- Selection of the appropriate assay concerning logistic aspects:
  i. Laboratory equipment
  ii. Personnel competences
  iii. Costs
  iv. Turnaround time

III. Quality management system for molecular laboratory performance.
- Management of the pre-analytical phase in collaboration with clinicians and pathologists:
  i. Preparation of a suitable biological starting material by the pathologist
  ii. Preparation of an accompanying document with useful information (specimen identification, sample preparation and any special characteristics, requested test and motivation)
  iii. Identification of critical samples (e.g. small or poorly preserved material and the presence of interfering substances)
- Evaluation of analytical performances:
  i. Definition of the own properties and limitations of available methodologies
  ii. Availability of multi-methodological approaches to obtain complementary and/or confirmatory results
  iii. Systematic examination of the literature study and comparison with methodological upgrade
- Internal quality control materials:
  i. Routine use of reference materials (positive and negative control samples)
  ii. Collection and storage of samples with different types of mutations
  iii. Preparation of series of samples with different percentages of mutated allele
  iv. Verification of the performance with samples from different biological matrices
- External quality control:
  i. Establishment of a peer inspection system among local laboratories with analogous specialties
  ii. Participation to international proficiency testing (external quality assessment programs)
- Elaboration of a laboratory specific report for clinicians

IV. Post-examination considerations.
- Correlation of molecular results with the clinical context
- Clinical validation of the new molecular test
- Evaluation of clinical and financial impact of the results

and monitored in relation to not only the patient benefit but also considering the impact on the entire public health sector. Molecular testing, used for the stratification of patients in relation to the choice of a therapy, can in fact bring a considerable savings due to the reduction of inappropriate or non-effective therapeutic prescriptions. The continuous emerging of new technologies has led to the development of HTA (Health Technology Assessment) agencies in the several countries for defining the criteria which should be used in the evaluation of a diagnostic test and moreover for regulating the conscious use of technology in the medical field, particularly in relation to the equitable distribution of the resources in the society and the impact of personalized medicine on the economic system.

Conflict of interest disclosure

The authors declare no conflict of interest.

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