



MOLECULAR DIAGNOSTIC METHODS FOR EARLY DETECTION OF BREAST IMPLANT-ASSOCIATED ANAPLASTIC LARGE CELL LYMPHOMA IN PLASTIC SURGERY PROCEDURES

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Abstract – Background: *Genotyping is crucial to the identification of genetic markers underlying development of neoplastic T cell diseases and individual molecular monitoring of minimal residual disease. However, these methods need to fulfill the principles of analytical validation to determine their suitability to assess single point mutation in target genes.*

Methods: *This review outlines current knowledge of the genetics of various forms of ALCL and new genetic markers in ALK-negative ALCL frequently recurred in Breast Implant-Associated Anaplastic Large Cell Lymphoma (BIA-ALCL). For the appropriate choice of any method, several criteria must be considered: i) known genetic variants in a given cancer gene; ii) diffusion and availability of large platforms and required equipment; iii) suitability of tests for routine diagnostics; iv) capacity of methods to offer a specific and sensitive detection of mutant alleles within great excess of wild-type alleles in a given sample; v) cost-effectiveness.*

Conclusions: *This review is intended to provide the reader with a better understanding of the various technologies available in the routine clinical laboratory for early detection of genetic markers of cancer T cell. Considerations about ways to most properly assess the analytical performance of these methods, are also given.*

Based on the criteria proposed here, lab managers, surgeons and clinicians may evaluate advantages and limitations of the different analytical platforms and possibly identify the most appropriate one according to specific operative settings for management of BIA-ALCL patients.

KEYWORDS: *T cell lymphoma, Genotyping methods, Analytical validations, Molecular diagnostics.*

List of abbreviations: Single nucleotide polymorphisms (SNPs), Single-strand Conformational Polymorphism (SSCP), Allele Specific Amplification (ASA), Amplification Refractory Mutation System (ARMS), Restriction fragment length polymorphism analysis (RFLP), Peptide Nucleic Acid (PNA), Matrix-Assist-

ed Laser Desorption/Ionization time of flight (MALDI TOF), Fluorescent Resonance Energy Transfer (FRET), Locked nucleic acid (LNA), Oligonucleotide ligation assay (OLA), High Resolution Melting (HRM), Quality Controls (QC), External Quality Assurance Programs (EQAP).



INTRODUCTION

The neoplastic nature of breast implant associated ALCL (BIA-ALCL) is reflected in the morphology of the anaplastic cells, an aberrant T-cell immunophenotype, and the finding of monoclonal rearrangements of the T-cell receptor genes. Moreover, clonal cytogenetic abnormalities were detected in a few instances in which cells were cultured ¹.

BIA-ALCL is a rare type of Lymphoma; it is not a cancer of the breast tissue itself. Very limited clinical follow-up is available, and therefore the natural history of this disease is unknown. Some studies have suggested that BIA-ALCL is clinically indolent presenting a late seroma after several years from implants with a median of 8 years ranging from 2 to 28 years ^{2,3}. Differently, some case reports have raised concerns that this lymphoma can be aggressive⁴. However, for these issues, it is essential to early detect T cell developing lymphoma. Phenotypically, BIA-ALCL is defined a neoplasm of large lymphoid cells with abundant cytoplasm and pleomorphic nuclei of T-cell lineage that uniformly expresses CD30 and is negative for anaplastic lymphoma kinase (ALK) protein or translocations involving the ALK gene at chromosome 2q23. In most patients, the tumor presents as an effusion around the breast implant. The tumor is not identified grossly but neoplastic cells are detected microscopically within the effusion or lining the fibrous capsule surrounding the implant. In a minority of patients, the tumor

cells form a mass that is detected by radiologic or gross pathologic examination⁵.

Actually, the best approach for managing patients with breast implant associated ALCL is controversial. A review of management modalities applied to these patients could help in elucidating appropriate management such as longer clinical follow-up of these patients might also be useful to better define the natural history of breast implant associated ALCL.

In our study, we will report a panel of somatic mutation known to be oncogenetic for the transformation of normal T-lymphocytes to anaplastic large T cells (Table 1).

To date, the genetics of T-cell lymphomas is poorly clarified. The only well-characterized abnormality is the translocation involving ALK, absent in ALK negative lymphomas. The majority of cases (74-90%) shows clonal rearrangement of TCR genes and other genetic variants ⁶. CGH studies indicate a tendency of ALCL-ALK negative to differ both from PTCL-NOS and from ALCL-ALK positive⁷. Similarly, gene expression profiling studies suggest that ALCL-ALK negative has a distinct profile. Recurrent IRF4 (interferon regulatory factor-4) translocations were recently found in PTCL-NOS and cutaneous ALCL and may represent a diagnostic tool to distinguish these entities from ALK-negative lymphomas, which lacked this translocation⁸. Recently, the translocation t(6;7)(p25.3;q32.3) was found in ALCL-ALK negative. The 6p25.3 disrupted DUSP22, a dual specificity phosphatase that

TABLE 1. Most common genetic abnormalities found in anaplastic large cells lymphoma.

GENES	Molecular effect	Clinical Effect
DUSP22	Rearranged in about 30% of cases; variable partners (FRA7H most common); associated with favorable prognosis	Clinico-pathologic features, including presentation as localized crops of papules in the elderly, large transformed cells in the dermis with smaller atypical cells infiltrating the epidermis in a pagetoid reticulosis-like fashion, and biphasic staining intensity for CD30 (weaker in the epidermal component).
TP63	Rearranged in about 8% of cases; variable partners	(TBL1XR1 most common); associated with poor prognosis
Higher Expression BATF3, CCND2, CCR7, CD80, CD86, CNTFR, IL21, IL22, MSC, POPDC3, TMEM158, TMOD1, TNFRSF8, ZNF267		Gene expression profiling has not yet found routine clinical use for ALCL, it has had a clear impact on elucidating ALCL biology.
Low expression CDKN2D		As above
TCR re-arrangements	Rearranged in all BIA-ALCL patients	It is a test suitable to identify a clonal T cells expansion. The test TCR ab/gd is available routinely in specialized molecular diagnostic laboratory.

inhibits T-cell antigen receptor signaling in reactive T-cells by inactivating the MAPK, ERK2⁹. DUSP22 expression has a tumor suppressor function and the translocation resulted in DUSP22 deregulation. Large screening of late seromas recruited from follow-up of patients with BIA-ALCL, as well as newly identified patients who were suspected to ALCL, will drive the development of new genotyping panel assay suitable to early detection of the neoplastic T cells. We believe that these data could contribute to:

- a) an early diagnosis of BIA-ALCL in seromas samples;
- b) a better understanding of transformation of normal T-Lymphocytes into ALCL of patients with BIA-ALCL;
- c) may be helpful in designing the most appropriate approach for patient management and personalized therapy.

Here, we are highlighting some of the most useful technologies able to detect low mutant DNA in a large excess of wild type DNA, currently used in specialized laboratory, making the transition from the research setting to the clinical laboratory¹⁰.

GENOTYPING METHODOLOGIES FOR DETECTION OF KNOWN MUTATIONS

The type of mutation and its allele heterogeneity define the choice of a specific genotyping detection assay for identification of mutations. In case of BIA-ALCL patients, methodology platform must be able to detect low mutant T cells in the wide range of wild type cells. A recent breakthrough in high-throughput strategies is represented by Next Generation Sequencing (NGS) technology, which allows the combined detection and identification of mutations. However, for many applications an appropriate platform will be available in the forthcoming years. Thus, conventional screening methods for point mutations and small deletions will most probably keep their place in the diagnostic laboratory for a reasonable amount of time. Costs, however, are projected to be high and assay performance and results interpretation will remain strictly dependent on the availability of highly qualified and well-trained personnel¹¹.

Mutation-detecting technologies can be divided into two major categories depending on the capacity to screen for new mutations or to identify already known mutations. Moreover, all genotyping technologies must fulfill two necessities: i) discrimination between alternative alleles (i.e. mutant vs. wild type); ii) detection of both alleles (i.e. mutant and wild type) in a given DNA sample. The only plat-

form able to fulfill both tasks in a single step assay is the Matrix-Assisted Laser Desorption/Ionization time of flight (MALDI TOF). Alternatively, several allele discrimination methods combine PCR-based methods with hybridization probes.

The most used platforms for the detection of known SNPs can be operatively classified in three major categories of PCR-based methods (Table 2): i) agarose gel-based detection; ii) fluorescence-based detection; iii) high throughput platforms.

The techniques listed in Table 2 show many overlaps and attempts to compare each other may result difficult and unproductive. In almost all assays, DNA amplification is required. In addition to sample pre-treatment for mutation detection by other methods, PCR is directly used in a variety of detection assays, such as allele-specific primer extension (ASA), PNA mediated clamping-PCR and allele-specific probe hybridization.

In each case, subsequent re-sequencing represents the unique method for the definitive confirmation of results.

Promises of recent high-throughput NGS technologies are represented by platforms such as: ISeq by Illumina (Cambridge, UK), SOLiD by Life Technologies (Foster City, CA, USA), Ion Torrent PGM by (Thermo Fisher, Waltham, MA, USA) and FLX by Roche Diagnostics (Brandfort, CT, USA). These kinds of ultrafast DNA sequencers needed several steps of DNA amplification and purification, generation of a single-stranded template DNA library, big amount of data results elaborated by specific bioinformatics tools, but finally they provide a consensus base accuracy of 99%.

TABLE 2. Widely used methods for genotyping at molecular level.

<i>Methods for detection of known mutations</i>
<i>Gel based detection</i> – Allele Specific Amplification (ASA) – Restriction Fragment Length Polymorphism (RFLP) – Single strand conformation polymorphism (SSCP) – Peptide nucleic acid- mediated Clamping PCR
<i>Fluorescent-based detection</i> – FRET probe Allelic Discrimination (Hyb Probe®, TaqMan®, Beacons®, Scorpions®) – Locked Nucleic Acid (LNA) probe – Invader® Assay – Pyrosequencing* – High resolution melting (HRM)
<i>High-throughput sequencing</i> – Next Generation Sequencing – Maldi-TOF Mass Spectroscopy – Sanger-based Conventional sequencing

*Required pre-PCR step.



When a large panel of SNPs assays needs to be developed, and budget for instruments is limited, methods based on conventional PCR followed by a gel-based detection assay should be preferred over fluorescent hybridization-based methods. Another advantage of electrophoretic detection systems is the possibility to check for the appropriate size of amplicons. We describe these methods as low-throughput, due to their time-consuming and labor-intensive characteristics. However, fluorescent-based detection systems have been developed for application in routine laboratories, due to their high specificity, high sensitivity and medium/high-throughput.

Gel loading-based detection.

LOW- THROUGHPUT ALLELE SPECIFIC AMPLIFICATION (ASA)

The method is based on a PCR performed in two parallel reactions. In the first reaction, the 5' primer is complementary to the wild-type sequence; in the second reaction, the 5' primer is complementary to the mutant or polymorphic sequence. Assuming that elongation occurs only when primer and target sequence match completely, only one allele of either mutant or wild-type DNA is amplified. Two different approaches have been described. The first approach is characterized by the lack of primer elongation due to a mismatch at the far 3'-end of the primer. This method has been named ARMS "Amplification Refractory Mutation System"¹² and developed by DxS Diagnostics (Manchester, UK). In the second approach, the mismatch is detected within the primer, avoiding primer annealing when mis-pairing occurs; methods based on this principle are defined AS-PCR "Allele Specific PCR"¹³. The specificity and sensitivity of the method is strongly influenced by the ratio of mutant to wild-type DNA. Limit of detection and identification of a homozygous or heterozygous state is the primary application of the ASA. Performance and quality assessment is crucial, since the possibility of false positive or negative results is the major limitation of ASA. False-positive results may be due to contamination or artificial point mutation introduced by mis-annealing of the primers specific for the mutated allele.

PCR-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS (RFLP)

This is a commonly used method including a gel electrophoresis-based technique, such as PCR, coupled with RFLP analysis. With this method, specific DNA sequences can be amplified, the PCR products are then digested with appropri-

ate restriction enzymes and visualized by gel electrophoresis. It is clear that major limitation of the PCR-RFLP method is the absolute requirement that the point mutation modifies a restriction enzyme cutting site¹⁴. For same point mutation or SNPs that reside in sequences one nucleotide away from endonuclease restriction sites, allele-specific primers introducing a point mutagenesis may be used to generate artificial mutation sites for RFLP¹⁵. Detection limit of simple RFLP analysis is of one mutant cell out of 50 to 100 non-mutant cells¹⁶. For RFLP analysis, a specificity of 100% can be achieved when appropriate restriction enzymes are used.

PNA-MEDIATED CLAMPING PCR

The peptide nucleic acid (PNA)-based PCR procedure has been developed for the selective enrichment of mutant allele-specific amplicons within a large excess of wild type alleles¹⁷. PNA is a synthetic DNA analog in which the normal phosphodiester backbone is replaced by a non-polar 2-aminoethylglycine chain¹⁸. Two essential features make PNA a superior PCR clamp oligonucleotide for specific alleles: i) PNA cannot be useful as a primer for polymerization, nor it can be a substrate for exonuclease activities of Taq polymerase; ii) the melting temperature (T_m) of a perfectly matched PNA-DNA duplex is higher than that of DNA-DNA of the same length, but a single mismatch destabilizes the PNA-DNA hybrids, causing a T_m shift of 10-18°C. Therefore, PNA can specifically block primer annealing and/or chain elongation on a perfectly matched template without interfering with templates carrying mismatched bases¹⁹. In this way, a target mutant DNA can be specifically detected in a large excess of wild type DNA. Detection limit of analysis is of one mutant cell over one hundred-thousand wild type cells²⁰. To improve sensitivity and throughput, it should be possible to carry out a large-scale screening in an automated manner by using matrix-assisted laser desorption/ionization time of flight (MALDI TOF) mass spectrometry²¹. Moreover, the large T_m difference between perfectly matched and mismatched hybrids makes PNA a good sensor of point mutations²².

Recently, fluorescent PNA probe was combined with PNA-mediated PCR clamping for detection of variant BCR/ABL allele in leukemia²³ and of K-Ras mutation in pancreatic cancer²⁴.

The key feature of this procedure is that a PNA oligomer bound with fluorophore serves both as PCR clamp and sensor probe, which allows the discrimination of sequence alterations in mutant codons from the wild-type sequence²⁵.

Fluorescent-based detection. Medium/high- throughput

FLUORESCENT RESONANCE ENERGY TRANSFER (FRET) BASED ALLELIC DISCRIMINATION

Many of the probe-based systems rely on the principle of FRET for signal generation. FRET involves the non-radioactive transfer of energy from a donor molecule to an acceptor fluorophore molecule causing its excitation and then light emission. Using FRET hybridization probes, a donor and an acceptor fluorophore present in two different probes, co-localize, after hybridization, to an adjacent region on the target molecule. Based on this chemistry several platforms for genotyping have been developed (as major players): Hyb-Probe® (Roche Diagnostic, Indianapolis, IN, USA) TaqMan® (Applied Biosystems, Foster City, CA, USA), Beacons® (Public Health Research Institute, Inc. NJ, USA) and Scorpions® (DxS Manchester, UK). Comparing Hyb-Probe chemistry to conventional RFLP, researchers obtained a 100% concordance test results and concluded that Hyb-probe assay is reliable, economical, and can be performed by less trained technologists²⁶. An alternative method for polymorphism detection is the TaqMan-based allelic discrimination assay, which combines the use of a standard pair of PCR primers, designed to amplify the region containing the point mutation of interest. Each detection probe is labeled on 5'-end by a "reporter" emitting fluorescent dye (different from the other) and a "quencher" on its 3'-end. During PCR, the probes specifically annealed to their complementary sequence, are cleaved by Taq DNA polymerase (5' exonuclease), causing the separation of the reporter dye from the quencher. The relative fluorescence emitted from both probes (wt and mutated) is detected by the instrument and plotted in a 2D cluster plot allowing quantization of the amount of each specific allele present in the analyzed samples. This method can be considered of medium/high throughput; the ability to analyze multiplex samples in single tube is limited by the restricted number of fluorescent dyes with non-overlapping spectra²⁷. However, probe design is mostly empirical and optimization times are significantly increased. Therefore, the optimal use of this platform is probably achieved when a relatively small number of SNPs must be assayed on a large number of samples. Molecular beacons are oligonucleotide probes with two complementary DNA sequences flanking the target DNA sequence and with a donor-acceptor dye pair at opposite ends of each probe. When the probe is not hybridized to the target, it adopts a hairpin-loop conforma-

tion so that the reporter and quencher dyes are close together, so that no donor fluorescence is generated. When the probe hybridizes to the target sequence, the two dyes are separated and the fluorescence is dramatically increased²⁸. Since the mismatched probe-target hybrids dissociate at a consistently lower temperature than matched ones, the different T_m increases the specificity of molecular beacons. In a typical SNP genotyping, two molecular beacons with sequence matching to the wild-type and variant alleles respectively, are used in the same PCR reaction.

Scorpion is a single bi-functional molecule containing a PCR primer covalently linked to a probe. The molecules are oligonucleotide with a "Stem-Loop" tail containing a fluorophore, which interacts with a quencher to reduce fluorescence.

The possibility to use Scorpion primers for each possible mutations in a single multiplex reaction, reduce scoring mistakes in the presence of a negative results²⁹. Moreover, Scorpion chemistry, suitable for several thermal cycling platforms, is cheaper, because it only requires a conventional PCR machine combined to a fluorescent plate reader. Therefore, Scorpion technology can be easily adapted to high throughput analysis for large-scale screening programmes by using ≥ 96-well plate formats and kits standardized are likely to become available in the next future.

LOCKED NUCLEIC ACID (LNA)

The LNA is a DNA mimic displaying a very high affinity towards complementary DNA and RNA. Because the difference of melting temperature between a perfect match and a single-nucleotide mismatch is larger for LNA-DNA heteroduplex than DNA-DNA homoduplex, the discrimination of a single point mutation is easier using this chemistry³⁰. LNA technique allows the sensitive detection of rare mutations in a tissue sample containing an excess of wild-type DNA. The assays have been carefully validated and results were highly consistent with DNA sequencing. Immobilized LNA probes may also be successfully used in a multiplex SNP genotyping assay performed on a microarray platform³¹.

PCR-INVADER® ASSAY

The PCR-Invader® (developed by Third Wave Technologies) is a homogeneous assay. It is a robust genotyping method that does not require allele-specific dye-labeled probe for each every SNP marker. The use of two generic dye-labeled probes is sufficient for all SNP markers. This detection method is based on the FRET signal generated by cleavage of a doubly labeled fluorescent probe³². One of the major disadvantages



of the current technology is the need to assay the two alleles of each SNP in single reaction wells; it allows good performances for allelic discrimination between polymorphism, but it cannot discriminate low mutant DNA in a large excess of wtDNA. This reaction format makes this assay time-consuming and labor-intensive.

PYROSEQUENCING

Pyrosequencing detects *de novo* incorporation of nucleotides. The incorporation process releases a pyrophosphate, which is converted to ATP in the presence of adenosine 5'-phosphosulfate that in turn stimulates luciferase³³. A charge couple device (CCD) camera detects the light produced by the luciferase-catalyzed reaction. The novel DNA strand, complementary to the target template, is built up and the nucleotide sequence is defined from the signal peak in the pyrogram. One more advantage of Pyrosequencing is the ability to detect additional sequence flanking the polymorphic site (typically 6-30 bases). Therefore, this method can be applied for discovering unknown SNPs and turns useful for analyzing those sequences containing complex secondary structures that render difficult the application of conventional sequencing approaches. The major disadvantage of this method lays in the requirement a post-PCR cleanup step for removing unincorporated nucleotides, primers, and salts. Moreover the presence of >10 nucleotides homopolymer tracts, could complicate the analysis due to the non-linear light response after incorporation of 5-6 identical dNTPs.

HIGH RESOLUTION MELTING (HRM)

HRM is a heteroduplex DNA dissociation-melting assay using an intercalating double strand DNA Dye³⁴. Wild type and mutant DNA samples are distinguished by melting temperature (T_m) shift. The advantages of this approach are that labeling of each primer (with dye) is not needed and PCR amplification and melting analysis can be performed in the same tube/capillary, minimizing specimen handling and reducing the possibility of error and sample contamination. HRM is easy, rapid, and not expensive and has a relevant accuracy for mutational analysis in clinical practice, mainly for genotyping of genetic disorders and for the identification of somatic mutations in human cancers as: i) large sequence aberrations of FLT3 gene in AML³⁵; ii) of c-Kit mutation in GIST³⁶; iii) mutation of EGFR and HER2 gene, in lung and in head-neck cancer³⁷ and c-Kit and BRAF gene activating mutations in melanoma³⁸. Finally, HRM was also proposed as a rapid and sensitive technique for the assessment of DNA methylation³⁹.

NGS Platforms

Firstly, this is a high-throughput method, as it permits massive parallel sequencing consisting of simultaneous sequencing of multiple targeted genomic regions in multiple samples in order to reveal concomitant mutations in the same run. The reduced turnaround time of analysis, which carries to reduce clinical reporting time, is another important advantage in routine tumor sequencing. Moreover, in contrast to traditional sequencing methods, an analysis in NGS requires very low input of DNA/RNA. A variety of genomic aberrations, such as single/multiple-nucleotide variants, small and large insertions and deletions, copy-number variations (CNVs) and fusion transcripts, can be screened simultaneously with high accuracy and sensitivity. NGS allows quantitative evaluation of the mutated allele and its sensitivity is higher than Sanger sequencing (detection of 2-10% vs. 15-25% allele frequency, respectively). NGS technologies have been applied to hematological disorders in a variety of contexts: guiding diagnosis and could also be used to identify TCR gene rearrangement to establish T-cell clonality⁴⁰. Although the method is largely used, a high level of expertise is required to interpret the data precisely when it comes to rare-event detection, such as MRD. According to the type of disorder, the panel of antibodies used, the number of cells analyzed, the expertise of the laboratory and clinician called to interpret genomic results, the sensitivity for the detection of malignant cells varies^{41,42}. NGS approaches are very useful both to investigate known mutations/translocations and all clonal gene mutations and rearrangements present in diagnostic samples in order to better understand the possible evolution of MRD. Unfortunately, NGS for this purpose is not yet routinely used in clinical practice; to the best of our knowledge to date, no one diagnostic panel is commercially available for detection of genetic variants listed in Table 1.

Comparison of genotyping methodologies

Wider available methods for the detection of known point mutations and small deletions or insertions are summarized in Table 3.

None of the genotyping methods appears ideal for all situations, then the technique used must be driven by project requirements. Each assay has advantages and disadvantages and reaction conditions must be standardized for each technique.

Fluorescent-based detection systems have been developed for most of the assays described,

TABLE 3. Comparison of methods for detection of known point mutation is based on analytical validation.

Genotyping methods	Specificity	Sensitivity (Mut/Wt)	Assay* stability	Equipment# required
ASA	-Medium -High§ (sequencing)	1/100 1/10000	High	Gel electrophoresis system
RFLP	-Medium -High (sequencing)	1/1000 1/10000	Low (restriction enzyme)	Gel electrophoresis system
PNA-mediate PCR clamping	-High -Very High (Maldi-Tof)	1/100000 1/1000000	High	Gel electrophoresis system
FRET Allelic Discrimination				
a) LC probe	High	1/100	Middle (probe fluoro-labeled)	Dedicate instrumentation and software
b) TaqMan 5'nuclease (End point detection)				
c) Beacons probe				
d) Scorpions probe Hyb probe				
LNA probe	Very High	1/10000	Middle (probe fluoro-labeled)	Common Fluorescent-detecting instrumentation or plate reader
Invader Assay	Very High	1/100	Middle (cleavase enzyme)	
Pyrosequencing	Very High	1/10000	Middle (luciferase related enzyme)	Pyrosequencer dedicate software
HRM	Medium	1/100	High	Common Fluorescent-detecting
NGS platforms	High	1/10	Middle (library constructions)	Dedicate instrumentation and software

§High/very high specificity, if combined to other detection platform (i.e. sequencer or MALDI-TOF).

*Referred to reagent stability: low = restriction enzyme; Middle= dye-labeled oligonucleotide; High= basic oligonucleotide.

#PCR thermal cycler and other common diffuse instrument, are not included in the estimate equipment.

resulting in an easy application in routine laboratories, due to their high specificity, low detection limit, moderate assay stability (decreasing fluorophore activity) and medium/high-throughput. Furthermore, these systems are specific, sensitive and highly reproducible, but usually require more fluorescently labeled probes, with the increase of cost and limitation in multiplexing capabilities. When a large number of assays must be developed and optimization time is limited, methods based on simple gel electrophoresis detection are preferable. The flexibility of the system may also be an important factor to be evaluated, if other types of assays or applications are desired. For example, the throughput of Pyrosequencing is limited by the time required for the luminescence cascade after the addition of each nucleotide. On the other hand, this method is more flexible than other assays because it can be used in a wider variety of polymorphisms including, short insertions/deletions, and CpG methylation analysis.

Nearly all methods require a separate PCR amplification step for the highest specificity and sensitivity. This requirement limits throughput and increases the cost per reaction. Multiplex PCR helps to increase throughput and decrease cost, but it is still not possible to develop robust and highly multiplexed reactions quickly. At present, PNA-mediated PCR Clamping analysis appears the technique best suited for the amplification of low abundance mutated alleles in great excess of non-mutated ones (very high sensitivity). In addition, this technique is flexible and could be coupled with Real-time-PCR⁴³ and other post PCR methods like sequencing, MALDI-TOF. AS-PCR analysis, if compared to RFLP, is technically simpler and sufficiently specific (higher specific if combined to sequencing of PCR products). Both of them do not require any specialized equipment or reagents, but suffer from a concomitant significant decrease in throughput and are laborious. No attempt has been made to evaluate the cost per



reaction for each of the described methods, due to the difference in manufacturer instrumentation model and reagents. Moreover, the costs may consistently vary between different laboratories due to the ability to produce “in house” many of the reagents needed⁴⁴.

CONCLUSIONS

All the technologies described here present advantages and disadvantages as summarized in Table 4. The comparisons among the different genotyping approaches are only made to

highlight the differences in performance among these platforms and to draw attention to a core set of selected criteria, before developing genotyping assay on a given platform. Decision criteria for the rational selection of a homogeneous platform for SNP detection, mainly depend on specific aims of the different diagnostic laboratories¹⁰. If the lab is built up to genotype a large number of SNPs in a small number of samples and if it has specialized personnel with a low budget, the most suitable platforms can be those for which the required reagents can be produced “in house” (i.e. AS-PCR, RFLP and PNA). Otherwise, if genotyping testing needs additional

TABLE 4. Advantages and disadvantages of described platforms.

<i>Platforms</i>	<i>Advantages</i>	<i>Disadvantages</i>
AS-PCR	Larger diffuse methods Combining capabilities Automation feasible Low cost “in house” set up	Low specificity and sensitivity Low throughput
RFLP	Low cost Simple instrumentation Allelic discrimination	Data interpretation Low specificity and sensitivity Low throughput Intense time-labor
PNA-mediate PCR clamping	Combining capabilities Automation feasible Low cost “in house” set up High sensitivity	No allelic discrimination Low throughput
FRET Allelic Discrimination a) LC probe b) TaqMan 5'nuclease c) Beacons probe d) Scorpions probe	Common instrumentation Medim/high throughput Allelic discrimination Quantification feasible	Low sensitivity Moderate multiplex Capabilities Possible data mis-interpretation
LNA probe	Combining capabilities Automation feasible High sensitivity Common instrumentation	No allelic discrimination Labor intense for optimization protocol
Invader assay	High throughput Allelic discrimination (2 tube assay) High sensitivity	Dedicate instrument, software and reagents No combining capabilities
Pyrosequencing	High throughput Allelic discrimination High sensitivity Short sequence context for each sample, good for deletion and insertion	Dedicate instrument, software and reagents No combining capabilities No multiplex
HRM	Common instrumentation Medium/high throughput Allelic discrimination Low cost	Low sensitivity No multiplex capabilities Possible data mis-interpretation
NGS platforms	High throughput Allelic discrimination High sensitivity	Dedicate software and reagents No combining capabilities Very high cost

Major selected criteria include: analytical validation, larger diffuse methods and instruments, allelic discrimination, platform combine capabilities, instrumentation cost, intense time-labor, methods which allow the specific and sensitive detection of mutant alleles in great excess of wt alleles, throughput.

information on mutant allele burden quantification, allele-specific detection platforms are to be preferred (i.e. HybProbe, TaqMan, Beacons, and Scorpions). Finally, if the genotyping panel is narrow and genotyping samples numerous, high-throughput platforms appear more suitable (Invader assay). Pyrosequencing is the only platform available for the detection of short sequence context (i.e. nucleotide deletion or insertion). PNA and LNA chemistries are ideal to be used for detection of a rare mutant allele in a large volume of wt alleles. Each technology designed to detect genetic abnormalities continues to evolve quickly. All present and future technological implementations for the detecting mutations, which yield early cancer cells detection, will always be amenable to analytical validation. The PCR-based methods described in this review have been validated and are well known in the world of molecular diagnostic. The expected performance of an assay can be estimated and each test can be monitored by validated Quality Controls (QCs) procedures in the global context of external Quality control assurance programs.

Recently, the WHO has identified a unique form of ALCL ALK-negative arising in association with breast implants⁴⁵. It consists of a late seroma fluid between the implant itself and the surrounding fibrous prosthesis in both saline- and silicone-filled implants⁴⁶.

The early detection is finalized to prevent the risk of lymph node involvement and systemic spread. Currently, the factors leading to anaplastic cell progression are still unclear, but the identification of new genetic trait marking ALCL should delineate these new issues. In this scenario, the present process is a multifaceted task that needs the successful cooperation of the diagnostic, pharmaceutical, surgical and public health fields⁴⁷. At the present genotyping testing is a small and specialized sector in the context of global diagnostics industry, comprising less than 5% of molecular diagnostics segment¹¹. Over the next few years, the emergence of new molecular markers and new therapies as results of genomic alteration (i.e. kinase inhibitors), will drive diagnostics companies to develop new tests able to produce results indicative for tailoring patient's treatment. Hopefully, the future implementation of the methods for genotyping will result in personalized treatment and eventually, in shifting the balance from disease relapse towards disease eradication. Therefore, medical, surgical and clinical lab branches join together, in order to develop diagnostic strategies suitable for personalized anticancer approach.

CONFLICT OF INTERESTS:

The Authors have no conflicts of interest to disclose.

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